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# Arteriosclerosis, Thrombosis, and Vascular Biology



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## **CCL20 Is Increased in Hypercholesterolemic Subjects and Is Upregulated By LDL in Vascular Smooth Muscle Cells : Role of NF- $\kappa$ B**

Olivier Calvayrac, Ricardo Rodríguez-Calvo, Judith Alonso, Josune Orbe, Jose Luis Martín-Ventura, Anna Guadall, Maurizio Gentile, Oriol Juan-Babot, Jesus Egido, Oscar Beloqui, José A. Paramo, Cristina Rodríguez and José Martínez-González

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# CCL20 Is Increased in Hypercholesterolemic Subjects and Is Upregulated By LDL in Vascular Smooth Muscle Cells

## Role of NF- $\kappa$ B

Olivier Calvayrac, Ricardo Rodríguez-Calvo, Judith Alonso, Josune Orbe, Jose Luis Martín-Ventura, Anna Guadall, Maurizio Gentile, Oriol Juan-Babot, Jesus Egido, Oscar Beloqui, José A. Paramo, Cristina Rodríguez, José Martínez-González

**Objective**—Our aim was to analyze the regulation of CC Chemokine ligand 20 (CCL20) by LDL in human vascular smooth muscle cells (VSMC).

**Methods and Results**—In asymptomatic subjects, circulating CCL20 levels were higher in patients with hypercholesterolemia ( $18.5 \pm 3.2$  versus  $9.1 \pm 1.3$  pg/mL;  $P < 0.01$ ). LDL induced the expression of CCL20 in VSMC in a dose- and time-dependent manner. Increased levels of CCL20 secreted by LDL-treated VSMC significantly induced human lymphocyte migration, an effect reduced by CCL20 silencing. The upregulation of CCL20 by LDL was dependent on the activation of kinase signaling pathways and NF- $\kappa$ B. By site-directed mutagenesis, electrophoretic mobility shift assay, and chromatin immunoprecipitation, we identified a NF- $\kappa$ B site (−80/−71) in CCL20 promoter critical for LDL responsiveness. Lysophosphatidic acid mimicked the upregulation of CCL20 induced by LDL, and minimal oxidation of LDL increased the ability of LDL to induce CCL20 through a mechanism that involves lysophosphatidic acid receptors. CCL20 was overexpressed in atherosclerotic lesions from coronary artery patients, colocalizing with VSMC. CCL20 was detected in conditioned media from healthy human aorta and its levels were significantly higher in secretomes from carotid endarterectomy specimens.

**Conclusion**—This study identifies CCL20 in atherosclerotic lesions and recognizes this chemokine as a mediator highly sensitive to the inflammatory response elicited by LDL. (*Arterioscler Thromb Vasc Biol.* 2011;31:2733-2741.)

**Key Words:** atherosclerosis ■ gene expression ■ lipoproteins ■ molecular biology ■ vascular biology

Atherosclerosis is essentially an inflammatory chronic disease.<sup>1–3</sup> Inflammation is a necessary response to injury and infection. Virtually all cardiovascular risk factors are capable of promoting an inflammatory response; among them, however, elevated levels of plasma cholesterol, in particular LDL, are recognized as one of the most important risk factors for atherosclerosis.<sup>4,5</sup> The inflammatory response involves the coordinated regulation of cell adhesion and migration and the establishment of a chemotactic gradient that guides inflammatory cells to damaged tissues. Key elements in this communication network are cytokines and chemokines, which orchestrate the recruitment, survival, expansion, and effector function of inflammatory cells.<sup>6–8</sup>

Chemokines are a superfamily of structurally related small chemotactic cytokines that control leukocyte function through interactions with their cognate 7-transmembrane-domain G protein-coupled receptors. Monocytes/macro-

phages and T lymphocytes are the most abundant inflammatory cells found in atherosclerotic plaques,<sup>9,10</sup> but also B cells, dendritic cells, and neutrophils contribute to the pathogenesis of atherosclerosis.<sup>9,11,12</sup> Native and modified LDL modulate the expression of key genes involved in the recruitment and trafficking of inflammatory cells including cellular adhesion molecules and chemokines such as monocyte chemotactic protein 1.<sup>4,5,13–15</sup> Recent studies have implicated other chemokines in atherosclerosis and have extended the knowledge about the regulation of chemokines/chemokine receptors on vascular cells,<sup>6–8</sup> but the complete picture of these molecules involved in atherogenesis is not completely understood.

Increasing data involving innate and adaptive immunity in atherosclerosis,<sup>9,16,17</sup> and recent reports that emphasize the role of LDL in the response of T cells<sup>18</sup> prompt us to study the regulation of CCL20 (CC Chemokine ligand 20) by LDL. CCL20 is a chemokine that selectively attracts immature

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**Table. Patients' Characteristics and Cardiovascular Risk Factor**

Characteristics	Patients With Total Cholesterol $\leq 239$	Patients With Total Cholesterol $\geq 240$	
N	54	53	
Age (years)	50.4 $\pm$ 12.7	54.3 $\pm$ 11.0	NS
Men, n (%)	40 (74.1)	41 (77.4)	
Body mass index (kg/m <sup>2</sup> )	26.8 $\pm$ 4.9	27.7 $\pm$ 3.9	NS
Systolic blood pressure (mm Hg)	123.5 $\pm$ 21.8	129.2 $\pm$ 21.2	NS
Diastolic blood pressure (mm Hg)	78.8 $\pm$ 11.9	81.6 $\pm$ 8.8	NS
Diabetes mellitus, n (%)	6 (11.1)	7 (13.2)	
Glucose level (mg/dL)	105.5 $\pm$ 35.9	100.3 $\pm$ 19.5	NS
Dyslipidemia, n (%)	34 (63.0)	53 (100)	
Total cholesterol (mg/dL)	192.8 $\pm$ 24.7	266.5 $\pm$ 25.2	$P<0.0001$
Triglyceride (mg/dL)	105.7 $\pm$ 71.9	129.9 $\pm$ 62.9	NS
HDL-cholesterol (mg/dL)	48.5 $\pm$ 10.3	50.6 $\pm$ 13.2	NS
LDL-cholesterol (mg/dL)	123.4 $\pm$ 22.7	189.6 $\pm$ 26.1	$P<0.0001$
Smokers, n (%)	16 (29.6)	16 (30.2)	
Biochemical markers			
Fibrinogen (mg/dL)	278.3 $\pm$ 65.6	316.8 $\pm$ 64.7	$P=0.0029$
Interleukin-6 (pg/mL)	1.20 $\pm$ 0.89 (n=40)	1.86 $\pm$ 1.45 (n=43)	$P=0.0207$
vWF (activity %)	111.8 $\pm$ 50.41	104.38 $\pm$ 49.85	NS
hs-CRP (mg/dL)	0.11 $\pm$ 0.07	0.38 $\pm$ 0.31	$P<0.0001$
Mean carotid-IMT (mm)	0.75 $\pm$ 0.22	0.74 $\pm$ 0.18	NS
PROCAM risk score (%)	0.51 $\pm$ 0.8	1.51 $\pm$ 1.66	$P=0.0002$

hs-CRP indicates high-sensitivity C-reactive protein; IMT, intima-media thickness; NS, not significant; PROCAM, Prospective Cardiovascular Münster; vWF, von Willebrand factor.

dendritic cells, effector/memory T lymphocytes, and naive B cells.<sup>19,20</sup> As far we know, only one recent study has described the expression of CCL20 by cultured vascular smooth muscle cells (VSMC) from vessels susceptible to atherosclerosis.<sup>21</sup> In the present study, we identify CCL20 as a LDL-responsive gene and dissect the molecular mechanisms underlying the regulation by LDL of this chemokine that is increased in the plasma of hypercholesteremic patients and is upregulated in atherosclerotic lesions from coronary artery patients.

## Methods

Detailed explanation of the different experimental procedures is available online at <http://atvb.ahajournals.org>.

### Subjects Characteristics and Assessment of Cardiovascular Risk

A total of 107 apparently healthy subjects, free from clinically manifest atherosclerotic vascular disease, were recruited in the Internal Medicine Department at the University Clinic of Navarra. All subjects underwent ultrasonography of common carotid arteries to assess the carotid intima-media thickness as a direct measure of subclinical atherosclerosis.<sup>22</sup> The institutional ethics committee approved this study, and written informed consent was obtained from all patients. The research was performed in accordance with the Declaration of Helsinki.

### Human Artery Sampling and Preservation

Human coronary artery samples were collected from patients undergoing heart transplant surgery at the Hospital de la Santa Creu i Sant Pau (HSCSP) in Barcelona, Spain with the approval of the ethics

committee of the HSCSP. Carotid samples were obtained from patients undergoing endarterectomy at the Fundación Jimenez Díaz (FJD) with the approval of the ethics committee of the FJD. Aorta samples (kindly provided by Dr Michel and Dr Meilhac) were obtained from organ donors with the authorization of the French Biomedicine Agency and the approval of the institutional ethics committee CPP Paris Ile de France IV. To obtain secretome, tissues were incubated in serum-free RPMI medium for 24 hours at 37°C. Informed consent was obtained from patients and all studies were in accordance with the Declaration of Helsinki.

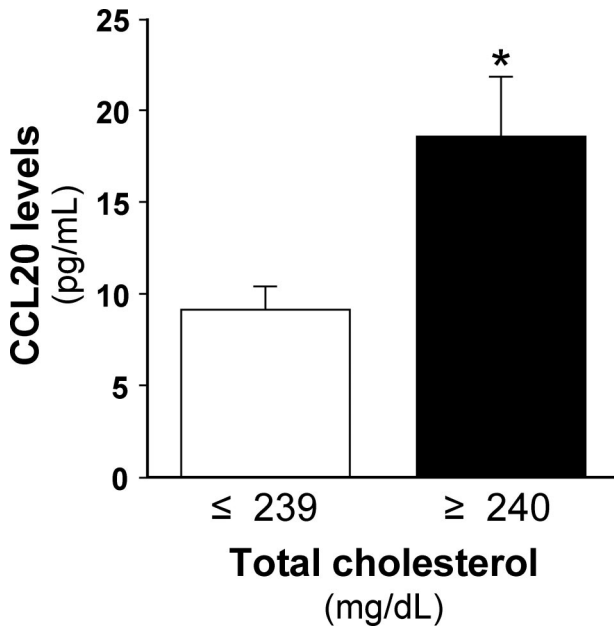
## Statistical Analyses

Data were expressed as mean $\pm$ SD (unless otherwise stated). Significant differences between groups were established by Student *t* test or 1-way ANOVA, according to the number of groups compared, using the GraphPad Instat program (GraphPad Software V2.03). Differences were considered significant at  $P<0.05$ . Associations between circulating CCL20 levels and atherosclerotic risk factors were examined by Pearson correlation test for continuous variables, and by unpaired Student *t* test for categorical variables. Multivariate linear regression analysis was performed to evaluate factors related to CCL20 and atherosclerotic risk factors that were selected by previous statistical evidence of a univariate association.

