RESEARCH ARTICLE

Pharmacological inhibitors of the mevalonate pathway activate pro-IL-1 processing and IL-1 release by human monocytes

Benoit Massonnet[1,6]*, Sylvain Normand[1]*, Reinhard Moschitz[1], Adriana Delwail[1], Laure Favot[1], Martine Garcia[1], Nicolas Bourmeyster[2], Laurence Cuisset[3], Gilles Grateau[4], Franck Morel[1], Christine Silvain[1,5], Jean-Claude Lecron[1,6]

[1] Laboratoire Inflammation, tissus épithéliaux et cytokines; EA 4331, Université de Poitiers; France
[2] Laboratoire de Génétique moléculaire de l'adressage et de la signalisation, CNRS UMR 6187, Poitiers, France
[3] Department of Biochemical Genetics, Pavillon Cassini, CHU and Institut Cochin, Université Paris 5, Paris, France
[4] Service de médecine interne, Hôpital Tenon; Université Paris 6, Paris, France
[5] Service d'hépato-gastroentérologie, CHU de Poitiers, France
[6] Service immunologie-inflammation, CHU de Poitiers, France

Correspondence: Dr J.-C. Lecron, Laboratoire inflammation, tissus épithéliaux et cytokines, EA 4331, Pôle biologie santé, 86022 Poitiers Cedex, France
<jean-claude.lecron@univ-poitiers.fr>

Accepted for publication August 19, 2009

ABSTRACT. ***Objective.*** **The effects of statins (3-hydroxy-3-methylglutaryl coenzyme A reductase-HMGR-inhibitors) on the inflammatory response remain unclear. HMGR is implicated in the mevalonate pathway, directly upstream of cholesterol biosynthesis. We studied the impairment by this pathway of cytokine production by peripheral blood mononuclear cells (PBMCs) and THP-1 cells. The aim was to identify a specific cytokine "signature" of cells under simvastatin treatment in order to link pharmacological inhibition of the mevalonate pathway and inflammation.** ***Methods.*** **Normal human PBMCs and THP-1 cells were cultured with inhibitors of HMGR (simvastatin), geranylgeranyltransferase (GGTI-298), farnesyltransferase (FTI-277), and/or caspase-1 (Z-VAD(Ome)-FMK). Following culture, cytokine production, caspase-1 activity, IL-1β mRNA and Rac-1 activity were determined.** ***Results.*** **Pharmacological inhibition of the mevalonate pathway specifically enhanced the release of IL-1α, IL-1β and IL-18 and inhibited IL-1ra production by LPS-activated PBMCs and THP-1 cells. Simvastatin did not modify pro-IL-1β expression, but enhanced caspase-1 activity, the enzyme responsible for IL-1β and IL-18 maturation. GGTI-298 also enhanced IL-1-family cytokine production, showing that geranylgeranylation is involved in caspase-1 activation. Additionally, simvastatin enhanced Rac-1 activity.** ***Conclusion.*** **Pharmacological inhibition of the mevalonate pathway by statins highlighted the specific induction of the proinflammatory cytokines of the IL-1 family whose maturation is either directly (*i.e.* IL-1β and IL-18), or indirectly (*i.e.* IL-1α) dependant on caspase-1.**

Keywords: caspase-1, simvastatin, IL-1 family cytokines, THP-1, peripheral blood mononuclear cells

Statins are potent inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), and are extensively prescribed to reduce atherosclerotic, cardiovascular events by lowering low-density lipoprotein cholesterol [1-4]. Statins impair cholesterol synthesis by inhibiting the rate-limiting step in the mevalonate pathway by preventing the reduction of HMG-CoA to mevalonate. Inhibition of the mevalonate pathway is also accompanied by an increase in low-density lipoprotein receptors in the liver, leading to increased uptake and clearance of cholesterol from the plasma [3, 4]. Downstream of mevalonate and upstream of cholesterol, the mevalonate pathway also produces a number of non-sterol isoprenoids, especially geranylgeranyl-pyrophosphate (GGPP) and farnesyl-pyrophosphate (FPP), which are essential for intracellular signaling. Indeed, FPP and GGPP are precursors for the isoprenylation of small G proteins [5]. This enzymatic process is necessary for small G proteins to localize at the plasma membrane, allowing their signal transduction activity. FPP allows farnesylation of Ras-family proteins, while most Rho-family proteins are geranylgeranylated [5]. In addition to a direct effect of statins on the reduction of cardiovascular events through the inhibition of cholesterol synthesis, indirect effects have been attributed to the ability of statins to modulate inflammation.

Interestingly, both anti-inflammatory and proinflammatory properties have been attributed to statins [6-11]. Statins inhibit *in vitro* expression of IL-1β and TNF-α in monocytes from patients with hypercholesterolemia [12], and reduce IL-6 expression and secretion by

* These authors contributed equally to this work.

doi: 10.1684/ecn.2009.0162

human adipocytes [13]. An *ex vivo* study of proinflammatory cytokine production by LPS-activated whole peripheral blood from pravastatin-treated patients with hypercholesterolemia showed a decrease in TNF-α and IL-6 production after treatment, whereas IL-1β, IL-8 and IL-1 receptor antagonist (IL-1ra) were not affected [14]. Other studies reported the induction of secretion of IL-1β, TNF-α and IL-6 by LPS or anti-CD2/CD28-stimulated peripheral blood mononuclear cells (PBMCs) in the presence of statins [15, 16], or by monocytes from statin-treated mice [16]. It has been suggested that such increases in cytokine release were due specifically to a lack of isoprenoids [15, 17].

Interestingly, this pharmacological model has physiopathological counterparts, as two genetics diseases characterised by the inhibition of the mevalonate pathway and recurrent inflammation. Indeed, the hereditary periodic fevers hyperimmunoglobulinemia D, periodic fever syndrome (HIDS; OMIM #260920) and the more severe mevalonic aciduria (MA; OMIM #310377), are rare, autoinflammatory diseases characterized as mevalonate kinase deficiencies (MKD) and are caused by recessive mutations in the *MVK* gene (locus 12q24) [18, 19]. These mutations lead to inactivation of mevalonate kinase (MK), the enzyme directly downstream of HMGR in the mevalonate pathway. The non-sterol isoprenoids GGPP [20] and FPP [18, 21] metabolites synthesized downstream of MK have been thought to play a crucial role in the inflammatory manifestations of the MKD patients.

In HIDS patients, plasma levels of IL-6 and TNF-α are increased during attacks, whereas IL-1α , IL-1β and IL-10 are unchanged [22]. Another study has shown that *ex vivo*, unstimulated or LPS-activated PBMCs from patients release more IL-1β, IL-6 and TNF-α than PBMCs from control subjects [23].

