



Pravastatin attenuates allergic airway inflammation by suppressing antigen-sensitization, IL-17 production, and antigen-presentation in the lung

Mitsuru Imamura, Katsuhide Okunishi, Hiroshi Ohtsu, et al.

Thorax published online October 3, 2008

doi: 10.1136/thx.2007.094540

Updated information and services can be found at:

<http://thorax.bmj.com/content/early/2008/10/03/thx.2007.094540>

These include:

**Supplemental
Material**

<http://thorax.bmj.com/content/suppl/2009/01/16/thx.2007.094540.DC1.html>

References

Article cited in:

<http://thorax.bmj.com/content/early/2008/10/03/thx.2007.094540#related-urls>

P<P

Published online October 3, 2008 in advance of the print journal.

**Email alerting
service**

Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Notes

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include the digital object identifier (DOIs) and date of initial publication.

To order reprints of this article go to:

<http://thorax.bmj.com/cgi/reprintform>

To subscribe to *Thorax* go to:

<http://thorax.bmj.com/subscriptions>

Pravastatin attenuates allergic airway inflammation by suppressing antigen-sensitization, IL-17 production, and antigen-presentation in the lung

Mitsuru Imamura,¹ Katsuhide Okunishi,¹ Hiroshi Ohtsu,² Kazuyuki Nakagome,¹ Hiroaki Harada,¹ Ryoichi Tanaka,¹ Kazuhiko Yamamoto,¹ and Makoto Dohi¹

¹ Department of Allergy and Rheumatology, ² Department of Clinical Trial Data Management, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Corresponding Author:

Dr. Makoto Dohi

University of Tokyo

Department of Allergy and Rheumatology, Graduate School of Medicine

7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

Phone: 81-3-3815-5411

Fax: 81-3-3815-5954

E-mail: mdohi-tky@umin.ac.jp

Key words: statins, bronchial asthma, airway inflammation, IL-17, antigen-presentation

Word count: 3083

ABSTRACT

Background: Statins are widely used to treat hyperlipidemia. Their immunosuppressive effect has recently been confirmed in various immune-mediated disease models. However, relatively few studies have been conducted on allergic inflammation, so the precise mechanisms of their actions against allergies have not been fully clarified. On the other hand, the role of interleukin (IL)-17 in immune responses has been recently highlighted, but whether statins affect IL-17 production has not been well studied. We examined the effect of pravastatin on allergic airway inflammation in a mouse model, then elucidated the mechanism of action, focusing on its effect on IL-17 production.

Methods: BALB/c mice were immunized with ovalbumin (OVA) and then challenged with OVA aerosol. Pravastatin was delivered by intraperitoneal injection during either the sensitization or the challenge.

Results: When delivered during systemic sensitization, pravastatin suppressed OVA-induced proliferation and production of Th2-type cytokines such as IL-5 in spleen cells *ex vivo* and *in vitro*. IL-17 production was also suppressed. Further, pravastatin delivered during the inhalation of OVA attenuated eosinophilic airway inflammation, OVA-specific IgE production in serum, and OVA-induced IL-17 production in the thoracic lymph node. We also found that pravastatin attenuated the antigen-presenting capacity of CD11c⁺ cells obtained from the OVA-challenged lung.

Conclusion: Pravastatin suppresses the systemic sensitization to allergen with a down-regulation of IL-17 production. It also suppresses an ongoing immune response in the airway partly by suppressing antigen-presentation in the lung. Therefore, statins could be a novel therapeutic option for treatment of asthma.

Abbreviation:

HMG-CoA = 3-hydroxy-3-methylglutaryl coenzyme A

DCs = dendritic cells

APCs = antigen-presenting cells

OVA = ovalbumin

BALF = bronchoalveolar lavage

Alum = aluminum hydroxide

INTRODUCTION

Statins are inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and have been widely prescribed to treat hyperlipidemia. Large clinical trials have demonstrated that statins can be used for primary and secondary prevention of cardiovascular diseases.¹

In addition to their conventional lipid-lowering properties, statins are now recognized to possess a variety of immunomodulatory effects.² Statins down-regulate various functions of antigen-presenting cells (APCs), such as dendritic cells (DCs)³⁻⁵ and B cells.⁶ Further, statins promote *in vitro* Th2 polarization from naïve CD4⁺ T cells via suppression of Th1 development.⁷ In animal models of Th1-biased autoimmune disorders such as experimental autoimmune encephalomyelitis^{5,8} and collagen-induced arthritis,⁹ statin attenuates disease severity with a shift from a Th1 to Th2-type cytokine profile.

Allergic bronchial asthma is a Th2-mediated disease, and statins would exaggerate allergic inflammatory responses in the airway. However, in several reports, statins suppressed the Th2-type airway inflammation.¹⁰⁻¹³ Simvastatin and pravastatin reduced production of Th2-type cytokines, thus attenuating allergic airway inflammation.¹⁰⁻¹² However, the precise mechanisms of action have not yet been fully clarified.¹³ Further detail should be clarified for the clinical application.

A significant role for interleukin (IL)-17-producing CD4⁺ T cells (Th17 cells) has recently been highlighted.¹⁴⁻¹⁶ Generally, IL-17 is critical to host defense against various infections.^{17,18} Further, IL-17 affects various immune responses. Many immunological processes have recently been re-evaluated in the context of Th17 and regulatory T cells (Tregs).¹⁴ For example, both the encephalomyelitis model and collagen-induced arthritis model have been considered Th17-mediated models rather than conventional Th1 models.^{14, 19, 20} IL-17 also plays an important role in neutrophil-dominated inflammatory responses in the lung.²¹ Recently, the role of IL-17 in asthma also has been intensively studied.²²⁻²⁷ For example, an elevated sputa IL-17 concentration correlates with clinical severity.²² The role of IL-17 in an animal model of allergic airway inflammation has been studied in several reports.²³⁻²⁵ In one report, IL-17 was essential for sensitization to the antigen, whereas it attenuated airway inflammation in the effector phase.²³ At present, the precise role of IL-17 in allergic airway inflammation is not fully clear. In addition, whether statins affect IL-17-mediated responses has not been well studied.

