Enhanced Levels of Oxidized Low-Density Lipoprotein Prime Monocytes to Cytokine Overproduction via Upregulation of CD14 and Toll-Like Receptor 4 in Unstable Angina

A. Fratta Pasini, M. Anselmi, U. Garbin, E. Franchi, C. Stranieri, M.C. Nava,
V. Boccioletti, C. Vassanelli, L. Cominacini

Objectives—The purpose of this study was to establish whether oxidized low-density lipoprotein (oxLDL) contributes to cytokine overproduction via upregulation of CD14 and toll-like receptor-4 (TLR-4) expression on circulating monocytes of unstable angina (UA) patients.

Methods and Results—Expression of CD14 and TLR-4 on circulating monocytes, and the concentration of plasma oxLDL, (interleukin [IL])-6, IL-1 beta, IL-8, tumor necrosis factor (TNF)-alpha, monocyte chemoattractant protein-1 (MCP-1) were measured in 27 control (C) subjects, 29 patients with stable angina (SA), and 27 with UA. CD14 and TLR-4 expression on monocytes and circulating IL-6, IL-1 beta, and oxLDL were higher in UA than in SA and C subjects ($P<0.001$). In in vitro experiments, oxLDL increased CD14 and TLR-4 expression ($P<0.001$) in control monocytes as well as IL-6, IL-1 beta, and at a lower extent TNF-α and MCP-1 levels in the supernatant (P from <0.05 to <0.001). The preincubation of sera derived from UA patients but with control monocytes also induced a significant increase of CD14 and TLR-4 expression ($P<0.001$) and of IL-6 and IL-1 beta production ($P<0.001$) in the supernatant.

Conclusions—In UA patients oxLDL may contribute to monocyte overproduction of some cytokines by upregulating CD14 and TLR-4 expression. (*Arterioscler Thromb Vasc Biol*. 2007;27:000-000.)

Key Words: oxidized LDL ■ IL-6 ■ IL-1 beta ■ CD14 ■ toll-like receptors ■ monocytes ■ unstable angina

We found that plasma IL-6 and IL-1 beta as well as oxidized low-density lipoprotein (oxLDL) were higher in unstable than in stable angina ($P<0.001$). We showed that circulating monocytes contributed to this difference as they may be primed by oxLDL to IL-6 and IL-1 beta overproduction via upregulation of CD14 and toll-like receptor-4.

Recent findings suggest that inflammation may play an important role in the pathogenesis of acute coronary syndromes.[1] It is known that monocytes/macrophages participate in several critical aspects of coronary artery disease[1] and that the magnitude of the acute-phase response may also be the result of the behavior of circulating inflammatory cells.[1] Circulating monocytes have been found to be activated in patients with unstable angina (UA).[2] These monocytes are characterized by upregulation of adhesion molecules,[3] increased production of cytokines,[4] and procoagulant substances[5] and activation of nuclear factor-kB.[6] Furthermore, these cells have been demonstrated to exhibit an enhanced production of IL-6 in response to low-dose lipopolysaccharide (LPS).[7]

A markedly increased expression of CD14 and Toll-like receptor 4 (TLR-4) has recently been reported in the monocytes of UA patients.[8] CD14 is the receptor that binds LPS.[9,10] It lacks a transmembrane and cytoplasmic domain and therefore it is unlikely to have direct signaling capabilities.[9,10] Signaling associations occur with member of the TLR family.[9,10] This receptor family is made up of at least 9 members, but TLR-4 has been described as the likely receptor in LPS recognition and activity.[11]

oxLDL levels have been shown to be elevated in acute coronary syndromes and in particular in UA[6] and to upregulate the in vitro expression of CD14 and TLR-4 in monocytes/macrophages.[12,13] CD14/TLR-4 complex may therefore provide a potential pathophysiological link between lipids and infection/inflammation and atherosclerosis. However it has to be emphasized that studies done with TLR-4, the adaptor molecule myeloid differentiation factor-88, TLR-2, and CD14-deficient mouse models showed that all except CD14 contributed to atherosclerotic disease.[14–16]

This study was aimed to evaluate whether in UA patients oxLDL contributes to monocytic overproduction of cytokines via upregulation of TLR-4 and CD14.

Methods

Study Population

The study was approved by the hospital ethical committee and informed consent was obtained from all the patients before their enrolment.

Major requirements for the enrolment in all the groups were: absence of infectious or acute/chronic inflammatory diseases, known malignancy, absence of acute/chronic renal failure, or hepatic failure.

Three groups of patients were studied: control (C) subjects (patients without clinical history of CAD, who underwent coronary angiography for a presurgical evaluation, because affected by valvular or congenital heart disease), patients with stable angina (SA), and patients with UA. The subjects were enrolled on the basis of previously established criteria.[6,17]

For both SA and UA patients exclusion criteria were: prior coronary artery bypass grafting (CABG), recent (<6 months) myocardial infarction (MI), recent (<6 months) percutaneous coronary intervention (PCI), congestive heart failure (CHF), coronary tree free of significant coronary artery disease (CAD; defined as at least one stenosis with minimal luminal diameter >70% of the arterial lumen by visual estimate) detected at coronary angiography.

The following data were obtained from all the patients: age, sex, presence of CAD risk factors (hypertension, cigarette smoking, diabetes mellitus, hypercholesterolemia, family history of CAD), use of medications, previous MI, previous revascularization (PCI or CABG).