The aim of the present study was to analyze *in vitro* the influence of the inhibition of the mevalonate pathway by simvastatin on the proinflammatory cytokine profile secreted from control donors' PBMCs or the LPS-treated THP-1 monocytic cell line. We further studied whether farnesylation, geranylgeranylation and Rac-1 (a protein of the Rho-family), as well as post-translational mechanisms of cytokine maturation and release, are involved in these processes.

Abbreviations

FPP	farnesyl-pyrophosphate
FTI	farnesyltranferase inhibitor
GGPP	geranylgeranyl-pyrophosphate
GGTI	geranylgeranyltransferase inhibitor
HIDS	Hyper IgD and periodic fever syndrome
HMGR	3-hydroxy-3-methylglutaryl-CoA reductase
IL	interleukin
LPS	lipopolysaccharide
mAb	monoclonal antibody
HMBS	hydroxymethylbilane synthase
MA	mevalonic aciduria
MK	mevalonate kinase
MKD	mevalonate kinase deficiency
PBMC	peripheral blood mononuclear cell
PMA	phorbol 12-myristate 13-acetate
TNF	tumor necrosis factor

METHODS AND MATERIALS

Cell culture and reagents

Normal human PBMCs were isolated by Ficoll-Hypaque (Biochrom AG, Berlin, Germany) centrifugation. For all experiments, PBMCs and THP-1 cells were grown in culture medium made up of RPMI 1640 (Invitrogen Life Technologies, Cergy Pontoise, France), supplemented with Glutamax-I, 10% heat-inactivated fetal calf serum (Sigma Aldrich, Saint-Quentin Fallavier, France), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen Life Technologies). THP-1 cells were seeded in this medium at a concentration of 0.3 10^6/mL, 72 h prior to experimentation. One million PBMCs or THP-1 cells were cultured in 1 mL of medium in the presence or absence of 1 μg/mL *E. coli* LPS (SigmaAldrich), 10 μg/mL anti-CD3 mAb (OKT3, ATCC, Rockville, MA, USA) and 1 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma ldrich), 10 μM simvastatin (Merck Pharmaceuticals, West Drayton, Middlesex, UK), 0.5 μM FTI-277 (Calbiochem, Darmstadt, Germany), 15 μM GGTI-298 (Calbiochem), 100 μM mevalonate (Mevanolactone, Sigma Aldrich) and/or 10 μM Caspase-1 inhibitor (Z-VAD(Ome)-FMK, Alexis Biochemicals, Switzerland).

The use of PBMCs for research studies was approved by the Ethics Committee of the Poitiers Hospital.

ELISA for cytokine measurements

Cytokines were measured by ELISA in 48-hour culture supernatants. Commercial kits were used to measure IL-1β, IL-18, IL-1RA (matched antibody pairs for ELISA, RTOTOD systems), IFN-γ, IL-1α, IL-2, IL-4, IL-6, TNF-α (Eli-pair, Diaclone, Besançon, France), and IL-4 (OptEIA, Pharmingen, San Diego, CA). ELISAs were performed according to manufacturers' instructions. All assays were performed in duplicate, in 96-well, flat-bottomed Maxisorp microtiter plates (Nunc, Rochester, NY, USA).

Caspase-1 activity measurement

Caspase-1 activity was measured using a Caspase-1 Fluorometric Assay Kit according to the manufacturer's instructions (BioVision, Mountain View, CA, USA) in 48 hour, THP-1 culture supernatants concentrated 50-fold with a 10 kDa cut-off centrifugal concentrator (Vivaspin 2, Sartorius, Palaiseau, France). Relative caspase-1 activity was expressed as a ratio between the fluorescence intensities determined from treated cells/control cells.

Real-time RT-PCR analysis

Total cellular RNA from THP-1 cells was extracted using TRIzol reagent (Invitrogen Life Technologies) and treated with DNase I (0.05 U/μL; Roche, Basel, Switzerland). cDNA was synthesized from 4 μg of total RNA using random hexamer primers (GE Healthcare, Stockholm, Sweden). Quantitative RT-PCR was conducted using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche) and the following primers: pro-IL-1β (forward, 5'-CTGTCCTGCGTGTTGAAAGA-3', and reverse,

5'-CTGGGCAGACTCAAATTCCA-3'; designed with Primer 3 Output software) and hydroxymethylbilane synthase (HMBS) [24] as a housekeeping gene.

Subcellular fractionation pull-down assays

The cells were lysed on ice by successive passages through a 25 G needle in a fractionation buffer (20 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 200 mM saccharose, 1 mM Na_3VO_4, and 1% protease inhibitor cocktail (Sigma-Aldrich). Cells were cleared by centrifugation (750 g for 10 min); the supernatant was ultra-centrifuged (100 000 g for 1 h) to separate cytoplasmic and membranous fractions. The resulting supernatant was removed and saved as the cytoplasmic fraction. The lipophylic membrane-containing pellet and the cytoplasmic fraction were then resuspended in Laemmli sample buffer for western blotting analysis. The pull-down assays for detection of activated Rac-1 were performed with GST-PAK-CRIB-domain bound to glutathione-sepharose beads as previously described [25].

Western blot analysis

Proteins were separated by 12% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes (GE Healthcare). Membranes were blocked for two hours in Tris-buffered saline (150 mM NaCl and 20 mM Tris, pH 7.5) containing 5% non-fat dry milk and 0.1% Tween 20, and incubated with a mouse anti-human Rac-1 mAb (murine IgG2b, 23A8 clone, Upstate, Lake Placid, NY, USA), with a rabbit anti-human IL-1β polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA), with a rabbit anti-human caspase-1 p20 subunit Ab (Sigma-Aldrich), with a rabbit anti-human phospho-p38 pAb or with a rabbit anti-human total p38 pAb (GE Healthcare) overnight at 4°C. Blots were then incubated with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies (Sigma Aldrich), and immunoreactive proteins were visualized using the enhanced chemiluminescence assay (ECL, GE Healthcare). Ponceau red staining was used to control loading homogeneity. Images of western blot were scanned (Perfection 1670, Epson, Tokyo, Japan) and analyzed using NIH ImageJ software for densitometric representation.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc, San Diego, CA, USA). Significance was determined using one-way ANOVA, followed by the Newman-Keuls test.

RESULTS

Blocking of the mevalonate pathway by simvastatin enhances the release of IL-1 family cytokines by LPS-activated PBMCs

To study the effects of mevalonate pathway inhibition on cytokine production by T cells, normal human PBMCs were activated with anti-CD3 mAb and PMA, and cultured for 48 hours in the presence or absence of simvastatin, and/or mevalonate. Whereas mevalonate had no effect on the secretion of the set of cytokines tested, simvastatin decreased the secretion of IFN-γ and IL-4 but did not significantly alter the secretion of IL-6, IL-2, IL-10, TNF-α, IL-1β, IL-1α and IL-18 *(figure 1)*. This effect was specific for the inhibition of HMGR, since the addition of mevalonate restored secretion to control levels. GGTI-298, an inhibitor of type-I geranylgeranyl transferase, decreased the secretion of IFN-γ by activated PBMCs, whereas FTI-277, an inhibitor of the farnesyl transferase, had no significant effect.

