Statin-Induced Proinflammatory Response in Mitogen-Activated Peripheral Blood Mononuclear Cells through the Activation of Caspase-1 and IL-18 Secretion in Monocytes

William R. Coward, Ayman Marei, AiLi Yang, Mariuca M. Vasa-Nicotera and Sek C. Chow

J Immunol 2006; 176:5284-5292; doi: 10.4049/jimmunol.176.9.5284
http://www.jimmunol.org/content/176/9/5284

References
This article cites 44 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/176/9/5284.full#ref-list-1

Subscription
Information about subscribing to J Immunol is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Statin-Induced Proinflammatory Response in Mitogen-Activated Peripheral Blood Mononuclear Cells through the Activation of Caspase-1 and IL-18 Secretion in Monocytes

William R. Coward,* Ayman Marei,* AiLi Yang,* Mariuca M. Vasa-Nicotera,† and Sek C. Chow2*

Statins, which inhibit 3-hydroxy-3-methylglutaryl CoA (HMG-CoA)1 reductase, have been shown recently to promote proinflammatory responses. We show in this study that both atorvastatin and simvastatin induced proinflammatory responses in mitogen-activated PBMCs by increasing the number of T cells secreting IFN-γ. This is abolished by the presence of mevalonate, suggesting that statins act specifically by blocking the mevalonate pathway for cholesterol synthesis to promote the proinflammatory response. Both statins at low concentrations induced a dose-dependent increase in the number of IFN-γ-secreting T cells in mitogen-activated PBMCs, whereas at higher concentrations the effect was abolished. The proinflammatory effect of statins was not seen in purified T cells per se activated with mitogen. However, conditioned medium derived from statin-treated PBMCs enhanced the activated PBMCs, whereas at higher concentrations the effect was abolished. The proinflammatory effect of statins was not seen in purified T cells per se activated with mitogen. However, conditioned medium derived from statin-treated PBMCs enhanced the number of IFN-γ-secreting cells in activated purified T cells. This effect was not blocked by mevalonate, but was abolished by neutralizing Abs to IL-18 and IL-12. Similarly, the up-regulation of IFN-γ-secreting T cells in PBMCs costimulated with statins and mitogens was blocked by the neutralizing anti-IL-18 and anti-IL-12. We showed that simvastatin stimulates the secretion of IL-18 and IL-1β in monocytes. Active caspase-1, which is required for the processing and secretion of IL-18 and IL-1β, was activated in simvastatin-treated monocytes. This was blocked by mevalonate and the caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone. Taken together, the proinflammatory response mediated by statins in activated PBMCs is mediated mainly via the activation of caspase-1 and IL-18 secretion in the monocytes and to a lesser extent by IL-12. The Journal of Immunology, 2006, 176: 5284–5292.

Statins are inhibitors of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA)1 reductase, a rate-limiting enzyme in the mevalonate pathway for the biosynthesis of cholesterol (1), and widely prescribed to lower cholesterol level in hyperlipidemic patients to reduce the risk of cardiovascular diseases (2, 3). Several large-scale intervention trials and epidemiological studies have shown that statin treatments significantly reduced the risk of developing arteriosclerosis and cardiovascular morbidity (2, 3). Although the improved prognosis in patients was initially thought to be due to the lipid-lowering properties of statins, accumulating evidence from a number of clinical studies and in vitro experiments suggests that the anti-inflammatory effects of statins may have contributed to its beneficial effects (4).

Although the molecular mechanisms underlying the anti-inflammatory properties of statins are still poorly understood, recent findings from a number of studies may help to explain some of these anti-inflammatory properties (5, 6). Statins have been shown to inhibit IFN-γ-induced up-regulation of MHC class II molecules on APCs such as macrophages, endothelial cells, and smooth muscle cells by suppressing CIITA, the promoter of the MHC CIITA (6). Although the constitutively expressed MHC class II molecules in professional APCs like B and dendritic cells are unaffected by statin treatment, the reduction of MHC class II expression in other APCs could result in a reduction in T cell proliferation and differentiation. Statins also selectively block interaction of the β2 integrin, LFA-1, with ICAM-1 (5). Besides playing an important role in leukocyte adhesion and extravasations, LFA-1 is also involved in costimulation of T cells (5, 7). Recently, compelling evidence from in vivo studies demonstrated that atorvastatin prevents or reverses experimental autoimmune encephalomyelitis in mice (8, 9). These preclinical studies suggest that the beneficial effects of atorvastatin were due to its specific induction of a Th2 bias in vivo by promoting the secretion of Th2 cytokines and suppressing the production of inflammatory Th1 cytokines by T cells. In addition, simvastatin inhibits collagen-induced arthritis in mice by attenuating the development and expansion of Th1 cells (10), and blocks the infiltration of T cells and inhibits eosinophilic inflammation in the lungs of a murine model of allergic asthma (11). These studies suggest that statins have beneficial effects in a broad range of inflammatory conditions and autoimmune diseases.

