



Make your **mark.**

Discover reagents that make  
your research stand out.

DISCOVER HOW



The Journal of  
**Immunology**

This information is current as  
of August 5, 2022.

## **Simvastatin Augments Lipopolysaccharide-Induced Proinflammatory Responses in Macrophages by Differential Regulation of the c-Fos and c-Jun Transcription Factors**

Makoto Matsumoto, Derek Einhaus, Elizabeth S. Gold and  
Alan Aderem

*J Immunol* 2004; 172:7377-7384; ;  
doi: 10.4049/jimmunol.172.12.7377  
<http://www.jimmunol.org/content/172/12/7377>

**References** This article **cites 42 articles**, 23 of which you can access for free at:  
<http://www.jimmunol.org/content/172/12/7377.full#ref-list-1>

**Why *The JI*? Submit online.**

- **Rapid Reviews! 30 days\*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*\*average*

**Subscription** Information about subscribing to *The Journal of Immunology* 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>

*The Journal of Immunology* is published twice each month by  
The American Association of Immunologists, Inc.,  
1451 Rockville Pike, Suite 650, Rockville, MD 20852  
Copyright © 2004 by The American Association of  
Immunologists All rights reserved.  
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Simvastatin Augments Lipopolysaccharide-Induced Proinflammatory Responses in Macrophages by Differential Regulation of the c-Fos and c-Jun Transcription Factors<sup>1</sup>

Makoto Matsumoto, Derek Einhaus, Elizabeth S. Gold, and Alan Aderem<sup>2</sup>

The 3-hydroxyl-3-methylglutaryl-coenzyme A reductase inhibitors, or statins, are a widely used class of drugs for cholesterol reduction. The reduction in mortality and morbidity in statin-treated patients is incompletely explained by their effects on cholesterol, and an anti-inflammatory role for the drug has been proposed. We report in this work that, unexpectedly, simvastatin enhances LPS-induced IL-12p40 production by murine macrophages, and that it does so by activating the IL-12p40 promoter. Mutational analysis and dominant-negative expression studies indicate that both C/EBP and AP-1 transcription factors have a crucial role in promoter activation. This occurs via a c-Fos- and c-Jun-based mechanism; we demonstrate that ectopic expression of c-Jun activates the IL-12p40 promoter, whereas expression of c-Fos inhibits IL-12p40 promoter activity. Simvastatin prevents LPS-induced c-Fos expression, thereby relieving the inhibitory effect of c-Fos on the IL-12p40 promoter. Concomitantly, simvastatin induces the phosphorylation of c-Jun by the c-Jun N-terminal kinase, resulting in c-Jun-dependent activation of the IL-12p40 promoter. This appears to be a general mechanism because simvastatin also augments LPS-dependent activation of the TNF- $\alpha$  promoter, perhaps because the TNF- $\alpha$  promoter has C/EBP and AP-1 binding sites in a similar configuration to the IL-12p40 promoter. The fact that simvastatin potently augments LPS-induced IL-12p40 and TNF- $\alpha$  production has implications for the treatment of bacterial infections in statin-treated patients. *The Journal of Immunology*, 2004, 172: 7377–7384.

The 3-hydroxyl-3-methylglutaryl-coenzyme A (HMG-CoA)<sup>3</sup> reductase is the rate-limiting enzyme in cholesterol biosynthesis (1). The drugs that competitively inhibit HMG-CoA reductase and that reduce cholesterol biosynthesis are collectively known as statins. Statins have been widely prescribed to lower cholesterol in hyperlipidemic patients at risk for cardiovascular disease.

Large-scale clinical trials have demonstrated significant reductions in cardiovascular events following statin therapy. However, the observed benefit of statin therapy is greater in these trials than would be expected from lowering lipid levels alone (2, 3). Statins are now believed to exert pleiotropic effects that include improvement of endothelial function, stabilization of atherosclerotic plaques, thrombosis control, and anti-inflammatory effects (4).

Accumulating evidence supports the assumption that statins have anti-inflammatory properties and thus may regulate important molecules in both immune and vascular biology. Pravastatin lowered the level of C-reactive protein and eliminated the higher risk of cardiovascular events associated with this inflammatory marker

(5). Pravastatin or simvastatin therapy reduced the incidence of rejection and improved survival after heart transplantation (6).

Several recent observations suggest a mechanism for the anti-inflammatory effects of statins. Statins suppressed IFN- $\gamma$ -inducible expression of MHC class II on monocyte-derived macrophages and endothelial cells, but not constitutive expression of MHC-II in peripheral blood-derived dendritic cells (7). Statin pretreatment of monocyte-derived macrophages and endothelial cells repressed subsequent activation of allogenic T cells; this effect was due to inhibition of the inducible promoter IV of the MHC class II transactivator (7). Statins have also been shown to bind directly to the  $\beta_2$  integrin LFA-1, thereby blocking LFA-1-mediated adhesion and costimulation of lymphocytes (8). In addition, atorvastatin prevented and reversed chronic and relapsing paralysis in experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (9). In these studies, atorvastatin induced the secretion of Th2 cytokines, but inhibited secretion of Th1 cytokines, thereby suppressing Ag-specific T cell activation (9). This effect was thought to be due to enhanced STAT6 phosphorylation, and decreased STAT4 phosphorylation. By contrast, Aktas et al. (10) suggested that atorvastatin-induced down-regulation of cyclin-dependent kinase 4 and up-regulation of p27<sup>kip1</sup> caused the decrease of T cell proliferation.

Contrary to these anti-inflammatory effects, lipophilic statins, including simvastatin, atorvastatin, lovastatin, and fluvastatin, have been shown to have proinflammatory effects on PBMC, endothelial cells, monocytes, or bone marrow-derived dendritic cells. Montero et al. (11) reported that fluvastatin activated caspase-1, and enhanced secretion of IL-1 $\beta$ , IL-18, and IFN- $\gamma$  from PBMC stimulated with *Mycobacterium tuberculosis*. Unexpectedly, simvastatin potentiated TNF- $\alpha$ - or IL-1 $\beta$ -induced expression of E-selectin, ICAM-1, and VCAM-1 in HUVECs (12). The authors also demonstrated that pertussis toxin mimicked this simvastatin effect, suggesting the participation of a G $_{i\alpha}$  protein. Recently, Sun and Fernandes (13) reported that lovastatin enhanced IL-6, IL-12,

Institute for Systems Biology, Seattle, WA 98103

Received for publication December 1, 2003. Accepted for publication April 12, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported by National Institutes of Health Grants AI25032 (to A.A.) and 1K08HL71582 (to E.S.G.). M.M. was supported by postdoctoral fellowships from Uehara Memorial Foundation and Mochida Pharmaceutical Foundation.

<sup>2</sup> Address correspondence and reprint requests to Dr. Alan Aderem, Institute for Systems Biology, 1441 North 34th Street, Seattle, WA 98103-8904. E-mail address: aderem@systemsbiology.org

<sup>3</sup> Abbreviations used in this paper: HMG-CoA, 3-hydroxyl-3-methylglutaryl-coenzyme A; CSK<sub>4</sub>, Cys-Ser-(Lys)<sub>4</sub>; EAE, experimental autoimmune encephalomyelitis; EF-1 $\alpha$ , elongation factor-1 $\alpha$ ; ELAM, endothelial leukocyte adhesion molecule; IP-10, IFN- $\gamma$ -inducible protein-10; JNK, c-Jun N-terminal kinase; MK, mevalonate kinase; Pam<sub>3</sub>, N-palmitoyl-S-dipalmitoylglycerol; R.L.U., relative luciferase unit.

and TNF- $\alpha$  production in bone marrow-derived dendritic cells, and Monick et al. (14) reported that lovastatin increased TNF- $\alpha$  production by inhibition of Rho family GTPases in a murine macrophage cell line.

Thus, statins have both anti-inflammatory and proinflammatory effects; however, the molecular mechanisms underlying these properties are poorly understood. In this study, we define the molecular mechanism underlying the proinflammatory response to statins. We demonstrate that simvastatin pretreatment enhances LPS-induced IL-12p40 production through activation of the IL-12p40 promoter, and that C/EBP and AP-1 binding sites are essential for this effect. Furthermore, we have shown that c-Fos repression and c-Jun N-terminal kinase activation underlie this mechanism. Simvastatin also augments TNF- $\alpha$  expression, which is also controlled by the C/EBP and AP-1 transcription factors.