## Results

### Circulating CCL20 Levels in Hypercholesterolemic Subjects

After exclusion criteria, 107 subjects free from clinical cardiovascular disease, 54 normocholesterolemic (total cholesterol  $\leq 239$  mg/dL), and 53 hypercholesterolemic (total cholesterol  $\geq 240$  mg/dL) were included (Table). No significant differences in the prevalence of cardiovascular risk



**Figure 1.** Circulating CCL20 levels in normo- and hypercholesterolemic subjects. Circulating CCL20 levels in normocholesterolemic (total cholesterol  $\leq 239$  mg/dL;  $n=54$ ) and hypercholesterolemic subjects (total cholesterol  $\geq 240$  mg/dL;  $n=53$ ). Data are expressed as mean  $\pm$  SEM ( $P < 0.01$ ; \*vs normocholesterolemic subjects).

factors other than hypercholesterolemia were observed between both groups. Circulating levels of CCL20 were significantly higher in subjects with hypercholesterolemia (Figure 1). Hypercholesterolemic subjects also exhibited a higher cardiovascular risk as determined by the PROCAM (Prospective Cardiovascular Münster) score and higher circulating levels of inflammatory markers including fibrinogen, hs-CRP, and IL-6. CCL20 levels were positively correlated with total cholesterol ( $r=0.20$ ,  $P < 0.05$ ), LDL-cholesterol ( $r=0.20$ ,  $P < 0.05$ ), and IL-6 ( $r=0.28$ ,  $P < 0.01$ ). The association between CCL20 and hypercholesterolemia remained significant in multiple regression analysis after adjustment for other atherosclerotic risk factors (Beta=0.26,  $P=0.006$ ).

### LDL Induce CCL20 Expression in Human VSMC

CCL20 expression was upregulated by LDL in a time-dependent manner, reaching a maximum after 4 hours (Supplemental Figure IA). The effect was dose-dependent, was significant at 50  $\mu\text{g/mL}$ , and maximal at 300  $\mu\text{g/mL}$  (Supplemental Figure IB). We choose 4 hours of treatment and 300  $\mu\text{g/mL}$  LDL for further experiments. LDL also induced CCL20 expression in endothelial cells from human umbilical veins with a similar temporal pattern to that observed in VSMC (Supplemental Figure IC).

### LDL Induce CCL20 Secretion in Human VSMC

Levels of CCL20 secreted by human VSMC treated with LDL were significantly higher than those detected in control cells (Figure 2A). In cell migration assays, supernatants from LDL-treated VSMC promoted human lymphocytes migration in a similar extent than human recombinant CCL20 at a concentration equivalent to that found in these media (40 pg/mL) (Figure 2B and 2C). Cell supernatants from VSMC treated with siRNA

that efficiently prevented the upregulation of CCL20 expression and the secretion of CCL20 induced by LDL (Figure 2D, left and middle panels) significantly reduced lymphocyte migration (Figure 2D, right panel).

### LDL Increase CCL20 Transcriptional Activity in VSMC Through a NF- $\kappa$ B Response Element

Pretreatment of human VSMC with a transcriptional inhibitor prevented the increase in CCL20 mRNA levels elicited by LDL (Figure 3A). Accordingly, in transient transfection assays LDL increased CCL20 promoter activity (Figure 3B). Analysis of the CCL20 promoter sequence revealed several conserved putative binding sites for transcription factors potentially regulated by LDL among them a cAMP response element, an AP-1 and a NF- $\kappa$ B. Parthenolide and BAY 11-7082 (2 NF- $\kappa$ B inhibitors) but not NDGA (an AP-1 inhibitor) significantly prevented the upregulation of CCL20 by LDL (Figure 3C). LDL significantly activate NF- $\kappa$ B signaling in human VSMC, promoting a decrease in cytosolic levels of I $\kappa$ B $\alpha$  that parallels the translocation of p65 from the cytosol to the nucleus, effect that was prevented by parthenolide (Figure 3D and 3E).

We analyzed a series of promoter deletions and delimited LDL responsiveness to a proximal promoter region that contains a putative NF- $\kappa$ B site ( $-80/-71$ ). Mutation of this response element abrogated LDL-induced CCL20 promoter activity (Figure 4A and 4B). Consistent with this, EMSA analysis showed that LDL increase the binding to this NF- $\kappa$ B site (Figure 4C, left panel). The CCL20 probe formed several complexes with nuclear proteins from human VSMC. These DNA-binding complexes disappeared in competition experiments, and addition of an antibody against p65 supershifted complexes I and II (Figure 4C, middle and right panel). Finally, chromatin immunoprecipitation assays confirmed that, in vivo, NF $\kappa$ B binds to this site, and that LDL significantly increased NF $\kappa$ B binding (Figure 4D).

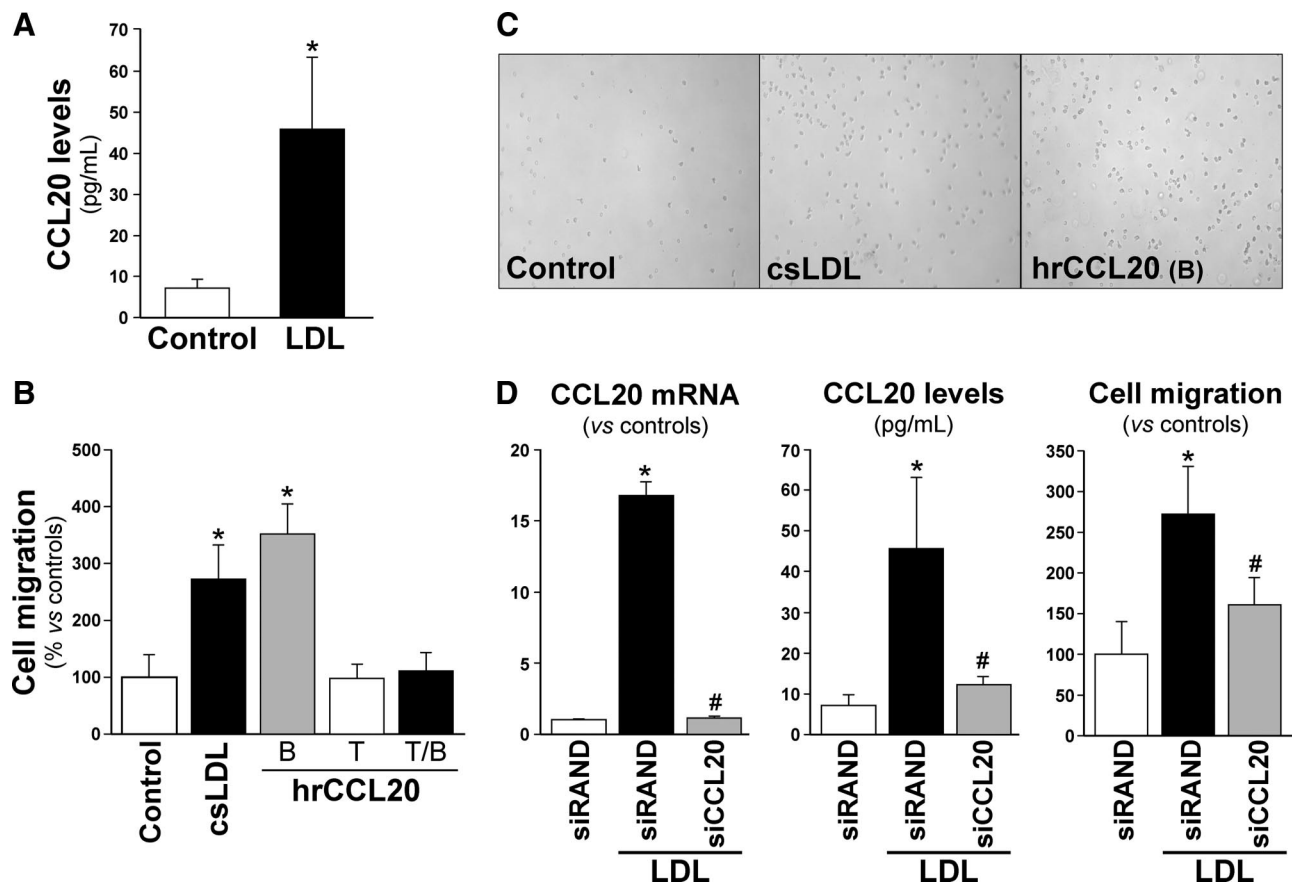
### Signaling Pathways Involved in CCL20 Induction by LDL

We used specific inhibitors in order to identify the pathways involved in the upregulation of CCL20 by LDL. LDL-induced CCL20 expression was dependent on calcium mobilization (inhibited by BAPTA-AM), PKC (inhibited by GF10933X), ERK1/2 (inhibited by U0126), and p38 MAPK activation (inhibited by SB203580) (Supplemental Figure IIA). The activation of ERK1/2 and p38 MAPK by LDL in VSMC as well as the inhibition exerted by U0126 and SB203580 is shown in Supplemental Figure IIB.

### Upregulation of CCL20 by LDL Is Mediated by Lysophosphatidic Acid Receptors

Blocking antibodies against different lipoprotein receptors including the LDL receptor, scavenger receptor class A type I, lectin-like oxidized LDL receptor 1, and CD36 did not significantly modified the upregulation of CCL20 induced by LDL (Supplemental Figure III). By contrast, pertussis toxin, an inhibitor of receptors coupled to G $_{i/o}$ -proteins, significantly reduced CCL20 mRNA levels induced by LDL (data not shown) suggesting the involvement of a bioactive component carried by LDL. Products of LDL oxidation





**Figure 2.** LDL induce the secretion of biologically relevant levels of CCL20 protein in VSMC. A, Vascular smooth muscle cells (VSMC) were stimulated with LDL (300  $\mu$ g/mL for 24 hours) and CCL20 release was assessed by EIA ( $n=6$ ;  $P<0.05$ : \*vs controls). B, Quantitative data from cell migration assays of human lymphocytes induced by supernatants from human VSMC cultured under control conditions (control), supernatants from human VSMC treated with LDL (csLDL; 300  $\mu$ g/mL for 24 hours) or human recombinant CCL20 (hrCCL20; 40 pg/mL in the bottom chamber [B]) ( $n=6$ ;  $P<0.05$ : \*vs controls). As a negative control CCL20 was added only in the top chamber (T) or in both chambers (T/B) of the transwell system. C, Representative images showing lymphocyte migration. D, VSMC were transfected with a CCL20 specific siRNA (siCCL20) or a control siRNA (siRAND), and the effect of LDL treatment on CCL20 mRNA levels, CCL20 secretion, and lymphocyte migration induced by cell supernatants were determined [ $n=6$ ;  $P<0.05$ : \*vs controls (unstimulated cells transfected with siRAND); #versus cells transfected with siRAND and stimulated with LDL].

(7-ketocholesterol, 25-hydroxycholesterol, and 4-HNE), even at concentrations that largely exceed those that could be found in native LDL,<sup>23</sup> did not affect CCL20 expression (Supplemental Figure IV). By contrast, low concentrations of lysophosphatidic acid (LPA) induced CCL20 expression mimicking the effect of LDL. Minimally oxidation of LDL, a modification that increase LPA content,<sup>24,25</sup> potentiated the ability of LDL (100  $\mu$ g/mL) to induce CCL20 expression (Supplemental Figure V). Interestingly, Ki-16425 (an antagonist of LPA receptors) impaired MAPK pathways involved in CCL20 modulation by LDL and reduced both CCL20 mRNA levels and CCL20 transcriptional activity induced by LDL and moxLDL (Supplemental Figure V).