However, in contrast to the anti-inflammatory properties of statins, a number of recent studies have shown that the lipophilic statins such as fluvastatin, simvastatin, atorvastatin, and lovastatin...
are able to induce proinflammatory responses in a number of model systems (12–15). Fluvastatin was shown to activate caspase-1 and enhances the secretion of inflammatory cytokines such as IL-1β, IL-18, and the Th1 cytokine, IFN-γ, in PBMCs stimulated with Mycobacterium tuberculosis (12). The induction of a Th1 bias in activated PBMCs by fluvastatin is in sharp contrast to the Th2 bias mediated by atorvastatin and simvastatin in mice in vitro and in vivo (8, 9). Lovastatin has been shown to enhance the production of IL-6, IL-12, and TNF-α in bone marrow-derived dendritic cells (14) and promote TNF-α secretion in a macrophage cell line by inhibiting the Rho family of GTPase (16). More recently, simvastatin was found to augment LPS-induced proinflammatory response in macrophages by differential regulation of transcription factors (13). These findings suggest that statins can have both anti-inflammatory as well as proinflammatory properties.

In the present study, we examined whether atorvastatin and simvastatin are able to induce proinflammatory response in mitogen-activated PBMCs. Our results showed that both atorvastatin and simvastatin promote a proinflammatory or Th1 response in activated PBMCs by increasing the number of IFN-γ-secreting T cells. This response now characterized is mainly mediated indirectly via the activation of caspase-1 and IL-18 secretion in monocytes, and to a lesser extent by IL-12 in response to the inhibition of HMG-CoA reductase by statins.

Materials and Methods

Reagents

mAb against CD3 (clone OKT3) was purified from hybridoma (American Type Culture Collection) culture supernatants. Atorvastatin and simvastatin (open ring active form) were purchased from Calbiochem. Recombinant IL-12 and anti-CD3 mAb (clone 37407) were from PeproTech, and IL-18 was from MBL. Rabbit anti-caspase-1 (Sc-515) was from Santa Cruz Biotechnology. IFN-γ secretion assays, and MACS beads conjugated with anti-CD4, anti-CD8, or anti-CD14 were acquired from Miltenyi Biotec. Lymphoprep was from Axis-Shield PoCAS, and RPMI 1640 and FCS were purchased from Invitrogen Life Technologies. Benzoxylcarbonyl-Val-Ala-Asp (OME) fluoromethylketone (z-VAD-FMK) was obtained from Bachem. Purified protein derivative (PPD) was obtained from Evans Vaccines.

Cell isolation

Peripheral venous blood was obtained from normal healthy volunteers and collected into heparinized tubes. PBMCs were purified using density gra- dient centrifugation with Lymphoprep. In brief, heparinized blood was layered onto Lymphoprep (density gradient of 1.077), and centrifuged at 800 × g for 30 min. The mononuclear cells at the interface were collected, washed, and resuspended in RPMI 1640 containing 10% (v/v) FCS. The viability of the lymphocyte population obtained was 99 ± 1% (n = 10), as assessed by trypan blue exclusion. The PBMCs were kept on ice until use. CD4+ and CD8+ T cells were isolated from PBMCs using anti-CD4 and anti-CD8 mAb-conjugated MACS beads. This resulted in purities of CD4+ and CD8+ T cells of 97 and 98%, respectively. Peripheral blood monocytes were isolated from PBMCs using anti-CD14-conjugated MACS beads. Following isolation, the purity of the monocytes was determined by flow cytometry and found to be greater than 95%. Cord blood mononuclear cells (CBMCs) were isolated from placental cord blood obtained during routine caesarean delivery from the Leicester Royal Infirmary (Leicester National Health Trust) with ethical approval. The isolation of CBMCs is essentially the same as PBMCs from peripheral blood.