Blood Samples

Venous blood samples were obtained from SA patients and C subjects the morning after the day of admission together with the routine laboratory tests. In UA patients samples were drawn within 24 hours from the index event (the last episode of anginal pain or admission if angina occurred previously). Blood was collected from each patient and drawn into pyrogen-free blood collection tubes without additives. Multiple aliquots of serum were placed into sterile 1-mL screw-capped polypropilene vials and stored at −80°C in plastic bags. Samples were kept frozen for no longer than 30 days with an average of 21 days. For thawing, serum tubes were placed overnight in a refrigerator at 2 to 8°C. The samples were frozen and thawed only once.

Total cholesterol, high-density lipoprotein (HDL) cholesterol, LDL cholesterol, triglycerides, glucose, white blood cell (WBC) count, were measured with standard methods. IL-6, IL-1 beta, TNF-α, IL-8, and MCP-1 were measured with commercial assay kits (Quantikine, R&D System). CRP was measured by a high sensitivity method.

Peripheral Blood Mononuclear Cells and Monocyte Isolation

Peripheral blood mononuclear cells (PBMCs) were separated as previously described.[18] A detailed description of the method is available in the supplemental materials (available online at http://atvb.ahajournals.org).

Stimulation of Whole Blood With LPS and Measurement of IL-6 and IL-1 Beta

Aliquots of 1-mL of heparinized whole blood (with or without LPS) were placed in sterile 1.5-mL centrifuge tubes and placed on a rotator and incubated under sterile conditions at 37°C in an atmosphere containing 5% CO_2. The samples were treated with 1 ng/mL of LPS (Sigma Chemical) for 4 hours. The supernatant was then removed and stored at −80°C for the measurement of IL-6 and IL-1 beta.

Stimulation of Monocytes With LPS and Measurement of IL-6 and IL-1 Beta

Freshly isolated monocytes (5×10^5) were incubated for 4 hours at 37°C in RPMI-1640 supplemented with 8% fetal calf serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 0.2 mmol/L L-glutamine with or without LPS (1 ng/mL). Then the culture supernatant was removed and stored at −80°C for the measurement of IL-6 and IL-1 beta.

Flow Cytometry and Western Blotting Analysis

CD14 and TLR-4 protein expression was analyzed in blood samples and in pools of separated monocytes by flow cytometry and Western blotting analysis. A detailed description of the 2 methods is available in the supplemental materials.

Circulating Plasma oxLDL Measurement

Circulating plasma oxLDL levels were measured with the enzyme-linked immunosorbent assay Mercodia Oxidized LDL ELISA (Mercodia AB) as previously described.[6] Cu^{2+}-modified LDL ranging from 50 to 500 ng/mL was used as a standard solution.

LDL Isolation and Oxidation

LDL and Cu^{2+}-modified LDL were prepared as reported.[6]

Real-Time Quantitative RT-PCR Analysis and Protein Expression of CD14 and TLR-4 in Separated Monocytes

A detailed description of the method is available in the supplement materials.

oxLDL-Dependent Expression of CD14/TLR-4 in Monocytes and Production of IL-6, IL-1 Beta, TNF-α, and MCP-1

Purified monocytes (3×10^5/mL, 200 μL/well) from healthy donors were cultured in RPMI 1640 with L-glutamine (GIBCO) for 20 hours at 37°C with increasing amounts of oxLDL (from 20 to 40 μg/mL medium). CD14 and TLR-4 expression and IL-6, IL-1 beta, TNF-α, and MCP-1 concentration in the supernatant were measured as described. In some experiments, blocking anti-CD14 monoclonal antibody (mAb; 20 μg/mL) or control mouse IgG (50 μg/mL) were also added.

Serum-Dependent Expression of CD14/TLR-4 in Monocytes and Production of IL-6, IL-1 Beta, TNF-α, and MCP-1

Purified monocytes (3×10^5/mL, 200 μL/well) from healthy volunteers were cultured in RPMI 1640 with L-glutamine (GIBCO) for 20 hours at 37°C with 40% serum from: (1) 10 UA patients with the highest oxLDL levels, (2) 10 UA patients with the lowest oxLDL levels, (3) 10 C subjects with the highest oxLDL levels, and (4) 10 C subjects with the lowest oxLDL levels. For processing of serum, 80 μL of serum from each patient or C subject was added to monocyte culture immediately after thawing, at the start of the culture period. As a further control, monocytes were also incubated with the corresponding UA lipoprotein-deprived serum (LPDS) in which all the lipoproteins were taken away by ultracentrifugation at a density >1.21 g/mL.[6] CD14 and TLR-4 expression and IL-6, IL-1 beta, TNF-α, and MCP-1 concentration in the supernatant were measured as described. In some experiments anti-CD14 mAb (20 μg/mL) or control human IgG (50 μg/mL) were also added.

Endotoxin contamination of cell cultures involving the use of oxLDL, serum, or LPDS was routinely excluded with the chromogenic Limulus amebocyte lysate assay (Sigma).

Statistical Analysis

A detailed description of the statistical methods is available in the supplement materials.