### CCL20 Is Induced in Human Atherosclerotic Coronary Arteries

The expression of CCL20 in human coronary arteries from patients with coronary artery disease (CAD) was analyzed and compared with vessels from patients without atherosclerosis. The mRNA of this chemokine was weakly expressed in nonatherosclerotic arteries but significantly upregulated in atherosclerotic lesions ( $\approx 20$ -fold induction,  $P<0.05$ ) (Figure

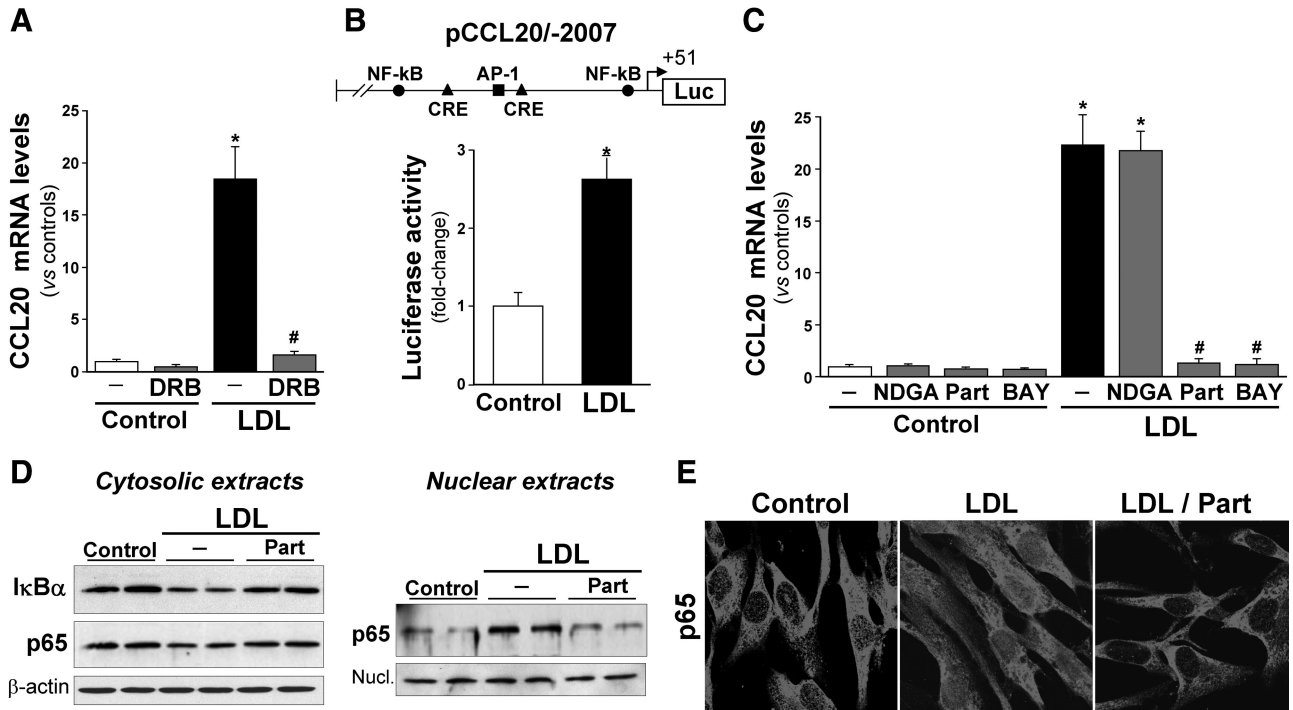
5A). Upregulation of CCL20 expression was as high as that of monocyte chemoattractant protein 1, a well-known chemokine involved in inflammation/atherogenesis; moreover, we observed a significant increase in mRNA levels of its receptor (CCR6). EMSA using a probe containing the NF- $\kappa$ B response element present in CCL20 promoter, showed higher DNA-binding activity in atherosclerotic arteries compared with nonatherosclerotic ones (Figure 5B), and by immunohistochemistry, CCL20 was mainly colocalized with VSMC in the intima of atherosclerotic coronary arteries (Figure 5C).

### CCL20 Is Released By Vascular Tissues

To assess whether vascular cells could be a source of CCL20, we analyzed CCL20 levels in secretomes from intima-media layers of human vessels. CCL20 was detected in conditioned media from healthy human aorta and its levels were significantly higher in secretomes from human carotid endarterectomy specimens (Figure 5D).

### Discussion

The onset and progression of atherosclerosis parallels the influx of inflammatory cells into the vessel wall.<sup>6,9</sup> LDL play



**Figure 3.** LDL induce CCL20 expression through a transcriptional mechanism that involves NF- $\kappa$ B. **A**, Vascular smooth muscle cells (VSMC) were induced with 300  $\mu$ g/mL LDL for 4 hours in the presence or in the absence of 5,6-dichloro-1-( $\beta$ -D-ribofuranosyl)-benzimidazol (DRB) and CCL20 expression was analyzed ( $n=9$ ;  $P<0.05$ : \*vs control cells; #vs cells treated with LDL alone). **B**, VSMC were transfected with the pCCL20/-2007 construct and treated with LDL (300  $\mu$ g/mL for 7 hours) ( $n=9$ ;  $P<0.05$ : \*vs control cells). **C**, Effect of NDGA, parthenolide (Part) and BAY 11-7082 (BAY) on CCL20 mRNA levels induced by LDL (300  $\mu$ g/mL for 4 hours) ( $n=9$ ;  $P<0.05$ : \*vs controls; #vs LDL alone). **D**, VSMC were treated with LDL (300  $\mu$ g/mL) in the presence or absence of parthenolide, and cytosolic or nuclear extracts were analyzed by Western blot. Representative immunoblots using antibodies against I $\kappa$ B $\alpha$  and p65 are shown. Beta-actin and nucleolin (Nucl.) were used as a loading control for cytosolic and nuclear extracts, respectively. **E**, Confocal microscopy analysis showing the mobilization of p65 to the nuclei in cells stimulated with LDL and the preventive effect exerted by parthenolide (LDL/Part).

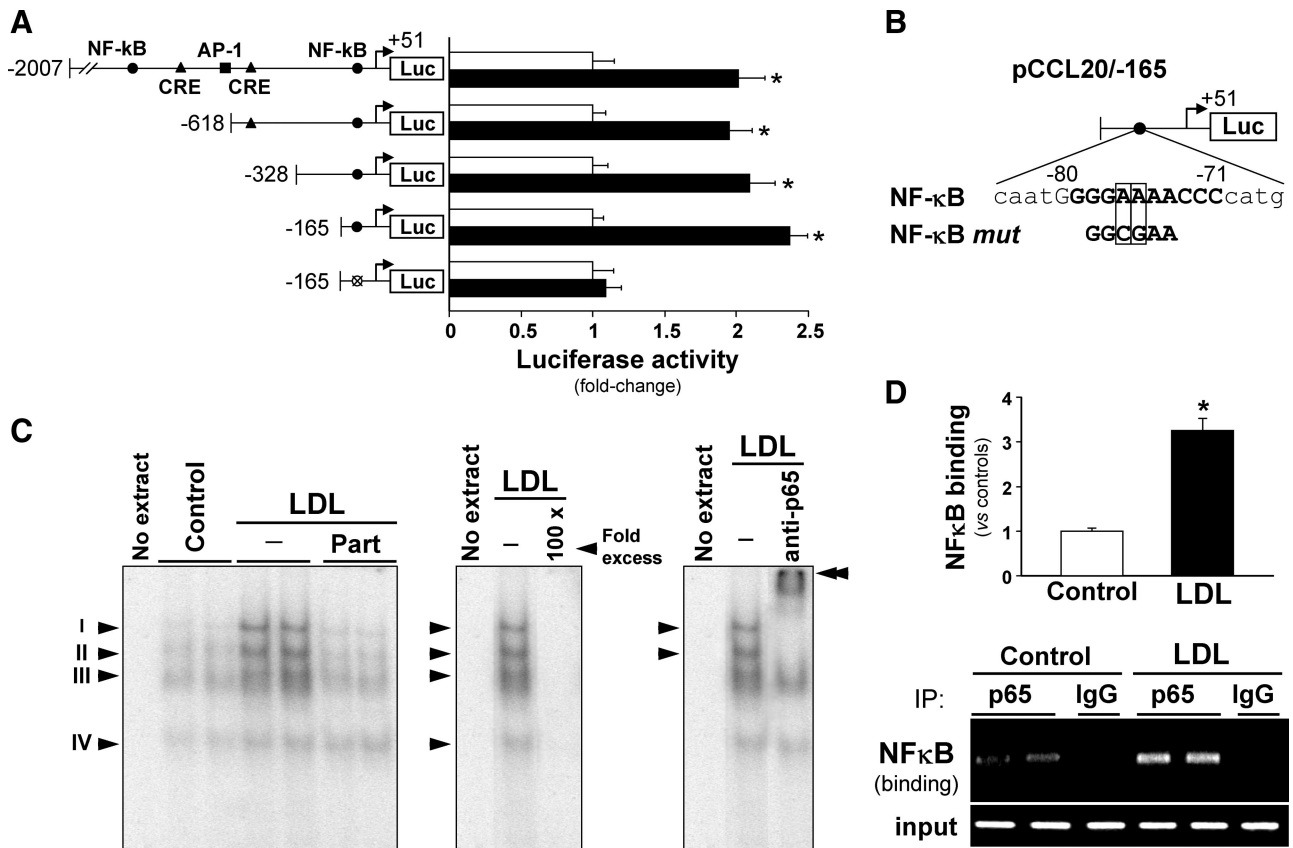
a key role in this “call-effect” as these lipoproteins modulate the expression of vascular cell adhesion molecules, chemokines, and chemokine receptors involved in the recruitment and trafficking of inflammatory cells.<sup>4,5</sup> Despite the well-documented presence of active T cells,<sup>16</sup> and dendritic cells<sup>11</sup> in human plaques, chemokines involved in the recruitment of inflammatory cells other than monocytes/macrophages remain incompletely understood. Moreover, there is no information concerning CCL20 regulation in VSMC and atherosclerosis. In the present study, we found increased CCL20 levels in serum from hypercholesterolemic patients; moreover, CCL20 was significantly upregulated in atherosclerotic lesions. In cultured human vascular cells, LDL induced biologically relevant levels of this chemokine through a transcriptional mechanism mediated by NF- $\kappa$ B.

CCL20 was identified in 1997 by 3 independent groups in screens of human cDNA libraries from liver, monocytes, and pancreatic cells and was called liver and activated-regulated chemokine, macrophage inflammatory protein-3 $\alpha$ , and Exodus, respectively.<sup>19</sup> CCL20 is a constitutive/homeostatic and inducible/inflammatory chemokine. Indeed, CCL20 is expressed both constitutively and inducibly in response to proinflammatory stimuli in lymphoid and nonlymphoid tissues and cells.<sup>19,20</sup> CCL20 is the unique chemokine ligand for CCR6, a receptor with a restricted distribution in tissues and cells such as immature dendritic cells, effector/memory T

lymphocytes, and naive B cells.<sup>19,20,26</sup> CCL20 is typically expressed at low basal levels but can be strongly induced by diverse proinflammatory stimuli.<sup>19,26</sup> In fact, CCL20 is up-regulated in inflamed areas, in particular in epithelial surfaces, in pathologies such as arthritis or cancer.<sup>19,27</sup> Here, we show that circulating levels of CCL20 were significantly higher in subjects with hypercholesterolemia, and besides total cholesterol or LDL-cholesterol, they were positively correlated with serum levels of IL-6, an inflammatory mediator regulated by LDL in vascular cells.<sup>28</sup> This is the first study showing that this chemokine, recently proposed as a potential biomarker in inflammatory diseases including rheumatoid arthritis and certain carcinomas,<sup>29,30</sup> could be a new inflammatory marker associated to hypercholesterolemia.