Cell cultures and treatments

PBMCs (5 × 10⁶ cells/ml), CBMCs (5 × 10⁶ cells/ml), or purified CD4+ and CD8+ T cells (1 × 10⁶ cells/ml) in RPMI 1640 supplemented with 10% FCS were stimulated with either PPD, anti-CD3 plus anti-CD28, or PHA in the presence or absence of atorvastatin or simvastatin for 16 h. The activated T cells were then analyzed for IFN-γ secretion using the IFN-γ capture assay (Miltenyi Biotech). In whole blood experiments, 1 ml of blood was stimulated with PHA for 16 h in the presence or absence of atorvastatin. After the incubation, 14 ml of lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA (pH 7.3)) was added to the blood samples and incubated for 5 min at room temperature to lyse the erythrocytes. The cells were washed with PBS supplemented with 2% FBS before analysis using the secretion assays. In experiments involving the use of PPD, the PBMCs were isolated from normal healthy volunteers that had previously been vaccinated with bacillus Calmette-Guerin. For conditioned medium preparation, PBMCs (5 × 10⁶ cells) were incubated with 1 μM atorvastatin for 24 h. The supernatants were collected by centrifugation and sterile filtered before use in subsequent cell cultures. Aliquots (150 μl) of the conditioned medium were taken for IL-18 and IL-12 determination using ELISA. To block the activity of IL-18 and IL-12, neutralizing Abs to IL-18 or IL-12 were added to PBMCs stimulated with PHA in the presence or absence of statins. To reverse the effect of statins, t- mevalonate (100 μM) was added to PBMCs costimulated with statin and PHA or anti-CD3 plus anti-CD28. The pan-caspase inhibitor, z-VAD-FMK (25 μM), was used to block caspase-1 in PBMCs and monocytes. For monocyte treatments, 1 × 10⁶ cells/ml monocytes in RPMI 1640 supplemented with 10% FCS were treated with 1 μM simvastatin in the presence or absence of either mevalonate (100 μM) or z-VAD-FMK (25 μM) for 48 h. After treatments, the supernatants were collected and stored at −80°C before IL-12, IL-18, and IL-1β analysis using ELISA. The cells were scraped and washed, and the pellets were analyzed for the activation of caspase-1 using immunoblotting following SDS-PAGE separation of the proteins.

Detection of IL-4- and IFN-γ-secreting cells

T cells secreting IFN-γ and IL-4 were determined using cytokine capture assays (Miltenyi Biotech), as previously reported (17, 18), and recently validated for their sensitivity and specificity (19). Resting T cells were activated with either PHA (5 μg/ml), PPD (10 μg/ml), or plate-bound anti-CD3 (1 μg/ml) and anti-CD28 mAbs (10 μg/ml) for 16 h. The activated T cells were washed with PBS and incubated with either IFN-γ or IL-4 capture reagents at 37°C for 45 min. The cells were washed with PBS, and the IFN-γ and IL-4 were detected using secondary conjugated anti-IFN-γ and anti-IL-4. The cells were then analyzed using flow cytometry.

ELISA

The concentrations of cytokines (IL-1β, IL-12, and IL-18) in culture supernatants or conditioned medium were determined using ELISA kits, according to the manufacturer’s instruction. Supernatants and conditioned medium were collected and kept frozen at −80°C until cytokine analysis.

Immunoblot analysis

Following treatments, cell lysates were prepared from the cell pellets by three consecutive freeze-thaw cycles in lysis buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, supplemented with 1 mM PMSF), and the protein concentration was determined using the Bio-Rad Protein Assay Kit (Bio-Rad). Protein equivalent to 30 μg of cell lysates was separated using 16% SDS-PAGE. The separated proteins were transferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with 10% skim milk in TBST (20 mM Tris, 137 mM NaCl, 0.1% Tween 20 (pH 7.6)) for 1 h at room temperature and probed with Abs to caspase-1. Detection was conducted by chemiluminescence using Super signal regents (Pierce). Following caspase-1 detection, the membrane was incubated with stripping buffer (Pierce) and reprobed with Abs to β-actin.