This study was performed to examine the hypothesis that pravastatin would attenuate

allergic airway inflammation through suppression of IL-17 production. We conducted research in vitro and in vivo using an animal model. We found that pravastatin suppressed systemic sensitization to the antigen with a down-regulation of IL-17 production, and thus suppressed antigen-induced eosinophilic airway inflammation, partly by regulating antigen-presentation in the lung.

METHODS

Mice

Male BALB/c mice 7 wk of age were obtained from Charles River Japan (Kanagawa Japan). Male BALB/c T-cell receptor-transgenic DO11.10 mice aged 6-8 wk were purchased from The Jackson Laboratory (Bar Harbor, ME). They were maintained under conventional animal housing conditions in a specific pathogen-free setting. All experiments were approved and performed according to the guidelines of University of Tokyo.

Study protocol in vivo

BALB/c mice were immunized with an intraperitoneal (i.p.) injection of 2 µg of OVA (Sigma-Aldrich) / 2 mg of aluminum hydroxide (alum) on days 0 and 11. Control mice received saline instead of the OVA/alum solution. The animals were challenged with an aerosolized solution of 3% w/v OVA in PBS for 10 min from day 18 to day 20. Pravastatin (10mg/kg, kindly provided by Sankyo Co (Tokyo, Japan)) dissolved in 0.5ml of saline, or saline alone was given by i.p. injection 30 min before the inhalation of OVA on days 18, 19, and 20. The control mice inhaled PBS on days 18-20. On day 21, samples of serum, bronchoalveolar lavage (BALF), and lungs were obtained. The lungs were cut out, and used for analyses of antigen-presentation. In another experiment, left lungs were homogenized in 1.0ml of PBS containing 0.5% Triton X-100 and complete protease inhibitor cocktail (Roche). The lung homogenates were cleared of debris and cells by centrifugation at $10,000 \times g$ for 10 min. Thoracic lymph nodes were also obtained and used for ex vivo analyses. Cell counts and cell differentials of BALF were determined as previously reported.²⁸⁻³⁰ Cytokine concentrations in BALF, lung homogenates, and immunoglobulin levels in sera were measured by Enzyme-linked immunosorbent assay (ELISA). The numbers of animals were 6 in non-sensitized group, and 8-14 in saline-treated- or pravastatin-treated-sensitized groups, respectively. We conducted the experiment more than four times,

and confirmed the reliability of the data.

Quantification of cytokines, cell proliferation, and cholesterol

Cytokine concentrations in BALF, serum, and cell culture supernatants were determined by ELISA³¹ (see methods in the online data supplement).

Preparation of single cell suspensions of spleen and lymph node cells

Spleens were collected and incubated at 37 °C for 15 min after treatment with 0.1% (w/v) collagenase (Sigma-Aldrich, St Louis, MO)/complete DMEM solution, then minced. Lymph node cells were collected and minced, then incubated at 37 °C for 30 min with 0.033% collagenase /complete DMEM solution. Single-cell suspensions were prepared with a cell strainer. RBCs were removed by hypotonic lysis. After two washes, the cells were used for experiments. Complete DMEM was used for incubation throughout the study.²⁸⁻³⁰

Response of spleen and lymph node cells to OVA

Spleen or lymph node cells (2.5×10^6 cells/ml) were cultured in a 96-well, flat-bottom microtiter assay plate with OVA (100 µg/ml) in an incubator (37 °C, 5% CO₂, 90% humidity). Cell Proliferation was measured on day 3. On day 4, cytokine production was measured by ELISA.

Animal preparation for ex vivo analyses

BALB/c mice were immunized with OVA / alum on days 0 and 11. On days 11-17, some mice received pravastatin (10mg/kg) dissolved in 0.5 ml of saline or saline alone by i.p. injection. The control mice received saline on days 0 and 11. On day 18, spleens were collected and cell proliferation and cytokine production were measured.

Effects of pravastatin on antigen-presentation in the lung

On day 21, CD11c⁺ antigen-presenting cells in the lungs of mice were positively selected as reported.²⁸⁻³⁰ In brief, lung tissues were minced and then treated with 0.033% (w/v) collagenase /complete DMEM solution for 30 min. Single-cell suspensions of the tissues were obtained, and CD11c⁺ cells were positively selected using MACS CD11c microbeads (Myltenyi Biotec, Auburn, CA). The populations of cells selected from lung tissues were routinely ~70% CD11c⁺, and there was no significant difference in the purity of CD11c⁺ cells between the groups of mice. Preliminary experiments demonstrated that almost 70% of the CD11c⁺ cells expressed