Data on Cytokines and Chemokines in the Three Groups of Patients

	C (n=27)	SA (n=29)	UA (n=26)	P
IL-6, pg/ml	0.72 (0–2.12)	1.18 (0.82–4.44)	7.22 (4.21–31.1)*	<0.001
IL-1 beta, pg/ml	0.14 (0.04–0.25)	0.21 (0.12–0.42)	0.62 (0.22–1.11)*	<0.001
TNF-alpha, pg/ml	4.97 (2.61–12.41)	4.11 (1.96–10.52)	4.19 (2.90–10.21)	NS
MCP-1, pg/ml	141.2 (82.3–181.4)	153.3 (96.6–204.6)	145.8 (102.3–202.7)	NS
IL-8, pg/ml	15.88 (2.44–40.58)	18.42 (3.72–48.53)	17.92 (3.47–39.62)	NS
IL-6 response to LPS in blood, pg/ml	634 (186–1430)	938 (189–1942)	3457 (1510–8609)*	<0.001
IL-6 response to LPS in monocytes, pg/ml	447 (126–1094)	655 (189–1751)	2318 (998–4601)*	<0.001
IL-1 beta response to LPS in blood, pg/ml	659 (82–1211)	905 (156–1600)	2103 (609–4311)*	<0.001
IL-1 beta response to LPS in monocytes, pg/ml	190 (80–742)	382 (120–1091)	1612 (620–1950)*	<0.001

Data are presented as median (ranges).

*UA vs SA and C. C indicates controls; SA, stable angina; UA, unstable angina; LPS, lipopolysaccharide.

Results

Baseline Characteristics of the Patients
Along a period of 14 months, 102 patients were enrolled in the study; of these only 82 (27 C, 29 with SA, and 27 with UA) fully satisfied the enrolling criteria. Baseline clinical characteristics of the patients are described in supplemental Table I.

Laboratory Data
Data on total, LDL, HDL cholesterol, triglycerides, glucose plasma levels, and on WBC number and CRP are shown in supplemental Table II. Total WBC number and CRP were higher in UA patients than in SA or C patients ($P<0.001$). Data on plasma IL-6, IL-1 beta, TNF-α, IL-8, and MCP-1 and on the concentrations of IL-6 and IL-1 beta stimulated in blood and in separated monocytes with LPS are indicated in the Table. There were no significant differences in TNF-α, IL-8, and MCP-1 plasma levels; both basal and stimulated IL-6 and IL-1 beta levels were higher in UA patients than in SA or C patients ($P<0.001$).

CD14 and TLR-4 Expression on Circulating Monocytes
Figure 1A shows the mean expression of CD14 and TLR-4 in peripheral blood monocytes of the 3 groups of patients as measured by flow cytometry. CD14 and TLR-4 were higher in UA patients than in SA and in C subjects ($P<0.001$). A representative histogram of the flow cytometry analysis is presented in supplemental Figures I and II. Data on TLR-4 were confirmed by Western blotting analysis on separated monocytes (Figure 1B).

Circulating Plasma oxLDL
oxLDL differed significantly among groups (Figure 2A). In particular, post-hoc tests revealed that oxLDL levels were higher in UA than in SA and C subjects and in SA than in C subjects (P from <0.001 to <0.01).

In the whole population of subjects studied, a statistically significant nonlinear sigmoidal regression ($r=0.983$; $P<0.001$) was detected between plasma oxLDL and TRL-4 (Figure 2B). oxLDL levels also correlated linearly with CD14 ($r=0.81$, $P<0.001$; data not shown).

oxLDL-Dependent Expression of CD14 and TLR-4 and Cytokine Production in Monocytes Derived From Healthy Volunteers
Different amounts of Cu^{2+}-oxLDL but not of native-LDL (n-LDL) dose-dependently increased cell mRNA and protein expression of CD14 and TLR-4 as evaluated by flow-cytometry and Western analysis (data on n-LDL not shown) in monocytes derived from healthy volunteers ($P<0.001$; supplemental Figure IIIA through IIIC).

Similarly increasing amounts of oxLDL but not of n-LDL dose-dependently increased the production of IL-6, IL-1 beta, TNF-α and MCP-1 ($P<0.001$; Figure IVA through IVD). The effect of oxLDL on TNF-α and MCP-1 was much lower than on IL-6 and IL-1 beta ($P<0.001$ for any amount of oxLDL). On average with the highest dose of oxLDL (40 μg/mL), IL-6 and IL-1 beta increased respectively 23.3 ± 3.1 and 10.2 ± 1.8 times whereas TNF-α and MCP-1 respectively 2.6 ± 0.31 and 3.2 ± 0.41 times when compared with control. The presence of anti-CD14 blocking mAb significantly reduced the oxLDL-dependent increase of IL-6, IL-1 beta, TNF-α, and MCP-1 (P from <0.01 to <0.001; supplemental Figure IVA through IVD).