## Materials and Methods

### Reagents

Simvastatin, c-Jun N-terminal kinase (JNK) inhibitor I, and negative control peptide were purchased from Calbiochem (La Jolla, CA). Simvastatin was converted to open acid form before use, as previously described (15). Briefly, 20 mg of simvastatin was suspended in 0.4 ml of ethanol, and 2.4 ml of 0.1 M NaOH was added. The solution was heated to 50°C for 2 h. To this heated solution, 3.6 ml of an aqueous solution containing 81 mM Na<sub>2</sub>HPO<sub>4</sub> and 15 mM NaH<sub>2</sub>PO<sub>4</sub> was added. The solution was heated to 40°C for 30 min, and the pH was adjusted to 7.3 with concentrated HCl. The carrier was used as vehicle control in the same dilution as simvastatin. Mevalonate, farnesylpyrophosphate, geranylgeranylpyrophosphate, and squalene were purchased from Sigma-Aldrich (St. Louis, MO). We used 0.7 mg/ml methanol and 3 mM NH<sub>4</sub>OH that dissolved farnesylpyrophosphate and geranylgeranylpyrophosphate as mock in Fig. 5. LPS O55:B5 was purchased from List Biological Laboratories (Campbell, CA). *N*-palmitoyl-*S*-dipalmitoyl-glyceryl (Pam<sub>3</sub>) Cys-Ser-(Lys)<sub>4</sub> (CSK<sub>4</sub>) was purchased from EMC Microcollections (Tuebingen, Germany). R848 was purchased from 3M Pharmaceuticals (St. Paul, MN). Rabbit polyclonal Abs recognizing C/EBP $\alpha$  (14AA), C/EBP $\beta$  (C-19), C/EBP $\delta$  (C-22), C/EBP $\epsilon$  (C-22), c-Jun (H-79), JunB (N-17), JunD (329), c-Fos (4), FosB (102), Fra-1 (R-20), and Fra-2 (Q-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-c-Jun (Ser<sup>63</sup>) and JNK Abs were purchased from Cell Signaling Technology (Beverly, MA).

### Plasmid constructs

The murine IL-12p40 promoter region was amplified by PCR from the genomic DNA of C57BL/6 mouse. Appropriate 5' primers and a common 3' primer were used to amplify varying lengths of the promoter region. The following primers were used to generate the -352, the -194, and the -127 constructs: 5' primers, -352; 5'-GGCAAGTCCTTCCTTTTCTGCA GTCTGTGG-3', -194; 5'-TCTATATCCCTCTGTAT-3', -127; 5'-CCC CCAGAATGTTTTGAC-3', 3' primer, +55; 5'-CTTGCTTGCGA GCTGCC-3'.

Amplified products were inserted into pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA), digested with *SacI/XhoI*, and subcloned into pGL3 basic vector (Promega, Madison, WI). The -81 construct was generated by self ligation after excising -352 to -81 region with *SpeI* digestion. Mutants with 2-bp mutations in NF- $\kappa$ B, C/EBP, or AP-1 binding site were generated for the -352 construct by QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutagenic primers for NF- $\kappa$ B, C/EBP, and AP-1 binding sites (with altered nucleotides underlined) were 5'-CTTCTAAAAT TCGGCCAGAAATGTTTTG-3', 5'-GTTTTTCAGTGTGCCCCTTGAGACT AGTC-3', and 5'-GCAATTGAGACTAGTTGGTTTCTACTTTGGGTTTC CATC-3', respectively. The mouse JunB was amplified by RT-PCR from resident macrophages and cloned into the expression vector pcDNA3.1<sup>+</sup> (Invitrogen). All constructs generated by PCR were confirmed by sequencing. The expression plasmids for the C/EBP dominant-negative protein A-C/EBP and the AP-1 dominant-negative protein A-Fos were kindly provided by C. Vinson (National Cancer Institute, Bethesda, MD) (16, 17). The expression plasmids for rat c-Jun and c-Fos (CMV-c-Jun and CMV-c-Fos) were gifts from T. Curran (St. Jude Children's Research Hospital, Memphis, TN) (18). The expression plasmid for mouse FosB (pcDNA3.1-FosB) was a gift from S. Okada (Kumamoto University, Kumamoto, Japan) (19). The expression plasmid for rat Fra-2 (pcDNA3.1-Fra-2) was a gift from R. Balcer (National Institutes of Health, Bethesda, MD) (20). pGL2-mouse TNF- $\alpha$  promoter was a gift from R. Ulevitch (The Scripps Research Institute, La Jolla, CA) (21). The

TNF- $\alpha$  promoter region (-1072 to +131) was excised with *KpnI/HindIII* and cloned into pGL3 basic vector for this study. pGL3-human endothelial leukocyte adhesion molecule (ELAM) promoter was described previously (22). Numbers described above indicate positions relative to the transcriptional start site.

### Macrophage cultures and generation of stable transfectants

Resident peritoneal macrophages were harvested from specific pathogen-free female C57BL/6 mice (Charles River Breeding Laboratories, Wilmington, MA) by peritoneal lavage with 8 ml of ice-cold PBS. The cells were plated at  $1 \times 10^6$  cells/ml and washed three times in PBS to remove nonadherent cells after incubation for 2 h. Resident peritoneal macrophages and RAW264.7 macrophages (ATCC TIB-71; American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 supplemented with 10% FBS (HyClone Laboratories, Logan, CT), 2 mM L-glutamine, and 1% of penicillin/streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The pooled stable transfectant of RAW264.7 cells expressing human ELAM promoter-firefly luciferase was described previously (23). The pooled stable transfectant of RAW264.7 cells expressing mouse IL-12p40 promoter-firefly luciferase was generated by cotransfection of the pGL3-IL-12p40-352 construct and pcDNA3.1<sup>+</sup> vector. Both stable transfectants were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, and 400  $\mu$ g/ml geneticin (Invitrogen).

### Luciferase assay

RAW264.7 cells were seeded at  $1.5 \times 10^5$  cells/well into 48-well plate on the day before transfection. pGL3-IL-12p40 promoter constructs and pHRG-TK (Promega) were transiently transfected by Fugene6 (Roche Diagnostics, Indianapolis, IN), following the manufacturer's instruction. Cells were subsequently treated with vehicle or 8  $\mu$ M simvastatin for 20 h and stimulated with or without 10 ng/ml LPS for 5 h. Luciferase assays were done by dual-luciferase assay kit, according to the manufacturer's instruction (Promega). The pooled stable transfectants of RAW264.7 cells, expressing either the ELAM or IL-12p40 promoter-driven firefly luciferase, were seeded at  $5 \times 10^4$  cells/well into 96-well plates on the day before stimulation. Cells were subsequently treated with vehicle or 8  $\mu$ M simvastatin for the indicated time periods and stimulated with or without 1 ng/ml LPS for 6 h. Luciferase assays were performed using the luciferase 1000 assay system (Promega). The protein concentration of the cell lysate was determined by the bicinchoninic acid method (Pierce, Rockford, IL) in parallel. The luciferase values were normalized to protein concentration. All assays were done in triplicate, and each experiment was repeated at least three times. The luciferase activity was calculated for each individual assay. Fold activation was calculated by dividing the luciferase values for the test conditions by the relative luciferase value for the control condition. In all cases, values are the mean  $\pm$  SD of triplicate wells and are representative of at least three separate experiments.

### ELISA

The concentration of IL-12p40 and TNF- $\alpha$  was measured with DuoKit ELISA system (R&D Systems, Minneapolis, MN). The production of IFN- $\gamma$ -inducible protein-10 (IP-10) was determined with IP-10 ELISA system (Cedarlane Laboratories, Hornby, Ontario, Canada).