LDL upregulated CCL20 expression in human VSMC and increased the release of CCL20 to levels that significantly induced human lymphocyte migration in a transwell assay, supporting the biological significance of these findings. This effect involved Ca<sup>2+</sup> mobilization, the activation of PKC and MAPK (ERK1/2 and p38 MAPK), interrelated pathways commonly activated by LDL in vascular cells,<sup>14,31,32</sup> that have been associated to the CCL20 induction by other stimuli in diverse cell types.<sup>33–35</sup>

In transient transfection assays we showed that LDL increased the transcriptional activity of human CCL20 promoter. In silico analysis of CCL20 promoter identified

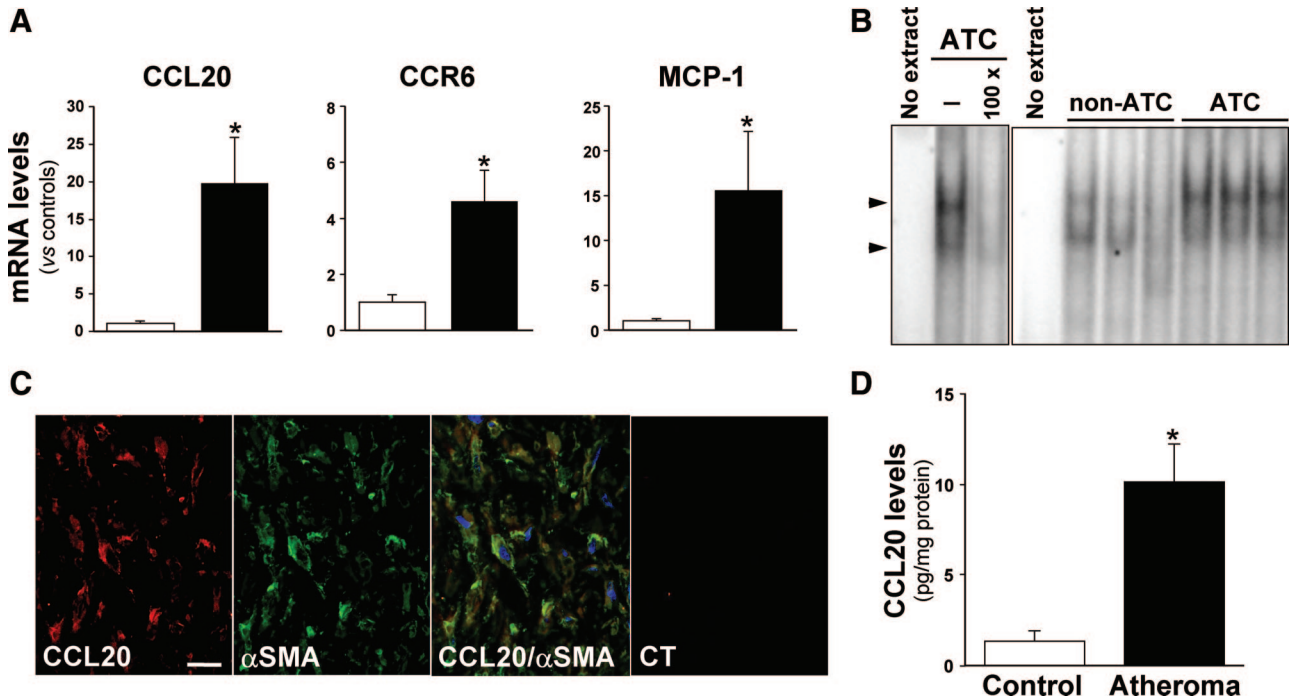


**Figure 4.** NF- $\kappa$ B is involved in LDL-induced CCL20 upregulation. **A**, Vascular smooth muscle cells (VSMC) were transiently transfected with various CCL20 promoter deletion mutants and promoter activity in the absence (white bars) or presence of LDL (black bars; 300  $\mu$ g/mL for 7 hours) was assessed. The location of the putative response elements is indicated. The activity of pCCL20/-117 mutated in the NF- $\kappa$ B site (-80/-71; deleted white circle) is also shown ( $n=7$ ;  $P<0.05$ ; \*vs control cells transfected with the same construct). **B**, Schematic representation of pCCL20/-165. The core consensus of the NF- $\kappa$ B site is indicated in bold, and changes introduced by mutagenesis are boxed. **C**, Representative autoradiogram of EMSA performed with the CCL20-88/-65 probe and nuclear protein extracts from controls and cells treated with LDL in the presence or absence of 1  $\mu$ mol/L parthenolide (Part). The position of 4 complexes (I to IV), whose upregulation by LDL was prevented by parthenolide (left panel) and competed by a molar excess of unlabeled probe (100-fold) (middle panel) is indicated. The supershifted bands on addition of a specific antibody against p65 (anti-p65) are indicated (right panel; double arrowhead). **D**, Chromatin immunoprecipitation (ChIP) from control cells and cells treated with LDL (300  $\mu$ g/mL for 2 hours) using an anti-p65 antibody (IP: p65) or a nonspecific rabbit IgG (IP: IgG). Top: The enrichment of NF- $\kappa$ B was quantified by real-time PCR using CCL20 promoter specific primers. Data were normalized to the total input DNA and are represented as means $\pm$ SEM of 2 independent experiments performed in duplicate ( $P<0.05$ ; \*vs controls). Bottom: Agarose gel electrophoresis of PCR products.

several elements corresponding to transcription factors potentially activated by LDL through the signaling pathways mentioned above, among them CREB, AP-1 and NF- $\kappa$ B.<sup>31,36,37</sup> Because these transcription factors have been involved in the regulation of inducible chemokines including CCL20,<sup>34,35</sup> as a first approach we showed that inhibitors of NF- $\kappa$ B signaling prevented the upregulation of CCL20 mRNA levels. The involvement of a NF- $\kappa$ B site located in the proximal region of CCL20 promoter was demonstrated by serial deletion analysis of promoter-luciferase constructs, site-directed mutagenesis, EMSA, and chromatin immunoprecipitation assays. It is noteworthy that NF- $\kappa$ B has also been involved in the modulation of CCL20 by other mediators such as TNF $\alpha$  in a human cancer cell line,<sup>38</sup> prolactin in keratinocytes,<sup>35</sup> or hypoxia in primary human monocytes/macrophages.<sup>39</sup> Taken together these results emphasize the role of NF- $\kappa$ B in the modulation of CCL20 by lipoproteins and other inducers. Concerning the mechanism involved in

such effect, lipoprotein receptors do not seem to play a major role. By contrast, several data suggest the involvement of a bioactive component of LDL (LPA) and its receptors. Indeed, minimal oxidation of LDL, a process that increases the content of LPA<sup>24,25</sup> without significantly affecting apolipoprotein B,<sup>40,41</sup> increased the ability of LDL (moxLDL) to upregulate CCL20 expression. Moreover, low LPA concentrations mimicked this lipoprotein effect while other products of LDL oxidation, at concentration that largely exceed those likely to be present in native LDL preparations,<sup>23</sup> did not. Finally, Ki-16425, an antagonist of LPA receptors (mainly LPA1 and LPA3 and in a lesser extent LPA2), was able to prevent the activation of signaling pathways involved in the upregulation of CCL20 by LDL, as well as the increase in CCL20 expression and CCL20 transcriptional activity induced by LDL. Therefore, CCL20 modulation by LDL seems to be associated to the activation of LPA receptors, receptors that have been involved in a number of biological activities of





**Figure 5.** Atherosclerotic coronary arteries show increased CCL20 mRNA levels and NF- $\kappa$ B activity. **A**, mRNA levels of CCL20, CCR6, and monocyte chemoattractant protein 1 (MCP-1) in human nonatherosclerotic coronary arteries (non-ATC;  $n=7$ ; white bars) and human atherosclerotic coronary arteries (ATC;  $n=7$ ; black bars). Data are expressed as mean  $\pm$  SEM ( $P<0.05$ ; \*vs non-ATC). **B**, Representative EMSA performed with the CCL20-88/-65 probe and whole-protein extracts from non-ATC ( $n=6$ ) and ATC ( $n=6$ ). Arrowheads indicate the main complexes upregulated in ATC and competed with a molar excess of unlabeled probe ( $\times 100$ ). **C**, High-power views showing immunostaining corresponding to CCL20,  $\alpha$ -SMA (marker of VSMC), and the merge image (nuclei staining in blue) demonstrating their colocalization in the intima of a representative ATC ( $n=4$ ). CT indicates control immunostaining of a consecutive section incubated with nonimmune goat IgG. Bar=20  $\mu$ m. **D**, CCL20 levels in conditioned media from human vascular tissues. Intima-media layers from carotid endarterectomy specimens (atheroma;  $n=15$ ) and healthy aortas (control;  $n=8$ ) were incubated in serum-free medium (24 hours at 37°C), and CCL20 levels were analyzed by EIA. CCL20 levels were normalized by protein concentration. Data are expressed as mean  $\pm$  SEM (\* $P<0.001$  vs aorta controls).

LDL,<sup>24,25,42,43</sup> and that seem to participate in atherosclerosis as suggested a recent study showing that Ki-16425 is able to reduce diet-induced atherosclerosis in apoE-deficient mice.<sup>44</sup>

CCL20 and its receptor seem to be critical for the arrest of rolling lymphocytes under flow conditions.<sup>45</sup> However, studies that early identified the expression of several chemokines chemoattractants for lymphocytes in carotid specimens from patients subjected to endarterectomy<sup>46</sup> failed to detect CCL20 by conventional PCR or in situ hybridization. Most recently, however, Yilmaz et al<sup>47</sup> described the presence of CCL20 in carotid plaques associated with inflammatory cells. In the present study, we detected a strong upregulation of CCL20 in human atherosclerotic lesions from CAD patients suggesting an active role of the CCL20/CCR6 system in atherosclerosis. In fact, the upregulation of CCL20 in these atherosclerotic lesions was as higher as that of monocyte chemoattractant protein 1, a well-known NF- $\kappa$ B-regulated chemokine involved in inflammation/atherogenesis. CCL20 mainly colocalized with VSMC in atherosclerotic coronary arteries and was associated with an enhanced NF- $\kappa$ B binding activity in these lesions, in agreement with the key role of this pathway in vascular inflammation. Finally, CCL20 was detected in conditioned media from healthy human vessels and its levels were significantly higher in secretomes from human atherosclerotic lesions, suggesting that vascular cells could be a source of this chemokine. A limitation of our study, however,

is the low number of human artery specimens analyzed due to the limited access to these samples. Furthermore, the potential of CCL20 as a biomarker should be validated in largest studies with patients affected by different pathological disorders associated with vascular inflammation.

In summary, in the present study we identify CCL20 in human coronary atherosclerotic lesions and recognize this chemokine as a mediator highly sensitive to the inflammatory response elicited by hypercholesterolemia in vivo and by LDL in vitro. CCL20 could be regarded as a new player in atherogenesis and a NF- $\kappa$ B downstream target potentially useful for strategies aimed to improve the balance between pro- and antiinflammatory mediators. Indeed, emerging evidence from experimental studies and clinical trials support the use of inflammation status as a clinical tool to aid prevention and guide the therapy and management of cardiovascular disease.<sup>3</sup> Moreover, handling the chemokine system could open new therapeutic perspectives.<sup>48</sup> The strict selectivity of CCL20 for CCR6, one of the exceptions to the common promiscuity among chemokines and their receptors, makes this tandem attractive for selective interventions addressed to an inflammatory subset of cells involved in atherogenesis. Interestingly, recently Wan et al<sup>49</sup> have shown that genetic deletion of the CCL20 receptor (CCR6) decreases atherogenesis in apoE-deficient mice. Further studies are warranted to better understand the pathophysiological role

of CCL20/CCR6 in vascular homeostasis and human atherosclerosis.

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### Disclosures

None.

