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A Novel Anti-Inflammatory Role of Simvastatin in a Murine Model of Allergic Asthma[1]

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Statins, the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, are effective serum cholesterol-lowering agents in clinical practice, and they may also have anti-inflammatory properties. Asthma is characterized by chronic eosinophilic inflammation in the airways, which is thought to be regulated by the activity of T lymphocytes. We therefore examined the anti-inflammatory activity of simvastatin in a murine model of allergic asthma. In mice previously sensitized to OVA, simvastatin treatment, either orally or i.p., reduced the total inflammatory cell infiltrate and eosinophilia in bronchoalveolar lavage fluid in response to inhaled OVA challenge. Simvastatin therapy i.p. was also associated with a reduction in IL-4 and IL-5 levels in bronchoalveolar lavage fluid and, at higher doses, a histological reduction in inflammatory infiltrates in the lungs. OVA-induced IL-4, IL-5, IL-6, and IFN-γ secretion was reduced in thoracic lymph node cultures from simvastatin-treated mice. Simvastatin treatment did not alter serum total IgE or OVA-specific IgG1 and IgG2a levels. These data demonstrate the therapeutic potential of statin-sensitive pathways in allergic airways disease. *The Journal of Immunology*, 2004, 172: 2903–2908.

Statins are inhibitors of the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA)[3] reductase, in cholesterol biosynthesis. As such, they have been widely used in clinical practice as cholesterol-lowering agents to reduce morbidity and mortality from coronary artery disease (1, 2). There is evidence from clinical studies (3, 4) and in vitro experiments (5, 6) that statins have additional anti-inflammatory properties in atherosclerotic disease that are unrelated to their lipid-lowering activity. There are likely to be several molecular mechanisms through which statins exert their immunomodulatory effects (7), but these have not yet been fully elucidated. Statin treatment has the potential to modify T lymphocyte-driven disease through the ability to allosterically inhibit the interaction between the cellular adhesion molecules LFA-1 and ICAM-1 (8) and decrease IFN-γ-induced expression of MHC-II on APCs (9). Also, by inhibiting the production of L-mevalonic acid and its metabolites, statins prevent the isoprenylation of signaling molecules such as Ras and Rho, which are involved in lymphocyte activation (10, 11). Statins may therefore have beneficial effect in a broad range of inflammatory conditions.

Atherosclerotic plaques contain large numbers of lymphocytes that are mainly of the Th1 type (12), which characteristically secrete IFN-γ. As statins inhibit inflammation in plaques, recent studies have focused on the potential ability of statins to modulate Th1-predominant disease, such as rheumatoid arthritis (13) and multiple sclerosis (14). In animal models of both these conditions, statins have had an immunosuppressive action (15–17). In contrast, evidence is lacking as to whether statins might modulate inflammation in which Th2 lymphocytes, secreting IL-4, IL-5, and IL-13, are important.

Asthma is a chronic inflammatory condition of the airways characterized by airway hyper-responsiveness, inflammatory infiltrates in the bronchial walls containing eosinophils, and elevated serum IgE levels. Th2 lymphocytes are thought to play a key role in the initiation and perpetuation of this airway inflammation (18–20). Treatments targeted at inhibiting the function of LFA-1 have been effective in reducing airway eosinophilia in a murine model of allergic asthma (21) and sputum eosinophilia after allergen challenge in asthmatic patients (22). As simvastatin can inhibit LFA-1/ICAM-1 interaction, we sought to establish whether this drug could modulate inflammatory responses in a murine model of allergic asthma, a Th2-driven condition. In this study we show for the first time that simvastatin can effectively suppress acute eosinophilic airway inflammation and Th2 cytokine secretion.

Materials and Methods

Preparation of simvastatin

Simvastatin (Merck, Sharp & Dohme, Middlesex, U.K.) was prepared as a 4 mg/ml stock. Briefly, 4 mg of simvastatin was dissolved in 100 μl of ethanol and 150 μl of 0.1 N NaOH and incubated at 50°C for 2 h, then the pH was adjusted to 7, and the total volume was corrected to 1 ml. The stock solution was diluted to the appropriate concentration in sterile PBS immediately before use.