### Real-time PCR analysis

After stimulation, total RNA was isolated with an RNeasy minikit (Qiagen, Valencia, CA). Approximately 2  $\mu$ g of total RNA was treated with DNase I (Fisher Scientific, Pittsburgh, PA) and reverse transcribed using Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers (Promega). Quantitative real-time PCR was performed on an ABI 7700 (Applied Biosystems, Foster City, CA) using TaqMan Universal PCR Master Mix (Applied Biosystems). All data were normalized to elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) expression in the same cDNA set. Each experiment was performed independently at least three times, and the results of one representative experiment are shown. Sequences of TaqMan probes and forward and reverse primers for TNF- $\alpha$  and EF-1 $\alpha$  were described previously (24). Sequences for IL-12p40 and IP-10 are as follows: IL-12p40 core, CTCGAGGAAACACATGC CCACTTG; forward primer, GCTCAGGATCGCTATTACAATCC; reverse primer, TCTCCTTAATGTCTCCACTTTTCTT. IP-10 probe, TCACTGGCCCCGTCATCGATATGGA; forward primer, GACGGTC CGCTGCAACTG; reverse primer, GCTTCCCTATGGCCCTCATT.

### EMSA

The nuclear extracts were prepared, as described previously (25). The protein concentration was determined by bicinchoninic acid method (Pierce).

Probes were made by annealing single-stranded oligonucleotides and labeled by filling in with [ $\alpha$ - $^{32}$ P]dCTP using Klenow fragment (Promega). Five micrograms of nuclear extracts were mixed with  $5 \times 10^4$  cpm probe in 20 mM HEPES (pH 7.9), 50 mM KCl, 1 mM EDTA, 0.2 mM EGTA, 5% glycerol, 1 mM DTT, and  $1 \times$  protease inhibitor mixture (Roche Diagnostics) at room temperature for 30 min. Bound and free DNAs were then resolved by electrophoresis through a 5% polyacrylamide gel (0.5  $\times$  Tris-glycine-EDTA buffer) at 25 mA for 90 min. For supershift analysis, Abs were incubated with the nuclear extracts for 1 h on ice, followed by an additional incubation for 30 min with labeled probes. Binding activities were measured by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

#### Immunoblot analysis

Forty micrograms of nuclear extracts or 100  $\mu$ g of whole cell lysates were denatured in SDS, electrophoresed on a 12% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA). Ponceau S staining was performed to ensure equivalent gel loading. Membranes were then incubated with the indicated rabbit polyclonal Abs, followed by HRP-conjugated goat anti-rabbit IgG (Zymed Laboratories, South San Francisco, CA), which were detected using West-Pico chemiluminescence reagent (Pierce).

#### In vitro kinase assay

RAW264.7 cells ( $2 \times 10^6$  cells in a 35-mm dish) were washed twice with ice-cold PBS and lysed in lysis buffer (1 $\times$  PBS, 0.2% IGEPAL CA630, 0.1% SDS, 0.25% sodium deoxycholate, 2 mM  $\text{Na}_3\text{VO}_4$ , 1 $\times$  complete protease inhibitor). Lysates were passed through a 26-gauge needle five times, clarified by centrifugation, and assayed for protein content (Pierce). Five hundred micrograms of whole cell lysates were suspended in JNK lysis buffer (25 mM HEPES, pH 7.5, 0.2% IGEPAL CA630, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM DTT, 2 mM  $\text{Na}_3\text{VO}_4$ , 20 mM  $\beta$ -glycerophosphate, 10 mM *p*-nitrophenyl phosphate, and 1 $\times$  complete protease inhibitor) and incubated with 20  $\mu$ l of GST-c-Jun1-89 beads (Cell Signaling Technology) on a rotator for 1 h. The beads were washed and transferred to JNK assay buffer containing 10  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP, and JNK activity was determined (26).

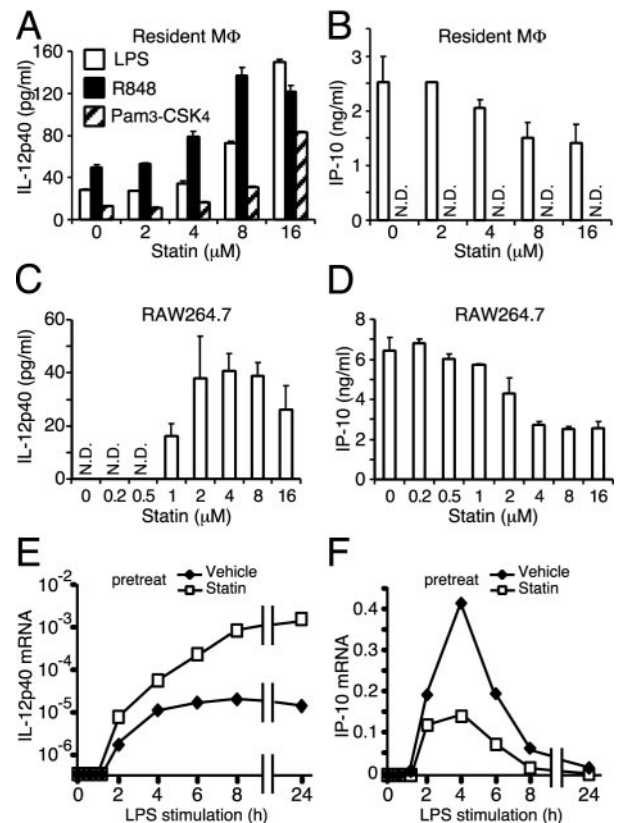
## Results

### Simvastatin augments LPS-induced IL-12p40 production by macrophages

To study the effect of simvastatin on IL-12 production by macrophages, we pretreated murine resident peritoneal macrophages with simvastatin for 20 h and stimulated them with LPS for 8 h. As shown in Fig. 1A, simvastatin primed the cells for enhanced LPS-induced IL-12p40 production, and this occurred in a dose-dependent manner. Simvastatin also augmented IL-12p40 production in response to other Toll-like receptor agonists, such as R848 and Pam<sub>3</sub>-CSK<sub>4</sub> (Fig. 1A). The effect was not general, because LPS-induced IP-10 secretion was decreased in simvastatin-treated cells (Fig. 1B). Because mechanistic studies are not possible in primary macrophages, we decided to focus on the murine macrophage cell line RAW264.7. The primary cell data were recapitulated in the cell line, thereby validating the model (Fig. 1, C and D). We next examined whether simvastatin regulates IL-12p40 production at the mRNA level. RAW264.7 macrophages were pretreated with simvastatin for 20 h and stimulated with LPS, and the levels of IL-12p40 and IP-10 mRNA were measured by quantitative PCR. As shown in Fig. 1E, during the early time points (2–6 h), simvastatin pretreatment enhanced LPS-induced IL-12p40 mRNA levels by 5- to 10-fold. Interestingly, at later time points (12–24 h), the increased IL-12p40 mRNA levels persisted, hinting at additional forms of regulation. By contrast, simvastatin pretreatment inhibited LPS-induced IP-10 mRNA levels (Fig. 1F). Taken together, these data indicate that simvastatin pretreatment enhances LPS-induced IL-12p40 production, whereas it inhibits LPS-induced IP-10 production.

### Simvastatin activates IL-12p40 promoter-luciferase constructs

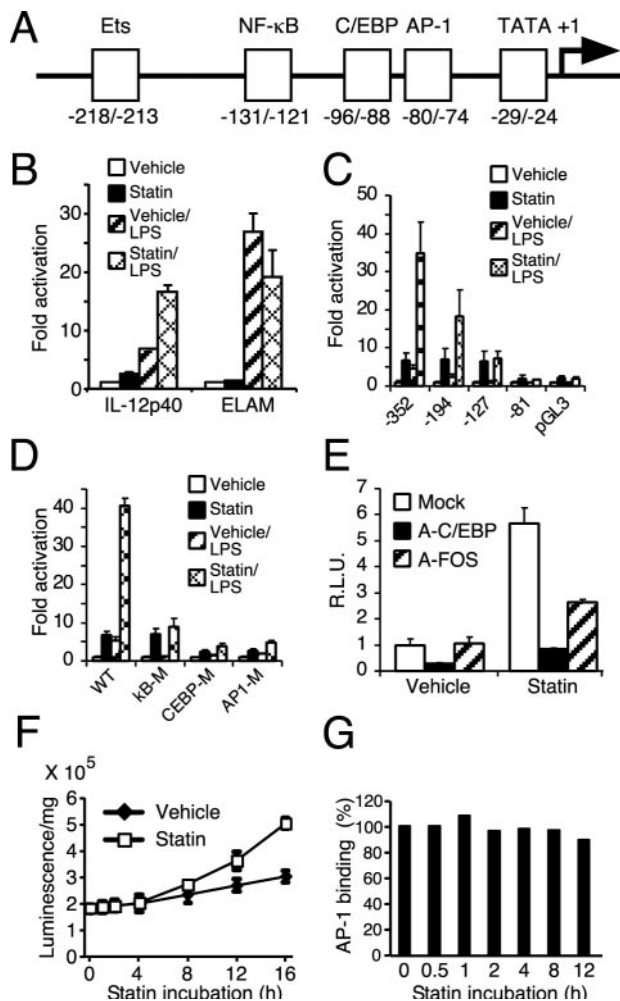
To investigate whether simvastatin enhances IL-12p40 production at the transcriptional level, we compared the effect of simvastatin