Statistical analysis

All the data were analyzed using one-way ANOVA, followed by the Tukey test, except for the dose-response studies in which Dunnett’s test was used. Value of p < 0.05 was taken as statistically significant.

Results

Atorvastatin and simvastatin enhanced the number of activated IFN-γ-secreting T cells in PHA-treated PBMCs.

We evaluated whether lipophilic statins such as atorvastatin and simvastatin would enhance IFN-γ secretion in PBMCs following T cell activation. The secretion of IFN-γ by activated T cells was determined using the IFN-γ capture assay, which allows us to identify the phenotype and the percentage of cells secreting IFN-γ. As illustrated in Fig. 1, both atorvastatin and simvastatin at 1 μM each markedly potentiated the number of IFN-γ-secreting CD4+ and CD8+ T cells in PHA-stimulated PBMCs. The effect of both
statins was abolished when mevalonate (100 μM) was added to the PBMCs to bypass the inhibition of HMG-CoA reductase. This indicates that the effects of atorvastatin and simvastatin are mediated via the inhibition of the mevalonate pathway for cholesterol synthesis and not through other nonspecific effects. Statins or mevalonate on their own had no effect on IFN-γ-secreting T cells, suggesting that the T cells in PBMCs have to be activated to produce this enhancing effect. Both atorvastatin and simvastatin were found to have no effect on IL-4-secreting CD4\(^+\) and CD8\(^+\) T cells (results not shown). Dose-response studies revealed that both atorvastatin and simvastatin at low concentrations induced a concentration-dependent increase in the number of IFN-γ-secreting cells in PBMCs stimulated with PHA, but the effect was abolished at higher concentrations (Fig. 2). The enhancing effect of atorvastatin and simvastatin was abolished when mevalonate (100 μM) was added where indicated to bypass the inhibition of statins. The CD4\(^+\) and CD8\(^+\) T cells secreting IFN-γ were determined using cytokine capture assays, as outlined in Materials and Methods. Results are the means ± SEM from eight (A) or three (B) separate experiments. *, Significantly different (p < 0.05) from PHA alone. **, Significantly decreased (p < 0.05) compared with PHA + statins.

FIGURE 1. Effects of atorvastatin and simvastatin on IFN-γ-secreting cells in PBMCs treated with PHA. PBMCs (5 x 10\(^6\) cells) were stimulated with PHA in the presence of atorvastatin (Ator) (A) or simvastatin (Sim) (B) for 16 h. Mevalonate (Mev) was added where indicated to bypass the inhibition of statins. The CD4\(^+\) and CD8\(^+\) T cells secreting IFN-γ were determined using cytokine capture assays, as outlined in Materials and Methods. Results are the means ± SEM from eight (A) or three (B) separate experiments. *, Significantly different (p < 0.05) from PHA alone. **, Significantly decreased (p < 0.05) compared with PHA + statins.

Effect of atorvastatin on purified CD4\(^+\) and CD8\(^+\) T cells activated with PHA

We next examined whether atorvastatin was able to induce an increase in IFN-γ-secreting T cells when the PBMCs were co-stimulated with anti-CD3 and anti-CD28 and PPD from M. tuberculosis. Like PHA, co-stimulation with anti-CD3 and anti-CD28 is a polyclonal stimulus and activates all the T cells, whereas PPD stimulation only activates a small fraction of memory T cells that recognize PPD in a recall Ag manner. As illustrated in Fig. 3A, simvastatin induced an increase in the number of IFN-γ-secreting cells in both CD4\(^+\) and CD8\(^+\) T cell populations in PBMCs co-treated with anti-CD3 and anti-CD28. Accordingly, the effect of simvastatin was reversed by mevalonate, and the IL-4-secreting T cells were unaffected (results not shown). With PBMCs stimulated with PPD, there were less cells secreting IFN-γ, which is expected from the recall Ag assay. Although atorvastatin consistently induced a small increase in the number of IFN-γ-secreting CD4\(^+\) and CD8\(^+\) T cells in PPD-treated PBMCs (Fig. 3B), the results are not significant. We also found that atorvastatin has a similar enhancing effect on IFN-γ-secreting T cells in PHA-treated whole blood and cord blood mononuclear cells (Fig. 3, C and D), suggesting that both naive and memory T cells can respond to this effect of statins.