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## Supplement Material

### Supplementary Methods

**Subjects' characteristics and assessment of cardiovascular risk.** A total of 107 apparently healthy subjects were recruited at the time of attending the outpatient clinic for vascular risk assessment in the Internal Medicine Department at the University Clinic of Navarra. Subjects were free from clinically manifest atherosclerotic vascular disease on the basis of the following criteria: (i) absence of history of coronary disease, stroke or peripheral arterial disease; and (ii) normal electrocardiogram and chest X-ray results. Coronary heart disease was defined by: (i) self-reported myocardial infarction, angina, or use of nitroglycerin; and (ii) self-reported history of coronary angioplasty or coronary artery bypass surgery. Cerebrovascular disease was defined as self-reported stroke, transient ischemic attack, or carotid endarterectomy. Patients were questioned about symptoms of intermittent claudication in a questionnaire, and in the physician's interview. Exclusion criteria were the presence of severely impaired renal function (glomerular filtration rate < 60 mL/min), chronic inflammatory conditions, and administration of anti-inflammatory, antithrombotic, or hormonal therapy in the previous 2 weeks. Patients with significant acute infection, according to clinical criteria applied by the attending physician, were also excluded. In addition, information was obtained about myocardial infarction in the family history and smoking. Smoking status was categorically evaluated based on self-reports, with a smoker defined by the history of smoking ( $\geq 10$  cigarettes per day > 1 year). Blood pressure was measured on the right upper arm with a random-zero mercury sphygmomanometer, with patients in a seated position (average of two measurements). Fasting serum and plasma samples were collected by venipuncture, centrifuged (20 min at  $1200 \times g$ ) and stored at  $-80^\circ\text{C}$  until analysis. Serum levels of glucose, total cholesterol, LDL, HDL and triglycerides were determined on a modular autoanalyzer (Analytics, Roche Diagnostics). Plasma fibrinogen activity and von Willebrand factor (vWF) were determined by clotting assay (Clauss) and ELISA (Asserachrom, Diagnostica Stago) respectively. Serum levels of CCL20 were measured by EIA (Boster Biological Technology, LTD), high-sensitivity (hs)-CRP by an enzyme-amplified chemiluminescence assay (Immulyte<sup>TM</sup>; Diagnostic Product Corporation) and interleukin-6 (IL-6; Quantikine<sup>TM</sup>, R&D Systems) by ELISA following the manufacturers' instructions. Diabetes mellitus was defined by fasting glucose levels > 126 mg/dL, or by the use of glucose-lowering agents. The European global vascular risk score (PROCAM: Prospective Cardiovascular Münster) was calculated as described.<sup>1</sup> All subjects underwent ultrasonography of common carotid arteries (CCA) to assess the carotid intima-media thickness (IMT) as a direct measure of subclinical atherosclerosis as previously described.<sup>2</sup> Ultrasonography was performed with a 5–12-MHz linear array transducer (ATL 5000 HDI; Philips). Carotid IMT was measured 1 cm proximal to the carotid bulb of each CCA at plaque-free sites. For each individual, the IMT was determined as the average of near and far-wall measurements of each common carotid artery. Subjects were examined by the same two certified sonographers, who were blinded to all clinical information. The reproducibility of IMT measurements between and within sonographers had previously been checked in 20 individuals who returned 2 weeks later for a second examination. Intra and inter-observer coefficients of variation were 5% and 10% respectively.

**Human artery sampling and preservation.** Human coronary artery samples were collected from patients undergoing heart transplant surgery at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). Immediately after surgical excision, arteries were dissected, immersed in cell maintenance media, and cleaned of connective tissue and fat under low magnification



with a zoom stereo microscope (SZH10; Olympus). This examination allowed us to classify coronary arteries as atherosclerotic, assessed by the presence of evident atherosclerotic lesions, and non-atherosclerotic arteries as deduced from absence of fibro-fatty tissue or visible plaques. Vessel samples were split and processed for either conventional staining or frozen in liquid nitrogen and stored at -80°C for later protein or RNA extraction. The specimens for conventional staining were immersed in fixative solution (4% paraformaldehyde/0.1 M phosphate-buffered saline, pH 7.4) within 30 min of surgical excision. After overnight treatment, they were sectioned into blocks and embedded in paraffin. Vessels were cut into 5- $\mu$ m-thick sections that were collected on chromopotassium-gelatin coated slides and stored at -20°C until tested. The absence/presence of atherosclerotic lesions was confirmed by conventional Masson's trichrome or hematoxylin and eosin staining. Carotid samples were obtained from patients undergoing endarterectomy (carotid stenosis >70%) at the Fundación Jimenez Díaz (FJD). Samples were collected in saline buffer and processed in a safety culture cabinet. In a sterile Petri dish, endarterectomy samples were dissected separating the stenosing complicated zone (origin of the internal carotid artery) from the adjacent plaque (common and external carotid endartery) using a surgical scalpel as described.<sup>3</sup> For this study, we selected this adjacent area since it was composed of fibrous tissue enriched in VSMC as previously shown.<sup>4</sup> Control aortas were obtained from organ donors. These control aortic samples were macroscopically normal and devoid of early atheromatous lesions. For control aortas, the adventitia was removed. Carotid and aortic tissues were cut into pieces of 1 mm<sup>3</sup> and incubated separately in serum-free RPMI 1640 medium (Life Technologies) for 24 h at 37°C in 24-well cell culture plates. The conditioned media from vascular tissue samples were collected and centrifuged, protein concentration was determined by Bradford's method, and samples were aliquoted and stored at -80°C for later analysis. CCL20 levels were analyzed by EIA and results were normalized by protein content.

**Cell culture.** VSMC were obtained from human coronary arteries of hearts removed in transplant operations by using a modification of the explant technique.<sup>5</sup> VSMC (from 3rd to 5th passages) were cultured in M199 (Gibco) supplemented with 20% fetal calf serum (FCS), 2% human serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin). Cells were seeded in multi-well plates and arrested in medium containing 0.4% FCS for 48 hours. Arrested cells were stimulated with increasing concentrations of LDL (expressed in  $\mu$ g cholesterol/mL). When needed, cells were pretreated with inhibitors for 30 min (unless otherwise stated). The inhibitors used were: 1,2-bis(2-aminophenoxy)ethano-N,N,N',N'-tetraacetic acid tetrakis acetoxymethyl ester (BAPTA-AM, a Ca<sup>2+</sup> chelator; 25  $\mu$ M; Sigma), bisindolylmaleimide I (GF10933X, a protein kinase C [PKC] inhibitor; 15  $\mu$ M; Sigma), SB203580 (a p38 mitogen-activated protein kinase [MAPK] inhibitor; 10  $\mu$ M; Oxford Biomedical Research Inc.), U0126 (a mitogen-activated protein kinase kinase [MEK1/2] inhibitor that prevents extracellular signal-regulated kinase1/2 [ERK1/2] activation; 10  $\mu$ M; Calbiochem), parthenolide (a NF $\kappa$ B inhibitor, 1  $\mu$ M, Sigma),<sup>6</sup> BAY-11-7082 (a NF $\kappa$ B inhibitor, 10  $\mu$ M, 1 h pre-incubation, Sigma), NDGA (an AP-1 inhibitor, 1  $\mu$ g/mL, Sigma),<sup>7</sup> pertussis toxin (100 ng/mL, overnight pre-incubation; Sigma), and Ki-16425 (at 10  $\mu$ M antagonizes of lysophosphatidic acid [LPA] receptor-1, -2 and -3; Cayman chemical).<sup>8</sup> To determine whether LDL affect CCL20 transcription, cells were incubated (30 min) with 5,6-dicloro-1-(b-D-ribofuranosil)-benzimidazol (DRB; 50  $\mu$ M; Sigma) before LDL exposure.

In some experiments, blocking antibodies (10  $\mu$ g/mL) to LDL receptor (LDLR, R&D Systems), scavenger receptor class A type I (SR-AI, R&D Systems), lectin-like oxidized



LDL receptor 1 (LOX-1, R&D Systems), and CD36 (Abcam) were applied to VSMC 2 h before the treatment as described.<sup>9,10</sup> Control cells were pretreated with non-specific mouse or goat immunoglobulins G. The average of controls is used because there was no difference between the two controls.

Endothelial cells from human umbilical veins (HUVEC) were isolated and cultured as previously described.<sup>11</sup> Cells were seeded in six-well plates in M199 medium containing 5 % FCS (without heparin and endothelial cell growth factor) overnight prior LDL treatment.

Lymphocytes were isolated from human blood buffy coats from the Hospital of the Vall d'Hebron Blood Bank (Barcelona) as described.<sup>12</sup> 5 volumes of Buffy coats were layered on top of 3 volumes of Ficoll-paque plus (GE Healthcare) and spun for 45 min at 400 x g. The PBS-Ficoll interphase containing platelets, lymphocytes, and monocytes was collected and washed three times in PBS (150 x g, 10 min). The lymphocyte-monocyte fraction was free of other type of cells, as judged by microscopic examination and coulter counting (Multisizer 3<sup>TM</sup> COULTER COUNTER, Beckman Coulter). Cells were resuspended in RPMI 1640 (Gibco) supplemented with 10% human serum AB (Invitrogen), 10 mM Hepes, 2 mM L-glutamine and antibiotics, and seeded in multi-well plates. After 24 hours supernatants containing lymphocytes were collected and used in migration assays.

All the procedures were approved by the Reviewer Institutional Committee on Human Research of the Hospital de la Santa Creu i Sant Pau and conforms to the Declaration of Helsinki.

**Lipoprotein isolation and characterization.** LDL were isolated from pooled plasma of healthy blood donors of the Barcelona area.<sup>13</sup> Briefly, pooled plasma was centrifuged (80,000 x g for 30 min at 4°C) to remove chylomicrons. LDL (d=1.019-1.063 g/mL) were isolated by potassium bromide density-gradient ultracentrifugation using a Beckman-Coulter Optima<sup>TM</sup> L-100 XP ultracentrifuge and a Beckman 50.2 Ti rotor (Beckman Coulter) at 36,000 rpm for 18 h at 4°C (gmax= 156,000). The LDL fraction was dialyzed four times against 200 volumes of buffer (150 mM NaCl, 20 mM Tris·HCl and 1 mM EDTA, pH 7.4) and once against 200 volumes of 0.9% NaCl at least 2 hours. All solutions were deoxygenated by N<sub>2</sub> bubbling. LDL were sterilized by filtration through a low protein-binding non-pyrogenic filter (Millex-GV, Millipore), stored under N<sub>2</sub> at 4°C and protected from exposure to light. These lipoprotein preparations were used within 2 days after isolation, and are referred to LDL (or native LDL) in the present study. The content of protein (BCA protein assay<sup>TM</sup>, Pierce) and cholesterol (Cholesterol assay kit<sup>TM</sup>, RefLab) was determined by colorimetric assays. The absence of contamination by other lipoproteins was determined by electrophoresis on agarose gels (Paragon Electrophoresis kit, Beckman). Thiobarbituric acid-reactive substances (TBARS) content of LDL were used as an indirect evaluation of lipid peroxidation. TBARS levels were determined as previously described.<sup>5</sup> No oxidation of LDL was observed within 2 days after LDL preparation as assessed by measurement of TBARS content (TBARS between 0.3 and 0.6 nmol malonaldehyde (MDA)/mg LDL) and electrophoretic mobility in agarose gels. Extensively dialyzed LDL (7 mg/mL) were subjected to spontaneous oxidation during 30 days at 4°C in the dark.<sup>13</sup> This spontaneously oxidized lipoprotein preparation is referred as minimally oxidized LDL (moxLDL). TBARS values for moxLDL were between 1.8 and 2.1 nmol MDA/mg protein. This minimal oxidation did not modify the content of free amino groups analyzed by 2,4,6-trinitrobenzenesulfonic acid method, using alanine as a standard.<sup>13,14</sup> Lipoproteins were free of endotoxin (as determined by a Limulus assay) and the absence of any effect attributable to endotoxin contamination was discarded pre-incubating cells with polymyxin B before native (LDL) and moxLDL treatment (Supplemental figure VI).