Serum-Dependent Expression of CD14 and TLR-4 and Cytokine Production in Monocytes
Monocytes from healthy volunteers were evaluated for CD14 and TLR-4 expression after they were cultured for 20 hours in medium supplemented with either 40% serum from UA patients with the highest (n=10) and lowest (n=10) oxLDL plasma concentrations (respectively 36.4 ± 3.25 and 26.4 ± 3.21 μg/mL) or 40% serum from C subjects with the highest (n=10) and lowest (n=10) oxLDL levels (respectively 17.7 ± 2.7 and 9.73 ± 3.1 μg/mL). The incubation of monocytes with the sera derived from UA patients with the highest and lowest oxLDL levels induced a significant increase in the expression of CD14 and TLR-4 compared with the corresponding sera derived from the C subjects (Figure 3A and 3B as evaluated by flow cytometry and Western analysis respectively; $P<0.001$). The incubation of monocytes with the LPDS of UA patients with the highest oxLDL levels (oxLDL undetectable) induced a reduced increase in CD14 and TLR-4 when compared with the corresponding sera in toto (Figure 3A and 3B; $P<0.001$). The
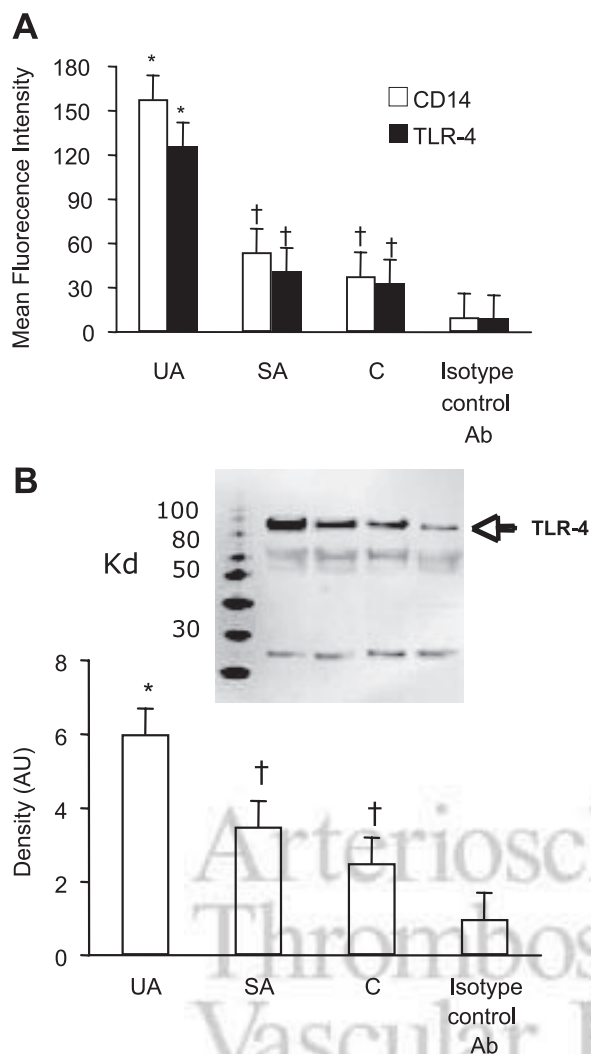
A

B

Figure 1. CD14 and toll like receptor-4 (TLR-4) protein expression in peripheral blood cells as evaluated by flow cytometry (A) and TLR-4 protein expression as evaluated by Western analysis (B) in separated monocytes of unstable (UA) and stable angina (SA) patients and in control subjects (C). Results of flow cytometry are expressed as mean fluorescence intensity and are the means±SD of measurements performed in triplicate. As controls isotypes of TLR-4 and CD14 antibodies were used. *<0.001 vs SA, C, and isotype control antibody (Ab); †<0.001 vs isotype control Ab. Results of Western analysis were expressed as density in arbitrary units (AU), as determined by densitometric analysis, and are the means±SD of measurements performed in triplicate. *P<0.001 vs SA and C subjects; †P<0.01 vs isotype control Ab.

A

B

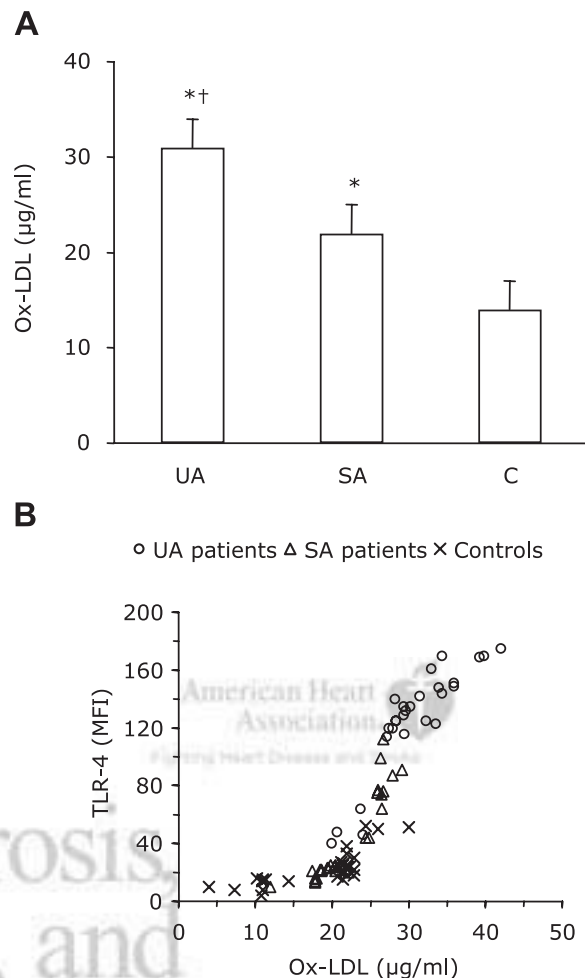○ UA patients △ SA patients × Controls

Figure 2. Concentrations of plasma oxLDL in unstable (UA) and stable angina (SA) patients and in control subjects (C) (A) and nonlinear sigmoidal regression between plasma oxLDL and circulating concentration of toll-like receptor-4 (TLR-4) in the whole population of subjects studied ($r=0.983$, $P<0.001$) (B). Results on oxLDL are the means±SD of measurements performed in triplicate. *$P<0.001$ vs C; †$P<0.01$ vs SA.

concentrations of IL-6, IL-1 beta, TNF-α, and MCP-1 were significantly higher in the monocytes incubated with the sera of UA patients with highest and lowest oxLDL levels than in those incubated with the corresponding sera of C subjects ($P<0.001$; Figures 4A, 4B, 5A, and 5B). The effect on TNF-α and MCP-1, however, was negligible, and post-hoc test revealed that the changes of IL-6 and IL-1 beta were much higher than those of TNF-α and MCP-1 ($P<0.001$). The presence of anti-CD14 blocking mAb significantly reduced the sera-dependent increase of IL-6 and IL-1 beta ($P<0.001$), but not of TNF-α and MCP-1 (Figures 4A, 4B, 5A, and 5B). In the LPDS-stimulated monocytes, only the

production of IL-6 and IL-1 beta was lower than that induced by UA sera in toto ($P<0.001$; Figures 4A, 4B, 5A, and 5B).

