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Statins (HMG-CoA reductase inhibitors) reduce CD40 expression in human vascular cells

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Abstract

Objective: HMG-CoA reductase inhibitors (statins) possess anti-inflammatory and immunomodulatory properties that are independent of their lipid-lowering action. As the CD40–CD40L signaling pathway is implicated in the modulation of inflammatory responses between vascular cells, involving adhesion molecules, pro-inflammatory cytokines, chemokines, we sought to investigate the potential role of statins in regulating the expression of CD40. **Methods and Results:** Using Western blot, flow cytometry and immunohistochemistry analyses, we observed that four different statins reduced IFN-γ-induced CD40 expression in human vascular cells (endothelial cells, smooth muscle cells, macrophages and fibroblasts). This effect was dose-dependent (from 5 μM to 80 nM) and reversed by addition of L-mevalonate. Activation of vascular cells by human recombinant CD40L, as measured by ELISA for IL-6, IL-8 and MCP-1, was strongly reduced when cells were treated with statins. Immunostaining of human carotid atherosclerotic lesions of patients subjected to statin treatment revealed less CD40 expression on a 'per vascular cell' basis compared to control patients. Although many pleiotropic effects of statins are mediated by nitric oxide synthase (NOS)- or peroxisome proliferator-activated receptor (PPAR)-dependent signaling pathways, we observed similar statin-induced reduction of CD40 expression using NOS inhibitors or different PPAR ligands. **Conclusion:** Statins decrease CD40 expression and CD40-related activation of vascular cells. These effects are partially reversed by the HMG-CoA reductase product L-mevalonate and are mediated by NOS- or PPAR-dependent pathways. Altogether, these findings provide mechanistic insight into the beneficial effects of statins on atherogenesis. They also provide a scientific rationale for the use of statins as immunomodulators after organ transplantation.

Keywords: Atherosclerosis; Cytokines; Immunology; Lipid metabolism; Receptors

1. Introduction

Statins are effective lipid-lowering agents, extensively used in medical practice [1]. They are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme crucial for cholesterol synthesis. Several large clinical trials have demonstrated that these drugs greatly reduce cardiovascular-related morbidity and mortality in patients with and without coronary disease [2,3]. Recently, in vitro and in vivo findings have indicated that statins, beside their lipid-lowering effects, possess anti-inflammatory properties [4]. In addition, we recently demonstrated that these drugs might also be recognized as a new type of immunosuppressor [5].

Atherosclerosis is considered as an immuno-inflammatory disease [6,7] and increasing evidence suggests a

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central role for the CD40–CD40L signaling pathway in the pathogenesis of this disease [8,9]. Recently, two reports demonstrated that hypercholesterolemia, one of the major cardiovascular risk factors, increases CD40 and CD40L expression on platelets and monocytes [10] as well as plasma levels of soluble CD40L [11]. In addition, it has been shown that blocking CD40–CD40L interactions significantly prevents the development of atherosclerotic plaques and reduces already pre-established lesions [12,13]. Moreover, CD40 signaling has been implicated in other chronic disorders such as rheumatoid arthritis, multiple sclerosis and allograft rejection following organ transplantation [14].

CD40L (recently renamed CD154) and CD40 are members of the tumor necrosis factor (TNF) and TNF-receptor family, respectively. The original function of CD40L in T cell-dependent humoral immunity involves the activation and differentiation of B-lymphocytes, the switching of immunoglobulin classes as well as the formation of germinal center and memory cells. More recently, activation of vascular cells such as macrophages (MΦ), endothelial cells (ECs) and smooth muscle cells (SMCs) via CD40 signaling has been shown to induce inflammatory responses with expression of adhesion molecules and secretion of pro-inflammatory cytokines, chemokines, matrix metalloproteinases and tissue factor [15]. All these molecules are known to be crucial in the pathogenesis of atherosclerosis as well as during graft-versus-host disease.

In view of the large clinical beneficial effects of statins on cardiovascular morbidity and mortality, and the emergence of numerous non-lipid lowering anti-inflammatory effects of these drugs, we sought to investigate whether statins regulate the expression of CD40 in vascular cells.

2. Methods

2.1. Reagents

Human and mouse recombinant IFN-γ were obtained from Endogen (Cambridge, MA, USA) and R&D Systems (Abingdon, UK), respectively. The statins used in these studies, i.e. atorvastatin, simvastatin, lovastatin and pravastatin were obtained from commercial sources. Because ECs lack lactonases, to process simvastatin and lovastatin to their active forms, these agents were chemically activated before their use as previously described [16]. Mouse anti-human CD40 monoclonal antibody fluorescein isothiocyanate-conjugated (FITC) and mouse anti-human HLA-DR monoclonal antibody (FITC) were obtained from Pharmingen (San Diego, CA, USA). Rabbit anti-human CD40 polyclonal antibody, anti-rabbit IgG FITC, and HRP (horseradish peroxidase) goat anti-rabbit IgG were purchased from Santa Cruz (Santa Cruz, CA, USA), Jackson ImmunoResearch (West Grove, PA, USA) and Vector Laboratories (Burlingame, CA, USA), respectively. Antibodies for IL-6, IL-8 and MCP-1 were obtained from R&D. L-Mevalonate was purchased from Sigma (St. Louis, MO, USA). Human recombinant CD40 ligand (rCD40L) was generated as described previously [17]. L-NAME was purchased from Sigma. 15d-PGJ_2 was obtained from Calbiochem (La Jolla, CA, USA), ETYA from Sigma, WY14643 from Biomol (Plymouth Meeting, PA, USA), and Troglitazone a gift from Park Davis Pharmaceuticals (Morris Plains, NJ, USA).

2.2. Cells

The investigation conforms with the principles outlined in the Declaration of Helsinki (Cardiovasc Res 1997;35:2-3) as well as the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH publication No. 85-23, revised 1996). Human vascular ECs and SMCs were isolated from saphenous veins and cultured as described previously [5,18]. Primary human fibroblasts were isolated from nasal polyps and cultured as previously described [19]. Cells were used at passages two to four for all experiments. Human monocytes were obtained from peripheral blood of healthy donors as described previously [20]. They were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS) for 10 days. The human Raji cell line was obtained from ATCC (Rockville, MD, USA).