Regarding the production of monocyte-derived cytokines by LPS-activated PBMCs, simvastatin and GGTI, in contrast, strongly and specifically enhanced the production of cytokines of the IL-1 family: IL-1α, IL-1β and IL-18, whereas FTI-277 had no effect *(figure 2)*. Mevalonate abrogated all the effects of simvastatin. Simvastatin, FTI-277 and GGTI-298 did not significantly alter the production of TNF-α, IL-6 and IL-10, and mevalonate alone had no significant effect on cytokine secretion by LPS-activated PBMCs.

We further analyzed the effects of simvastatin on IL-1α, IL-1β, IL-1ra, IL-18 and IL-6 production by the THP-1 monocytic cell line *(figure 3)*. The secretion of IL-1β and IL-1α by unstimulated cells was very low and was not further enhanced by simvastatin *(figure 3A, B)*. In the presence of LPS, we observed a release of IL-1β within the first hours of culture *(figure 3A)*, whereas IL-1α release was not modified *(figure 3B)*. Addition of simvastatin to LPS-treated THP-1 cells dramatically enhanced both IL-1β and IL-1α production after 24 hours of culture *(figure 3A, B)*. IL-1β production by LPS-activated THP-1 cells increased in a dose-dependent manner in response to simvastatin, ranging from 2.5 to 20 μM, and decreased at higher concentrations *(figure 3C)*. As observed with simvastatin-treated PBMCs, GGTI-298 enhanced IL-1β and IL-18 secretion by LPS-activated THP-1, while FTI-277 had no effect *(figure 3D, E)*. Otherwise, simvastatin significantly inhibited IL-1ra release, but did not affect IL-6 production *(figure 3F, G)*.

Simvastatin does not affect pro-IL-1β expression, but induces IL-1β maturation through caspase-1 activation

Our data showed a specific enhancement of the secretion of IL-1 family cytokines by monocytes in the presence of simvastatin. Interestingly, two members of this family, IL-1β and IL-18, have the peculiarity of being produced as inactive pro-cytokines, matured into active form by caspase-1. Therefore, we studied the expression levels of pro-IL-1β transcript and protein, and the maturation of pro-IL-1β to the active form under simvastatin treatment in THP-1 cells. Real-time quantitative RT-PCR *(figure 4A)* and western blot *(figure 4B)* experiments showed that although pro-IL-1β mRNA and intracellular protein levels were increased by LPS, they were not significantly modified by simvastatin. Whereas LPS induced the secretion of the 34kDa pro-IL-1β by THP-1 cells, the 17kDa mature IL-1β form was not detectable in the culture supernatant *(figure 4C)*. Interestingly, simvastatin

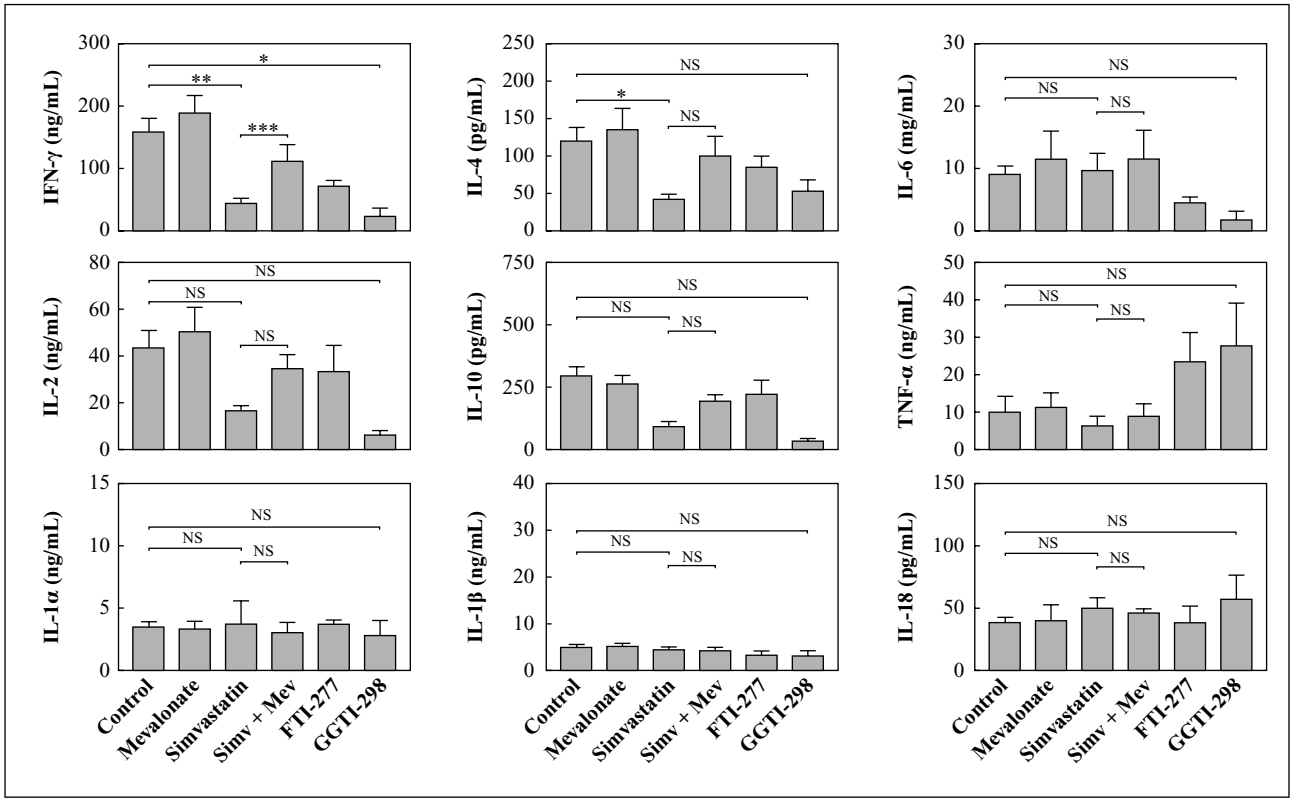

Figure 1

Effects of simvastatin and inhibition of protein prenylation on cytokine secretion by anti-CD3 mAb + PMA-stimulated human PBMCs. Normal PBMCs were cultured for 48 hours with 10 µg/mL anti-CD3 mAb and 1 ng/mL PMA, in the presence or absence of 10 µM simvastatin, 0.5 µM FTI-277, 15µM GGTI-298 and 100 µM mevalonic acid. Cytokines were assayed in culture supernatants by ELISA. Each bar represents mean ± SEM of six independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ based on one-way ANOVA followed by the Newman-Keuls test.