Discussion

The results of this study show that during unstable phases of angina, UA patients exhibited a greatly enhanced concentration of IL-6 and IL-1 beta in plasma as well as in whole blood and separated monocytes after LPS stimulation. The mechanisms leading to this rather selective increase of cytokines and to the abnormal production of IL-6 and IL-1 beta after LPS challenge in UA patients are unclear. Here we considered the possibility that oxLDL may contribute to the cytokine overproduction by upregulating CD14 and TLR-4 expression in circulating monocytes of UA patients.

In our study there was an expansion of CD14- and TLR-4–positive monocytes in UA patients and very interestingly statistically significant correlations were found between the plasma levels of circulating oxLDL and the circulating concentration of CD14 and TLR-4 indicating that oxLDL may be implicated in CD14 and TLR-4 expression in vivo.
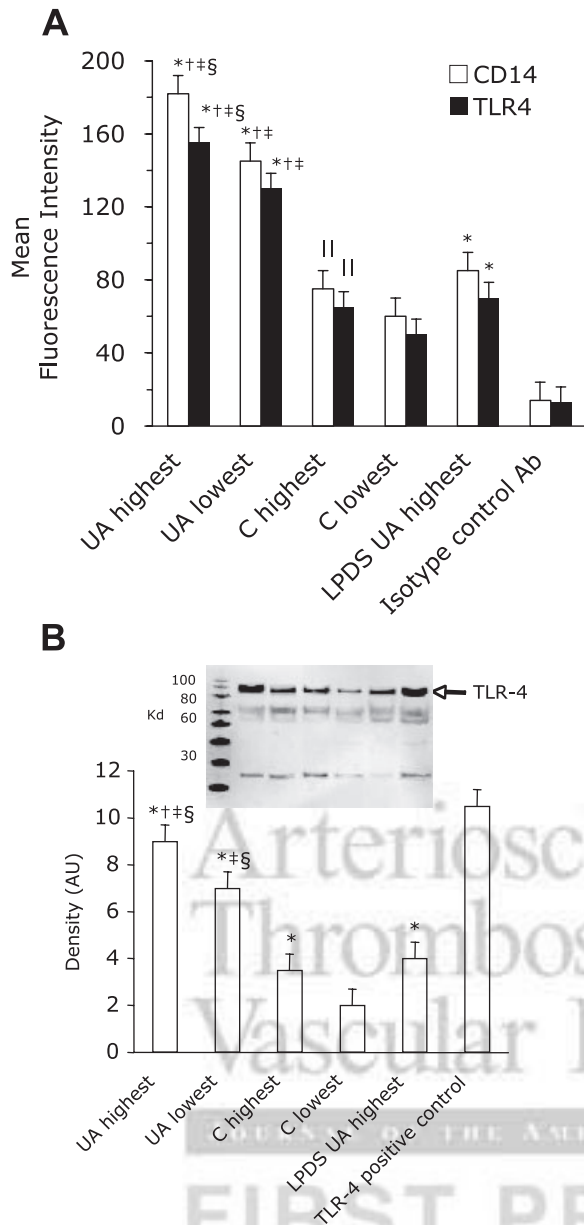
A

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Figure 3. Expression of CD14 and toll-like receptor-4 (TLR-4) as evaluated by flow cytometry (A) and of TLR-4 as evaluated by Western analysis (B) in monocytes derived from healthy volunteers incubated with serum of unstable angina (UA) patients with the highest (UA highest, n=10) and lowest (UA lowest, n=10) oxLDL levels and controls (C) with the highest (C highest, n=10) and lowest (C lowest, n=10) oxLDL levels. Monocytes were also incubated with lipoprotein deprived serum (LPDS) derived from UA patients with the highest oxLDL levels (LPDS UA highest; n=10). Results of flow cytometry are expressed as mean fluorescence intensity and are the means±SD of experiments performed in triplicate. As controls, isotypes of TLR-4 and CD14 antibodies were used. *$P<0.001$ vs C lowest; †$P<0.01$ vs UA lowest; ‡$P<0.001$ vs C highest; §$P<0.001$ vs LPDS UA highest; ‖$P<0.05$ vs C lowest. Results of Western analysis are expressed as density in arbitrary units (AU), as determined as densitometry analysis and are the means±SD of experiments performed in triplicate. *$P<0.001$ vs C lowest; †$P<0.01$ vs UA lowest; ‡$P<0.001$ vs C highest; §$P<0.001$ vs LPDS UA highest.
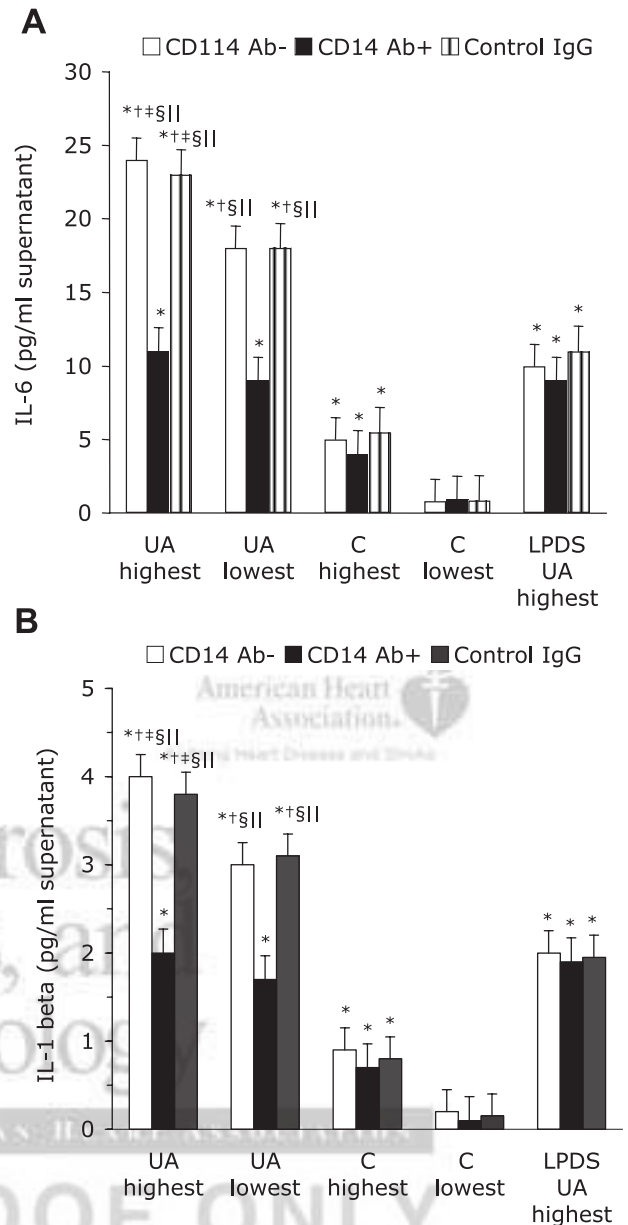
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B