2.3. Western blot analysis

Cells were harvested in ice-cold radio-immuno-precipitation assay (RIPA) solubilization buffer, and total amounts of protein determined using a bicinchoninic acid quantification assay (Pierce, Rockford, IL, USA). Western blotting was performed as described before [5]. Blots were blocked in 5% defatted dry milk/phosphate-buffered saline (PBS)/0.1% Tween, incubated for 1 h at room temperature with primary antibody (1:40; rabbit polyclonal anti-CD40), or mouse monoclonal anti-human β-actin (1:5000; Pharmingen) as control of loading, followed by 1 h incubation with secondary antibody (1:10,000; Jackson ImmunoResearch). Quantification was performed using AIDA software (Raytest, Urdorf, Switzerland).

2.4. Flow cytometry

Cells were incubated with FITC-conjugated specific antibody (60 min, 4 °C) and analyzed in a Becton Dickinson FACScan flow cytometer as described [5]. At least 100,000 viable cells were analyzed per condition. Data were analyzed using CELLQUEST software (Becton Dickinson).

2.5. Cytokine assays

Release of IL-6, IL-8 and MCP-1 was measured using a

sandwich-type enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D). Experiments were performed in the presence of polymyxin B (1 μg/ml). Optical density was measured at 450 nm using a Dynatech plate reader (Chantilly, VA, USA). The amount of IL-6, IL-8 and MCP-1 detected was calculated from a standard curve prepared with the recombinant protein. Samples were assayed in duplicate.

2.6. Fluorescence immunolabeling

Monocytes/macrophages grown on coverslips were rinsed and fixed for 15 min with paraformaldehyde (4%). Coverslips were rinsed and cells incubated successively in 0.5 M NH_4Cl/PBS for 15 min and PBS supplemented with 2% bovine serum albumin (Sigma) for a further 20 min. Human macrophages were incubated overnight with primary rabbit polyclonal anti-CD40 (1:50) in 10% normal goat serum (Sigma)/PBS. After rinsing, human macrophages were incubated with secondary antibodies FITC-conjugated (1:800) for 3 h. All steps were performed at room temperature and between incubation steps cells were rinsed with PBS. Finally, coverslips were mounted on slides in Vectashield (Vector Laboratories). Cells were examined using a Zeiss Axiophot microscope. Replacement of the primary antibody with PBS/10% normal goat serum was used to control the specificity of the immunolabeling of human macrophages.

2.7. Immunohistochemistry

Surgical specimens of human carotid atheroma were obtained by protocols approved by the Review Committee at the University Hospital of Geneva. All atherosclerotic carotid specimen analyzed were obtained from patients treated (for at least 3 months) or not with the simvastatin. Materials were directly embedded in OCT compound or snap-frozen in liquid nitrogen. There was no significant difference for age, gender, cardiovascular risk factors and other medications between patients treated or not with simvastatin (Table 1). Serial cryostat sections (5 μm) were cut, air dried onto microscope slides (Fisher Scientific, Wohlen, Switzerland), and fixed in acetone at −20 °C for 5 min. Sections were pre-incubated with blocking buffer (PBS/Tween with 8% normal horse serum) and then incubated successively with an anti-human CD40 antibody for 1 h. Thereafter, sections were incubated with biotinylated secondary antibody (45 min; Vector Laboratories) followed by avidin–biotin–alkaline phosphatase complex (Vectastain ABC kit). Antibody binding was visualized by alkaline phosphatase substrate (Vector Laboratories). Quantitative analyses for CD40 and CD68 expression were performed with a computer-based color image analysis system [21].

Table 1
Baseline characteristics of patients in the simvastatin and control groups

	Simvastatin ($n=18$)	Control ($n=14$)
Age (years)	68	65
Sex (F/M)	8/10	8/6
Diabetes (%)	32	38
Smokers (%)	34	30
Hypertension (%)	73	68
Medication (%)		
Aspirin	33	28
ACEIs	45	48
β-Blockers	24	22
Anti-diabetic agents	33	38
Diuretics	32	21
Total cholesterol (mmol/l)	5.3 (±1.3)	6.4 (±1.4)
LDL-c,	3.3 (±1.1)	4.1 (±1.2)
HDL-c	1.3 (±0.3)	1.2 (±0.3)
Triglycerides	1.9 (±1.4)	2.3 (±1.4)

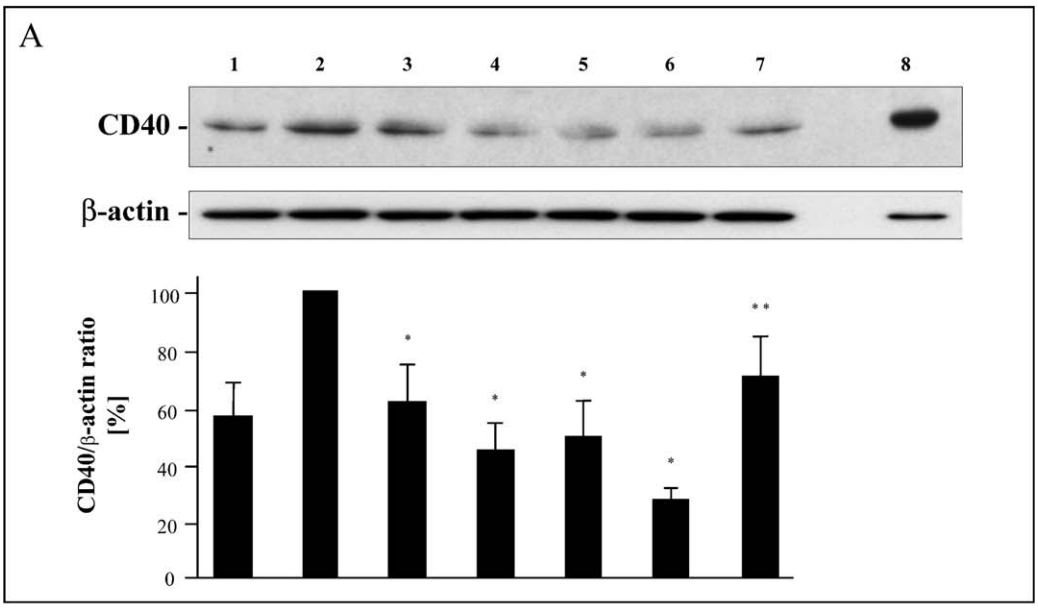
2.8. Statistical analysis

Data are presented as mean±S.E.M. Mean values between two groups were compared using a two-tailed Student's *t*-test, after having performed an *F*-test for homogeneity of variances. If data failed to meet requirements for use of the parametric *t*-test, a Mann–Whitney *U*-test was used. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. Statins reduce CD40 expression on vascular cells