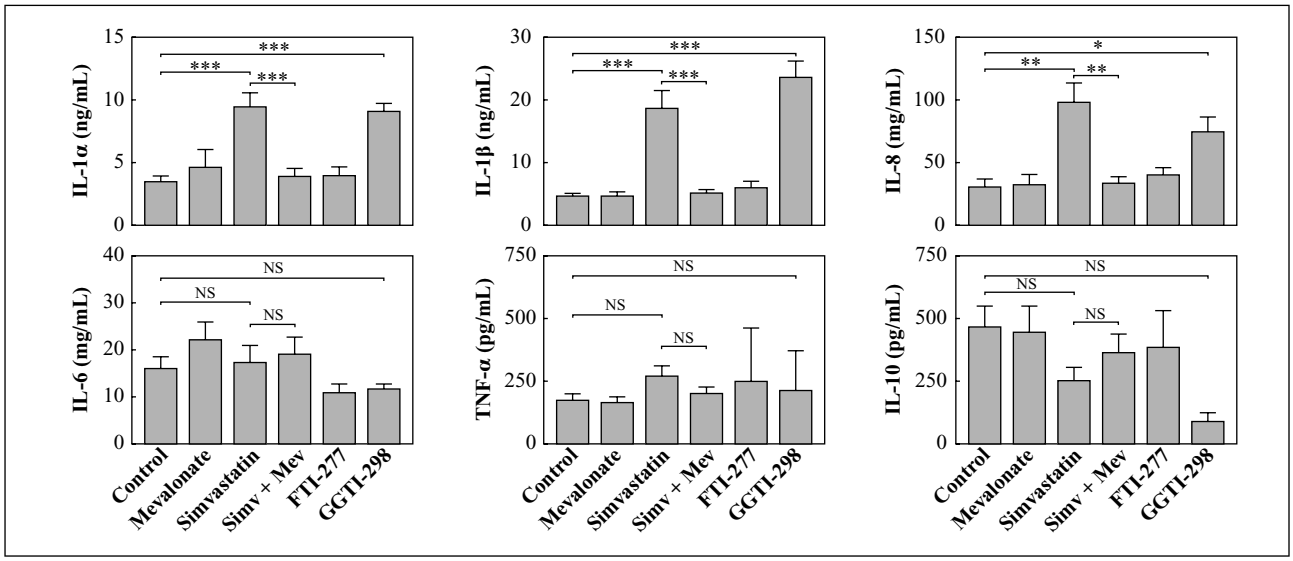

Figure 2

Effects of simvastatin and inhibition of protein prenylation on cytokine secretion by LPS-activated human PBMCs. Normal PBMCs were cultured for 48 hours with 1 µg/mL LPS, in the presence or absence of 10 µM simvastatin, 0.5 µM FTI-277, 15 µM GGTI-298 and 100 µM mevalonic acid. Cytokines were assayed in culture supernatants by ELISA. Each bar represents mean ± SEM of six independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ based on one-way ANOVA followed by the Newman-Keuls test.

strongly enhanced the maturation of pro-IL-1β to active IL-1β *(figure 4C)*. This effect was totally or partially abolished by the addition of mevalonate or by a caspase-1 inhibitor respectively *(figure 4C)*. Independently, we showed that the activity of caspase-1 was enhanced in culture supernatants from simvastatin-treated THP-1 cells *(figure 4D)*. Moreover, we showed that the active caspase-1 p20 subunit is enhanced by simvastatin in culture supernatants from THP-1 cells *(figure 4E)*.