the major histocompatibility complex (MHC) class II molecule I-A /I-E. Some CD11c⁺ cells co-expressed F4/80 or Gr-1 (1-2%), an indicator of macrophages and granulocytes, respectively. Lung CD11c⁺ cells had negligible expression of CD4, CD8 α , and CD19 (data not shown). These results suggest that although lung CD11c⁺ cells contain some subsets, the main population of CD11c⁺ cells were dendritic cells. These CD11c⁺ cells were incubated with mitomycin-C (10 μ g/ml; Sigma-Aldrich) for 35 min at 37 °C in a humidified atmosphere containing 5% CO₂, then washed four times with PBS. In addition, CD4⁺ T cells were obtained from spleen cells of the DO11.10 mice using anti-mouse CD4 colloidal superparamagnetic microbeads (Myltenyi Biotec), as previously reported.²⁸⁻³⁰ The purity of CD4⁺ cells, confirmed by flow cytometry, was >95%. For measuring antigen-presenting capacity, lung CD11c⁺ cells (0.25×10^4 , 0.8×10^4 , and 2.5×10^4 cells/ml) obtained from each group of mice were cocultured with CD4⁺ T cells (2.5×10^5 cells/ml) selected from spleens of DO 11.10 mice. After a 2-day coculture, proliferation of CD4⁺ T cells was measured.

Statistical analysis

We applied data from at least three sets of samples from individual animals to statistical analysis, while we did not apply data from individual culture wells. Values are expressed as the mean $\pm$ S.D or individual points. The Kolmogorov-Smirnov test was used to check the normal distribution of data. For all variables, the numbers of lymphocytes and IL-13 levels in the BALF rejected normality. Differences in variables between the groups were determined by Student's t-test or Mann-Whitney's U test for variables with or without normal distribution, respectively. Values of $p < 0.05$ were considered to be significant.

RESULTS

Pravastatin decreased eosinophilic inflammation in the lung, and suppressed total IgE, OVA-specific IgE, and IgG1 levels in serum

Treatment with pravastatin significantly suppressed eosinophilic airway inflammation induced in the OVA-treated mice (fig 1A and B). We conducted this experiment repeatedly, and the numbers of eosinophils in BALF of mice treated by pravastatin were 30-60% of those of OVA-treated mice. The concentrations of IL-13 and TGF- β in BALF were also significantly suppressed (fig 1C and D). Although the IL-17 content of the lung increased in the OVA-treated mice compared with the saline-treated control

mice, it did not seem to be affected by pravastatin (fig 1E). Pravastatin treatment significantly reduced serum total IgE, OVA-specific IgE, and OVA-specific IgG1 levels, while no significant difference was observed in the total IgG and OVA-specific IgG levels (fig 2A-E). Pravastatin did not affect total serum cholesterol levels (saline-treated mice: 110.4 ± 12.4 mg/dl, OVA-treated mice: 102.6 ± 7.0 mg/dl, pravastatin-treated mice: 106.3 ± 11.0 mg/dl). So, the suppressive effect was independent of serum cholesterol levels.

Treatment with pravastatin during systemic sensitization suppresses the Ag-induced immune response of spleen cells

Cell proliferation and production of IL-5, IL-10, and IFN- γ by splenocytes obtained from OVA-treated mice were increased compared with saline-treated mice (fig 3 A-D). IL-17 production was also increased in OVA-treated mice, and pravastatin seemed to suppress the IL-17 production (fig 3E). Further, pravastatin treatment tended to reduce these responses to OVA except the production of IFN- γ (fig 3A-E). In another experiment, pravastatin treatment in vitro tended to suppress cell proliferation and production of IL-5, IL-17, and IL-10 in a dose-dependent manner (see supplementary fig 1 with method online). So, treatment with pravastatin during a second systemic sensitization attenuated the Th2-type antigen-induced immune response with a suppression of IL-17 production.

Pravastatin treatment during OVA challenge suppresses the antigen-induced IL-17 production of thoracic lymph node cells

Pravastatin treatment during the OVA challenge suppressed production of IL-17 from immunocytes in the thoracic lymph nodes (fig 4). This result suggests that pravastatin suppresses the Th17 response in the thoracic lymph node as well.

Pravastatin suppresses the antigen-presentation in the lung

Finally, we examined the effect of pravastatin on antigen-presentation in vivo. Treatment with pravastatin during the effector phase seemed to suppress the antigen-presenting capacity of CD11c⁺ cells in the lung (fig 5). In our preliminary study, the expression of MHC class II and co-stimulatory molecules such as CD40, CD80, and CD86 on CD11c⁺ cells from lung tissues did not differ between saline-treated mice and OVA-treated mice (data not shown). So we could not further assess the effect of pravastatin on the expression of these molecules. In another experiment, we confirmed that treatment with pravastatin did not affect the migration of

APCs (see supplementary fig 2 with method online). These results suggest that pravastatin suppressed the immune response in the airway partly by suppressing antigen-presentation in the lung.

DISCUSSION

The results of the present study demonstrated that pravastatin suppresses sensitization to OVA antigen with a down-regulation of IL-17 production, and that it also attenuates the ongoing immune response in the airway. Pravastatin, delivered during systemic immunization, attenuated the OVA-induced Th2-type response with suppression of the production of IL-17. In addition, pravastatin delivered during the airway challenge with OVA suppressed eosinophilic airway inflammation by affecting IL-17 production, and suppressing the antigen-presenting capacity of CD11c⁺ cells in the lung. As far as we know, this is the first study to clarify the effect of any statin treatment in vivo on IL-17 production in an immune response and its effect on antigen-presentation in the lung.

The immuno-modulating effect of statins involves the inhibition of Th1-type responses.² In addition, recent reports demonstrate that they can also suppress Th2-type responses. So far, two studies with simvastatin and one report with pravastatin demonstrated that statins suppressed the Th2-type allergic airway inflammation.¹⁰⁻¹² In the current study, pravastatin tended to suppress OVA-induced cell proliferation and cytokine production by spleen cells both ex vivo (fig 3) and in vitro (see supplementary fig 1 with method online). Pravastatin also suppressed eosinophilic airway inflammation, IgE production in serum, and cytokine production from immunocytes in the thoracic lymph node (fig 1, 2, 4). These results are basically consistent with the findings of previous reports,¹⁰⁻¹² confirming that statins certainly suppress Th2-type allergic airway inflammation in an experimental system.