Sensitization and challenge with OVA

Female BALB/c mice (Harlan-Olac, Bicester, U.K) were used at 6–8 wk of age. Airway eosinophilia was elicited using a modification of a previously described protocol (23) with OVA (fraction V; Sigma-Aldrich, Poole, U.K.) as the allergen. Mice were immunized with OVA (100 μg) in an alum suspension (2% Alhydrogel; Brenntag Biosector, Fredriksund, Denmark) in a volume of 200 μl by i.p. injection on days 0 and 14. On day 14, mice were anesthetized with 50 μl of avertin (1,1,1-tribromoethanol dissolved 1/1 (w/v) in amyl alcohol, then diluted 1/40 in PBS) and 100 μg of OVA in 50 μl of PBS administered intranasally. Mice were again anesthetized before being challenged with 50 μg of OVA in 50 μl of PBS on each of days 25, 26, and 27. Control mice were given PBS in place of OVA in both the sensitization and challenge stages of the protocol. Mice were sacrificed on day 28 by administration of a fatal dose of avertin.

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[3] Abbreviations used in this paper: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; BAL, bronchoalveolar lavage; CIA, collagen-induced arthritis; o.g., oral gavage.

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Treatment protocols

Simvastatin (4 or 40 mg/kg) was given by i.p. injection 30 min before the OVA challenge on days 25, 26, and 27. Similarly, simvastatin (40 mg/kg) was given by oral gavage (o.g.) 1 h before each OVA challenge on these days. Control mice received PBS alone. The doses of simvastatin used are comparable to those used previously in murine (5, 15–17, 24, 25) studies in vivo and are necessarily higher than those used in man because of significant up-regulation of HMG-CoA reductase with statin treatment in rodents (26).

Bronchoalveolar lavage (BAL)

Immediately after the administration of a fatal dose of avertin, the thoracic cavity was opened by careful dissection. The trachea was then exposed, and a small transverse incision made just below the level of the larynx. BAL was then performed using two doses of 0.5 ml of PBS, ensuring that both lungs inflated during the lavage process and that there was no leakage of lavage fluid from the trachea. The lavage samples from each mouse were pooled and kept on ice until processing. BAL was centrifuged at 400 $\times$ *g* for 5 min, and the supernatant was removed. The volume of supernatant from each lavage was measured before storage at −70°C until assay of cytokines. To remove any contaminating RBC, the BAL cell pellet was resuspended in 1 ml of FACS Lysis Buffer (BD Biosciences, Oxford, U.K.), incubated for 10 min at 18°C, washed twice in PBS, and then resuspended in 1 ml of PBS. Cell number was then counted using a hemocytometer. Cytospin preparations were made using a Cytospin (Shandon, Pittsburg, PA), then were stained with Diff-Quik (Triangle Biomedical Sciences, Skelmersdale, U.K.), a rapid Romanowsky staining method. Differential cell counting was performed using standard morphological criteria.

Serum collection

Blood was collected by cardiac puncture immediately after the thoracic cavity was opened and before BAL was performed. Blood was allowed to clot, then was centrifuged, and aliquots of serum were stored at −70°C before analysis by ELISA for serum Igs.

Lung histology

After BAL sampling had been completed, the lungs were removed from the thoracic cavity by careful dissection. The lungs were inflated with 1 ml of 10% neutral-buffered Formalin and then fixed in 10% neutral-buffered Formalin for 72 h. After fixation, the left lung was dissected free and embedded in paraffin, and 6-μm sections were cut. Sections were then stained with H&E. Total lung inflammation was defined as the sum of the peribronchial and perivascular scores (27). Peribronchial and perivascular inflammation was quantified by a treatment-blind observer and graded on the following scale: 0, none; 1, mild; 2, moderate; 3, marked; and 4, severe. An increment of 0.5 was used when the inflammation fell between two levels.

Cell culture

Thoracic lymph nodes were obtained by careful dissection after the lungs had been removed from the thoracic cavity and passed through Nytex (Cadisch Precision Meshes Ltd., London, U.K.) to prepare a single-cell suspension. Cells were cultured at 2 $\times$ 10^6 cells/ml in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES buffer, and 10% heat-inactivated FCS (all from Life Technologies, Paisley, U.K.). Cells were cultured with 500 μg/ml OVA for 72 h, and the supernatants from parallel triplicate cultures were stored at −70°C until analysis of cytokine concentrations by ELISA. Proliferation assays were performed in triplicate in U-bottom, 96-well plates (Nunc, Roskilde, Denmark). Cells were cultured for 96 h, with 1 μCi/ml [^{3}H]thymidine (Amersham Pharmacia Biotech, Little Chalfont, U.K.) added for the last 8 h of culture.