Effect of atorvastatin on purified CD4\(^+\) and CD8\(^+\) T cells activated with PHA

To further characterize the mechanisms underlying the enhanced IFN-γ-secreting T cells in PBMCs co-treated with PHA and statins,
we examined the effect of atorvastatin on purified CD4⁺ and CD8⁺ T lymphocytes. To this end, the purified CD4⁺ and CD8⁺ T cells were stimulated with PHA in the presence of atorvastatin (0.001 to 1 μM) for 16 h, and the percentage of IFN-γ-secreting cells was determined. As shown in Fig. 4, both purified CD4⁺ and CD8⁺ T cells stimulated with PHA alone result in a moderate increase in cells secreting IFN-γ, as expected. However, the presence of atorvastatin, between 0.001 and 1 μM, has no effect on PHA-induced IFN-γ-secreting cells in purified CD4⁺ and CD8⁺ T cells, suggesting that the T cells are not the primary targets of the statins. These results also suggest that the enhanced number of activated T cells secreting IFN-γ mediated by statins may be due to soluble factors secreted from non-T cells in the PBMCs in response to statin treatments.

Involvement of soluble factors in statin-induced IFN-γ secretion in T cells from PHA-treated PBMCs

To determine whether soluble factors are involved in the increased IFN-γ-secreting T cells mediated by statins in activated PBMCs, we first prepared conditioned medium from PBMCs treated with atorvastatin for 24 h. Purified CD4⁺ T lymphocytes were then stimulated with PHA in either normal or conditioned medium. As illustrated in Fig. 5, the purified CD4⁺ T cells in normal medium respond to PHA stimulation with a moderate increase in cells secreting IFN-γ. In contrast, PHA-stimulated CD4⁺ T cells in conditioned medium showed a marked increase in the number of cells secreting IFN-γ. Mevalonate, which inhibits the effect of atorvastatin in PHA-treated PBMCs previously (Fig. 1), did not block the effect seen with conditioned medium. This indicates that soluble factors and not atorvastatin present in the conditioned medium are responsible for the increased IFN-γ-secreting CD4⁺ T cells. Because fluvastatin has previously been shown to activate caspase-1 in PBMCs (12), we examined IL-18, which is a substrate of caspase-1 as well as a potent inducer of IFN-γ, as possible soluble factor in the conditioned medium (22, 23). Another possible factor is IL-12, which is also known to induce IFN-γ secretion in activated T cells (24, 25). The possible involvement of IL-18 and IL-12 in the conditioned medium was examined using blocking Abs to IL-18 and IL-12. As illustrated in Fig. 5, blocking IL-12 Abs (2 μg/ml) consistently have a small reduction in the effect mediated by the conditioned medium, although this is not significant. However, IL-18-blocking Abs (10 μg/ml) markedly reduced the effect mediated by the conditioned medium. ELISA analysis showed that the conditioned medium contains 67.55 ± 35 pg/ml IL-18 (n = 3), but no IL-12 was detected. Medium from untreated cells has no detectable levels of IL-18 or IL-12. It is possible that the level of IL-12 present in the conditioned medium is below the detection limit of our ELISA. Nevertheless, the consistent small inhibition with IL-12-neutralizing Abs (Fig. 5) suggests that IL-12 is present in the conditioned medium, albeit in low levels.

**FIGURE 3.** Effect of statins on IFN-γ-secreting T cells in PBMCs, CBMCs, and whole blood activated with mitogens. PBMCs (5 × 10⁶ cells/ml) were treated with A, anti-CD3 plus anti-CD28, or B, 10 μg/ml PPD in the presence or absence of atorvastatin (Ator) or simvastatin (Sim), where indicated. CBMCs (5 × 10⁶ cells/ml) (C) and whole blood (D) were activated with PHA in the presence of atorvastatin. Mevalonate (Mev) was added to the cell cultures to reverse the effect of atorvastatin. IFN-γ-secreting CD4⁺ and CD8⁺ T cells were determined, as described in Materials and Methods. The results are the means ± SEM from three (C and D) or four (A and B) separate experiments. *, Significantly increased (p < 0.05) compared with PHA or cotreated with anti-CD3 and anti-CD28. **, Significantly decreased (p < 0.05) compared with mitogen treatment in the presence of statins.
IL-18- and IL-12-blocking Abs inhibit the increase in IFN-γ-secreting T cells in PBMCs cotreated with PHA and atorvastatin