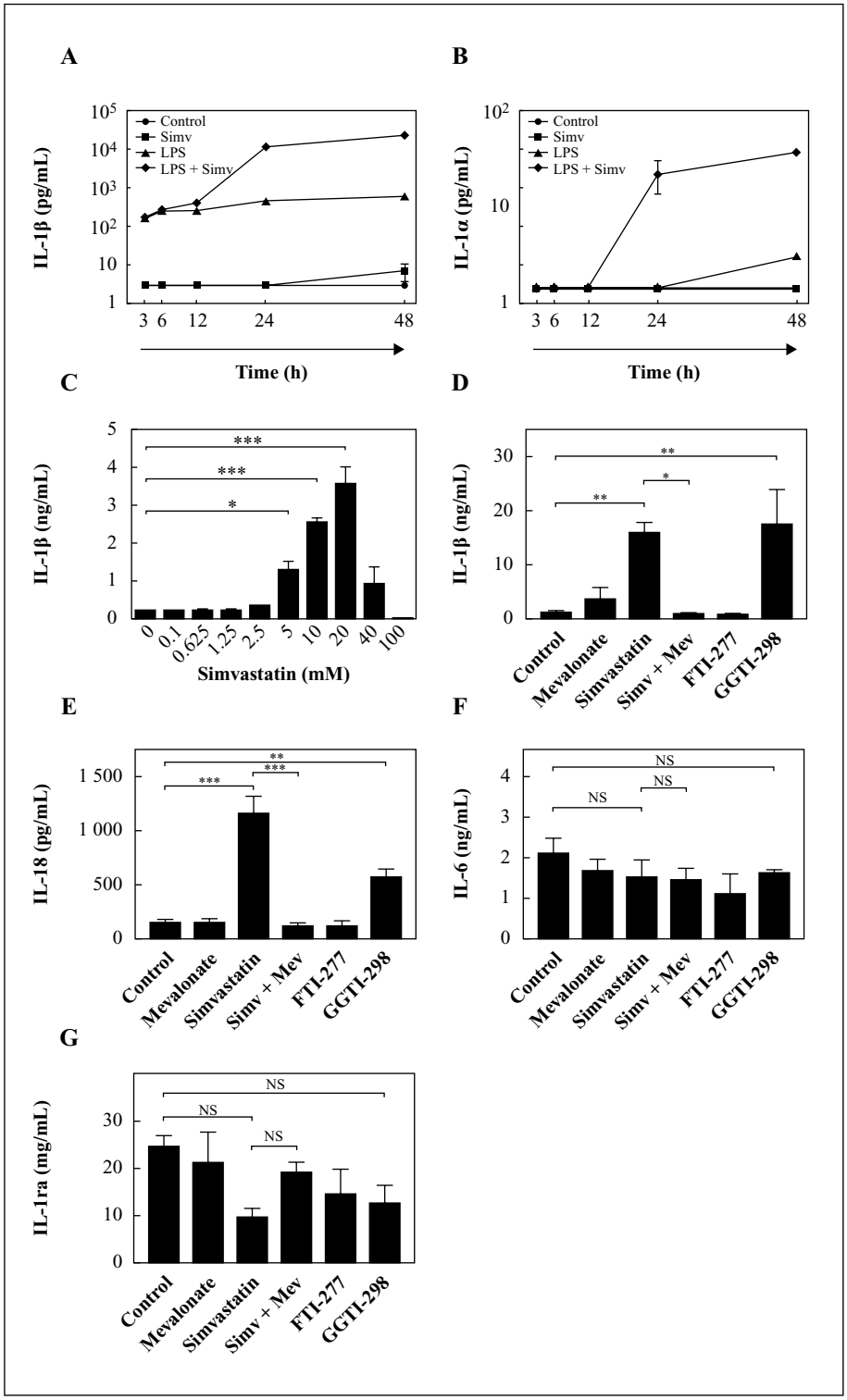

Figure 3

Effects of simvastatin and prenylation inhibitors on cytokine secretion by THP-1 cells. (**A, B**) THP-1 monocytes were cultured for 3, 6, 12, 24 or 48 hours with or without 1 µg/mL LPS in the presence or absence of 10 µM simvastatin. IL-1β (**A**) and IL-1α (**B**) were assayed in culture supernatants by ELISA. Each dot represents mean ± SD of two independent experiments. (**C**) THP-1 monocytes were cultured for 48 hours with 1 µg/mL LPS in the presence or absence of 0 to 100 µM simvastatin. IL-1β was assayed by ELISA in culture supernatants. Each bar represents mean ± SEM of three independent experiments. * $p < 0.05$, *** $p < 0.001$. (**D, G**) THP-1 cells were cultured for 48 hours with 1 µg/ml LPS, in the presence or absence of 10 µM simvastatin, 0.5 µM FTI-277, 15 µM GGTI-298 and 100 µM mevalonic acid. IL-1β (**D**), IL-18 (**E**), IL-6 (**F**) and IL-1ra (**G**) were assayed in culture supernatants by ELISA. Each bar represents mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ based on one-way ANOVA followed by the Newman-Keuls test.

We also showed that the caspase-1 inhibitor decreased the simvastatin-induced secretion of IL-1β and IL-1α by both LPS-activated THP-1 cells *(figure 4F)* and normal PBMCs *(figure 4G)*. In contrast, simvastatin decreased the secretion of IL-1ra by both LPS-activated normal PBMCs and THP-1 cells. As expected, this decrease

was abolished by the addition of mevalonate, but not by addition of a caspase-1 inhibitor *(figure 4F, G)*. Finally, neither simvastatin nor caspase-1 inhibitor significantly modulated the production of IL-6 by both LPS-activated THP-1 cells *(figure 4F)* and PBMCs *(figure 4G)*.

Simvastatin increases GTP-loading activity of Rac-1, with a discrete effect on Rac-1 localization

Our data suggested that the modulation of protein geranylgeranylation is responsible for the simvastatin-induced