To determine whether statins influence CD40 expression, human vascular endothelial cells (ECs) were activated with human recombinant IFN-γ (1000 U/ml/24 h) in the presence or absence of atorvastatin, lovastatin, pravastatin or simvastatin. As previously described, unstimulated vascular cells showed little expression of CD40, which was greatly induced by IFN-γ [18]. Western blot analysis (Fig. 1A) revealed that statin treatment reduced both, basal CD40 expression as well as IFN-γ-induced CD40. Simvastatin appeared to be the most potent inhibitor, followed by lovastatin, pravastatin and atorvastatin (Fig. 1A). Raji cells, known to express a large amount of CD40, were used as positive control (Fig. 1A, lane 8). The reduction of CD40 expression by statins was dose-dependent (Fig. 1B). The concentration range (0.08 to 10 μM) of statins used in these experiments was similar to earlier reports [5,22], and neither cell survival nor protein synthesis was affected at these concentrations (data not shown). Interestingly, simvastatin treatment significantly reduced CD40 expression (Fig. 1B) at a concentration of only 80 nM, which corresponds to the effective serum levels seen with statin treatment in clinical practice [23]. These effects of statins on CD40 expression were specific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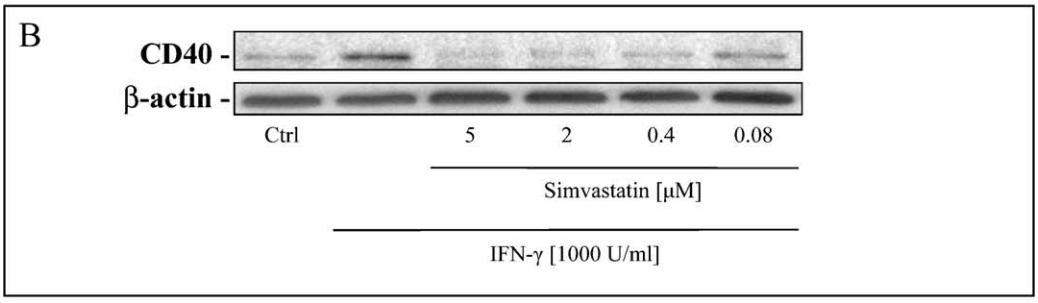
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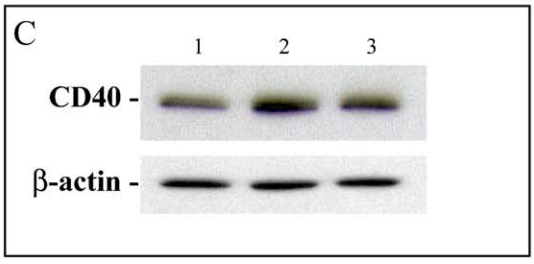
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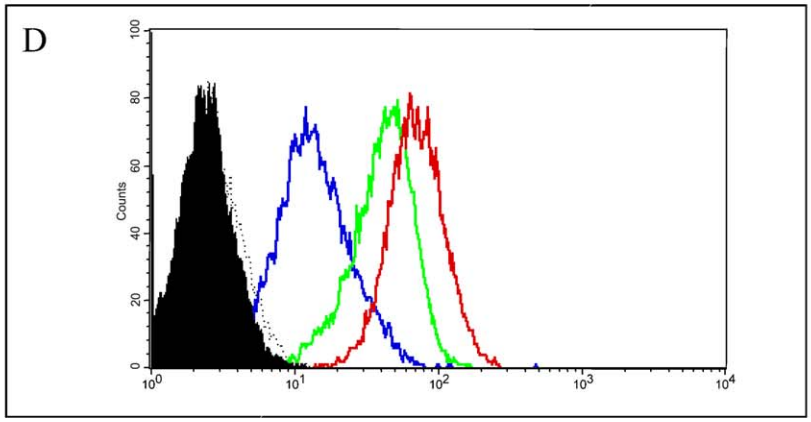
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for the inhibition of the HMG-CoA reductase enzyme, since addition of L-mevalonate largely reversed the statin-induced inhibition of CD40 expression (Fig. 1A). Similar results were obtained for human vascular SMCs (Fig. 1C). These results were confirmed using flow cytometry analysis (Fig. 1D). Western blot results were supported by immunohistochemical analysis on human monocyte–macrophages (Fig. 2A–F) and on human fibroblasts (Fig. 2G–I), two other cell types present in atherosclerotic lesions. Taken together, these experiments demonstrate that statin treatment decreased CD40 expression on all human vascular cells tested.

3.2. Statins inhibit functional CD40 signaling

To examine functional consequences of statin treatment on CD40 expression, we stimulated vascular cells with human recombinant CD40 ligand (rCD40L) in the presence or absence of the statins. As presented in Fig. 3 for ELISA experiments on ECs, rCD40L induced the secretion of the pro-inflammatory molecules IL-6, IL-8 and MCP-1, as previously described [17,24]. When stimulated in the presence of the statins, the synthesis and secretion of all these pro-inflammatory proteins were largely reduced. As previously described, statins also reduced constitutive cytokine secretion, these effects were however not statistically significant. The statin-induced reduction in cytokine/chemokine secretion was partially reversed by the addition of L-mevalonate (Fig. 3A–C). Specificity of these experiments was confirmed by blocking CD40–CD40L signaling with the addition of an anti-CD40L antibody (Fig. 3C).

3.3. Statin treatment decreases CD40 expression within human atherosclerotic tissue

In view of our findings that statins reduce CD40 expression and CD40 signaling in vitro, we decided to explore the expression of CD40 on vascular cells within human atherosclerotic lesions obtained from patients treated or not with simvastatin (20 mg/day for at least 3 months). Baseline patients characteristics were not statistically different and are shown in Table 1. Immunohistochemical in situ and Western blot analyses of human carotid arteries revealed that atherosclerotic lesions from patients under statin therapy expressed much less CD40 compared to control patients (Fig. 4A, B). In addition, we observed that individual macrophages within lesions from statin-treated patients showed markedly less CD40 expression compared to individual macrophages in lesions of control patients (Fig. 4C). This reduction was mainly detected within the shoulder region of the atherosclerotic plaques.