To corroborate the results in Fig. 5, varying concentrations of IL-18- and IL-12-blocking Abs were added to PBMCs cotreated with PHA and atorvastatin. As shown in Fig. 6, A and B, blocking anti-IL-12 (1-1000 ng/ml) dose dependently inhibited atorvastatin-induced increase in CD4+ and CD8+ IFN-γ-secreting T cells in PHA-treated PBMCs. However, anti-IL-18 at concentrations between 1 and 100 ng/ml has little effect, whereas at high concentration (1 μg/ml) the increase in IFN-γ-secreting cells induced by atorvastatin was inhibited. Both anti-IL-18 and anti-IL-12 at high concentrations were unable to reduce IFN-γ-secreting cells to levels induced by PHA alone: the latter effect required the simultaneous presence of both IL-12- and IL-18-blocking Abs (Fig. 6C). These results and those in Fig. 5 demonstrated that both IL-12 and IL-18 are important mediators involved in the increased IFN-γ-secreting cells induced by atorvastatin in PHA-treated PBMCs. Accordingly, rIL-12 (2 ng/ml) and rIL-18 (10 ng/ml), when added on their own to PHA-stimulated purified CD4+ T cells, induced an increase in the number of IFN-γ-secreting cells (Fig. 7A). On its own, IL-12 was less effective compared with IL-18 in promoting IFN-γ secretion in the activated T cells. However, when the two cytokines were added in combination, the effect on the PHA-activated CD4+ T cells was synergistic. Similar effect was observed with rIL-12 and rIL-18 in PHA-treated PBMCs; however, IL-18 alone appears to be capable of inducing most T cells to produce IFN-γ (Fig. 7B). The results in Fig. 7 suggest that the rIL-18 and rIL-12 are more effective in up-regulating IFN-γ secretion in PBMCs compared with purified CD4+ T cells. We observed that IL-12 and IL-18 alone or together have no effect on unstimulated purified CD4+ and CD8+ T cells (results not shown). This is in agreement with previous studies in which the IL-12R and IL-18R are only expressed in activated T cells (24, 25).

Simvastatin stimulates the secretion of IL-18 and IL-1β in monocytes and is blocked by mevalonate and z-VAD-FMK

As shown earlier (Fig. 5), the presence of IL-18 and IL-12 in the conditioned medium derived from atorvastatin-treated PBMCs came from non-T cells. In PBMCs, the cells most likely to secrete these two cytokines are the monocytes and dendritic cells. We therefore examine whether monocytes purified from PBMCs are able to secrete IL-18 and IL-12 when treated with simvastatin. Incubation of monocytes with simvastatin (1 μM) for 48 h resulted in a marked secretion of IL-18 (Fig. 8A) in the supernatants, but no IL-12 was detected (results not shown). Similar to the conditioned medium, the level of IL-12 may be below the detection limit of our ELISA. Alternatively, the IL-12 in conditioned medium (Fig. 5) could have come from other APCs such as dendritic cells and B cells (24). The secretion of IL-18 by simvastatin-treated monocytes was blocked by mevalonate (100 μM) and z-VAD-FMK (25 μM). The former suggests that statins are acting via the mevalonate pathway through the inhibition of HMG-CoA reductase. The latter confirms that maturation and secretion of IL-18 depend on caspase-1 activation (22, 23). To corroborate that caspase-1 was activated in simvastatin-treated monocytes, we assayed the supernatants for another cytokine that requires caspase-1 enzymatic cleavage for maturation, i.e., IL-1β. Indeed, IL-1β was detected in the supernatants of simvastatin-treated monocytes (Fig. 8B), and the secretion was inhibited by the presence of mevalonate and z-VAD-FMK. These results strongly suggest that caspase-1 is activated in monocytes when treated with simvastatin. To confirm this, the activation of caspase-1 in simvastatin-treated monocytes was analyzed using immunoblot. As shown in Fig. 8C, there was a decrease in the proform of caspase-1 (~45 kDa) in simvastatin-treated monocytes compared with untreated cells. Caspase-1 exists as an inactive proenzyme and is activated by proteolysis. The marked decrease in the proform of caspase-1 in simvastatin-treated monocytes is indicative of caspase-1 being processed into the active subunits. The lack of any noticeable processed caspase-1 fragments detected is likely to be due to the rapid turnover of the active enzyme (26). The loss of the proform of caspase-1 in simvastatin-treated monocytes was reversed by mevalonate, which correlates with the marked decrease in IL-1β and IL-18 secretion (Fig. 8, A and B). Similarly, z-VAD-FMK also inhibited the decrease in pro-caspase-1 in simvastatin-treated monocytes as well as the secretion of IL-1β and IL-18. Surprisingly, the presence of the z-VAD-FMK
further increased the level of procaspase-1 above the control level seen in untreated monocytes. This is not due to unequal amount of protein loaded because the β-actin level in each lane showed equivalent loading. The effect of z-VAD-FMK on the level of procaspase-1 suggests that caspase-1 may have a high turnover rate in resting monocytes, presumably mediated by caspase(s). Taken together, these results demonstrate that IL-18 and IL-1β are processed by caspase-1 in monocytes and then secreted following simvastatin treatment.