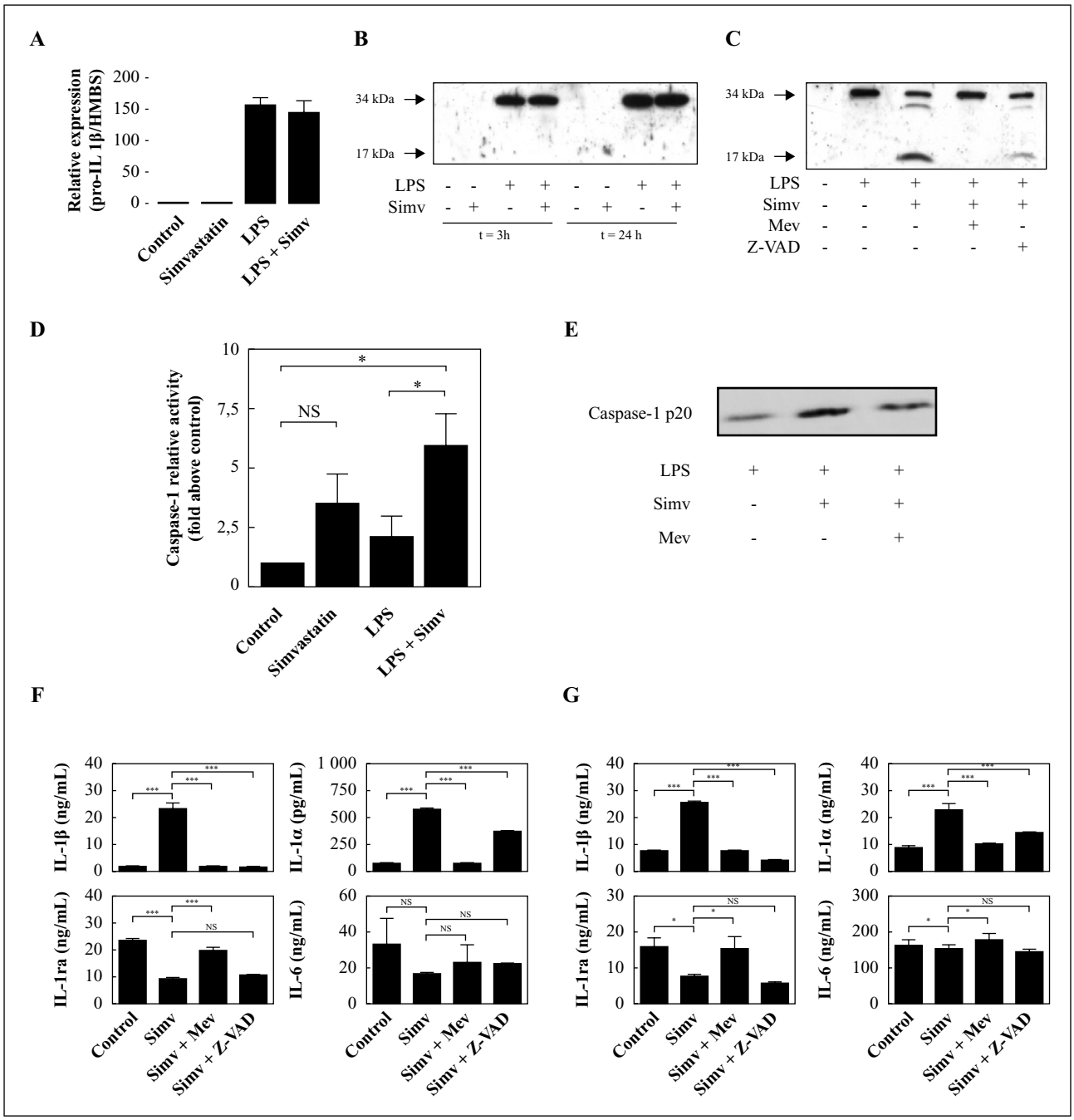

Figure 4

Involvement of caspase-1 on simvastatin-induced production of proinflammatory cytokines of IL-1 family in LPS-activated THP-1 cells and human PBMCs. (**A-F**) Human THP-1 monocytes or normal PBMCs (**G**) were cultured with or without 1 µg/mL LPS in the presence or absence of 10 µM simvastatin, 100 µM mevalonate and 10 µM Caspase-1 inhibitor (Z-VAD). (**A**) Relative pro-IL-1β mRNA expression was determined by quantitative real-time RT-PCR analysis after three hours of culture. Pro-IL-1β expression was normalized using HMBS mRNA expression level. Each bar represents mean ± SEM of three independent experiments. (**B**) Pro-IL-1β (34 kDa) and mature IL-1β (17 kDa) levels were analyzed by western blot in three- and 24-hour culture THP-1 lysates or in 48 hours THP-1 culture supernatants (**C**) (representative of two independent experiments). (**D**) Caspase-1 activity was measured in 50-fold concentrated, 48-hour culture supernatants using a fluorometric caspase-1 activity assay. Results are expressed as the relative activity detected in supernatants of stimulated cells as compared with control cells. Each bar represents mean ± SEM of four independent experiments. (**E**) Caspase-1 p20 subunit extracellular levels were assessed by western blot in 50-fold concentrated, 48-hour culture supernatants (representative of three independent experiments). (**F, G**) Cytokines were assayed in culture supernatants of LPS-stimulated THP-1 (**F**) or PBMCs (**G**) by ELISA. Each bar represents mean ± SEM of four independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ based on one-way ANOVA followed by the Newman-Keuls test.

activation of caspase-1 and subsequent maturation of IL-1 family cytokines. We studied the effects of simvastatin on the GTP-loading activity and localization of Rac-1, a geranylgeranylated small G-protein of the Rho family that requires geranylgeranylation to be localized to the plasma membrane. Interestingly, Rac-1 is directly implicated in the activation of caspase-1 [26, 27]. Using GST-PAK-CRIB-domain pull down, we observed that the active form of Rac-1 (the GTP-loaded Rac-1) was clearly enhanced in the presence of simvastatin, both in resting and LPS-activated THP-1 cells *(figure 5A)*. Moreover, we showed by western blot analysis that the level of Rac-1 was slightly increased in the cytoplasm, and decreased at the plasma membrane under simvastatin treatment *(figure 5B)*. We also showed that p38 phosphorylation, an indicator of Rac-1 activation [28], was increased by simvastatin *(figure 5C)*.

DISCUSSION

Simvastatin treatment of LPS-activated PBMCs and THP-1 cells strongly and specifically enhanced the secretion of IL-1α, IL-1β and IL-18, proinflammatory cytokines belonging to the IL-1 family. IL-1β and IL-18 have the common feature of being synthesized as inactive pro-cytokines that require enzymatic processing by caspase-1 in order to matured into the active form [29-31]. The involvement of post-translational events in IL-1β release induced by simvastatin was shown by the enhanced secretion of mature IL-1β by activated monocytes, without any effect on either pro-IL-1β mRNA or protein levels. Both caspase-1 activity and active caspase-1 p20 subunit level were increased in culture supernatants of simvastatin-treated THP-1 cells. In addition, inhibition of caspase-1 decreased simvastatin-induced maturation and release of IL-1β, whereas the production of the caspase-1-independent cytokines IL-6 and IL-1ra was not modified. These data are in agreement with previous studies showing that statins enhance caspase-1 activity and IL-1β release by *Mycobacterium tuberculosis*-primed PBMCs or by activated THP-1 cells [10, 17, 32]. Unlike IL-1β and IL-18, IL-1α is known to be synthesized as the biologically active cytoplasmic or membrane-bound pro-IL-1α, and is mainly secreted through a proteolytic cleavage by calpain [33]. Surprisingly, we showed herein IL-1α release under simvastatin treatment that is inhibited in the presence of a caspase-1