**FIGURE 1.** Simvastatin increases IL-12p40 production, but decreases IP-10 production by macrophages. *A* and *B*, Resident peritoneal macrophages ( $1 \times 10^6$ /ml) were treated with the indicated concentration of simvastatin for 20 h and stimulated either with 10 ng/ml LPS, 100 ng/ml R848, or 100 ng/ml Pam<sub>3</sub>-CSK<sub>4</sub> for 8 h. Concentrations of IL-12p40 (*A*) and IP-10 (*B*) were measured by ELISA. Results are the mean  $\pm$  SD of triplicate wells and are representative of three separate experiments. IP-10 production by R848 or Pam<sub>3</sub>-CSK<sub>4</sub> was not detected (N.D.). *C* and *D*, RAW264.7 cells ( $5 \times 10^5$ /ml) were treated with the indicated concentration of simvastatin for 16 h and stimulated with 10 ng/ml LPS for 24 h. Concentrations of IL-12p40 (*C*) and IP-10 (*D*) were measured by ELISA. Results are the mean  $\pm$  SD of triplicate wells and are representative of three separate experiments. *E* and *F*, RAW264.7 cells ( $5 \times 10^5$ /ml) were treated with vehicle (Vehicle) or 4  $\mu$ M simvastatin (Statin) for 16 h and stimulated with 10 ng/ml LPS for the indicated time periods. Induction of IL-12p40 mRNA (*E*), IP-10 mRNA (*F*), and the housekeeping gene EF-1 $\alpha$  was measured with quantitative real-time PCR. Data shown are relative mRNA amounts normalized to EF-1 $\alpha$ . Similar results were obtained in three independent experiments.

on RAW264.7 cells that stably express the IL-12p40 promoter linked to the firefly luciferase gene with cells that stably express the ELAM promoter-firefly luciferase gene. As shown in Fig. 2B, simvastatin activated the IL-12p40 promoter, and substantially augmented the LPS response. By contrast, simvastatin did not activate the ELAM promoter and did not augment LPS stimulation of the ELAM promoter. This clearly demonstrates that simvastatin regulates the IL-12p40 promoter. The promoter of the gene encoding IL-12p40 has been studied in great detail. The murine IL-12p40 promoter has an Ets, an NF- $\kappa$ B, a C/EBP, and an AP-1 binding site at -218 to -213, -131 to -122, -96 to -88, and -79 to -74 relative to the transcription start site, respectively (Fig. 2A). These transcription factors have been shown to functionally cooperate with each other for efficient transcription of IL-12p40 in response to LPS (27–30).

To delineate the statin-responsive elements, we made a series of luciferase reporter constructs in which we serially deleted the



**FIGURE 2.** The promoter assay for determining statin-responsive elements. *A*, Diagram of the important regulatory elements in the mouse IL-12p40 promoter. Ets (–218/–213), NF- $\kappa$ B (–131/–121), C/EBP (–96/–88), AP-1 (–80/–74), and TATA box (–29/–24) were detected in previous studies (summarized in Ref. 42). *B*, RAW264.7 cells, stably transfected with IL-12p40 promoter-firefly luciferase and ELAM promoter-firefly luciferase, were used to measure the promoter activities. Each pool of stable transfectant was treated with vehicle or 8  $\mu$ M simvastatin for 20 h and incubated with or without 1 ng/ml LPS for 6 h. The luciferase values were normalized to protein concentration. Each result is expressed as fold activation normalized to cells treated with vehicle alone. Vehicle, vehicle treatment only; Statin, simvastatin treatment only; Vehicle/LPS, vehicle treatment plus LPS stimulation; Statin/LPS, simvastatin treatment plus LPS stimulation. *C*, RAW264.7 cells were transiently transfected with either 180 ng of the IL-12p40 promoter constructs containing the indicated deletions, together with 20 ng of Renilla luciferase vector (pHRG-TK) as a control for transfection efficiency. Cells were subsequently treated with vehicle or 8  $\mu$ M simvastatin for 20 h and incubated with or without 10 ng/ml LPS for 5 h. The luciferase titers were measured by the dual-luciferase assay kit. Each result is expressed as fold activation normalized to cells treated with vehicle alone. The number indicates the 5' position for individual construct relative to the transcriptional start site. pGL3 means pGL3 empty vector. *D*, The 2-bp substituted mutants for NF- $\kappa$ B, C/EBP, or AP-1 binding site were transfected and examined, as described in *C*. WT, the –352 construct;  $\kappa$ B-M, NF- $\kappa$ B binding site mutant; CEBP-M, C/EBP binding site mutant; AP1-M, AP-1 binding site mutant. Each result is expressed as fold activation normalized to cells treated with vehicle alone. *E*, RAW264.7 cells were transfected with either 5 ng of empty vector (Mock), 5 ng of plasmid expressing A-C/EBP, or 5 ng of plasmid expressing A-Fos, together with 180 ng of the –127 construct and 20 ng of pHRG-TK. Cells were subsequently incubated with vehicle or 8  $\mu$ M simvastatin for 24 h, and luciferase activity was measured. A-C/EBP and

known *cis* elements of the IL-12p40 promoter (Fig. 2C). The –352 construct has all known *cis* elements for IL-12p40 induction. The –194 construct lacks the Ets-binding element; the –127 construct lacks the NF- $\kappa$ B-binding element; the –81 construct lacks the C/EBP binding site. RAW264.7 cells were transiently transfected with these constructs and then treated with simvastatin. After 20 h of incubation, the cells were stimulated for 5 h with 10 ng/ml LPS, and the luciferase activities were measured. The –352, the –194, and the –127 luciferase constructs were activated by 5- to 7-fold in response to simvastatin treatment (Fig. 2C). Both the –81 and the empty pGL3 constructs were activated ~2-fold by simvastatin, suggesting a small, promoter-independent effect.

Progressive deletion of the IL-12p40 promoter demonstrated the requirement for Ets, NF- $\kappa$ B, and C/EBP in LPS responsiveness in simvastatin-treated cells (Fig. 2C). By contrast, simvastatin alone activated the –352, –194, and –127 constructs equally, suggesting that the statin-responsive elements are contained within the –127 construct (Fig. 2C). To more precisely delineate the statin-responsive elements, we introduced a 2-bp substitution in either the NF- $\kappa$ B, C/EBP, or AP-1 binding sites in the –352 construct, and examined their response to simvastatin and/or LPS. As previously described, all mutants lost the response to stimulation with LPS alone (Fig. 2D) (29). By contrast, the NF- $\kappa$ B mutant showed a normal response to simvastatin treatment, whereas both C/EBP and AP-1 mutants were unresponsive (Fig. 2D). Taken together, the data demonstrate that NF- $\kappa$ B does not play a role in the simvastatin effects on the IL-12p40 promoter.

We next used dominant-negative inhibitors to address the involvement of C/EBP and AP-1 transcription factors in the simvastatin response. We used a strategy in which we introduced artificial homologues that form heterodimers with C/EBP and AP-1, thereby preventing them from activating target genes; C/EBP was inhibited with A-C/EBP (16), and AP-1 was inhibited by A-Fos (17). Either A-C/EBP or A-Fos substantially inhibited the simvastatin-mediated response. Moreover, A-C/EBP suppressed the basal activity of the IL-12p40 promoter by 70% (Fig. 2E). These data suggest that the C/EBP and AP-1 transcription factors have a role in the augmentation of the IL-12p40 promoter activity by simvastatin.