3.4. Statin reduction of CD40 expression is neither mediated via nitric oxide nor via PPARs

Due to numerous recent reports demonstrating that several anti-inflammatory effects of statins could be largely related to their ability to stabilize and increase nitric oxide synthase (NOS) [25], we investigated whether statins reduce CD40 expression via NOS pathways. To address this question, we stimulated human ECs with recombinant IFN-γ and simvastatin in the presence or absence of the NOS inhibitor L-NAME. As shown in Fig. 5 by flow cytometry analysis, the level of CD40 expression after statin treatment was similarly reduced with or without L-NAME treatment.

Furthermore, as statins have been recently reported to activate PPARs [26], we investigated whether PPAR ligands could regulate CD40 expression on human vascular cells. We did not detect any effect of either PPAR-α or PPAR-γ ligands on CD40 cell surface expression following IFN-γ activation (Fig. 6). These findings indicate that neither NOS nor PPARs are involved in the reduction of CD40 expression by statins on vascular cells.

4. Discussion

Increasing evidence supports the central role of the CD40–CD40L signaling pathway in atherosclerosis. Activation of vascular cells via CD40–CD40L interactions has been shown to induce inflammatory responses with expression of adhesion molecules, secretion of pro-inflammatory cytokines, matrix metalloproteinases, tissue factor and chemokines [8,9,18], molecules considered as crucial players in atherogenesis. Recent in vitro and in vivo findings suggest that statins also have anti-inflammatory properties [24,27]. Statins inhibit HMG-CoA reductase that

Fig. 1. Statins decrease IFN-γ-induced CD40 expression on human endothelial cells. (A–C) Western blot analysis for CD40. (A) Human vascular ECs under unstimulated conditions (1); treated with IFNγ (1000 U/ml, 24 h) alone (2); or in the presence of atorvastatin (10 μM) (3); lovastatin (10 μM) (4); pravastatin (20 μM) (5); simvastatin (10 μM) (6); or simvastatin (10 μM) and L-mevalonate (400 μM) (7); Raji cells under unstimulated condition as positive control (8). *$P<0.05$ (compared to 2); **$P<0.05$ (compared to 6). Quantification of Western blots is expressed as ratio of CD40/β-actin signal for each samples. (B) Human vascular ECs under unstimulated conditions (Ctrl), or treated with IFNγ (1000 U/ml, 24 h) alone; or in the presence of simvastatin (5 to 0.08 μM). β-actin is shown as loading control. (C) Human vascular SMCs under unstimulated conditions (1), treated with IFNγ (500 U/ml, 24 h) alone (2); or in the presence of simvastatin (10 μM) (3). β-actin is shown as loading control. (D) Flow cytometry analysis for CD40. Human vascular ECs treated with IFNγ (1000 U/ml, 24 h) alone (green line); or in the presence of simvastatin (5 μM) (blue line). Expression of MHC class II for ECs stimulated with IFNγ (1000 U/ml, 24 h) is shown as positive control (red line). Shown is a histogram representing cell numbers (*y*-axis) vs. CD40 log fluorescence intensity (*x*-axis) for 20,000 viable cells, and ECs under unstimulated conditions are shown (solid histograms) as well as isotype controls (dotted lines). For all analysis, similar results were obtained in separate experiments using cells from four different donors.