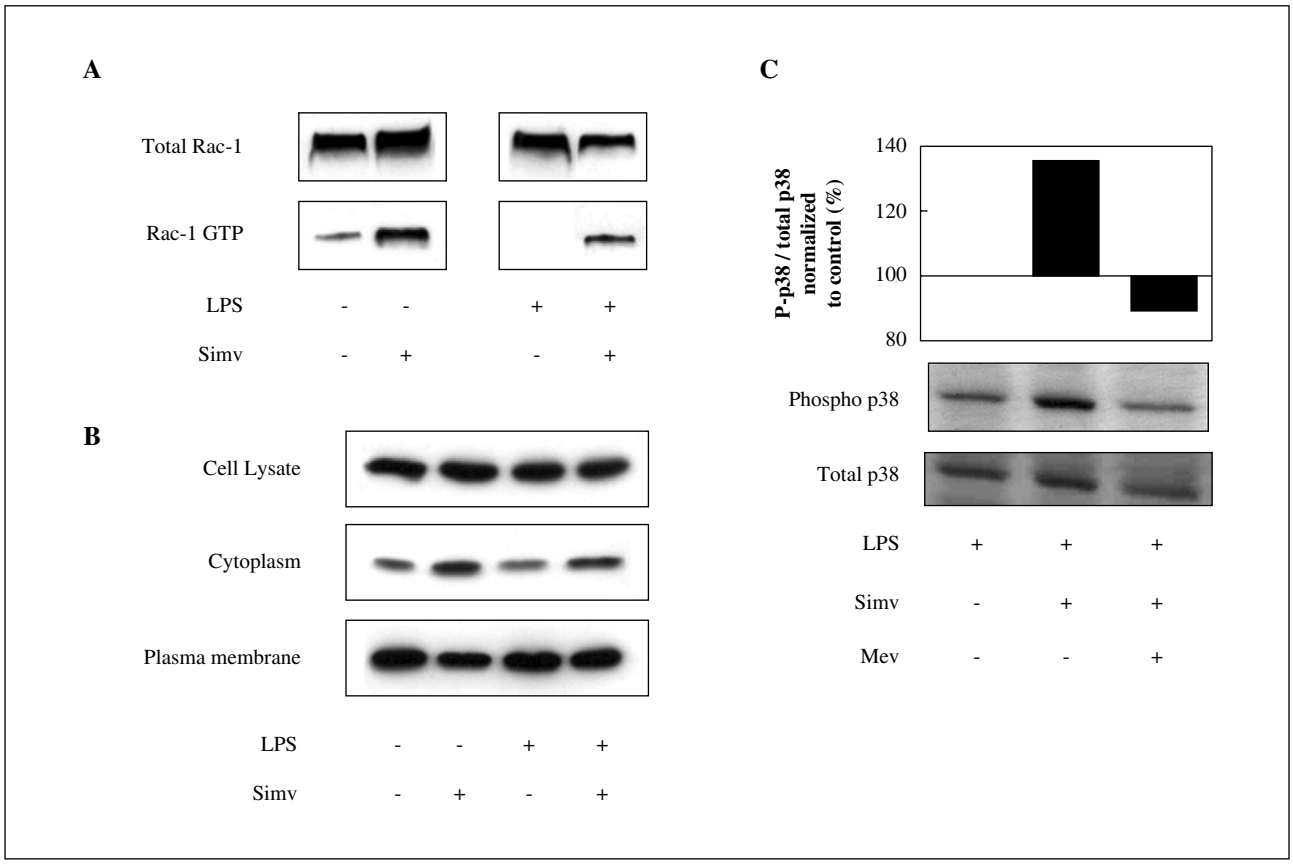

Figure 5

Effects of simvastatin on the GTP-loading activity and localization of Rac1, and p38 phosphorylation. THP-1 cells were cultured with or without 1 μg/mL LPS and/or 10 μM simvastatin for 24 hours. (**A**) Cells were lysed and GTP-loaded Rac1 was purified by GST-PAK-CRIB-domain pull-down. The presence of Rac-1 was revealed by western blot analysis (representative of four independent experiments). (**B**) Cytoplasm and membrane fractions were separated by 100 000 g, 1 h ultracentrifugation. Supernatants containing cytoplasm were collected and membrane pellets were solubilized in Laemmli sample buffer. Rac1 level was assessed by western blot analysis (representative of three independent experiments). (**C**) Total and phosphorylated p38 intracellular levels were assessed by western blot. Results are expressed as the relative phospho-p38/total p38 ratio detected in supernatants of stimulated cells as compared with control cells (representative of two independent experiments).

inhibitor. IL-1β-induced IL-1α expression could account for this effect. As IL-6 and TNF-α are not over-expressed in our culture conditions, we suggest that it is not the primary mechanism. Since a caspase-1-dependent activation of calpain has been reported [34, 35], we hypothesize that caspase-1 could indirectly enhance IL-1α maturation. In agreement, IL-1α secretion is impaired in caspase-1-deficient mice [36], and secretion of pro-IL-1α requires efficient caspase-1 activity [37].

In accordance with a previous study showing the involvement of geranylgeranylation on IL-1β production by LPS-activated PBMCs (20), simvastatin, GGTI-298, but not FTI-277, strongly induced the secretion of IL-1α, IL-1β and IL-18 by LPS-stimulated PBMCs. These results also suggest the involvement of geranylgeranylation in the simvastatin-induced activation of caspase-1. Geranylgeranylation seems to be associated with the activity of Rac-1, a small G-protein of the Rho family reported to play a critical role in caspase-1 activation [26, 27]. Rac-1-dependent phosphorylation of p38 was also demonstrated [28]. Under simvastatin treatment, Rac-1 was partially relocalized from the plasma membrane to the cytoplasm, but surprisingly, the level of GTP-bound Rac-1 is enhanced. In agreement, a simvastatin-induced Rac-1 activation has also been recently reported in the vascular wall [38] and in THP-1 cells [39] and, independently, a simvastatin-reduced expression of membranous Rac-1 has been observed in smooth muscle cells [40]. The increased Rac-1 signaling activity was supported by the enhancement of p38 phosphorylation. Such an increase of p38 phosphorylation after 24 h simvastatin treatment has been reported in H_2O_2-treated smooth muscle cells, but not in unstimulated cells [40]. In the absence of LPS-stimulation of THP-1 cells, simvastatin does not enhance p38 phosphorylation (data not shown), as reported [39]. Although the link between Rac-1 geranylgeranylation and caspase-1 activation remains to be further explored, these results clearly show the increase Rac-1 GTP loading activity under simvastatin stimulation. In accord with our observations, it has been recently and independently demonstrated that dysregulated isoprenoid biosynthesis activates the Rac-1/PI3K pathway, resulting in caspase-1 activation and increased IL-1β release [39].

The stimulatory effect of simvastatin on the enhancement of IL-1α, IL-1β and IL-18 production by macrophages *in vitro* is largely mediated *via* the activation of caspase-1. On the other hand, we and others have demonstrated that statins inhibit the release of cytokines such as IFN-γ, IL-2, IL-10 and IL-4 by *in vitro* activated-T lymphocytes [41, 42] or IL-10 by monocytes. *In vivo*, treatment with statins have anti-inflammatory effects associated with *ex vivo* inhibition of IL-1β, TNF-α and IL-6 production [14], suggesting that the inhibitory effect of statins on cytokine synthesis predominates over caspase-1 activation. These effects could account for the beneficial effect of statins as lipid-lowering agents in the reduction of atherosclerotic cardiovascular events.

Acknowledgments. *The authors thank Merck Pharmaceuticals for kindly providing simvastatin. We also thank Dr Bruce Koppelman for the careful review of the manuscript.*

Financial support. *This study was supported by grants from a clinical research program from Poitiers University Hospital, from the National Agency of Research (project ANR HEREF-EVER), and the Région Poitou-Charentes.*

REFERENCES

1. Borghi C, Dormi A, Veronesi M, *et al.* Use of lipid-lowering drugs and blood pressure control in patients with arterial hypertension. *J Clin Hypertens* 2002; 4: 277-85 (Greenwich).
2. Duriez P. Current practice in the treatment of hyperlipidaemias. *Expert Opin Pharmacother* 2001; 2: 1777-94.
3. Gaw A. A new reality: achieving cholesterol-lowering goals in clinical practice. *Atheroscler Suppl* 2002; 2: 5-8 (discussion 8–11).
4. Stein E. The lower the better? Reviewing the evidence for more aggressive cholesterol reduction and goal attainment. *Atheroscler Suppl* 2002; 2: 19-23; (discussion 23-5).
5. Seabra MC, James GL. Prenylation assays for small GTPases. *Methods Mol Biol* 1998; 84: 251-60.
6. Greenwood J, Steinman L, Zamvil SS. Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. *Nat Rev Immunol* 2006; 6: 358-70.
7. Ikeda U, Shimpo M, Ikeda M, *et al.* Lipophilic statins augment inducible nitric oxide synthase expression in cytokine-stimulated cardiac myocytes. *J Cardiovasc Pharmacol* 2001; 38: 69-77.
8. Kwak B, Mulhaupt F, Myit S, *et al.* Statins as a newly recognized type of immunomodulator. *Nat Med* 2000; 6: 1399-402.
9. Martinez-Gonzalez J, Alfon J, Berrozpe M, Badimon L. HMG-CoA reductase inhibitors reduce vascular monocyte chemotactic protein-1 expression in early lesions from hypercholesterolemic swine independently of their effect on plasma cholesterol levels. *Atherosclerosis* 2001; 159: 27-33.
10. Montero MT, Hernandez O, Suarez Y, *et al.* Hydroxymethylglutaryl-coenzyme A reductase inhibition stimulates caspase-1 activity and Th1-cytokine release in peripheral blood mononuclear cells. *Atherosclerosis* 2000; 153: 303-13.
11. Sadeghi MM, Collinge M, Pardi R, Bender JR. Simvastatin modulates cytokine-mediated endothelial cell adhesion molecule induction: involvement of an inhibitory G protein. *J Immunol* 2000; 165: 2712-8.
12. Ferro D, Parrotto S, Basili S, *et al.* Simvastatin inhibits the monocyte expression of proinflammatory cytokines in patients with hypercholesterolemia. *J Am Coll Cardiol* 2000; 36: 427-31.
13. van Harmelen V, Skurk T, Rohrig K, *et al.* HMG-CoA reductase inhibitor cerivastatin inhibits interleukin-6 expression and secretion in human adipocytes. *Horm Metab Res* 2003; 35: 466-70.
14. Rosenson RS, Tangney CC, Casey LC. Inhibition of proinflammatory cytokine production by pravastatin. *Lancet* 1999; 353: 983-4.
15. Frenkel J, Rijkers GT, Mandey SH, *et al.* Lack of isoprenoid products raises *ex vivo* interleukin-1beta secretion in hyperimmunoglobulinemia D and periodic fever syndrome. *Arthritis Rheum* 2002; 46: 2794-803.
16. Kiener PA, Davis PM, Murray JL, *et al.* Stimulation of inflammatory responses in vitro and in vivo by lipophilic HMG-CoA reductase inhibitors. *Int Immunopharmacol* 2001; 1: 105-18.
17. Montero MT, Matilla J, Gomez-Mampaso E, *et al.* Geranylgeraniol regulates negatively caspase-1 autoprocessing: implication in the Th1 response against Mycobacterium tuberculosis. *J Immunol* 2004; 173: 4936-44.