We explored the temporal effect of simvastatin on IL-12p40 promoter activity in stably transfected RAW264.7 cells. At early time points (up to 4 h), simvastatin had no effect on the IL-12p40 promoter activity (Fig. 2F). After 8 h, there was a gradual increase in IL-12p40 promoter activity (Fig. 2F). Importantly, there was no temporal difference in AP-1 binding to the IL-12p40 promoter (Fig. 2G). Taken together, these data clearly demonstrate that there is no initial spike in AP-1 activity.

A-Fos are dominant-negative forms of C/EBP and AP-1 transcription factors, respectively. The luciferase titers are shown as relative luciferase units (R.L.U.) compared with mock-transfected cells with vehicle treatment. *F*, The effect of simvastatin on IL-12p40 promoter activity. The pooled stable transfectant of RAW264.7 cells expressing the IL-12p40 promoter-driven firefly luciferase was treated with vehicle or 8  $\mu$ M simvastatin for the indicated time periods. Luminescence was measured and normalized to protein concentration. *G*, AP-1-binding activity was conducted by EMSA in RAW264.7 cells. The cells were incubated with vehicle or 8  $\mu$ M simvastatin for the indicated periods. Nuclear extracts (5  $\mu$ g/lane) were mixed with radiolabeled probe –88–56 in the presence of 0.5  $\mu$ g of poly(dI-dC). The probe –88–56 encompasses the AP-1 binding site on the IL-12p40 promoter. AP-1-binding activity was measured by PhosphorImager.

### Simvastatin reduces c-Fos binding to the IL-12p40 promoter

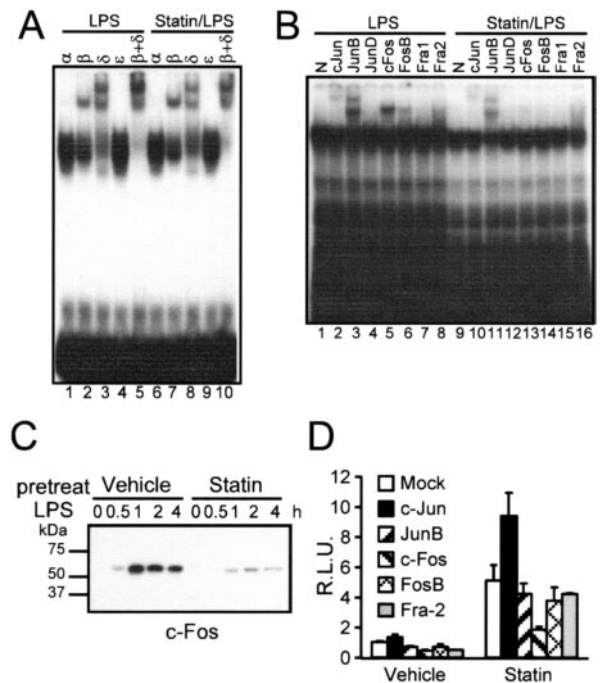
We examined the effect of simvastatin on the binding activities of the C/EBP and AP-1 transcription factors to the IL-12p40 promoter. Radiolabeled double-stranded oligonucleotide probes spanning the sequence from position  $-103$  to  $-76$ , and  $-88$  to  $-56$ , were prepared and used to detect the C/EBP and AP-1 complexes, respectively. LPS stimulation enhanced the binding activities of both C/EBP and AP-1, as previously reported (data not shown) (29, 30). Simvastatin pretreatment did not show any effects on either constitutive or LPS-induced C/EBP-binding activities (data not shown). C/EBP refers to a family of transcription factors; and the formal possibility existed that a different C/EBP family member might be induced to bind to the IL-12p40 promoter when the cells were treated with simvastatin. Supershift assays were performed using polyclonal Abs against C/EBP $\alpha$ ,  $-\beta$ ,  $-\delta$ , or  $-\epsilon$ , and nuclear extracts from LPS-stimulated RAW264.7 cells with or without simvastatin pretreatment. The majority of the C/EBP complex consisted of C/EBP $\beta$  and C/EBP $\delta$ , and this was not changed by the presence of simvastatin (Fig. 3A). Simvastatin pretreatment slightly inhibited the augmentation of AP-1-binding activities by LPS (data not shown). Supershift assays demonstrated that the AP-1 complex formed in response to LPS contained c-Jun, JunB, c-Fos, FosB, and Fra-2 (Fig. 3B). Importantly, simvastatin pretreatment decreased c-Fos dramatically from the AP-1 complex (Fig. 3B). This decrease in c-Fos is due to a simvastatin-mediated inhibition of LPS-induced c-Fos synthesis (Fig. 3C).

### Ectopic expression of c-Fos negatively regulates the IL-12p40 promoter, whereas expression of c-Jun positively regulates the promoter

The simvastatin-mediated inhibition of c-Fos binding appeared to be paradoxical. On the one hand, simvastatin clearly triggered IL-12p40 production through the AP-1 binding site (Fig. 2, D and E). In contrast, simvastatin drastically reduced LPS-mediated c-Fos induction and binding to the promoter (Fig. 3, B and C). This suggested that the reduction of c-Fos contributed to the activation of the IL-12p40 promoter. Ectopic expression of c-Fos reduced p40-127 promoter activity by 70% in the simvastatin-treated cells, while c-Jun expression enhanced it (Fig. 3D). Thus, these findings suggest a resolution to the paradox: c-Fos negatively regulates the IL-12p40 promoter activity, whereas c-Jun augments it. Ectopic expression of JunB, FosB, and Fra-2 had no effect on IL-12p40 promoter activity (Fig. 3D).

### Simvastatin induces c-Jun phosphorylation

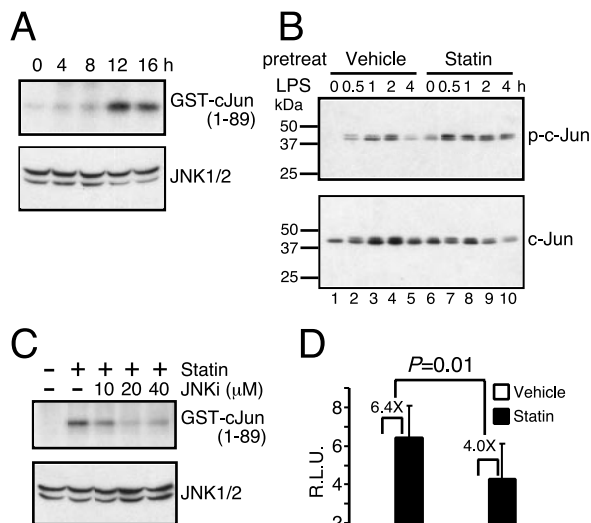
c-Jun is activated when c-Jun N-terminal kinase (JNK) phosphorylates Ser<sup>63</sup> and Ser<sup>73</sup>. An *in vitro* kinase assay showed that simvastatin pretreatment provoked JNK activation at 12 h in RAW264.7 cells (Fig. 4A). Moreover, simvastatin pretreatment significantly induced Ser<sup>63</sup> phosphorylation of the endogenous c-Jun protein (Fig. 4B, lane 6) and substantially augmented LPS-induced phosphorylation at the same site (Fig. 4B, lanes 7-10). Thus, JNK activation appeared responsible for the simvastatin-mediated IL-12p40 promoter activation. This was tested directly using a cell-permeable peptide inhibitor of JNK (31). Indeed, the JNK inhibitor diminished the simvastatin-mediated JNK activation in a dose-dependent manner (Fig. 4C), and this was accompanied by the partial inhibition of transcriptional activation of the IL-12p40 promoter (Fig. 4D). Taken together, simvastatin diminished the negative regulator, c-Fos, by inhibiting its induction, and augmented the positive regulator, c-Jun, through phosphorylation.