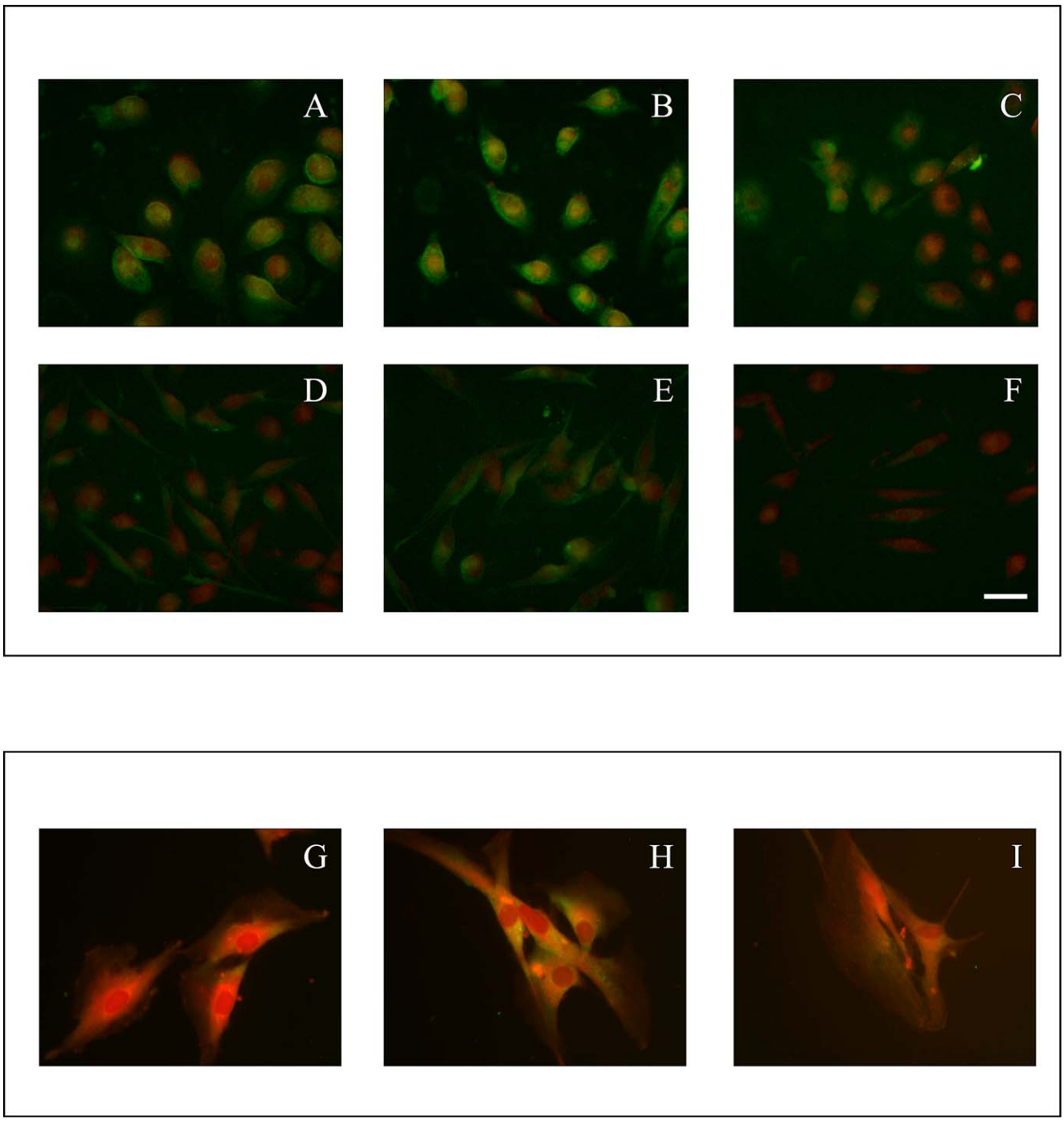


Fig. 2. Statins reduce CD40 expression on human macrophages and fibroblasts. Immunohistochemical analysis of CD40 expression (green fluorescence). Macrophages (A–F) and fibroblasts (G–I). Cells were counterstained with Evans Blue (red color). (A) Cells under unstimulated conditions. (B) Cells treated with IFN-γ alone (1000 U/ml, 24 h); or in the presence of simvastatin (5 μM) (C); simvastatin (1 μM) (D); or pravastatin (20 μM) (E). (F) Cells treated with IFN-γ (1000 U/ml, 24 h) and stained with secondary antibody only (negative control). (G) Cells under control conditions; treated with IFN-γ alone (500 U/ml, 24 h) (H); or in the presence of simvastatin (10 μM) (I). Scale bar represents 50 μm. Similar results were obtained in separate experiments using cells from three different donors.

transforms HMG-CoA into mevalonate. Indeed, as mevalonate is not only the precursor of cholesterol but also of many non-steroidal isoprenoids, statins may regulate the expression and activities of important molecules in the pathogenesis of atherosclerosis. Given the importance of CD40–CD40L in atherogenesis as well as the pleiotropic effects of statins in this disease, we sought to investigate in vitro and in vivo the effect of statins on CD40 expression as well as on related CD40 activation in human vascular cells.

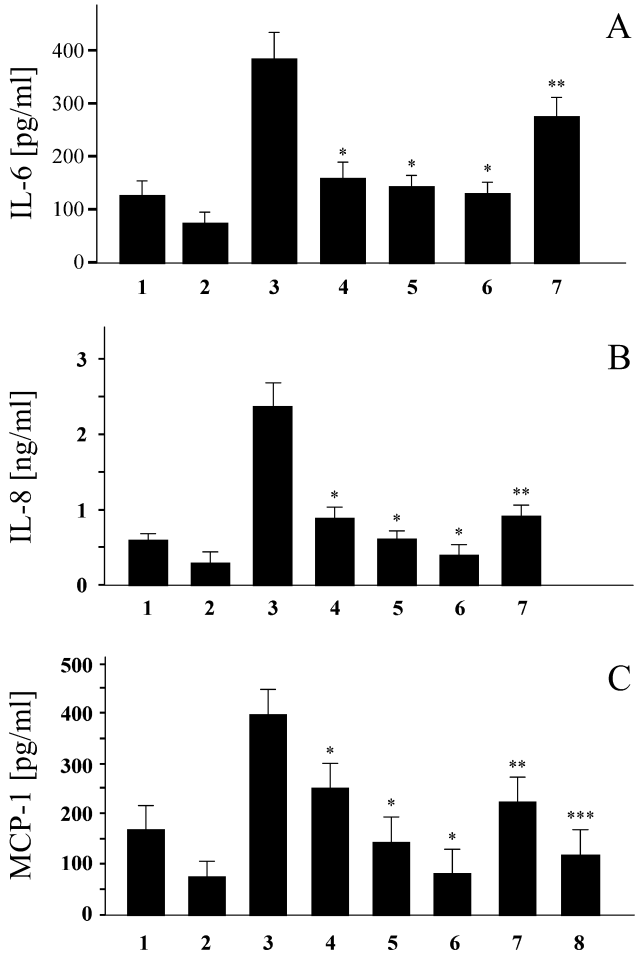


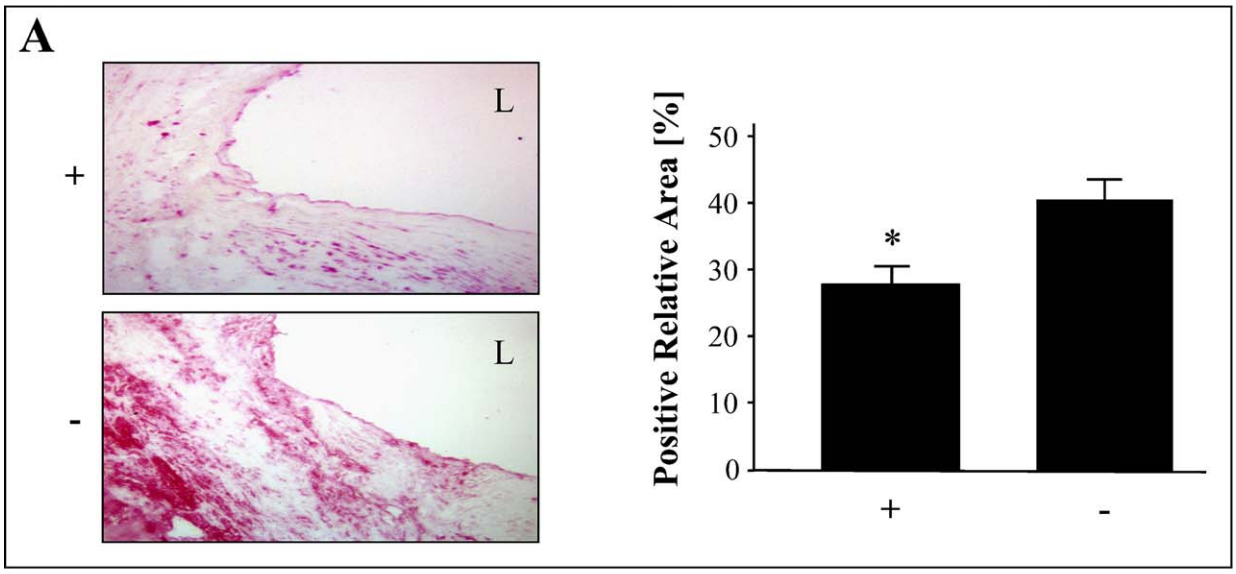
Fig. 3. Statins reduce CD40 function on CD40L-activated human vascular endothelial cells. IL-6 (A), IL-8 (B) and MCP-1 (C) releases measured by ELISA in supernatants of ECs exposed (24 h) to normal media (1); simvastatin (10 μM) alone (2); activated by rCD40L (5 μg/ml) alone (3); or in the presence of atorvastatin (10 μM) (4); lovastatin (10 μM) (5); simvastatin (10 μM) (6), or simvastatin (10 μM) and L-mevalonate (400 μM) (7); rCD40L (5 μg/ml) preincubated with polyclonal CD40L antibody (8). Similar results were obtained in independent experiments with ECs from four different donors. *$P<0.05$ (compared to 3); **$P<0.05$ (compared to 6); ***$P<0.05$ (compared to 3).