ELISA

Murine IL-4, IL-5, IL-6, IFN-γ (BD Biosciences), and eotaxin (R&D Systems, Oxon, U.K.) in BAL fluid and culture supernatants were assayed by ELISA using paired Abs according to the manufacturer's instructions. The lower limit of detection for IL-4, IL-5, IL-6, and eotaxin was 10 pg/ml, and that for IFN-γ was 40 pg/ml. Total serum IgE was measured using an OptiEIA ELISA kit (BD Biosciences) following the supplied protocol; the lower limit of detection of this assay was 6 ng/ml. Serum OVA-specific IgG1 and IgG2a titers were measured by ELISA as previously described (28), with modification of the dilution of sera as required.

Statistical analysis

BAL total cell counts, differential cell counts, cytokine levels, and serum Ab levels were compared by ANOVA. Cytokine and proliferation counts from lymph node cultures and histological score were compared by Student's *t* test.

Results

Simvastatin suppresses eosinophilic airway inflammation

To determine whether simvastatin could influence OVA-induced airway inflammation in this murine model of allergic asthma, mice were initially given simvastatin by i.p. injection before allergen challenges. Simvastatin at 40 mg/kg i.p., but not at 4 mg/kg, produced a significant reduction in BAL total cell count and eosinophilia (Fig. 1, *A* and *B*). The higher dose of i.p. simvastatin was also associated with a significant reduction in BAL macrophage count (Fig. 1*C*).

As simvastatin is orally administered in clinical use, it was important to determine whether a similar anti-inflammatory effect could be obtained by giving the drug enterally. A significant decrease in BAL total cell count and eosinophilia (Fig. 2, *A* and *B*) was also observed when simvastatin (40 mg/kg) was administered orally before allergen challenges, although reductions were proportionately less than those produced by the same dose of simvastatin given i.p..

Histological analysis demonstrated that there was a reduction in inflammatory infiltrates in the lungs of mice treated with 40 mg/kg

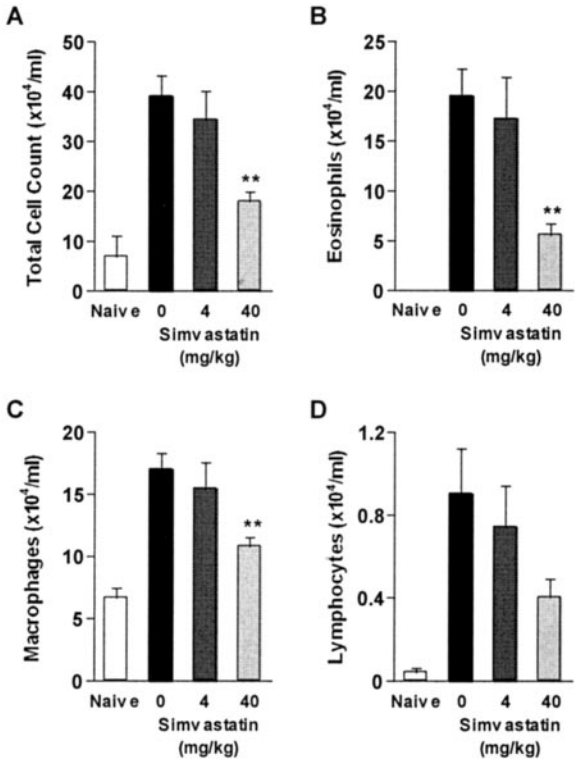

FIGURE 1. Total BAL cellularity and differential cell count after treatment with simvastatin i.p.. BALB/c mice were sensitized with OVA and then challenged with OVA intranasally on 3 consecutive days (days 25–27). Simvastatin (40 or 4 mg/kg i.p.) was given 30 min before each Ag challenge. Control mice were given PBS. BAL cell counts were performed on day 28. Treatment with simvastatin (40 mg/kg) resulted in a significant reduction in total cell count (*A*), eosinophilia (*B*), and macrophage number (*C*) compared with PBS-treated controls. No significant difference was seen in BAL lymphocyte levels (*D*). Normal mice challenged intranasally with OVA had lung cellularity similar to that of mice given PBS alone (not shown). Data are expressed as the mean ± SEM (n = 14). **, $p < 0.01$ (by ANOVA).