**FIGURE 3.** The effect of simvastatin on C/EBP and AP-1 DNA-binding activity, c-Fos expression, and the regulation of IL-12p40 promoter activity stimulated by ectopic expression of members of the AP-1 complex. *A*, RAW264.7 cells were pretreated with vehicle or 4  $\mu$ M simvastatin for 16 h and stimulated with 10 ng/ml LPS for 4 h. Nuclear proteins (5  $\mu$ g/lane) were prepared and incubated with 400 ng of the following polyclonal Abs on ice for 1 h in the presence of 1.5  $\mu$ g of poly(dI-dC): anti-C/EBP $\alpha$  (lanes 1 and 6), anti-C/EBP $\beta$  (lanes 2 and 7), anti-C/EBP $\delta$  (lanes 3 and 8), anti-C/EBP $\epsilon$  (lanes 4 and 9), and anti-C/EBP $\beta$  plus anti-C/EBP $\delta$  (lanes 5 and 10). Following this incubation, labeled probe  $-103$ – $76$  was added at room temperature for 30 min before electrophoresis. The probe  $-103$ – $76$  encompasses the C/EBP binding site on the IL-12p40 promoter. *B*, RAW264.7 cells were pretreated with vehicle or 4  $\mu$ M simvastatin for 16 h and stimulated with 10 ng/ml LPS for 4 h. Nuclear extracts (5  $\mu$ g/lane) were incubated with 400 ng of the following polyclonal Abs on ice for 1 h in the presence of 0.5  $\mu$ g of poly(dI-dC): normal IgG (lanes 1 and 9), anti-c-Jun (lanes 2 and 10), anti-JunB (lanes 3 and 11), anti-JunD (lanes 4 and 12), anti-c-Fos (lanes 5 and 13), anti-FosB (lanes 6 and 14), anti-Fra-1 (lanes 7 and 15), and anti-Fra-2 (lanes 8 and 16). After this incubation, labeled probe  $-88$ – $56$  was added at room temperature for 30 min before electrophoresis. The probe  $-88$ – $56$  encompasses the AP-1 binding site on the IL-12p40 promoter. *C*, Effect of simvastatin on c-Fos expression. RAW264.7 cells were pretreated with vehicle or 4  $\mu$ M simvastatin for 16 h and stimulated with 10 ng/ml LPS for the indicated time periods. Nuclear extracts (40  $\mu$ g/lane) were subjected to SDS-PAGE. Expression of c-Fos was determined by immunoblot analysis. *D*, The effect of ectopic expression of members of the AP-1 complex on IL-12p40 promoter activity. RAW264.7 cells were transfected with either 10 ng of empty vector (Mock), or 10 ng of plasmid expressing c-Jun, JunB, c-Fos, FosB, or Fra-2, together with 180 ng of the IL-12p40-127 promoter construct and 20 ng of pHRG-TK. Cells were incubated with vehicle or 8  $\mu$ M simvastatin for 24 h, and luciferase activity was measured. The luciferase titers are shown as R.L.U. compared with mock-transfected cells with vehicle treatment.

### The intermediates of the cholesterol biosynthetic pathway restore the normal response to LPS in simvastatin-pretreated cells

To show that the simvastatin effect was due to its inhibition of the HMG-CoA reductase, we demonstrated that mevalonate, farnesylpyrophosphate, and geranylgeranylpyrophosphate, intermediates in the cholesterol biosynthetic pathway, reversed the effect

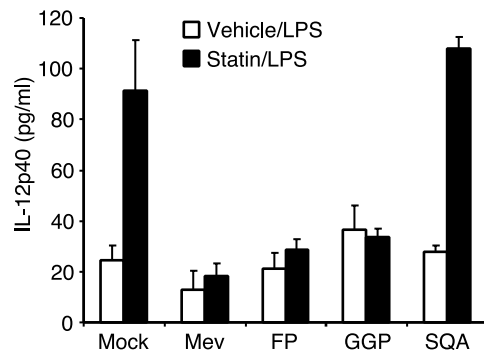


**FIGURE 4.** JNK activation plays an important role in the simvastatin-mediated IL-12p40 promoter augmentation. *A*, RAW264.7 cells were treated with 4  $\mu$ M simvastatin for indicated times and harvested for the preparation of whole cell lysates. Five hundred micrograms of whole cell lysates were subjected to in vitro kinase assay by using GST-c-Jun (1–89) as a substrate. *Bottom panel*, Expression of JNK1 and 2 as a loading control. *B*, RAW264.7 cells were pretreated with vehicle or 4  $\mu$ M simvastatin for 16 h and stimulated with 10 ng/ml LPS for the indicated time periods. Nuclear extracts (40  $\mu$ g/lane) were subjected to SDS-PAGE, and Ser<sup>63</sup>-phosphorylated c-Jun protein (upper panel) and total c-Jun protein (bottom panel) were detected by immunoblot analysis. *C*, RAW264.7 cells were treated with 4  $\mu$ M simvastatin with or without the indicated concentration of JNK inhibitor I (JNKi) for 16 h, and JNK activation was determined by in vitro kinase assay, as described above. *Bottom panel*, Expression of JNK1 and 2 as a loading control. *D*, RAW264.7 cells were transfected with the IL-12p40–127 promoter construct (180 ng/well) and pHRG-TK (20 ng/well) and subsequently treated with vehicle (Vehicle) or 8  $\mu$ M simvastatin (Statin) for 24 h. Twenty micromolar of negative control peptide (Control) or JNK inhibitor I (JNKi) were added twice to the culture medium at 0 and 16 h following simvastatin addition. Data are shown as R.L.U. compared with cells treated with vehicle and control peptide. The data represent the mean  $\pm$  SD of five independent experiments run in triplicate. Value of *p* was calculated by Student's *t* test.

(Fig. 5). Interestingly, squalene, a cholesterol precursor that is distal to geranylgeranylpyrophosphate, did not reverse the simvastatin effect on the IL-12p40 production. This suggests that the simvastatin effect on the IL-12p40 is not due to cholesterol deprivation, but rather on a proximal step, perhaps on protein prenylation.

#### Simvastatin also augments TNF- $\alpha$ production by transcriptional activation

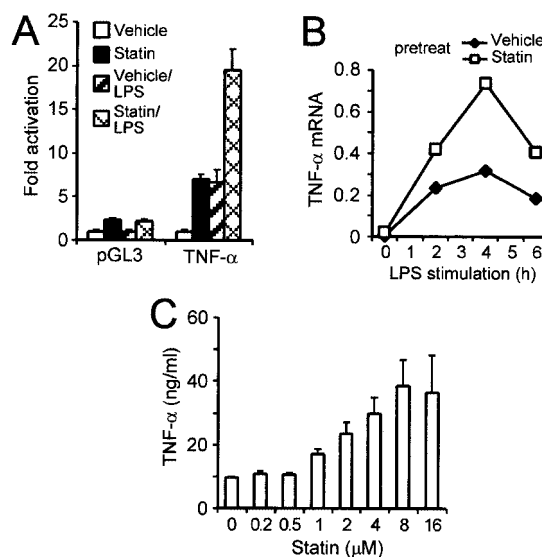
The promoter analysis indicates that both C/EBP and AP-1 binding sites are critical for the simvastatin-induced augmentation of IL-12p40 promoter. Other promoters containing both C/EBP and AP-1 binding sites might also be expected to be activated by simvastatin treatment. To confirm this hypothesis, we examined another inflammatory cytokine whose promoter has a similar structure to the IL-12p40 promoter. The unique feature of IL-12p40 promoter is that the C/EBP and AP-1 binding sites are located close to each other. The human TNF- $\alpha$  promoter has C/EBP and AP-1 binding sites similarly apposed (32–34), and, based on sequence homology, so does the murine TNF- $\alpha$  promoter. As expected, simvastatin induced TNF- $\alpha$  promoter-luciferase construct by 7-fold (Fig. 6A), and this was mirrored by increased TNF- $\alpha$  mRNA and secreted protein (Fig. 6, B and C).



**FIGURE 5.** The intermediates of the cholesterol biosynthetic pathway reverse the simvastatin effect on IL-12p40 production. Resident peritoneal macrophages ( $1 \times 10^6$ /ml) were treated with vehicle or 8  $\mu$ M simvastatin for 16 h. Mock (see *Materials and Methods*), 10  $\mu$ M Mevalonate (Mev), 10  $\mu$ M farnesylpyrophosphate (FP), 10  $\mu$ M geranylgeranylpyrophosphate (GGP), or 10  $\mu$ M squalene (SQA) was added simultaneously with vehicle or simvastatin. Cells were subsequently stimulated with 10 ng/ml LPS for 8 h. The concentration of IL-12p40 was measured by ELISA. Results are the mean  $\pm$  SD of triplicate wells and are representative of three separate experiments.