Figure 4. IL-6 (A) and IL-1 beta (B) production induced by serum of unstable angina (UA) patients with the highest (UA highest, n=10) and lowest (UA lowest, n=10) oxLDL levels and controls (C) with the highest (C highest, n=10) and lowest (C lowest, n=10) oxLDL levels in monocytes derived from healthy volunteers. Monocytes were also incubated with lipoprotein-deprived serum (LPDS) derived from UA patients with the highest oxLDL levels (LPDS UA highest, n=10). In some experiments, anti-CD14 antibody (Ab; 20 μg/mL) or control IgG (50 μg/mL) were also added to cell culture. Results are the means±SD of experiments performed in triplicate. *$P<0.001$ vs C lowest; †$P<0.001$ vs anti-CD14 Ab+; ‡$P<0.001$ vs UA lowest; §$P<0.001$ vs C highest; ‖$P<0.001$ vs LPDS UA highest.

The in vitro experiments definitely support this hypothesis because the amounts of oxLDL that increased the expression of CD14 and TLR-4 in monocytes were in the same order of concentrations found in the plasma of UA patients. The upregulation in CD14 and TLR-4 expression induced by oxLDL was associated with an increased production of IL-6 and IL-1 beta and at a lower extent of TNF-α and MCP-1. The fact that the presence of blocking anti-CD14 mAb
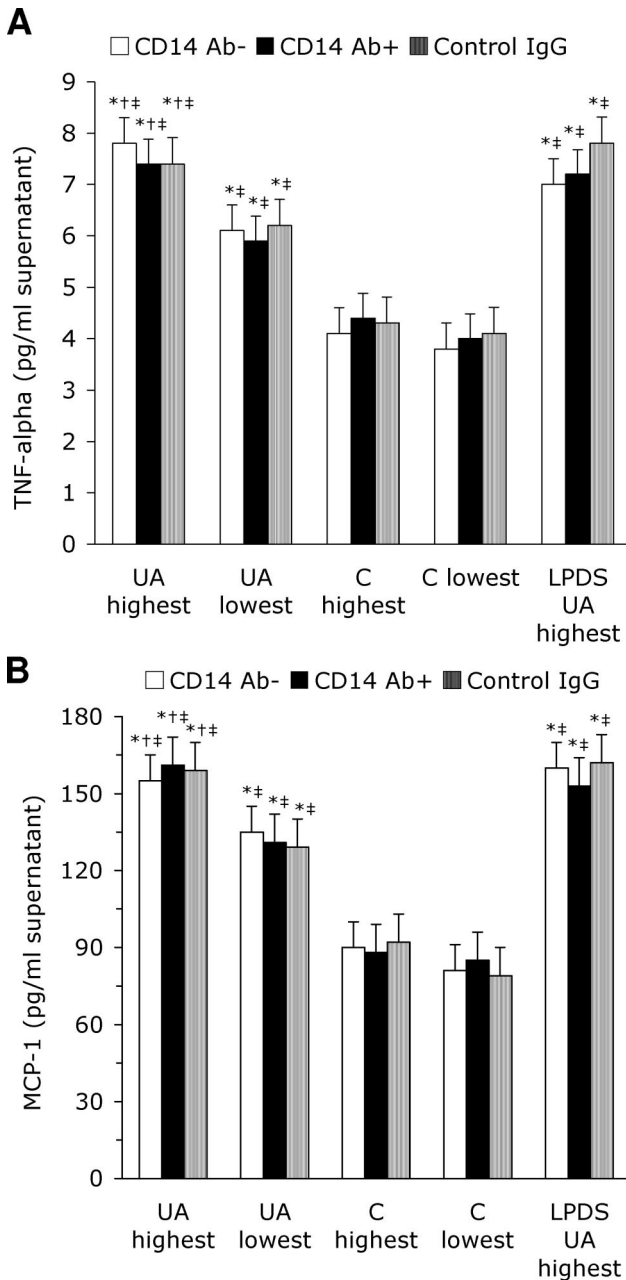
A