So far, only one study has examined the effect of pravastatin on allergic airway inflammation.¹² Yeh and co-workers reported that treatment of mice with pravastatin from 2 weeks before antigen-sensitization to after an antigen-challenge reduced airway eosinophilia.¹² In a preliminary experiment, however, we could not reconfirm their results (data not shown). The reason for this discrepancy is not clear at present.

In this study, pravastatin tended to suppress OVA-induced IL-17 production in the spleen ex vivo and in vitro (fig 3 and supplementary fig 1 with method online). A recent study reported that simvastatin treatment in vitro directly inhibits IL-17

production by inhibiting the expression of IL-17 transcription factor RORC in human CD4⁺ T cells.³² The role of IL-17-producing Th17 cells in various immune responses has been recently highlighted.¹⁴⁻²⁷ The role of IL-17 in asthma has been elucidated in humans,²² experiments in vitro,^{26, 27} and animal models.²³⁻²⁵ IL-17 induces cytokine/chemokine production by human bronchial fibroblasts²⁶ and by human airway smooth muscle cells.²⁷ These studies indicate that IL-17 may enhance allergic reactions in the airway. In animal studies, OVA-induced airway inflammation was reduced in IL-17-deficient mice²⁴ and in IL-17 receptor-deficient mice.²³ In the current study, suppression of the OVA-induced immune response by pravastatin was associated with suppression of IL-17 production (fig 3 and supplementary fig1 online). These findings support the idea that IL-17 plays a critical role in antigen sensitization.

In the effector phase of airway inflammation, lung IL-17 content was increased in the OVA-treated mice compared with the control (fig 1E). This is consistent with the findings of previous reports.^{23, 24} Two studies suggested that IL-17 may play a protective role in the lung in the effector phase.^{23, 25} In contrast, in the present study, pravastatin suppressed IL-17 production as well as Th2-type cytokine production by the lymph node (fig 4), and suppressed eosinophilic airway inflammation (fig 1). The role of IL-17 or Th17 cells in the effector phase in the lung is not fully clarified at present, and further studies should be conducted.

Finally, to elucidate a novel role of statins in the lung immune response, we examined the effect of pravastatin on antigen-presentation. Pravastatin tended to suppress the antigen-presenting capacity of lung CD11c⁺ cells (fig 5). So far, the effect of statins on APC function has been elucidated mostly in vitro.^{3, 33-34} Only a few studies have reported the effect of statins on APCs in vivo.⁵ The effect of statins on APCs especially in the lung has not been examined, and the current study for the first time confirmed that pravastatin attenuates antigen-presentation in the lung.

Our findings and previous studies with simvastatin^{10,11} indicate that statins could inhibit the progression of ongoing airway inflammation in patients with bronchial asthma. However, whether the results obtained with an animal model can be adapted to human asthma remains unclear. Although the animal model using ovalbumin is one of the most popular methods of analyzing allergic airway inflammation, there are some differences between this model and human asthma. First, ovalbumin is not a true antigen of asthma. Second, the sensitization by intraperitoneal injection of ovalbumin / alum is an artificial system. Further, the dose of pravastatin is important in the clinical situation. In a preliminary study, treatment with 2mg/kg/day of pravastatin did not attenuate allergic airway inflammation (data not shown). The dose of

10mg/kg/day of pravastatin in this study is higher than the dose clinically used in human. The clinical dose of pravastatin in Japan is about 0.2-0.4 mg/kg/day, and the concentration in serum is 0.1-1µM. On the other hand, the doses of statins used in most animal studies were higher than the clinical dose, and high doses of statins could suppress the activities of autoimmune disease, but did not show cholesterol-lowering effects in mice.²

As animal models have limitations to adaptation to human diseases, clinical studies need to be performed. So far, only one clinical study has examined the effect of statins on chronic asthma. Menzies and co-workers reported that simvastatin treatment for 1 month did not exhibit significant therapeutic anti-inflammatory effects.³⁵ However, in their study, the number of patients enrolled in the trial was small, and simvastatin was administered in a stable period of asthma. To draw a definite conclusion, further clinical studies in various clinical situations are necessary.

In conclusion, we demonstrated that pravastatin suppresses eosinophilic airway inflammation by attenuating the sensitization to allergens with suppression of IL-17 production and by regulating lung DCs in a murine model. Although further investigation is needed to confirm this possibility, our results indicate the therapeutic potential of statins as anti-inflammatory drugs for asthma.

ACKNOWLEDGEMENTS

We thank Kanae Kurosaki for technical assistance.

COMPETING INTERESTS

We declare that we have no conflicts of interest related to the publication of this article.

COPYRIGHT

The Corresponding Author has the right to grant on behalf of all authors and does grant on behalf of all authors, an exclusive licence (or non-exclusive for government employees) on a worldwide basis to the BMJ Publishing Group Ltd and its Licensees to permit this article to be published in Thorax editions and any other BMJ PGL products to exploit all subsidiary rights, as set out in our licence <http://thorax.bmjournals.com/ifora/licence.pdf>.