**Constructs of CCL20 promoter.** A 2.0 kb fragment corresponding to nucleotides –2007 to +51 of the human CCL20 promoter was generated by PCR and cloned into pGL3 vector (Promega) (pCCL20/-2007). The primers used were: 5'-ATGGAAGATCTGTTGGCCAGGTTAGAGG -3' (forward; *Bgl*III site is underlined) and 5'-ACTGACATCAAAGCAGCCAGGAGC-3' (reverse) placed downstream an internal *Bgl*III site (+46/+51). The PCR product was digested with *Bgl*III and cloned into pGL3 vector. A series of promoter deletions were generated using a common reverse primer 5'-GATCGCAGATCTGCTCAGTG-3' (*Bgl*III site is underlined) and the following forward primers (*Kpn*I site is underlined): (pGL3-CCL20/-618) 5'-CGATGGTACCATTAATCAAGGTGAAGCTG-3'; (pGL3-CCL20/-467) 5'-CGATGGTACCCATGTGATGGTAAATGTGT-3'; (pGL3-CCL20/-328) 5'-CGATGGTACCGTATCGCTGTTAATCCTCTA-3' and (pGL3-CCL20/-165) 5'-CGATGGTACCCGCACCTTCCCAATATGAG -3'. The putative NF- $\kappa$ B responsive element present in CCL20 promoter was mutated using the QuikChange<sup>TM</sup> Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The primers used were: forward 5'-GCCAGTTGATCAATGGGGcgAACCCCATGTGGCAACAC-3', and reverse 5'-GTGTTGCCACATGGGGTTcgCCCCATTGATCAACTGGC-3' (putative NF- $\kappa$ B site is underlined and changes are indicated in lower case letters). The new sequence was analyzed by different promoter analysis softwares to confirm that no new response elements were generated. Wild-type and mutated sequences were confirmed by DNA sequencing.

**Transient transfection and luciferase assays.** VSMC were transfected with luciferase reporter plasmids using Lipofectamine LTX<sup>TM</sup> and Plus Reagent (Invitrogen).<sup>15</sup> Briefly, transient transfections were performed in subconfluent cells seeded in 6 well/plates using 1  $\mu$ g/well of the luciferase reporter plasmid, 0.3  $\mu$ g/well of pSV $\beta$ -gal (Promega) as an internal control and 3  $\mu$ L of Lipofectamine LTX<sup>TM</sup> and Plus Reagent. The DNA/liposome complexes were added to the cells for 6 h. Cells were washed once with arrest medium, arrested overnight (1% FCS) and then treated with LDL for 7 h. Luciferase activity was measured in cell lysates using Luciferase assay Kit (Promega) and a luminometer (Orion I, Berthold Detection Systems) according to the manufacturer. Results were normalized by  $\beta$ -galactosidase activity.

**siRNA transfection.** Silencer predesigned small interfering RNA (siRNA) targeting CCL20 (Silencer<sup>TM</sup> Select s12604, Ambion) or a control siRNA (siRAND; Silencer<sup>TM</sup> Select Negative Control #1, 4390843, Ambion) were used in the knockdown experiments. Human VSMC were transfected with siRNAs using Nucleofector<sup>TM</sup> (Amaxa) and the corresponding kit for VSMC (VPC-1001, Amaxa) according to the manufacturer's instructions. Briefly, 1 x 10<sup>6</sup> cells were electroporated in 100  $\mu$ L of buffer containing 1  $\mu$ g of siRNA using the A-033 program. After electroporation, cells were resuspended in 1 mL of pre-warmed cell culture medium and seeded in 6-well plates (350,000 cells/well). After 24 h cells were arrested for 24 h and treated with LDL (300  $\mu$ g/mL). Gene knockdown was verified by real-time PCR (4 h after stimulus) and determining CCL20 by EIA into cell supernatants (24 h after stimulus).

**Gene expression: real-time PCR.** Total RNA was isolated using the Ultraspec reagent (Biotecx Laboratories) according to the manufacturer's recommendations. RNA integrity was determined by electrophoresis in agarose gels and was quantified by a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Total RNA (1  $\mu$ g) was reverse-transcribed using the High Capacity cDNA Archive kit (Applied Biosystems) and random hexamers. Levels of mRNA were assessed by real-time PCR on an ABI PRISM 7900 sequence detector (Applied

Biosystems). TaqMan<sup>TM</sup> gene expression assays-on-demand (Applied Biosystems) were used for human CCL20 (Hs00171125\_m1), human CCR6 (Hs01890706\_s1) and human MCP-1 (Hs00234140\_m1). Human glyceraldehyde-3-phosphate dehydrogenase (4326317E) was used as an endogenous control.

**Isolation of nuclear and cytosolic extracts from VSMC.** Nuclear and cytosolic extracts were obtained from VSMC stimulated with LDL for 120 and 60 min respectively, in presence or absence of parthenolide using the NucBuster<sup>TM</sup> Protein Extraction kit (Novagen), according to the manufacturer's recommendations. Cytosolic and nuclear extracts were aliquoted and stored at -80°C until used.

**Electrophoretic mobility shift assay (EMSA).** Nuclear extracts from VSMC were obtained as indicated above. Protein extracts from human coronary artery samples were obtained using an ice-cold lysis buffer containing 50 mM HEPES pH 7.4, 100 mM NaCl, 2.5 mM EGTA, 10 mM  $\beta$ -glycerol phosphate, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, 1 mM DTT, and complete protease inhibitor cocktail (Roche). Proteins were quantified by the BCA protein assay<sup>TM</sup> (Pierce) and protein extracts were aliquoted and stored at -80°C until used. EMSA was performed as described using 5  $\mu$ g of nuclear extracts from VSMC and 20  $\mu$ g of whole cell extract from coronary arteries.<sup>11</sup> The double-stranded DNA probe containing the putative NF- $\kappa$ B element present in human CCL20 promoter (CCL20/-88/-65 probe) was generated from the annealing of single-stranded complementary oligonucleotides (from -88 to -65; 5'-GATCAATGGGGAAAACCCCATGTG-3' and 5'-CACATGGGGTTTCCCCATTGATC-3'). DNA probes were labeled with [ $\gamma$ -<sup>32</sup>P]-ATP using T4 polynucleotide kinase (New England Biolabs, Inc) and purified on a Sephadex G-50 column (GE Healthcare). For super-shift assays, lysates were preincubated for 25 minutes with 1  $\mu$ g of an anti-p65 antibody (sc-109X, Santa Cruz Biotechnology) before adding radiolabeled probe. Protein-DNA complexes were resolved by electrophoresis at 4°C on 5% polyacrylamide gels in 0.5X TBE. Gels were dried and subjected to autoradiography using a Storage Phosphor Screen (GE Healthcare). Shifted bands were detected using a Typhoon 9400 scanner (GE Healthcare).

**Chromatin immunoprecipitation assay.** VSMC ( $3-5 \times 10^6$  cells) were treated with LDL (300  $\mu$ g/ml, 2 h) and fixed by supplementing the medium with formaldehyde (1% final concentration, 10 minutes). Cross-linked reaction was stopped by adding glycine (0.125 M final concentration). Then cells were extensively washed with ice-cold PBS and lysed for 10 min at 4°C in LB1 buffer (50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% NP-40, 0.3% Triton X-100, 10% glycerol) supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail, Roche Applied Science). After washing, nuclei were collected at 3,000 rpm in a microfuge and resuspended in LB2 buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-Deoxycholate, 0.5% N-lauroylsarcosine). Chromatin was sheared by sonication using Bioruptor<sup>TM</sup> UCD-200 (Diagenode, Liege, Belgium), centrifuged to pellet debris, and diluted ten times in dilution buffer (16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 1% Triton X-100, 0.01% SDS). At this stage, an aliquot (5%) was saved and stored as input DNA. Immunoprecipitation with 4  $\mu$ g of rabbit polyclonal anti-p65 or isotype IgG control antibody (sc-372 and sc-2027 respectively, Santa Cruz Biotechnology) was performed overnight at 4°C. Immune complexes were collected with salmon sperm DNA-saturated protein A and successively washed with low salt wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1 % SDS), high salt buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1 % SDS), LiCl buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% deoxycholate, 1% NP-40, 0.5 M LiCl) and TE buffer. Immune complexes were extracted in elution buffer (1%

SDS, 100 mM NaHCO<sub>3</sub>, 200 mM NaCl) and cross-link was reverted by heating at 65°C for 6 h. Finally, DNA was purified and concentrated using the QIAquick PCR purification kit (Qiagen, Valencia, CA). The purified DNA was analyzed by conventional PCR and real-time PCR with primers designed to amplify the human CCL20 promoter fragment from -209 to -11: 5'-CAGGATTCTCCCCTTCTCAAC-3' (forward), and 5'-GGGATGGCCCTATTTATAGCA-3' (reverse). Real-time PCR analyses were performed by triplicate with the Quantifast™ SYBR Green PCR kit (Qiagen). The relative abundance of specific sequences in immunoprecipitated DNA was determined using the  $\Delta\Delta C_t$  method. Results were normalized by input.

**Western blot analysis.** Human VSMC were washed with PBS. Nuclear and cytosolic proteins were obtained as described above. To obtain whole cellular protein extracts a lysis buffer containing 1% SDS in 10 mM Tris-HCl (pH 7.4) and 1 mM ortovanadate was used. Protein concentration was measured by the BCA protein assay™ and proteins were separated by SDS-PAGE (10% acrylamide:bisacrylamide) and electrotransferred onto Immobilon polyvinylidene difluoride membranes (Millipore). Western blot analysis was performed using antibodies against I $\kappa$ B $\alpha$  (#4814, Cell Signaling Technology), p65 (sc-109, Santa Cruz Biotechnology), phosphorylated ERK1/2 (#9106, Cell Signaling Technology), total ERK1/2 (#9102, Cell Signaling Technology), phosphorylated p38 MAPK (#9216, Cell Signaling Technology), and total p38 MAPK (sc-7194, Santa Cruz Biotechnology). Detection was performed using the appropriate horseradish peroxidase-labeled IgG and the Supersignal™ detection system (Supersignal West Dura™, Pierce). The size of detected proteins was estimated using protein molecular-mass standards (Fermentas).  $\beta$ -actin (ab8226, Abcam) and nucleolin (ab22758; Abcam) were used as a loading control for cytosolic and nuclear extracts respectively. To quantify relative protein levels immunoblots were digitalized (GS-800 Calibrated Densitometer; Bio-Rad) and images were analyzed with the Quantity One 4.6.3 software (Bio-Rad).

**Immunocytochemistry and immunohistochemistry analysis.** VSMC were cultured in glass-bottom dishes (Willco wells B.V.). Arrested cells were stimulated with LDL (300  $\mu$ g/ml for 2 h) in presence or absence of parthenolide. Cell monolayers were fixed with a 4% paraformaldehyde solution and were processed for immunocytochemistry as described.<sup>16</sup> After blocking, cells were incubated with the primary antibody [rabbit polyclonal anti-p65 antibody (sc-109, Santa Cruz Biotechnology)] for 1 hour at room temperature. Alexa Fluor 633 goat anti-rabbit immunoglobulin (Molecular Probes) was used as a secondary antibody. Controls incubated with non-immune rabbit  $\gamma$ -globulin and without the primary antibody were included in all procedures. Finally, cells were mounted with ProLong™ mounting medium (Molecular Probes) and analyzed by confocal microscopy (Leica TCS SP2-AOBS).

The presence of CCL20 in human coronary atherosclerotic lesions was assessed by immunohistochemistry analysis. Briefly, sections were deparaffinized, treated for unspecific binding and incubated with a goat polyclonal anti-CCL20 antibody (R&D Systems) overnight at 4°C. Double immunofluorescence analysis was used to analyze co-localization of CCL20 with VSMC [mouse monoclonal anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) antibody; clone 1A4, Dako]. Hoechst dye (#33342, Molecular Probes) was added with the primary antibodies for nuclear staining. As secondary antibodies, Alexa Fluor 633 rabbit anti-goat IgG (Molecular Probes) and Alexa Fluor 488 donkey anti-mouse IgG (Molecular Probes) were used. Finally, sections were mounted and analyzed by confocal microscopy as indicated above. Controls with non-immune goat IgG (as a control for the goat IgG against CCL20) or isotype-matched mouse IgG (as a control for mouse monoclonal anti- $\alpha$ -SMA antibody) were carried out.