## Discussion

We have shown that simvastatin augments IL-12p40 production by macrophages in response to LPS stimulation. Our promoter analysis indicates, for the first time, that C/EBP and AP-1 binding sites



**FIGURE 6.** Simvastatin enhances TNF- $\alpha$  production by activating its promoter. *A*, RAW264.7 cells were transfected with either pGL3 empty vector (180 ng/well) or TNF- $\alpha$  promoter construct (180 ng/well), together with pHRG-TK (20 ng/well), and treated with vehicle or 8  $\mu$ M simvastatin for 20 h, followed by 5-h stimulation with or without 10 ng/ml LPS. The luciferase titers were measured by dual-luciferase assay system, and each result is expressed as fold activation normalized to cells treated with vehicle alone. Vehicle, vehicle treatment only; Statin, simvastatin treatment only; Vehicle/LPS, vehicle treatment plus LPS stimulation; Statin/LPS, simvastatin treatment plus LPS stimulation. *B*, RAW264.7 cells were treated with vehicle (Vehicle) or 4  $\mu$ M simvastatin (Statin) for 16 h and stimulated with 10 ng/ml LPS for the indicated time periods. mRNA amounts of TNF- $\alpha$  and EF-1 $\alpha$  were measured with quantitative real-time PCR. Data shown are relative mRNA amounts of TNF- $\alpha$  normalized to EF-1 $\alpha$ . Similar results were obtained in three independent experiments. *C*, RAW264.7 cells were treated with the indicated concentration of simvastatin for 16 h and stimulated with 10 ng/ml LPS for 24 h. Concentrations of TNF- $\alpha$  in the culture supernatant were measured by ELISA. Results are the mean  $\pm$  SD of triplicate wells and are representative of three separate experiments.

are essential for simvastatin-mediated IL-12p40 enhancement. Previous studies reported that statins increase the production of proinflammatory cytokines by myeloid cells (13, 35), but the mechanism by which this occurs has not been addressed. Our data strongly suggest that statin-mediated c-Fos repression is responsible for increased proinflammatory cytokine production. Simvastatin inhibited the association of c-Fos with the AP-1 binding site of the IL-12p40 promoter, suggesting that c-Fos negatively regulates proinflammatory cytokine production. This was confirmed by the observation that ectopically expressed c-Fos inhibited IL-12p40 promoter activation. c-Fos is known to form stable heterodimers with Jun proteins, thereby enhancing their DNA-binding activities (36). Although c-Fos cannot homodimerize and bind DNA, c-Jun can. Although a number of studies have demonstrated that heterodimers of c-Fos and c-Jun can activate the transcription of proinflammatory genes, recent evidence demonstrates that this heterodimer also has the capacity to inhibit transcription (37). Furthermore, we show in this study that c-Jun and c-Fos have opposing effects on IL-12p40 promoter activity, suggesting that c-Fos/c-Jun heterodimers are inhibitory, whereas c-Jun/c-Jun homodimers are stimulatory. The JNK is known to phosphorylate c-Jun, resulting in the activation of this transcription factor. Consistent with this, we found that simvastatin stimulated JNK-dependent phosphorylation of c-Jun.

Although the AP-1 binding site was critical for simvastatin-mediated activation of the IL-12p40 promoter, so was the C/EBP binding site, and this was confirmed by the demonstration that dominant-negative C/EBP completely abolished the simvastatin effect. It is not clear how these sites functionally interact to mediate the simvastatin effect; one possible explanation is that AP-1 requires the assistance of C/EBP to enhance IL-12p40 transcription.

Two recent reports present compelling evidence that statins have potent anti-inflammatory effects in an EAE model. Atorvastatin induced STAT6 phosphorylation and secretion of Th2 cytokines such as IL-4, IL-5, IL-10, and TGF- $\beta$ . In contrast, STAT4 phosphorylation was inhibited, resulting in suppression of Th1 cytokine secretion (IL-2, IL-12, IFN- $\gamma$ , and TNF- $\alpha$ ) (9, 10). Although these observations appear to be at variance with our results, a number of important differences exist between the two experimental systems. Principally, Ag presentation is crucial for both the initiation and the progression of EAE (38), whereas we were examining LPS-induced cytokine production by macrophages.

There is also evidence for a proinflammatory effect of statins in human disease. Mevalonate kinase (MK) is the next enzyme after HMG-CoA reductase in the cholesterol biosynthetic pathway. Mutation of the MK gene results in two inflammatory diseases in humans, mevalonic aciduria and hyperimmunoglobulinemia D and periodic fever syndrome (39–41). The mutation of MK, like statin treatment, results in substantially reduced cholesterol biosynthesis, and both appear to elicit proinflammatory responses. This is supported by the observation that lovastatin treatment substantially exacerbated mevalonic aciduria (39).

In conclusion, simvastatin potentiates proinflammatory responses induced by bacterial products, and this occurs by a mechanism involving the AP-1 and C/EBP transcription factors. Given the prevalence of statin use, it may be important to monitor inflammatory responses when treated individuals experience bacterial infection.

## Acknowledgments

We thank C. Vinson, T. Curran, S. Okada, T. Tokuhisa, R. Baler, and R. Ulevitch for providing us plasmids. We also thank D. Underhill and A. Ozinsky for critical reading of the manuscript.