FIGURE LEGENDS

Figure 1 Treatment with pravastatin during OVA challenge significantly attenuates the development of allergic airway inflammation. Mice were sensitized with OVA on days 0 and 11. Then, mice were challenged with OVA aerosol on days 18-20. On days 18-20, they received i.p. injections of pravastatin (PR) or control vehicle (OVA) 30 min before the challenge. Control mice received saline injections on days 0 and 11, and PBS aerosol challenges on days 18-20 (saline). On day 21, BALF and lung tissues were obtained. A, Cell differentials in BALF. Each point represents an individual mouse. B, H&E staining of lung tissues. scale bar: 100 μ m. C, IL-13 concentration in BALF. Each point represents an individual mouse. D, E, TGF- β in BALF (D) and IL-17 in the lung homogenate (E). Data were expressed as the mean $\pm$ S.D. Data were obtained from 6 animals (saline), and 8-14 animals (OVA, PR) per group of mice. *, $p < 0.05$; **, $p < 0.01$ difference between OVA-sensitized / challenged mice (OVA-group) and OVA-sensitized / challenged and pravastatin-treated mice (PR-group). N.D. indicates not detectable. Data are representative of more than four independent experiments.

Figure 2 Treatment with pravastatin during OVA challenge significantly attenuates serum IgE and IgG1 production. Mice were subjected to analyses as described in Figure 1. On day 21, serum was obtained. Total IgE (A), OVA-specific IgE (B), total IgG (C), OVA-specific IgG(D), and OVA-specific IgG1(E) in the serum. Data were expressed as the mean $\pm$ S.D. Data were obtained from 6 animals (saline), and 8-14 animals (OVA, PR) per group of mice. *, $p < 0.05$ difference between OVA-group and PR-group. N.D. indicates not detectable. Data are representative of more than four independent experiments.

Figure 3 Treatment with pravastatin during sensitization in vivo decreases spleen cell responses to OVA restimulation. Mice received OVA/alum or saline injections on days 0 and 11. The OVA-sensitized mice received intraperitoneal injections of pravastatin (PR), or saline (OVA) on days 11-17. Spleen cells obtained on day 18 were restimulated in vitro with OVA. A, Cell proliferation was measured after 3 days of incubation. Data are expressed as a percentage of the response compared with that of spleen cells from OVA mice. B-D, Production of IL-5 (B), IL-10 (C), IFN- γ (D), and IL-17 (E) was measured after 4 days of incubation with OVA. Data were obtained from six wells per group of mice. N.D. indicates not detectable. All results are

representative of at least three independent experiments.

Figure 4 Treatment with pravastatin during OVA challenge attenuates Ag-induced IL-17 production of lymph node cells. A, B, On day 21, lymph nodes cells from OVA mice treated with pravastatin (PR) or control vehicle (OVA) were incubated with OVA. After 3 days of incubation, the production of IL-5 (A) and IL-17 (B) was measured. Data were obtained from six wells per group of mice. All results are representative of three independent experiments.

Figure 5 Treatment with pravastatin significantly suppresses Ag-presenting capacity of lung cells. On day 21, CD11c⁺ APCs in the lung of mice treated with pravastatin (PR) or saline (OVA) were positively selected, and cocultured with CD4⁺ T cells from the DO11.10 mice. After 2 days of coculture, cell proliferation was measured. Data are expressed as a percentage of the response compared with that induced by APCs obtained from saline-treated mice at an APC to CD4⁺ T cell ratio of 1:10. Data were obtained from six wells per group. Results are representative of three independent experiments.

REFERENCES

1. **Nakamura H**, Arakawa K, Itakura H, et al. Primary prevention of cardiovascular disease with pravastatin in Japan (MEGA Study): a prospective randomised controlled trial. MEGA Study Group. *Lancet* 2006;**368**:1155-63.
2. **Greenwood J**, Steinman L, Zamvil SS. Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. *Nat Rev Immunol* 2006;**6**:358-70.
3. **Yilmaz A**, Reiss C, Tantawi O, et al. HMG-CoA reductase inhibitors suppress maturation of human dendritic cells: new implications for atherosclerosis. *Atherosclerosis* 2004;**172**:85-93.
4. **Yilmaz A**, Reiss C, Weng A, et al. Differential effects of statins on relevant functions of human monocyte-derived dendritic cells. *J Leukocyte Biol* 2006;**79**:529-38.
5. **Youssef S**, Stuve O, Patarroyo JC, et al. The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature* 2002;**420**:78-84.
6. **Lawman S**, Mauri C, Jury EC, et al. Atorvastatin inhibits autoreactive B cell activation and delays lupus development in New Zealand black/white F1 mice. *J Immunol* 2004;**173**:7641-6.
7. **Hakamada-Taguchi R**, Uehara Y, Kuribayashi K, et al. Inhibition of hydroxymethylglutaryl-coenzyme A reductase reduces Th1 development and promotes Th2 development. *Circ Res* 2003;**93**: 948-56.
8. **Dunn SE**, Youssef S, Goldstein MJ, et al. Isoprenoids determine Th1/Th2 fate in pathogenic T cells, providing a mechanism of modulation of autoimmunity by atorvastatin. *J Exp Med* 2006;**20**:401-12.
9. **Leung BP**, Sattar N, Crilly A, et al. A novel anti-inflammatory role for simvastatin in inflammatory arthritis. *J Immunol* 2003;**170**:1524-30.
10. **Mckay A**, Leung BP, McInnes IB, et al. A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma. *J Immunol* 2004;**172**:2903-8.
11. **Kim DY**, Ryu SY, Lim JE, et al. Anti-inflammatory mechanism of simvastatin in mouse allergic asthma model. *Eur J Pharmacol* 2007;**557**:76-86.
12. **Yeh YF**, Huang SL. Enhancing effect of dietary cholesterol and inhibitory effect of pravastatin on allergic pulmonary inflammation. *J Biomed Sci* 2004;**11**:599-606.
13. **Hothersall E**, McSharry C, Thomson NC. Potential therapeutic role for statins in respiratory disease. *Thorax* 2006;**61**:729-34.
14. **Weaver CT**, Hatton RD, Mangan PR, et al. IL-17 family cytokines and the

expanding diversity of effector T cell lineages. *Annu Rev Immunol* 2007;**25**:821-52.