B

Figure 5. TNF-α (A) and monocyte chemoattractant protein-1 (MCP-1; B) production induced by serum of unstable angina (UA) patients with the highest (UA highest, n=10) and lowest (UA lowest, n=10) oxLDL levels and controls (C) with the highest (C highest, n=10) and lowest (C lowest, n=10) oxLDL levels in monocytes derived from healthy volunteers. Monocytes were also incubated with lipoprotein-deprived serum (LPDS) derived from UA patients with the highest oxLDL levels (LPDS UA highest, n=10). In some experiments, anti-CD14 antibody (Ab; 20 μg/mL) or control IgG (50 μg/mL) were also added to cell culture. Results are the means±SD of experiments performed in triplicate. *$P<0.001$ vs C lowest; †$P<0.01$ vs UA lowest; ‡$P<0.001$ vs C highest.

spreading of macrophages by binding to CD14 and signaling through the LPS receptor complex composed of TLR-4 and MD-2.[19] At variance with these results, Walton et al[20] showed that CD14 did not appear to mediate the action of oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (ox-PAPC) on IL-8 transcription in endothelial cells because it was not blocked by anti-CD14 neutralizing antibodies. These differences may be attributable to the fact that oxLDL is a mixture of possible ligands, including various proteins, cholesterol, and oxidized phospholipids, whereas ox-PAPC only contains oxidized phospholipids. Our conclusion is also in contrast with the results of Miller et al[21] who found that the induction of IL-6 by oxLDL was not dependent on TLR-4. These contrasting results may be attributable to the different oxLDL preparations or alternatively to differences in cell types.

Starting from these results we then evaluated the effect of sera derived from UA patients and C subjects on CD14 and TLR-4 expression in monocytes derived from healthy volunteers. The results show that the sera of UA patients increased at a much higher extent the expression of CD14 and TLR-4 in monocytes derived from healthy volunteers than in those of C subjects. Similarly to the results obtained with oxLDL where there was a prevalent increase of IL-6 and IL-1 beta, the overexpression of CD14 and TLR-4 caused by sera of UA patients, was associated with a clear-cut increase of IL-6 and IL-1 beta whereas the rise of TNF-α and MCP-1 was negligible. The reasons why oxLDL and sera of UA patients with the highest oxLDL circulating levels produced slight variations of TNF-α and MCP-1 are unclear. However, it is known that TRL engagement results in activation of mitogen-activated protein kinase (MAPKs), which, together with the NF-kB pathway, transduces extracellular signals to cellular responses.[22] Negative regulation of MAPK activity is affected primarily by MAPK phosphatase-1 (MKP-1), a member of the MAPK phosphatase family,[23] which has been recently reported to regulate the temporal response of pro- and antiinflammatory cytokines by TLRs.[24] oxLDL has been demonstrated to activate MKP-1,[25] and even though its role in TLR-mediated immune responses in vivo is far from being fully elucidated, a tentative explanation of our results could be that in our experimental conditions MKP-1 may have repressed to a greater degree some cytokines than others. Of course on the basis of present results we cannot exclude that other suppressing mechanisms that are normally evoked by TLR activation[26] may have contributed to the different cytokine production. Furthermore different serum components may have reduced the secretion of TNF-α and MCP-1 induced by the sera of UA patients. For instance, a C-C chemokine called regulated on activation normally T-cell express and secreted (RANTES), which has been reported to be high in acute coronary syndrome,[27] has been shown to modulate the TLR4-induced cytokine secretion in human peripheral blood monocytes.[28] These hypothetical counteracting mechanisms and others unknown may have therefore depressed not all but just some cytokines/chemokines and explain at least partially why basal circulating TNF-α and MCP-1 were not elevated in UA patients. In addition, the known very short half-life of some cytokines/chemochines

reduced the cytokine concentration in the supernatant of monocytes, may indicate that the cytokine increase and above all that of IL-6 and IL-1 beta is, at least partially, related to the binding of oxLDL to this receptor and therefore to TLR-4 engagement. This conclusion is in agreement with previous results showing that oxLDL induced actin polymerization and

and in particular of TNF-α may have contributed to the results.[29]

Because a number of circulating agents could potentially have increased monocytic CD14 and TLR-4 expression as well cytokine production, the LPDS of UA patients was then used to evaluate the magnitude of lipoprotein effect. In these conditions, ie, in absence of lipoproteins and with no detectable oxLDL, the increase in monocytic expression of CD14/TLR-4 and in the concentration of supernatant IL-6 and IL-1 beta were significantly lower than that obtained with the complete sera. These results of course have limitations because they have not been shown for oxLDL specifically because in LPDS all the lipoproteins and not only oxLDL were removed. Furthermore, because a considerable amount of TLR4/CD14 expression and of IL-6 and IL-1 beta production was not abolished by the absence of lipoproteins in the sera, it is likely that many other parameters and not just oxLDL or other lipoproteins may differ between the 2 groups and contribute to the results. These unknown parameters may have also been important in determining the slight increase of TNF-α and MCP-1 because there was no difference in the results obtained with sera in toto and LPDS.

Taken together these results suggest that the increased plasma levels of IL-6 and IL-1 beta in patients with UA may be at least in part related to the effect of circulating oxLDL on monocyte expression of CD14 and TLR-4 and provide new insights into the inflammatory origin of atherosclerosis.