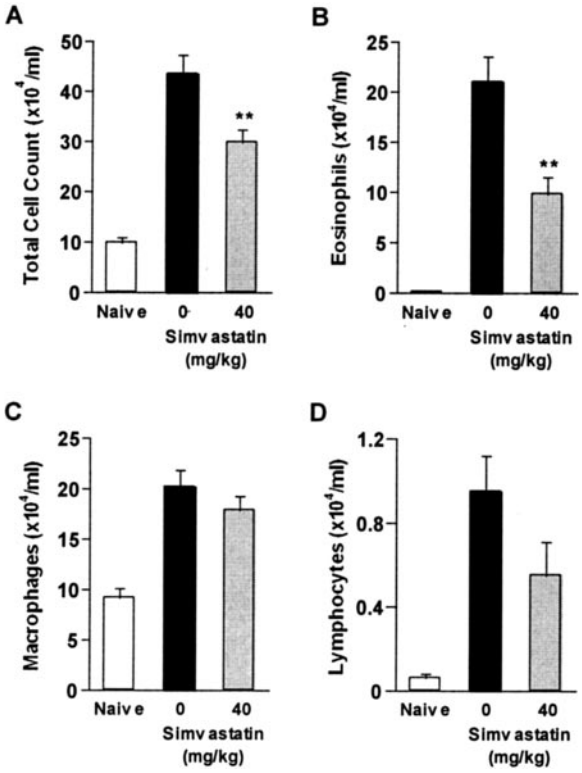

FIGURE 2. Total BAL cellularity and differential cell count after treatment with simvastatin orally. BALB/c mice were sensitized with OVA and then challenged with OVA intranasally on 3 consecutive days (days 25–27). Simvastatin (40 mg/kg) was given by o.g. 1 h before each Ag challenge. Control mice were given PBS. BAL cell counts were performed on day 28. Treatment with oral simvastatin resulted in a significant reduction in total cell count (*A*) and eosinophilia (*B*) compared with PBS-treated controls. There was no significant difference in macrophage (*C*) or lymphocyte (*D*) numbers. Data are expressed as the mean ± SEM ($n = 16$). **, $p < 0.01$ (by ANOVA).

simvastatin i.p. (Fig. 3), but not in those given 4 mg/kg simvastatin i.p. or 40 mg/kg simvastatin by o.g.

Simvastatin treatment reduces Th2 cytokine levels in BAL

Intraperitoneal administration of simvastatin produced a dose-related reduction in the levels of IL-4 and IL-5 in BAL fluid (Fig. 4). Simvastatin (40 mg/kg o.g.) did not significantly alter the levels of these cytokines in the lavage samples (data not shown). IFN-γ was not detectable, and there was a modest, but not significant ($p > 0.05$), reduction of eotaxin in the BAL fluid (data not shown). Serum levels of OVA-specific IgG1, Ig2a, and total IgE were not were not significantly different (data not shown).

Simvastatin suppresses OVA-specific responses in vitro

OVA-specific immune responses in thoracic lymph node cultures were performed to assess whether simvastatin treatment directly affected lymphocyte function. The OVA-specific production of IL-4 and IL-5 was significantly decreased in mice previously treated with simvastatin, either 40 or 4 mg/kg i.p., in a dose-dependent manner (Fig. 5, *A* and *B*). This was not associated with a decrease in OVA-induced cell proliferation (Fig. 5*C*). Mice treated with simvastatin orally had no significant difference in OVA-induced IL-4 or IL-5 levels in lymph node cultures (data not shown).

IFN-γ production was reduced in mice treated with i.p. simvastatin (40 and 4 mg/kg; Fig. 6*A*) and in those treated with simvastatin (40 mg/kg) by the o.g. route (Fig. 6*C*). IL-6 levels were also decreased in mice previously treated with simvastatin (40 mg/kg) both i.p. (Fig. 6*B*) and orally (Fig. 6*D*). IFN-γ and IL-6 secretion may therefore be more sensitive to the suppressive actions of simvastatin.