15. **Harrington LE**, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4⁺ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005;**6**:1123-32.
16. **Bettelli E**, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector T_H17 and regulatory T cells. *Nature* 2006;**441**:235-8.
17. **Happel KI**, Dubin PJ, Zheng M, et al. Divergent roles of IL-23 and IL-12 in host defense against *Klebsiella pneumoniae*. *J Exp Med* 2005;**202**:761-9.
18. **Khader SA**, Bell GK, Pearl JE, et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4⁺ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. *Nat Immunol* 2007;**8**:369-77.
19. **Cua DJ**, Sherlock J, Chen Y, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 2003;**421**:744-8.
20. **Murphy CA**, Langrish CL, Chen Y, et al. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med* 2003;**198**:1951-7.
21. **Laan M**, Cui ZH, Hoshino H, et al. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol* 1999;**162**:2347-52.
22. **Barczyk A**, Pierzchala W, Sozanska E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir Med* 2003;**97**:726-33.
23. **Schnyder-Candrian S**, Togbe D, Couillin I, et al. Interleukin-17 is a negative regulator of established allergic asthma. *J Exp Med* 2006;**203**:2715-25.
24. **Nakae S**, Komiyama Y, Nambu A, et al. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* 2002;**17**:375-87.
25. **Hellings PW**, Kasran A, Liu Z, et al. Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *Am J Respir Cell Mol Biol* 2003;**28**:42-50.
26. **Molet S**, Hamid Q, Davoine F, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* 2001;**108**:430-8.
27. **Dragon S**, Rahman MS, Yang J, et al. IL-17 enhances IL-1 β -mediated CXCL-8 release from human airway smooth muscle cells. *Am J Physiol Lung Cell Mol*

Physiol 2007;**292**:L1023-9.

28. **Okunishi K**, Dohi M, Nakagome K, et al. A novel role of cysteinyl leukotrienes to promote dendritic cell activation in the antigen-induced immune responses in the lung. *J Immunol* 2004;**173**:6393-402.
29. **Okunishi K**, Dohi M, Nakagome K, et al. A novel role of hepatocyte growth factor as an immune regulator through suppressing dendritic cell function. *J Immunol* 2005;**175**:4745-53.
30. **Nakagome K**, Dohi M, Okunishi K, et al. In vivo IL-10 gene delivery suppresses airway eosinophilia and hyperreactivity by down-regulating APC functions and migration without impairing the antigen-specific systemic immune response in a mouse model of allergic airway inflammation. *J Immunol* 2005;**174**:6955-66.
31. **Brandt EB**, Strait RT, Hershko D, et al. Mast cells are required for experimental oral allergen-induced diarrhea. *J Clin Invest* 2003;**112**:1666-77.
32. **Zhang X**, Jin J, Peng X, et al. Simvastatin inhibits IL-17 secretion by targeting multiple IL-17-regulatory cytokines and by inhibiting the expression of IL-17 transcription factor RORC in CD4⁺ lymphocytes. *J Immunol* 2008;**180**:6988-96.
33. **Pahan K**, Sheikh FG, Namboodiri AM, et al. Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. *J Clin Invest* 1997;**100**:2671-9.
34. **Kwak B**, Mulhaupt F, Myit S, et al. Statins as a newly recognized type of immunomodulator. *Nat Med* 2000;**6**:1399-1402.
35. **Menzies D**, Nair A, Meldrum KT, et al. Simvastatin does not exhibit therapeutic anti-inflammatory effects in asthma. *J Allergy Clin Immunol* 2007;**119**:328-35.

SUPPLEMENTAL FILE ONLINE REPOSITRY

Pravastatin attenuates allergic airway inflammation by suppressing antigen-sensitization, IL-17 production, and antigen-presentation in the lung

Mitsuru Imamura, Katsuhide Okunishi, Hiroshi Ohtsu, Kazuyuki Nakagome, Hiroaki Harada, Ryoichi Tanaka, Kazuhiko Yamamoto, and Makoto Dohi

METHODS

Quantification of cytokines, cell proliferation, and cholesterol

Concentrations of mouse IL-5, IL-10, IFN- γ , IgE (BD pharmingen, San Diego, CA), IgG (Bethyl Laboratories, Montgomery, TX), TGF- β , and IL-13 (R&D Systems, United Kingdom) were measured using commercial ELISA kits. We measured concentrations of mouse IL-17 by ELISA using anti-mouse IL-17 and biotinylated anti-mouse IL-17 antibodies (BD pharmingen). Recombinant mouse IL-17 (R&D Systems) was used as a standard. The minimum detectable concentration of IL-17 was less than 7.8 pg/ml. To measure OVA-specific IgE and IgG in sera, plates were coated with 100mg/ml OVA/0.1 M sodium carbonate solution at 4 °C overnight instead of each capture antibody, then subjected to the ELISA. For the analysis of OVA-specific IgG1, wells were coated with 100mg/ml OVA and exposed to serum samples, and HRP-conjugated anti-mouse IgG1 (BD pharmingen) was used for detection as previously reported.³¹ The titers of the samples were calculated by comparison with internal standards, which were obtained from the sera of five individual OVA-treated mice on day 21. These standards were calculated as 100 U/ml. Cell proliferation was evaluated by measuring the incorporation of BrdU using a BrdU cell proliferation ELISA kit (Roche, Mannheim, Germany). Total serum cholesterol was measured using a cholesterol test kit (Wako, Osaka, Japan). The data were analyzed with Microplate Manager III, version 1.45 (Bio-Rad, Tokyo, Japan).