18. Drenth JP, Cuisset L, Grateau G, *et al.* Mutations in the gene encoding mevalonate kinase cause hyper-IgD and periodic fever syndrome. International Hyper-IgD Study Group. *Nat Genet* 1999; 22: 178-81.

19. Houten SM, Kuis W, Duran M, *et al.* Mutations in MVK, encoding mevalonate kinase, cause hyperimmunoglobulinaemia D and periodic fever syndrome. *Nat Genet* 1999; 22: 175-7.

20. Mandey SH, Kuijk LM, Frenkel J, *et al.* A role for geranylgeranylation in interleukin-1beta secretion. *Arthritis Rheum* 2006; 54: 3690-5.

21. Houten SM, Schneiders MS, Wanders RJ, *et al.* Regulation of isoprenoid/cholesterol biosynthesis in cells from mevalonate kinase-deficient patients. *J Biol Chem* 2003; 278: 5736-43.

22. Drenth JP, van Deuren M, van der Ven-Jongekrijg J, *et al.* Cytokine activation during attacks of the hyperimmunoglobulinemia D and periodic fever syndrome. *Blood* 1995; 85: 3586-93.

23. Drenth JP, van der Meer JW, Kushner I. Unstimulated peripheral blood mononuclear cells from patients with the hyper-IgD syndrome produce cytokines capable of potent induction of C-reactive protein and serum amyloid A in Hep3B cells. *J Immunol* 1996; 157: 400-4.

24. Vandesompele J, De Preter K, Pattyn F, *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; 3: RESEARCH0034.

25. Harnois T, Constantin B, Rioux A, *et al.* Differential interaction and activation of Rho family GTPases by p210bcr-abl and p190bcr-abl. *Oncogene* 2003; 22: 6445-54.

26. Basak C, Pathak SK, Bhattacharyya A, *et al.* NF-kappaB- and C/EBPbeta-driven interleukin-1beta gene expression and PAK1-mediated caspase-1 activation play essential roles in interleukin-1beta release from Helicobacter pylori lipopolysaccharide-stimulated macrophages. *J Biol Chem* 2005; 280: 4279-88.

27. Schotte P, Denecker G, Van Den Broeke A, *et al.* Targeting Rac1 by the Yersinia effector protein YopE inhibits caspase-1-mediated maturation and release of interleukin-1beta. *J Biol Chem* 2004; 279: 25134-42.

28. Dunford JE, Rogers MJ, Ebetino FH, *et al.* Inhibition of protein prenylation by bisphosphonates causes sustained activation of Rac, Cdc42, and Rho GTPases. *J Bone Miner Res* 2006; 21: 684-94.

29. Cerretti DP, Kozlosky CJ, Mosley B, *et al.* Molecular cloning of the interleukin-1 beta converting enzyme. *Science* 1992; 256: 97-100.

30. Gu Y, Kuida K, Tsutsui H, *et al.* Activation of interferon-gamma inducing factor mediated by interleukin-1beta converting enzyme. *Science* 1997; 275: 206-9.

31. Thornberry NA, Bull HG, Calaycay JR, *et al.* A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* 1992; 356: 768-74.

32. Kuijk LM, Mandey SH, Schellens I, *et al.* Statin synergizes with LPS to induce IL-1beta release by THP-1 cells through activation of caspase-1. *Mol Immunol* 2008; 45: 2158-65.

33. Kobayashi Y, Yamamoto K, Saido T, *et al.* Identification of calcium-activated neutral protease as a processing enzyme of human interleukin 1 alpha. *Proc Natl Acad Sci USA* 1990; 87: 5548-52.

34. Barnoy S, Kosower NS. Caspase-1-induced calpastatin degradation in myoblast differentiation and fusion: cross-talk between the caspase and calpain systems. *FEBS Lett* 2003; 546: 213-7.

35. Brough D, Le Feuvre RA, Wheeler RD, *et al.* Ca^{2+} stores and Ca^{2+} entry differentially contribute to the release of IL-1 beta and IL-1 alpha from murine macrophages. *J Immunol* 2003; 170: 3029-36.

36. Kuida K, Lippke JA, Ku G, *et al.* Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science* 1995; 267: 2000-3.

37. Keller M, Ruegg A, Werner S, *et al.* Active caspase-1 is a regulator of unconventional protein secretion. *Cell* 2008; 132: 818-31.

38. Kou R, Sartoretto J, Michel T. Regulation of Rac1 by simvastatin in endothelial cells: differential roles of AMP-activated protein kinase and calmodulin-dependent kinase kinase-beta. *J Biol Chem* 2009; 284: 14734-43.

39. Kuijk LM, Beekman JM, Koster J, *et al.* HMG-CoA reductase inhibition induces IL-1beta release through Rac1/PI3K/PKB-dependent caspase-1 activation. *Blood* 2008; 112: 3563-73.

40. Negre-Aminou P, van Leeuwen RE, van Thiel GC, *et al.* Differential effect of simvastatin on activation of Rac(1) vs. activation of the heat shock protein 27-mediated pathway upon oxidative stress, in human smooth muscle cells. *Biochem Pharmacol* 2002; 64: 1483-91.

41. Danesh FR, Anel RL, Zeng L, *et al.* Immunomodulatory effects of HMG-CoA reductase inhibitors. *Arch Immunol Ther Exp (Warsz)* 2003; 51: 139-48.

42. Zeiser R, Maas K, Youssef S, *et al.* Regulation of different inflammatory diseases by impacting the mevalonate pathway. *Immunology* 2008.