The immunomodulatory action of simvastatin in vivo was OVA specific, as Con A-induced production of these cytokines was not affected (data not shown). In addition, thoracic lymph node class II *trans*-activator mRNA levels measured by quantitative (TaqMan; Applied Biosystems, Warrington, U.K.) PCR were not affected

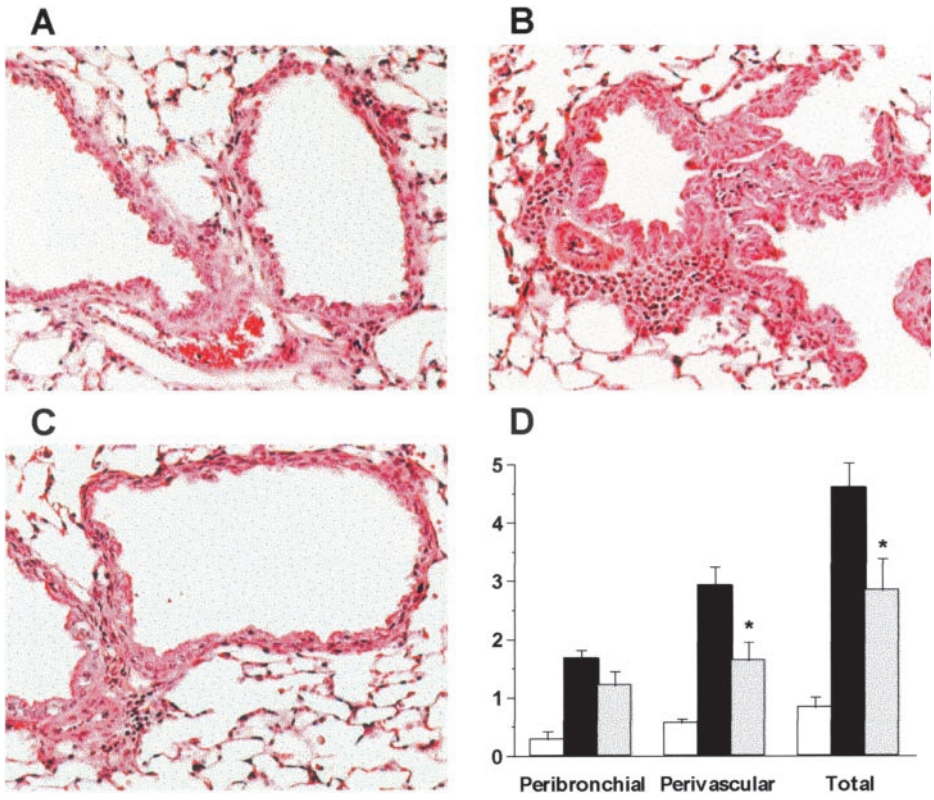

FIGURE 3. Histological evidence of decreased lung inflammation in mice treated with simvastatin (40 mg/kg i.p.). A representative section from each group of five mice is shown. *A*, Naive mouse, given PBS challenge. *B*, OVA-challenged mouse; peribronchial and perivascular inflammatory infiltrates are seen, with eosinophils present and mucosal hyperplasia. *C*, OVA-challenged mouse plus treatment with simvastatin (40 mg/kg i.p.); a reduction in inflammatory infiltrates is seen compared with *B*. H&E staining; magnification, ×200. *D*, Histological appearances were scored for the presence of peribronchial and perivascular inflammation, and these scores were added together to give a total lung inflammation score. □, Naive mouse; ■, OVA-PBS-treated mouse; ■, OVA-simvastatin-treated mouse (40 mg/kg i.p.). Data are expressed as the mean ± SEM ($n = 3$–7). *, $p < 0.05$ vs OVA-PBS (by Student's *t* test).

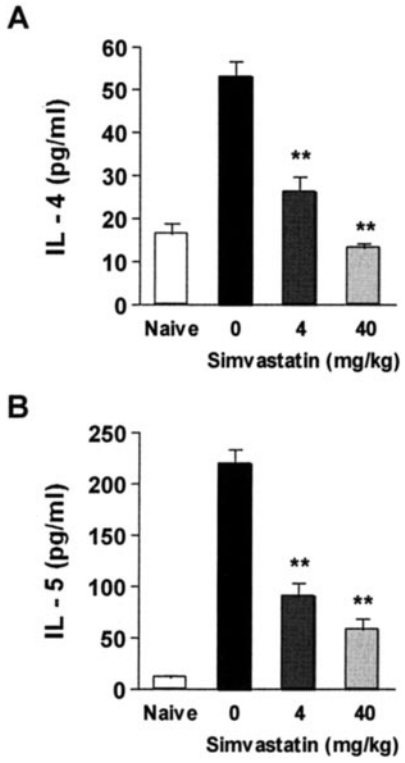

FIGURE 4. BAL IL-4 and IL-5 levels in mice treated with simvastatin i.p.. BAL IL-4 and IL-5 levels were determined by ELISA. Data are expressed as the mean ± SEM ($n = 14$). **, $p < 0.01$ by ANOVA).