Effects of pravastatin on spleen cells in vitro

BALB/c mice were immunized with OVA/alum on day 0. On day 10, spleen cells were obtained and cultured (2.5×10^6 cells/ml) with OVA (100 μ g/ml) in the absence or presence of different concentrations of pravastatin (0.01, 0.1, 1, and 10 μ M). Then, cell proliferation and cytokine production were measured.

Effect of pravastatin on migration of antigen-presenting cells

Mice were sensitized with OVA on days 0 and 11. On day 18, some mice received an i.p. injection of pravastatin (10 mg/kg) or saline, then were given FITC-labeled OVA (500 μ g; Molecular Probes, Eugene, OR) intratracheally 60 min later. On day 19, bronchial lymph nodes were excised, embedded in cryomolds containing Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan), and frozen in liquid nitrogen. Sections were cut 5 μ m thick by cryostat. The sections were subsequently observed under a fluorescence microscope.

FIGURE LEGENDS

Supplementary Figure 1

Treatment with pravastatin in vitro suppresses spleen cell responses to OVA restimulation. Spleen cells obtained from OVA mice or control mice on day 10 were restimulated in vitro with OVA. Several concentrations of pravastatin (PR) were added to each well. A, Cell proliferation was measured after 3 days of incubation. Data are expressed as a percentage of the response compared with that of spleen cells from OVA mice without pravastatin. B-E, The production of IL-5 (B), IL-10 (C), IFN- γ (D), and IL-17 (E) was measured after 4 days of incubation. Data were expressed as the mean $\pm$ S.D. Data were obtained from six wells per group of mice. N.D. indicates not detectable. All results are representative of at least three independent experiments.

Supplementary Figure 2

Fluorescence micrograph of a lymph node. Mice were sensitized with OVA on days 0 and 11. Mice were given FITC-labeled OVA (500 μ g) intratracheally on day 18. On day 18, they received i.p. injections of pravastatin (PR) or vehicle (OVA) 60 min before treatment with FITC-labeled OVA. On day 19, bronchial lymph nodes were excised and subjected to fluorescence microscopy. Magnification, 40 $\times$. Results are representative of three independent experiments.

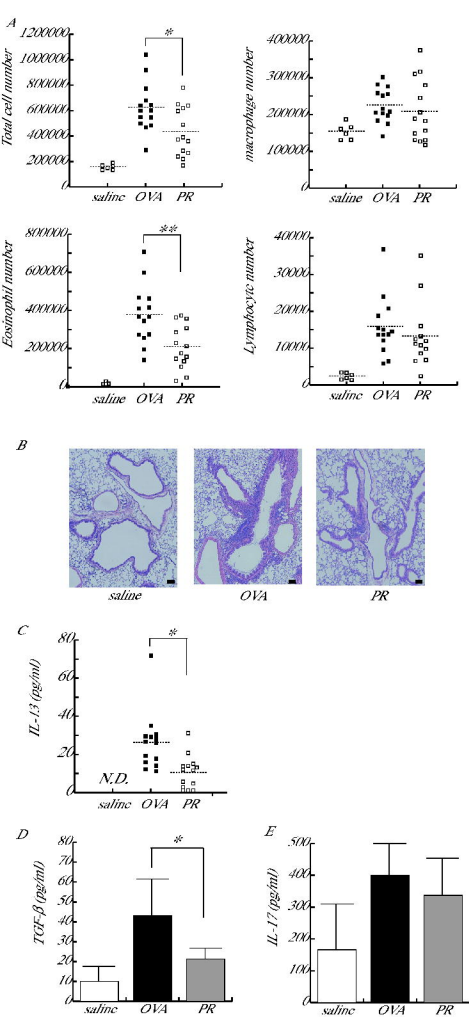


Fig 1.

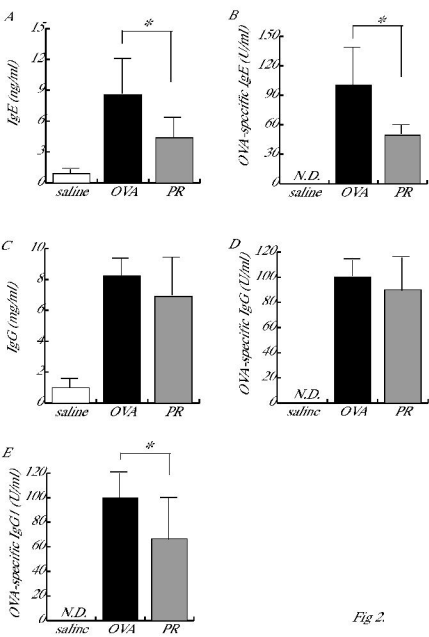


Fig 2.