Pro-inflammatory cytokines expressed within atheroma provide a chemotactic stimulus to adherent leukocytes, directing their migration into the intima. Our data show by different techniques that statin treatment largely reduces CD40 expression both on human ECs and on monocyte–macrophages, thereby potentially reducing EC–monocyte interactions. Similar results were obtained on murine macrophages (data not shown). Importantly, we observed a reduction of CD40 expression at a concentration of 80 nM, which is within the range of effective serum concentrations seen in clinical practice [23]. In addition, L-mevalonate largely reversed the statin-induced decreased in CD40 expression, indicating that indeed the inhibition of HMG-CoA reductase mediates the statin-induced repression of CD40 on vascular cells. These results are partly in contradiction with the data from Wagner et al. [28], which showed that the reduction of CD40 expression by statins is not mediated via mevalonate synthesis, but are in agreement with three other recent reports [10,29,30].

All four statins used in this study reduce IFN-γ-induced CD40 expression: simvastatin being the strongest, followed by lovastatin, pravastatin and atorvastatin. Although other in vitro studies have also revealed differences in potency of individual statins, these results should be interpreted with great care. One possible explanation for the strong effect of simvastatin on IFN-γ-induced CD40 expression might be that this specific statin possesses additional anti-inflammatory effects through inhibitory actions in other signaling pathways. Alternatively, simply due to different pharmacokinetic properties simvastatin may be more effectively processed by cells in culture. It is of importance to note in this respect that in vivo in mouse models both atorvastatin and simvastatin are effective treatments against multiple sclerosis and rheumatoid arthritis [30,31].

CD40 signaling plays a critical role in the initiation of the immune response by inducing inflammatory responses with secretion of pro-inflammatory cytokines and chemokines. These chemokines probably attract and direct T lymphocytes and macrophages to the atheroma, thus sustaining chronic inflammation [32,33]. Indeed, lack of chemoattractants or their receptors is known to significantly diminish atherosclerotic lesion progression in mice [34,35]. We found that statin treatment influences functional cellular CD40 signaling by reducing the release of cytokines and chemokines. By secreting these pro-inflammatory mediators and growth factors, vascular cells can promote the migration and proliferation of SMCs. In response to inflammatory stimulation, SMCs and MΦ secrete MMPs that will subsequently degrade matrix compounds of the plaque as well as other inflammatory mediators that inhibit collagen synthesis. As extracellular matrix components are considered crucial determinants of fibrous cap integrity and stability, statin-induced reduction of CD40 signaling with consequent reduction in secretion of pro-inflammatory cytokines and chemokines may not only exert beneficial actions on progression of atherosclerotic plaque development but on the composition of the plaque as well. Indeed, one of the major complications in clinical cardiology is plaque rupture with local thrombus formation, potentially leading to acute coronary syndromes.

Interruption of CD40–CD40L interactions by administration of anti-CD40L blocking antibody or gene targeting has been shown to prevent atherogenesis and limit the progression of established atherosclerotic lesions in mice [12,13]. Both strategies have resulted in a decrease in pro-inflammatory factors and an increase in collagen content of atherosclerotic lesions. Recently, it has been shown that in a short prospective treatment period statins reduced the pro-inflammatory state of human atheromatous lesions [36]. Our results showing that patients under statin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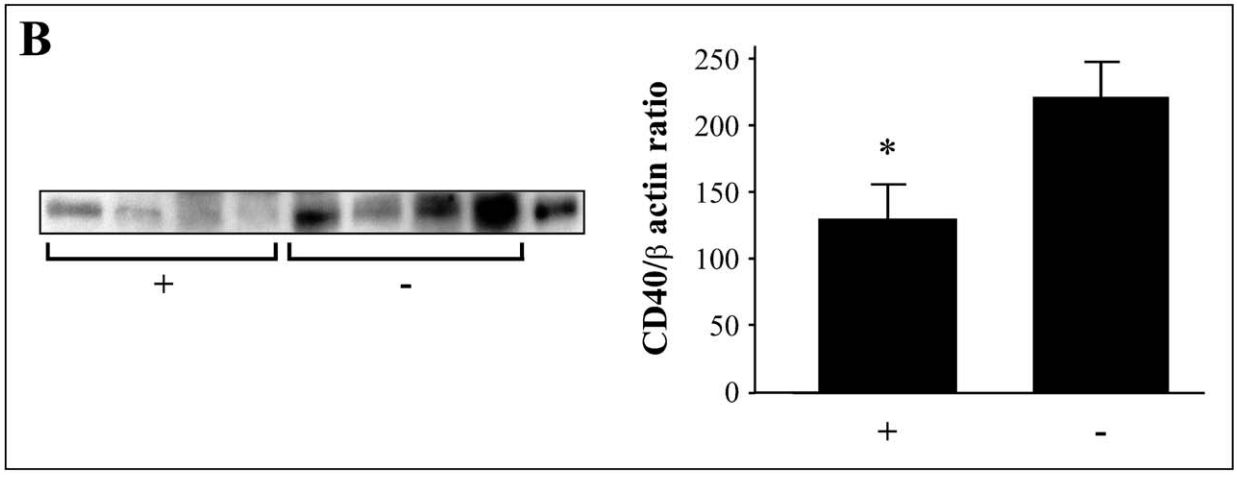
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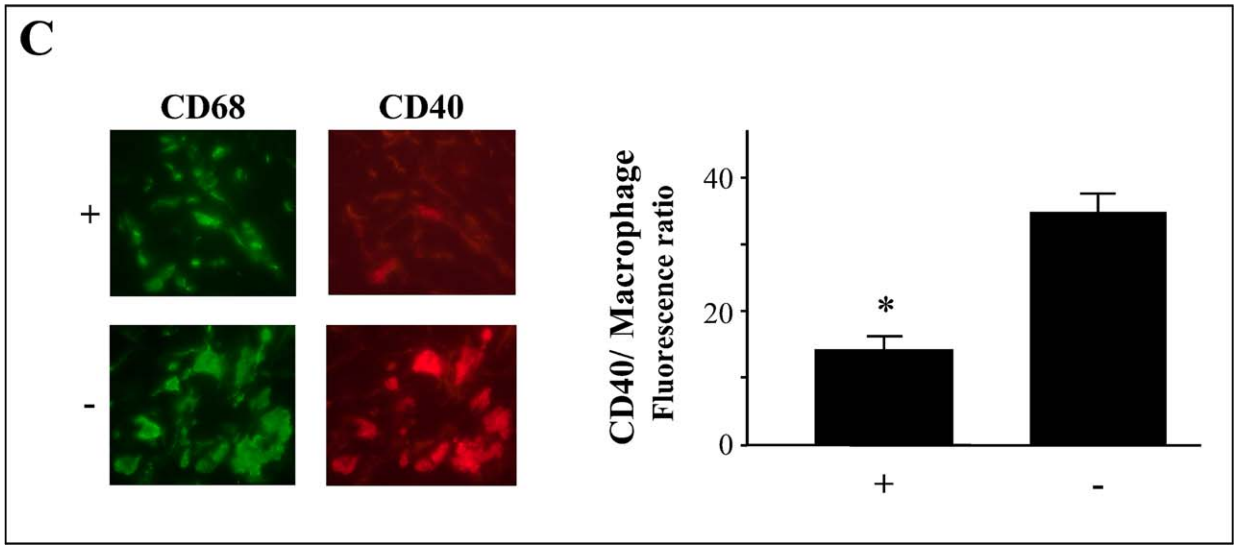
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therapy express less CD40-positive cells within atherosclerotic plaques compared to control patients without statin therapy. In addition, they show that individual macrophages express less CD40, suggesting direct effects of statins on macrophages in vivo.