## References

- Goldstein, J. L., and M. S. Brown. 1990. Regulation of the mevalonate pathway. *Nature* 343:425.
- Scandinavian Simvastatin Survival Study Group. 1994. Randomized trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet* 344:1383.
- Shepherd, J., S. M. Cobbe, I. Ford, C. G. Isles, A. R. Lorimer, P. W. MacFarlane, J. H. McKillop, and C. J. Packard. 1995. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia: West of Scotland Coronary Prevention Study Group. *N. Engl. J. Med.* 333:1301.
- Maron, D. J., S. Fazio, and M. F. Linton. 2000. Current perspectives on statins. *Circulation* 101:207.
- Ridker, P. M., N. Rifai, M. A. Pfeffer, F. Sacks, and E. Braunwald. 1999. Long-term effects of pravastatin on plasma concentration of C-reactive protein: the Cholesterol and Recurrent Events (CARE) Investigators. *Circulation* 100:230.
- Keogh, A., P. Macdonald, A. Kaan, C. Aboyoun, P. Spratt, and J. Mundy. 2000. Efficacy and safety of pravastatin vs simvastatin after cardiac transplantation. *J. Heart Lung Transplant.* 19:529.
- Kwak, B., F. Mulhaupt, S. Myit, and F. Mach. 2000. Statins as a newly recognized type of immunomodulator. *Nat. Med.* 6:1399.
- Weitz-Schmidt, G., K. Welzenbach, V. Brinkmann, T. Kamata, J. Kallen, C. Bruns, S. Cottens, Y. Takada, and U. Hommel. 2001. Statins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site. *Nat. Med.* 7:687.
- Youssef, S., O. Stuve, J. C. Patarroyo, P. J. Ruiz, J. L. Radosevich, E. M. Hur, M. Bravo, D. J. Mitchell, R. A. Sobel, L. Steinman, and S. S. Zamvil. 2002. The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature* 420:78.
- Aktas, O., S. Waiczies, A. Smorodchenko, J. Dorr, B. Seeger, T. Prozorovski, S. Sallach, M. Endres, S. Brocke, R. Nitsch, and F. Zipp. 2003. Treatment of relapsing paralysis in experimental encephalomyelitis by targeting Th1 cells through atorvastatin. *J. Exp. Med.* 197:725.
- Montero, M. T., O. Hernandez, Y. Suarez, J. Matilla, A. J. Ferruelo, J. Martinez-Botas, D. Gomez-Coronado, and M. A. Lasuncion. 2000. Hydroxymethylglutaryl-coenzyme A reductase inhibition stimulates caspase-1 activity and Th1-cytokine release in peripheral blood mononuclear cells. *Atherosclerosis* 153:303.
- Sadeghi, M. M., M. Collinge, R. Pardi, and J. R. Bender. 2000. Simvastatin modulates cytokine-mediated endothelial cell adhesion molecule induction: involvement of an inhibitory G protein. *J. Immunol.* 165:2712.
- Sun, D., and G. Fernandes. 2003. Lovastatin inhibits bone marrow-derived dendritic cell maturation and up-regulates proinflammatory cytokine production. *Cell. Immunol.* 223:52.
- Monick, M. M., L. S. Powers, N. S. Butler, and G. W. Hunninghake. 2003. Inhibition of Rho family GTPases results in increased TNF- $\alpha$  production after lipopolysaccharide exposure. *J. Immunol.* 171:2625.
- Liu, L., P. Moesner, N. L. Kovach, R. Bailey, A. D. Hamilton, S. M. Sebt, and J. M. Harlan. 1999. Integrin-dependent leukocyte adhesion involves geranylgeranylated protein(s). *J. Biol. Chem.* 274:33334.
- Krylov, D., M. Olive, and C. Vinson. 1995. Extending dimerization interfaces: the bZIP basic region can form a coiled coil. *EMBO J.* 14:5329.
- Olive, M., D. Krylov, D. R. Echlin, K. Gardner, E. Taparowsky, and C. Vinson. 1997. A dominant negative to activation protein-1 (AP1) that abolishes DNA binding and inhibits oncogenesis. *J. Biol. Chem.* 272:18586.
- Sonnenberg, J. L., F. J. Rauscher III, J. I. Morgan, and T. Curran. 1989. Regulation of proenkephalin by Fos and Jun. *Science* 246:1622.
- Okada, S., S. Obata, M. Hatano, and T. Tokuhisa. 2003. Dominant-negative effect of the c-fos family gene products on inducible NO synthase expression in macrophages. *Int. Immunol.* 15:1275.
- Smith, M., Z. Burke, A. Humphries, T. Wells, D. Klein, D. Carter, and R. Baler. 2001. Tissue-specific transgenic knockdown of Fos-related antigen 2 (Fra-2) expression mediated by dominant negative Fra-2. *Mol. Cell. Biol.* 21:3704.
- Bohuslav, J., V. V. Kravchenko, G. C. Parry, J. H. Erlich, S. Gerondakis, N. Mackman, and R. J. Ulevitch. 1998. Regulation of an essential innate immune response by the p50 subunit of NF- $\kappa$ B. *J. Clin. Invest.* 102:1645.
- Schindler, U., and V. R. Baichwal. 1994. Three NF- $\kappa$ B binding sites in the human E-selectin gene required for maximal tumor necrosis factor  $\alpha$ -induced expression. *Mol. Cell. Biol.* 14:5820.
- Hume, D. A., D. M. Underhill, M. J. Sweet, A. O. Ozinsky, F. Y. Liew, and A. Aderem. 2001. Macrophages exposed continuously to lipopolysaccharide and other agonists that act via Toll-like receptors exhibit a sustained and additive activation state. *BMC Immunol.* 2:11.
- Gantner, B. N., R. M. Simmons, S. J. Canavera, S. Akira, and D. M. Underhill. 2003. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* 197:1107.
- Caivano, M., B. Gorgoni, P. Cohen, and V. Poli. 2001. The induction of cyclooxygenase-2 mRNA in macrophages is biphasic and requires both CCAAT enhancer-binding protein  $\beta$  (C/EBP  $\beta$ ) and C/EBP  $\delta$  transcription factors. *J. Biol. Chem.* 276:48693.
- Blank, J. L., P. Gerwins, E. M. Elliott, S. Sather, and G. L. Johnson. 1996. Molecular cloning of mitogen-activated protein/ERK kinase kinases (MEKK) 2 and 3: regulation of sequential phosphorylation pathways involving mitogen-activated protein kinase and c-Jun kinase. *J. Biol. Chem.* 271:5361.
- Gri, G., D. Savio, G. Trinchieri, and X. Ma. 1998. Synergistic regulation of the human interleukin-12 p40 promoter by NF $\kappa$ B and Ets transcription factors in Epstein-Barr virus-transformed B cells and macrophages. *J. Biol. Chem.* 273:6431.



28. Murphy, T. L., M. G. Cleveland, P. Kulesza, J. Magram, and K. M. Murphy. 1995. Regulation of interleukin 12 p40 expression through an NF- $\kappa$ B half-site. *Mol. Cell. Biol.* 15:5258.
29. Plevy, S. E., J. H. Gemberling, S. Hsu, A. J. Dorner, and S. T. Smale. 1997. Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. *Mol. Cell. Biol.* 17:4572.
30. Zhu, C., K. Gagnidze, J. H. Gemberling, and S. E. Plevy. 2001. Characterization of an activation protein-1-binding site in the murine interleukin-12 p40 promoter: demonstration of novel functional elements by a reductionist approach. *J. Biol. Chem.* 276:18519.
31. Bonny, C., A. Oberson, S. Negri, C. Sauser, and D. F. Schorderet. 2001. Cell-permeable peptide inhibitors of JNK: novel blockers of  $\beta$ -cell death. *Diabetes* 50:77.
32. Pope, R. M., A. Leutz, and S. A. Ness. 1994. C/EBP  $\beta$  regulation of the tumor necrosis factor  $\alpha$  gene. *J. Clin. Invest.* 94:1449.
33. Zagariya, A., S. Mungre, R. Lovis, M. Birrer, S. Ness, B. Thimmapaya, and R. Pope. 1998. Tumor necrosis factor  $\alpha$  gene regulation: enhancement of C/EBP $\beta$ -induced activation by c-Jun. *Mol. Cell. Biol.* 18:2815.
34. Liu, H., P. Sidiropoulos, G. Song, L. J. Pagliari, M. J. Birrer, B. Stein, J. Anrather, and R. M. Pope. 2000. TNF- $\alpha$  gene expression in macrophages: regulation by NF- $\kappa$ B is independent of c-Jun or C/EBP  $\beta$ . *J. Immunol.* 164:4277.
35. Kiener, P. A., P. M. Davis, J. L. Murray, S. Youssef, B. M. Rankin, and M. Kowala. 2001. Stimulation of inflammatory responses in vitro and in vivo by lipophilic HMG-CoA reductase inhibitors. *Int. Immunopharmacol.* 1:105.
36. Smeal, T., P. Angel, J. Meek, and M. Karin. 1989. Different requirements for formation of Jun:Jun and Jun:Fos complexes. *Genes Dev.* 3:2091.
37. Li, L., J. C. Chambard, M. Karin, and E. N. Olson. 1992. Fos and Jun repress transcriptional activation by myogenin and MyoD: the amino terminus of Jun can mediate repression. *Genes Dev.* 6:676.
38. Kuchroo, V. K., A. C. Anderson, H. Waldner, M. Munder, E. Bettelli, and L. B. Nicholson. 2002. T cell response in experimental autoimmune encephalomyelitis (EAE): role of self and cross-reactive antigens in shaping, tuning, and regulating the autopathogenic T cell repertoire. *Annu. Rev. Immunol.* 20:101.
39. Hoffmann, G. F., C. Charpentier, E. Mayatepek, J. Mancini, M. Leichsenring, K. M. Gibson, P. Divry, M. Hrebicek, W. Lehnert, K. Sartor, et al. 1993. Clinical and biochemical phenotype in 11 patients with mevalonic aciduria. *Pediatrics* 91:915.
40. Houten, S. M., W. Kuis, M. Duran, T. J. de Koning, A. van Royen-Kerkhof, G. J. Romeijn, J. Frenkel, L. Dorland, M. M. de Barse, W. A. Huijbers, et al. 1999. Mutations in MVK, encoding mevalonate kinase, cause hyperimmunoglobulinemia D and periodic fever syndrome. *Nat. Genet.* 22:175.
41. Drenth, J. P., L. Cuisset, G. Grateau, C. Vasseur, S. D. van de Velde-Visser, J. G. de Jong, J. S. Beckmann, J. W. van der Meer, and M. Delpech. 1999. Mutations in the gene encoding mevalonate kinase cause hyper-IgD and periodic fever syndrome: International Hyper-IgD Study Group. *Nat. Genet.* 22:178.
42. Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3:133.