z-VAD-FMK inhibits the increased IFN-γ-secreting T cells induced by atorvastatin in mitogen-treated PBMCs

Because z-VAD-FMK inhibits IL-18 secretion in monocytes following simvastatin treatment (Fig. 8), we examined whether the caspase inhibitor can prevent the up-regulation of IFN-γ-secreting cells by statins in mitogen-treated PBMCs. As shown in Fig. 9, z-VAD-FMK (25 μM) inhibited the increase in IFN-γ-secreting T cells induced by atorvastatin in PBMCs treated with PHA or co-stimulated with anti-CD3 and anti-CD28. The results confirm that caspase-1 and IL-18 secretion play a pivotal role in the up-regulation of activated IFN-γ-secreting T cells in PBMCs cotreated with PHA and statins.

Discussion

In the present study, we have shown that both atorvastatin and simvastatin promote proinflammatory responses by enhancing the number of IFN-γ-secreting T lymphocytes in activated PBMCs. This effect was mediated via the mevalonate pathway for de novo synthesis of isoprenoids, suggesting that other nonspecific side effects of statins are not involved. Both statins at low concentrations dose dependently increased the number of IFN-γ-secreting T cells in PHA-treated PBMCs, whereas at higher concentrations the effect of statins was abolished. This suggests that the proinflammatory response of statins only occurs within a narrow concentration window, above which no response is apparent. Interestingly, statins at these concentrations inhibited mitogen-induced T cell proliferation (20, 21), while mitogen-mediated IFN-γ secretion remained unaffected. Our results also suggest that atorvastatin was more effective compared with simvastatin, as lower concentrations of atorvastatin were needed to induce the proinflammatory response in activated PBMCs. This correlates well with the higher efficiency of atorvastatin in blocking HMG-CoA reductase compared with simvastatin (27), and lends further support for the role of blocking this enzyme or pathway in promoting the proinflammatory response.
The proinflammatory effect of statins was not evident in purified T cells activated with PHA, indicating that the T lymphocytes are not the primary target of these drugs. Furthermore, conditioned medium from statin-treated PBMCs readily enhanced the IFN-γ-secreting cells in PHA-stimulated purified T cells, and was blocked by neutralizing Abs to IL-18 and IL-12. Monocytes are capable of producing both the cytokines, but only IL-18 and IL-1β were detected when incubated with simvastatin. IL-12 was not detected in the conditioned medium or supernatants from statin-treated monocytes, suggesting that the level of IL-12 secreted is very low and likely to be below the detection limit of our ELISA. Although IL-18 and IL-1β exist in cells in the inactive form and require cleavage at aspartate residues by caspase-1 to become their respective active mature cytokines before secretion (22, 23). The activation of caspase-1 in monocytes when treated with statins was confirmed using immunoblot analysis and was blocked by mevalonate and z-VAD-FMK, which also inhibit the secretion of IL-18 and IL-1β. Although the activation of caspase-1 has been associated with apoptotic cell death in a number of cellular systems (29, 30), we did not detect any sign of apoptosis in statin-treated primary monocytes. It is unclear at present how statins induced the activation of caspase-1 in monocytes, although recent studies suggest that the inhibition of isoprenylation of proteins was involved (31). Isoprenoids are important intermediates for the synthesis of several groups of compounds, and these include sterols, dolichols, ubiquinone, and prenylated proteins. These compounds are known to be involved in many cellular processes, such as cell growth and differentiation, glycosylation, electron transport, and signal transduction (32).