Most of the anti-inflammatory effects of statins have been related to their ability to stabilize and increase NO synthase [4,22]. This endogenous vasodilator molecule has by itself anti-inflammatory properties [37]. However, our experiments illustrate that the reduction of CD40 expression after statin treatment does not involve the NOS signaling pathway. Recently, statins have been reported to activate PPARs [26]. In addition, we recently reported that PPAR-γ-induced repression of major histocompatibility complex class II (MHC-II), another molecule involved in the immune response [38]. We did not detect an effect of either PPAR-α or PPAR-γ ligands on CD40 cell surface expression following IFN-γ activation, thus excluding also these signaling pathways from mediating statin-induced reduction in CD40 expression.

One of the major problems following organ transplantation is the development of graft-versus-host disease. Indeed, clinical studies have suggested a better outcome after cardiac transplantation in patients subjected to statin therapy [39,40]. We recently demonstrated that statins are involved in the immune response by inhibiting IFN-γ induced MHC-II [5], and thus might be used as immunomodulators. In addition, CD40 has been shown to play a crucial role in graft-versus-host disease [41,42]. Our current findings indicating that statins decrease CD40 expression on antigen presenting cells may therefore provide a new scientific rationale for their use as immunomodulators following organ transplantation.

In summary, we show that statins decrease CD40 expression in vascular cells in vitro as well as in vivo. By reducing CD40 expression and signaling, statins may reduce inflammation and thus stabilize atherosclerotic plaques, features believed to account for the beneficial effects of statins on cardiovascular morbidity and mortality. Our findings not only provide an additional explanation for previously unknown non-lipid effects of statins in atherogenesis, but also suggest their application in further immuno-inflammatory diseases in which CD40 is involved, such as multiple sclerosis, systemic lupus erythe-

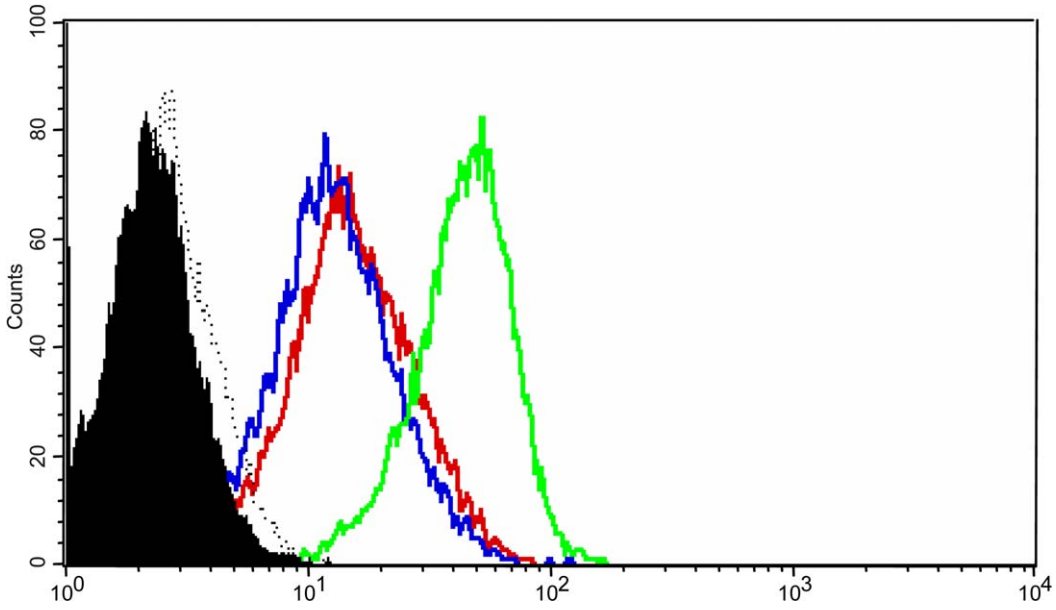

Fig. 5. Statins effect on CD40 expression is not mediated through NOS. Flow cytometry analysis for CD40 expression. Human vascular ECs treated with IFNγ (1000 U/ml, 24 h) alone (green line); or with simvastatin (5 μM) (blue line); or in the presence of simvastatin (5 μM) and L-NAME (5 mM) (red line). Shown is a histogram representing cell numbers (*y*-axis) vs. CD40 log fluorescence intensity (*x*-axis) for 20,000 viable cells, and ECs under unstimulated conditions are shown (solid histograms) as well as isotype controls (dotted lines). Similar results were obtained in independent experiments with ECs from three different donors.