**Migration assay.** Migration assays were performed in Costar transwell plates (Corning) containing 6.5 mm diameter, 5 µm pore-size polycarbonate membrane. Briefly,  $2 \times 10^5$  lymphocytes in 100 µL of RPMI 1640 (Gibco) supplemented with 10% human serum AB, 10 mM Hepes, 2 mM L-glutamine and antibiotics, were placed in the upper compartment of the transwell plate and 0.6 mL of cell supernatants collected from VSMC stimulated with 300 µg/mL of LDL-cholesterol were placed in the lower compartment. CCL20 recombinant protein (40 pg/mL) (Boster Biological Technology, LTD) was used as a positive control and cell supernatant from non-stimulated cells as a control of spontaneous migration. As negative controls CCL20 was added only in the top chamber or in both chambers. Following incubation for 30 min at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, cells that migrated into the lower chamber were monitored under a light microscope (Leica DMIRE2), photographed (Leica DFC 350 FX) and counted using a Neubauer hemocytometer.

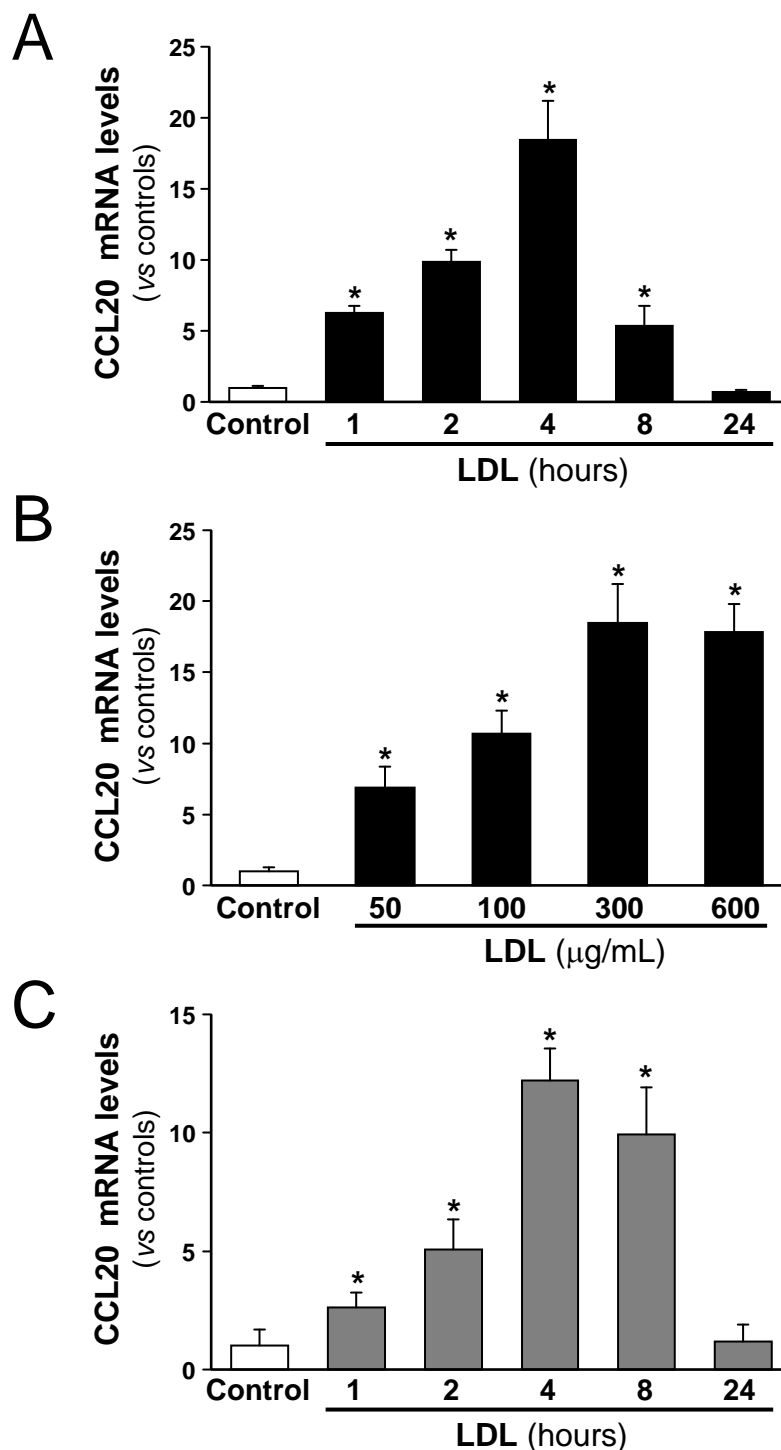
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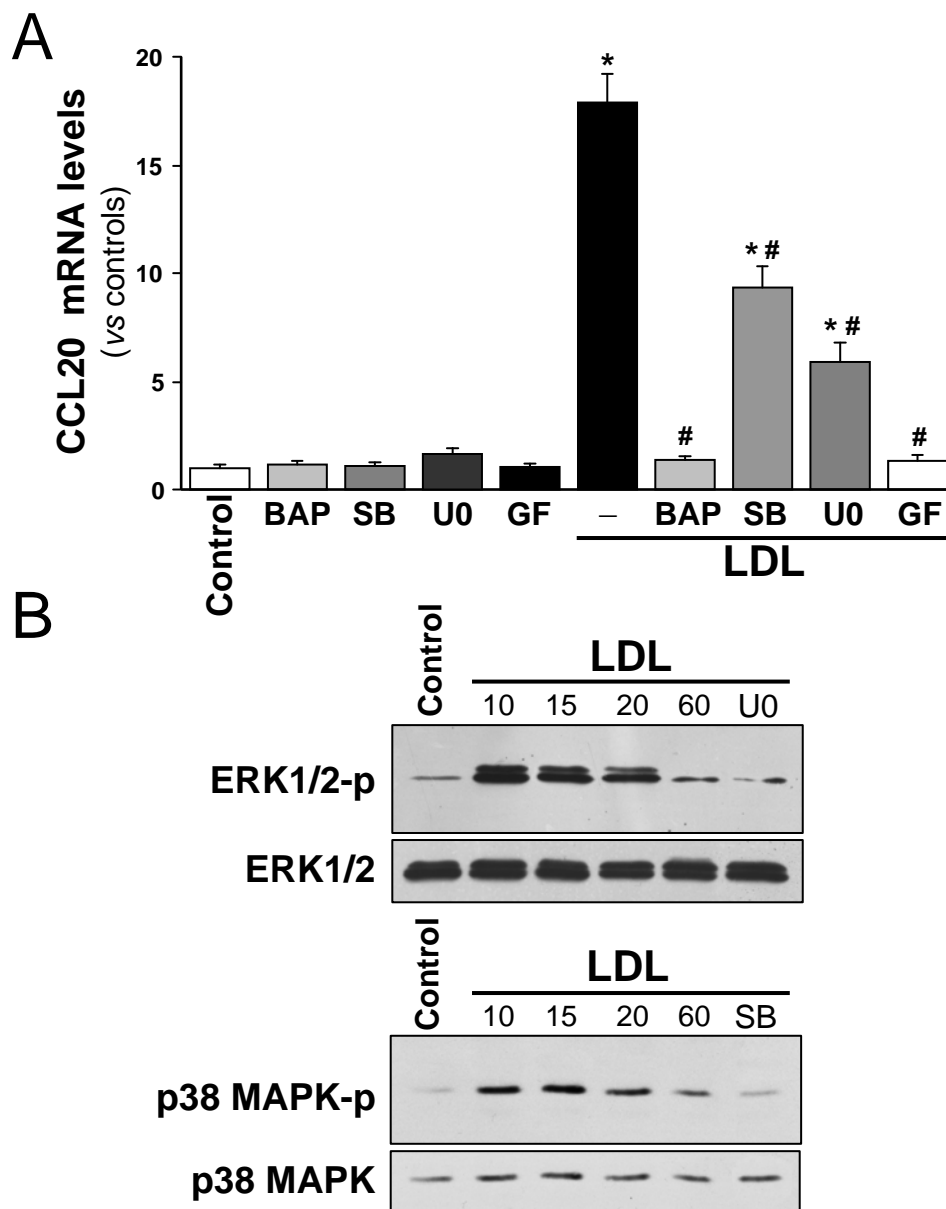
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## Supplementary Figure I



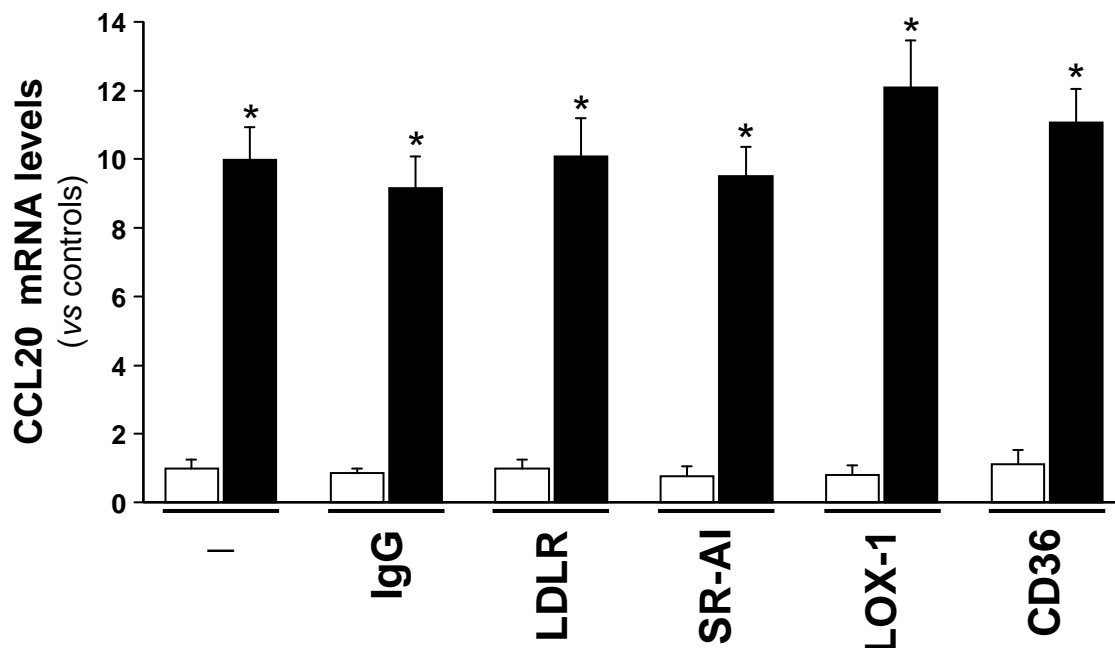
**LDL induce CCL20 expression in human VSMC.** (A) Arrested human VSMC were treated with LDL (300 µg/mL) at different times (from 1 to 24 hours) and CCL20 expression was analyzed by real-time RT-PCR. (B) Real-time PCR showing the increase of CCL20 mRNA levels after incubation of VSMC with increasing concentrations of LDL (50 to 600 µg/mL) for 4 hours. (C) LDL also induced CCL20 expression in endothelial cells (HUVEC) in a time-dependent manner. [n=9;  $P<0.05$ : \*, vs. control cells].