The proinflammatory response resulting from the inhibition of isoprenoid biosynthesis by statins may help to explain some of the clinical and biochemical phenotypes observed in human disorders associated with impaired isoprene biosynthesis. Periodic fever syndrome is often seen in hyperimmunoglobulinemia D (HIDS) and mevalonic aciduria (33–35). These disorders are caused by different mutations in the gene encoding mevalonate kinase (MK), the next enzyme after HMG-CoA reductase, which is inhibited by statins in the biosynthesis of isoprenoids. Because of these mutations, these patients exhibit different degree of deficiency in MK.
activity. HIDS patients have very low mevalonate kinase activity, and mevalonic aciduria patients have little or no activity of this enzyme (33–35). As a result, the mevalonate levels in the plasma of these patients are markedly elevated. The clinical phenotype of patients having mevalonic aciduria is more severe compared with HIDS, and it has been suggested that the differences between these two disorders are related to the level of metabolic disturbances in these patients. It has also been shown that the level of proinflammatory cytokines, such as IFN-γ and IL-6, was markedly increased during fever attacks in HIDS patients, and the PBMCs from these patients secrete large amounts of the proinflammatory cytokine, IL-1β (33). More recently, the increase in proinflammatory cytokines in HIDS patients’ PBMCs was shown to be due to a lack of isoprenoid products (36). Furthermore, oral statins have been shown to promote severe inflammatory attacks when given to patients suffering from mevalonic aciduria in an attempt to reduce the mevalonate level in the plasma (37). Our current findings provide a cellular mechanism and identified monocytes as cells susceptible to the inhibition of isoprenoid biosynthesis, which may play a major role in the proinflammatory response. Whether dendritic cells are involved is not clear, and further investigations are needed to establish this. Collectively, our present work and those published suggest that an insufficient synthesis of isoprenoids due to the deficiency in MK in HIDS and mevalonic aciduria patients leads to the activation of caspase-1 in monocytes and subsequently to an inflammatory response.

Given that the maximal atorvastatin and simvastatin plasma concentration reached following a therapeutically relevant dose of 40 mg/day is between 54 and 133 nM and 24 and 81 nM, respectively (38), this seems to be well within the concentration of statins shown in our in vitro studies that would promote a proinflammatory response, especially with atorvastatin. However, there is no indication of elevated Th1 activity or disorders associated with an increase in Th1 activity, despite the increasing use of statins. We believe this is because most statins, with the exception of atorvastatin, generally have a very short elimination t1/2 (38), and therefore are incapable of promoting inflammation. Although atorvastatin has a longer elimination t1/2, the level in plasma is so low that it is unlikely to cause any inflammation (39).

Even though statins are well tolerated, their systemic exposure can be markedly increased when coadministered with medications that shared their metabolic pathways (38). Statins are metabolized by cytochrome p450-3A4 isoenzymes in the liver. Their metabolism is markedly reduced by inhibitors and competing drugs of cytochrome p450 (40). Antiretroviral drugs are known inhibitors of cytochrome p450-3A4, and long-term antiretroviral therapy, as required by HIV patients, is associated with the unwanted side effects of cholesterololemia and hypertriglycerideremia (41) and increased risk of cardiovascular diseases. To counter this, statins are often administered to these individuals to reduce cholesterol and triglyceride levels (42). However, coadministration of retroviral protease inhibitor and statins has now been shown to increase several-fold the peak concentrations of statins in the plasma (43–45). Whether this abnormally high concentration of statins in the plasma of HIV patients will result in any inflammatory response is not known and deserves further attention.

In summary, we showed that statins promote proinflammatory response in activated PBMCs by activating caspase-1 in monocytes, which in turn process and promote the secretion of IL-18 and IL-1β. The secreted IL-18 and to a lesser extent also IL-12 are responsible for the increased number of activated IFN-γ-secreting T cells. Our findings suggest that the isoprenoid biosynthetic pathway in monocytes could be a target for therapeutic drug development to boost the Th1-type immune response for the treatment of Th2-mediated disorders.

Acknowledgments
We thank Dr. Paul Glynn for his helpful comments and reading of the manuscript.

Disclosures
The authors have no financial conflict of interest.

References