Fig. 4. Statin therapy decreases CD40 expression on human atherosclerotic lesions. (A) Representative photomicrographs (×100) of CD40 expression (red color) within human carotid atherosclerotic lesions in situ. Atherosclerotic lesion in patient treated (+) or not (−) by statin therapy. L, lumen of the vessel. Quantitative analysis of CD40 expression on atherosclerotic lesions obtained from patients treated with statin (+) ($n=18$), or without statin therapy (−) ($n=14$) is shown on the right side of this panel. *$P<0.05$. (B) Western blot analysis of human carotid atherosclerotic tissues obtained from patients treated (+) or not (−) with statin therapy. Quantitative analysis of CD40 expression in patients treated with statin (+) ($n=12$), or without statin therapy (−) ($n=10$) is shown on the right side of this panel. *$P<0.05$. (C) Double immunofluorescence stain for macrophages (CD68) in green and CD40 in red in patient treated (+) or not (−) by statin therapy. *$P<0.05$. Similar results were obtained in separate experiments using tissues from five different donors.

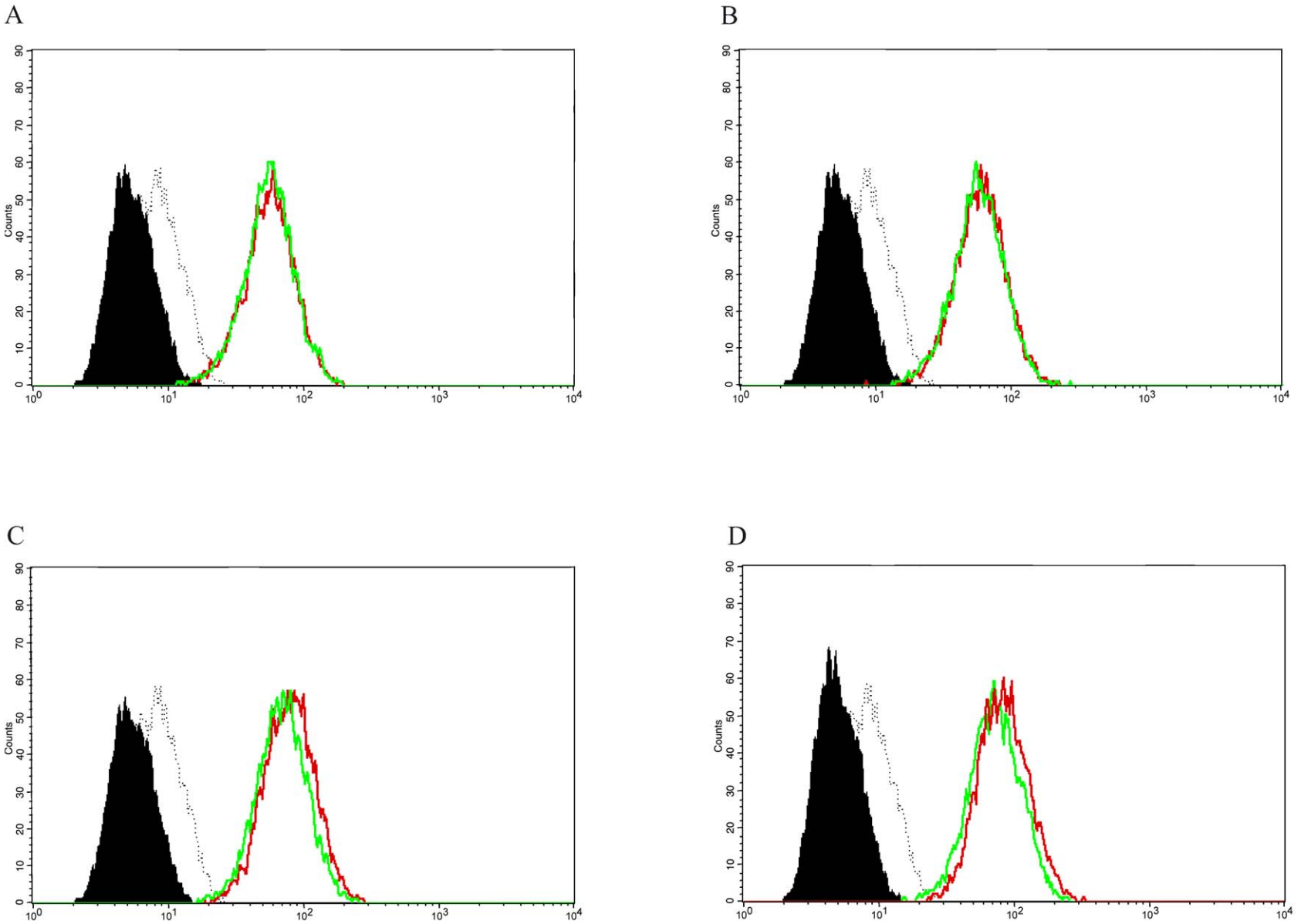

Fig. 6. Effects of PPAR ligands on IFN-γ-induced CD40 expression on human endothelial cells. Flow cytometry analysis for CD40 expression. Human vascular endothelial cells (ECs) treated with IFN-γ (500 U/ml, 24 h) alone (green line), or with the PPAR-γ ligands (red lines) 15d-PGJ_2 (10 μmol/l) (A), Troglitazone (50 μmol/l) (B); or with the PPAR-α ligands (red lines) ETYA (50 μmol/l) (C), and WY14643 (50 μmol/l) (D). Each panel is a histogram representing cell numbers (*y*-axis) vs. log fluorescence intensity (*x*-axis) for 15,000 viable cells, and ECs under unstimulated conditions are shown (solid histograms) as well as isotype controls (dotted lines). Similar results were obtained in independent experiments with ECs from five different donors.

matosus, autoimmune diabetes mellitus, chronic arthritis and graft-versus-host disease.

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