## Supplementary Figure II



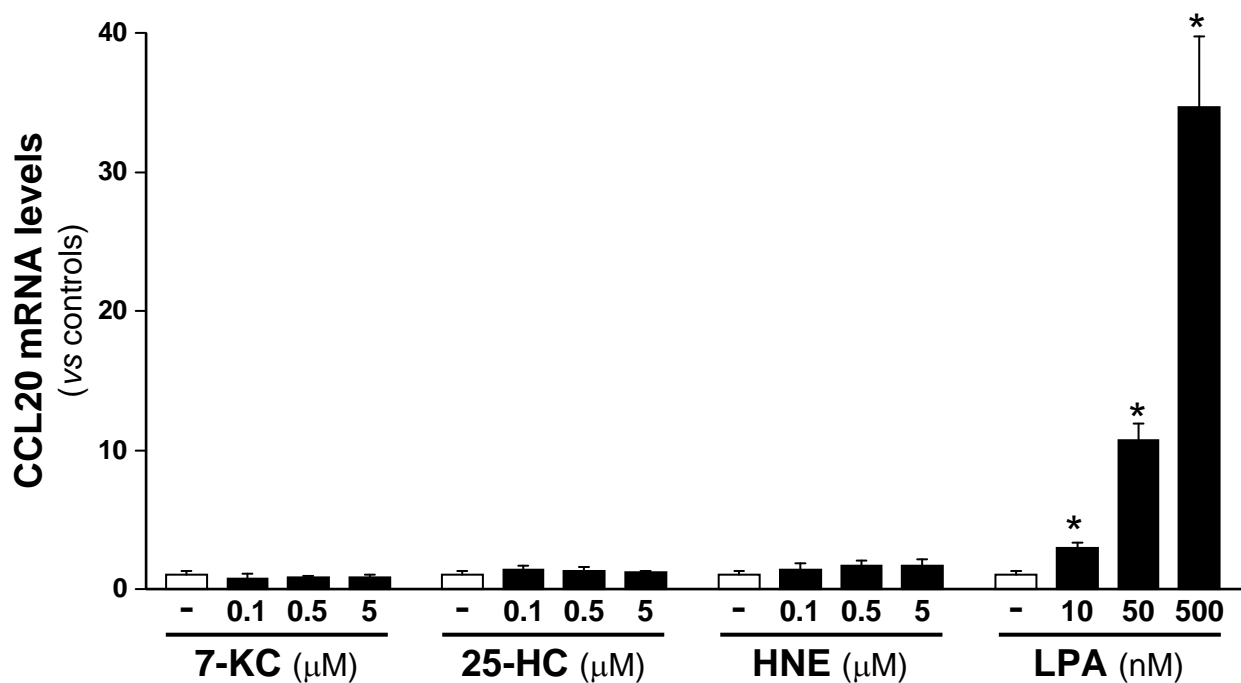
**Signaling pathways involved in the up-regulation of CCL20 by LDL.** (A) Real-time PCR showing CCL20 mRNA levels in human VSMC induced with LDL (300  $\mu\text{g/mL}$ ) for 4 hours in the presence of inhibitors of different signaling pathways: BAPTA-AM (BAP, 25  $\mu\text{M}$ ), SB203580 (SB, 10  $\mu\text{M}$ ), U0126 (U0, 10  $\mu\text{M}$ ) and GF10933X (GF, 15  $\mu\text{M}$ ). The effect of the inhibitors in the absence of LDL is also shown. [ $n=9$ ;  $P<0.05$ : \*, vs. control cells; #, vs. cells treated with LDL alone]. (B) Time-course showing the effect of LDL (300  $\mu\text{g/mL}$ ) on the activation of ERK1/2 (ERK1/2-p) and p38 MAPK (p38 MAPK-p). The inhibitory effect of U0126 (U0) and SB203580 (SB) on ERK1/2-p and p38 MAPK-p is shown. Unchanged total protein levels of both signaling kinases are shown as a loading control. Representative immunoblots from 2 independent experiments.

## Supplementary Figure III



**Classical lipoproteins receptors are not involved in the up-regulation of CCL20 expression by LDL in human VSMC.** VSMC were pre-incubated with 10  $\mu\text{g/mL}$  of individual lipoproteins receptors, and then were treated with LDL (100  $\mu\text{g/mL}$  for 4 hours). Bar graph showing CCL20 mRNA levels in control cells (white bars) and cells treated with LDL (black bars). [n=6;  $P<0.05$ : \*, vs. control cells pre-incubated with the same antibody]. LDLR: LDL receptor; scavenger receptor class A type I (SR-AI), lectin-like oxidized LDL receptor 1 (LOX-1).

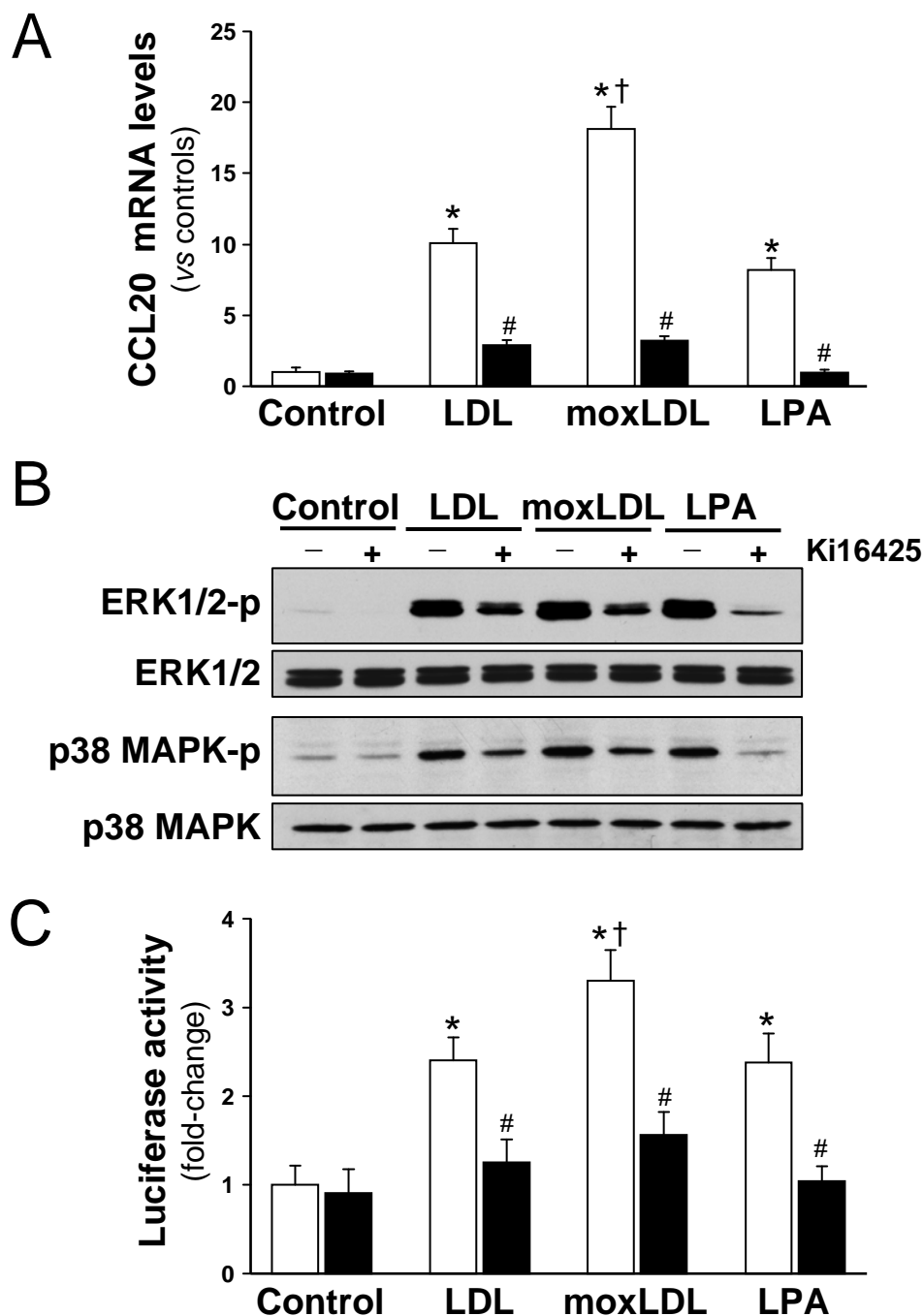
## Supplementary Figure IV



**Role of lysophosphatidic acid (LPA) and other LDL oxidation products on CCL20 expression.** CCL20 mRNA levels in VSMC treated with increasing concentrations of 7-ketocholesterol (7-KC), 25-hydroxycholesterol (25-HC), 4-hydroxynonenal (HNE) or LPA for 4 hours. [n=6;  $P<0.05$ : \*, vs. control cells].

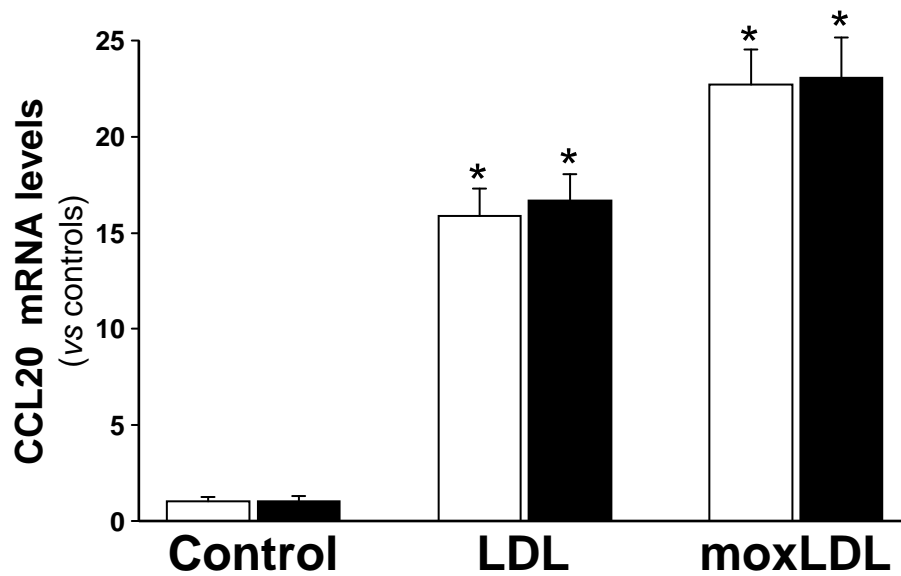


## Supplementary Figure V



Minimal oxidation of LDL increases the ability of these lipoproteins to up-regulate CCL20 through a mechanism dependent on lysophosphatidic acid (LPA) receptors. VSMC were pre-incubated (black bars) or not (white bars) with an antagonist of LPA receptors (10  $\mu$ M Ki-16425) and were treated with 100  $\mu$ g/ml of native (LDL) or minimally oxidized LDL (moxLDL) or LPA (50 nM) for 4 h (to analyze CCL20 mRNA levels; in A), 10 min (to analyze early MAPK activation; in B), or 7 h (to analyze CCL20 promoter activity using pCCL20/-165 construct; in C). [n=6;  $P<0.05$ : \*, vs. control cells; †, vs. cells treated with LDL or LPA; #, vs. cells treated with lipoproteins (or LPA) in the absence of inhibitor].

## Supplementary Figure VI



**Polymixin B did not affect the up-regulation of CCL20 expression induced by LDL in human VSMC.** VSMC were pre-incubated (black bars) or not (white bars) with polymixin B (5  $\mu\text{g/mL}$  for 30 min), and then treated with 300  $\mu\text{g/mL}$  of native (LDL) or minimally oxidized LDL (moxLDL) for 4 hours. Bar graph showing CCL20 mRNA levels in control cells (white bars) and cells treated with lipoproteins (black bars). [n=6;  $P < 0.05$ : \*, vs. control cells].