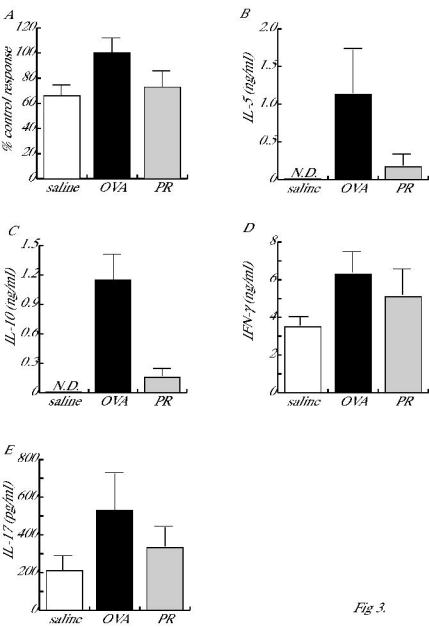


Fig. 3.

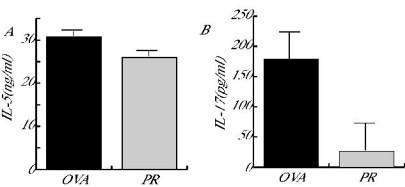


Fig. 4.

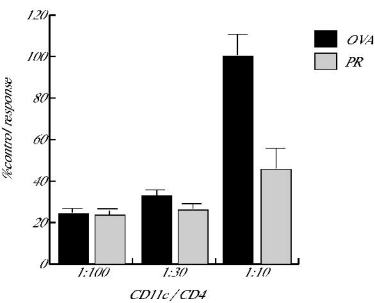
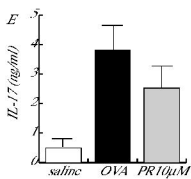
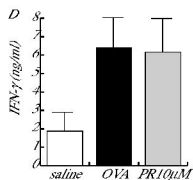
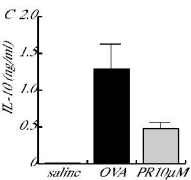
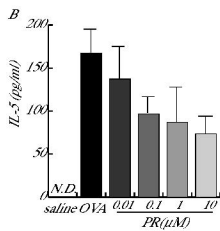
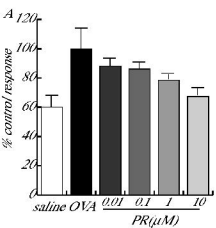
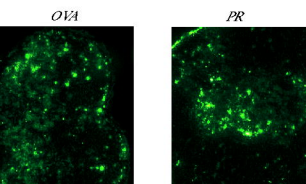
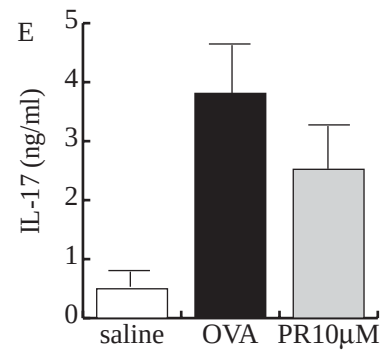
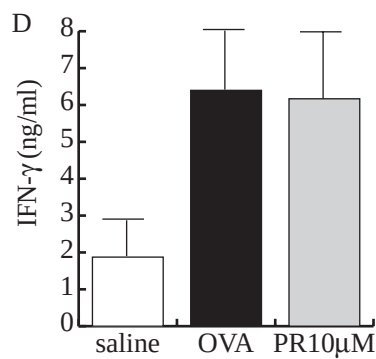
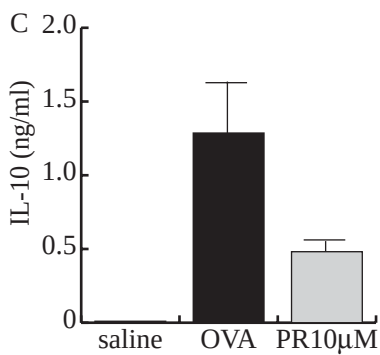
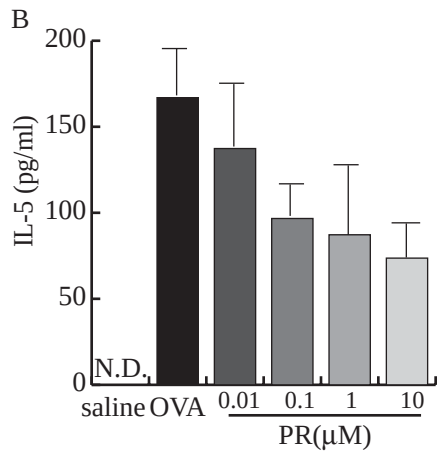
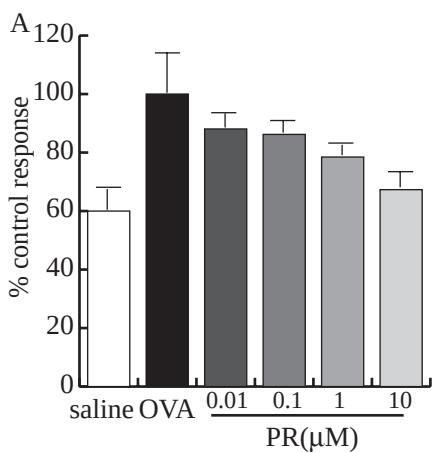


Fig 5.



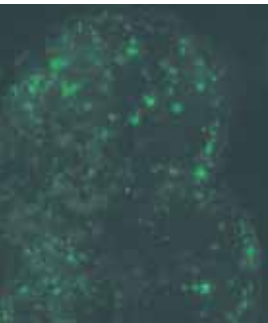
Sup. Fig 1.



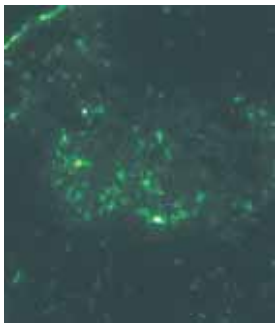


Sup. Fig 1.

OVA



PR



Sup. Fig 2.