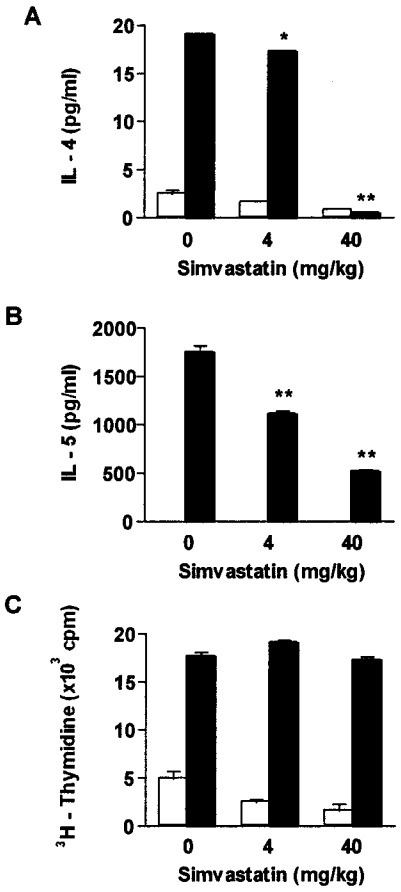

FIGURE 5. Reduced in vitro OVA-specific responses in mice treated with simvastatin i.p.. Thoracic lymph node cells ($n = 3$ mice/ group) were harvested from mice on day 38 and cultured for 72 h with medium alone (□) or OVA (■; 500 μg/ml). IL-4 (*A*) and IL-5 (*B*) concentrations in the culture supernatants were measured by ELISA. *C*, T cell proliferation was assayed by uptake of [^{3}H]thymidine after 96 h. Data are expressed as the mean ± SEM ($n = 3$) and are representative of three experiments. *, $p < 0.05$; **, $p < 0.01$ (simvastatin groups compared with PBS-treated controls, by Student's *t* test).

(data not shown), suggesting that general suppression of inducible class II MHC expression was not an important contributing factor to the production of these immunosuppressive actions.

Discussion

The prevalence of asthma is rising (29). Consequently, there is an increased need for the development of new agents for its treatment, especially for patients who respond poorly to conventional therapy. In this study we have shown that simvastatin has an effective anti-inflammatory action in a murine model of allergic inflammation. Therefore, statins or similar agents may have potential as therapeutic agents in human asthma.

Simvastatin has previously been shown to have an acute anti-inflammatory action in carageenin-induced footpad swelling in mice (5) and in thioglycolate-induced peritoneal inflammation (8). In both these models the inflammatory infiltrate is predominantly neutrophils. Statins have not previously been shown to have an inhibitory action on eosinophilic infiltration. In our study this anti-inflammatory effect is at least in part mediated through a suppressive action on T lymphocytes, as OVA-specific IL-4 and IL-5 secretions were reduced in thoracic lymph node cultures from mice treated with simvastatin i.p. A reduction in BAL fluid IL-4 and IL-5 levels was also observed in these mice. The reduction in Th2 cytokine production in thoracic lymph node cultures was not accompanied by an increase in the secretion of IFN-γ, a Th1 cytokine. Indeed, IFN-γ production was also reduced in lymph node cultures. There is now evidence that Th1 cells (30, 31) and IFN-γ (32) secretion may exacerbate airway inflammation in asthma. Therefore, there may also be suppression of Th1 cells, and hence IFN-γ levels, contributing to the decrease in inflammation seen. This result corresponds with our previous observation in murine collagen-induced arthritis (CIA), where a decrease in Th1 cytokines was not associated with an increase in Th2 cytokine secretion (15). This is in contrast to that seen in murine experimental allergic encephalitis, where statin treatment increased the Th2 bias in Ag-stimulated lymph node cultures (16, 17) while reducing the Th1 response. In these studies atorvastatin was used, and the immunomodulatory effects and plasma half-life of this drug may differ from those of simvastatin.

In contrast to the CIA and experimental allergic encephalitis inflammatory models, a reduction in Ag-induced cell proliferation in lymph node cells was not observed in our study. These other models were of chronic inflammatory conditions, and statin treatment was continued for at least 15 days after the last dose of Ag. In our study simvastatin was only given for 3 days and was not given after the last challenge with OVA. This shorter exposure time to statin therapy may explain the failure to suppress cell proliferation. This result suggests that there may be divergent mechanisms by which statins inhibit cytokine secretion and cell proliferation.

Simvastatin treatment at a dose of 40 mg/kg i.p. reduced BAL eosinophil and macrophage numbers. This might reflect the reduction in Th2 lymphocyte responses, but a direct suppressive effect of simvastatin on eosinophils and macrophages cannot be excluded. The migration of inflammatory cells from blood into the airways occurs through binding to specific adhesion molecules and

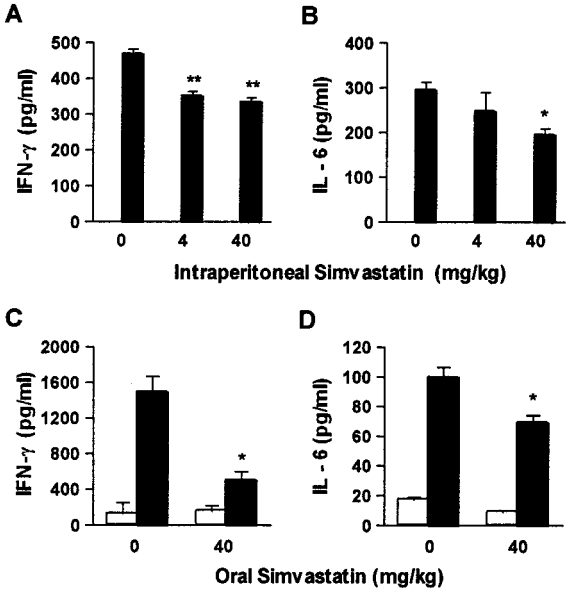

FIGURE 6. OVA-specific IFN-γ and IL-6 responses in vitro in mice treated i.p. (*A* and *B*) or orally (*C* and *D*) with simvastatin. Thoracic lymph node cells ($n = 3$ mice/group) were harvested from mice on day 28 and cultured for 72 h with medium alone (□) or OVA (■; 500 μg/ml). IFN-γ (*A* and *C*) and IL-6 (*B* and *D*) in culture supernatants were measured by ELISA. Data are expressed as the mean ± SEM of triplicate cultures and are representative of three experiments. *, $p < 0.05$; **, $p < 0.01$ (simvastatin groups compared with PBS-treated controls, by Student's *t* test).

the actions of chemokines. Eosinophils and macrophages both express LFA-1, so simvastatin may have a direct effect on trafficking of these cells into the airways (33, 34). IL-5-mediated Ras activation is important to eosinophil survival (35), and simvastatin may inhibit the activity of this signaling molecule in this model. Statins have also been shown to modify the secretion of proinflammatory cytokines, such as macrophage chemotactic protein-1 and IL-8, in macrophages (36).

It is important to establish that simvastatin could have an anti-inflammatory effect when administered orally, as this is the route of administration of the drug in clinical practice. Although oral therapy produced an anti-inflammatory effect, this was less pronounced than with i.p. administration of the same dose. This is probably due to first-pass hepatic metabolism of the drug after absorption from the gastrointestinal tract, where several metabolites may be produced (37), thus reducing the effective dose of simvastatin available.

The doses of simvastatin used in this study are higher than those used in man. Statin doses comparable to those used in this study are commonly used in rat/murine studies (5, 24, 25) as there is rapid up-regulation of HMG-CoA reductase with statin treatment in rodents (26). In our previous study in murine CIA, a dose of 40 mg/kg simvastatin i.p. did not produce a reduction in cholesterol levels, and liver function tests were similar in placebo- and simvastatin-treated mice (15). Our studies represent a proof of concept approach that illustrates not only the potential of statins to directly modulate airway inflammation, but indicates the future development potential for statin-like drugs selected for their anti-inflammatory, rather than lipid-lowering, activities alone.

In conclusion, we demonstrate that simvastatin treatment is effective in reducing BAL total cellularity and eosinophilia in a murine model of allergic asthma and also decreases IL-4 and IL-5 levels when given i.p.. This immunomodulatory effect is likely to occur through several different anti-inflammatory pathways, and these mechanisms require further elucidation. In particular, the effects of simvastatin on sensitization to Ag and on airway inflammation after prolonged administration will be of interest. Clinical studies are also necessary to assess whether simvastatin has therapeutic potential in allergic asthma.

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