Sources of Funding

Disclosures

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Arteriosclerosis, Thrombosis, and Vascular Biology

Enhanced Levels of Oxidized Low-Density Lipoprotein Prime Monocytes to Cytokine Overproduction via Upregulation of CD14 and Toll-Like Receptor 4 in Unstable Angina

A. Fratta Pasini, M. Anselmi, U. Garbin, E. Franchi, C. Stranieri, M. C. Nava, V. Boccioletti, C. Vassanelli and L. Cominacini

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MATERIALS AND METHODS

Peripheral blood mononuclear cells (PBMCs) and monocyte isolation

Whole blood was layered onto a sterile aqueous medium containing ficoll and sodium diatrizoate at a predetermined density of 1.007 g/ml at 25° C. Gentle centrifugation at room temperature resulted in the separation of PBMCs at the blood/ficoll interface, with the other white and red blood cells passing through the interface. Monocytes were then isolated from PBMCs by negative selection with a cocktail of hapten-conjugated antibodies and magnetic microbeads coupled to an anti-hapten monoclonal antibody (No touch monocyte isolation kit, Miltenyi Biotec) and depletion on a column in a magnetic field (VarioMACS, Miltenyi Biotec). Monocyte purity was greater than 97% as assessed by flow cytometry (data not shown).

Flow cytometry

Blood samples were stained within 30 minutes after sampling to prevent artificial activation of the cells. The cells were labelled with mouse anti-CD14 (UCH-M1, Santa Cruz Biotechnology, Inc.) and mouse anti-TLR-4 (HTA 125, Santa Cruz Biotechnology, Inc) antibodies as previously described (14). In order to distinguish positive antibody interactions from background fluorescence, control antibodies matching the immunoglobulin class and isotype of the primary antibodies were used (Santa Cruz Biotechnology, Inc). Immunofluorescence staining was analysed with a FACScan flow cytometer equipped with CellQuest software (Becton-Dickinson Biosciences, San Jose, CA, USA).

Western blotting analysis

Monocyte lysates were prepared by using T-PER lysis reagent (25 mM Bicine, 150 mM NaCl, pH 7.6) (Pierce) with a mortar and pestle. Samples were centrifuged at 10,000 rpm for 5 min to pellet cell debris, and supernatants were collected, aliquoted, and stored at –80°C. Protein concentrations were determined

by the BCA protein assay (Pierce). TLR-4 was immunoprecipitated from 1 mg of each protein lysate with a mouse monoclonal anti-TLR4 antibody (HTA 125; Santa Cruz Biotechnology). Immune complexes were captured with protein A/G-Sepharose beads (Pierce) for 2 h at 4°C, and the beads were washed four times with 100 mM NaCl. To ensure the specificity of protein-antibody interaction, lysates were incubated with beads in the absence of antibody as well as with an irrelevant P3 immunoglobulin G1 isotype control (Caltag). Proteins were resolved by sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) and electrotransferred to nitrocellulose membrane in Tris-glycine buffer, with 20% methanol. Immunoblots were washed with 1x phosphate-buffered saline-0.05% Tween 20 and blocked in 5% milk for 1 h at room temperature. TLR-4 was then detected by probing immunoprecipitates with rabbit polyclonal anti-TLR-4 antibody H-80 (Santa Cruz Biotechnology), followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Reactive antigens were visualized with Supersignal chemiluminescence substrate (Pierce). As positive control for TLR-4, the PC-3 lysate derived from human prostate adenocarcinoma was used (ProSci Incorporated, San Diego, CA, USA).

Real-Time quantitative RT-PCR analysis and protein expression of CD14 and TLR-4 in separated monocytes

Total RNA was extracted with a RNeasy Mini Kit (Qiagen) and was reverse transcribed using IScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time RT-PCR was conducted by iCycler thermocycler (Bio-Rad, Hercules, CA) using IQSYBR Green PCR SuperMix (Bio-Rad, Hercules, CA) and 300 pmol/ml each primer pair. Primer design and optimization in regard to primer dimmers, self priming formation, miss priming and amplicon length was done with the Beacon Design 4.0 (PREMIER Biosoft International, Palo Alto, CA, USA) primer design software. All primers were optimized to an equal annealing temperature of 60°C, a similar GC content and supplied by MWG Biotech (Ebersberg, Germany). The results were quantified as Ct values, where Ct is defined as the threshold

cycle of the polymerase chain reaction at which the amplified product is first detected, and expressed as the ratio of target to control (ß-actin).

Statistical analysis

Continuous data are expressed as mean ± SD values if normally distributed. Median (interquartile range) was used for variables not normally distributed. Normal distribution of the data was determined using Shapiro-Wilk test. Differences between continuous data were analysed by two-tailed unpaired Student's t-test. Statistical comparison between 3 groups was performed by one- or two-way ANOVA and post-hoc multiple comparison using Student-Newmann-Keuls' test, if a parametric distribution was assessed. If the data were not parametric, ANOVA on ranks and post-hoc Dunn's test was used. Relationship between variables was assessed by linear and non-linear regression. A probability value (p) <0.05 was considered to be statistically significant. All data were analyzed with SPSS 11.04 for Macintosh (SPSS, Chicago, Ill.)

Fratta Pasini et al., Data supplement 1

Table I. Baseline clinical characteristics of the three groups of patients.

	C (n=27)	SA (n=29)	UA (n=27)	p
Age (years)	66±11	61±9	61±9	NS
Females	9 (33%)*	3 (10%)	3 (11%)	<0.05
Risk factors:				
Family history	10 (37%)	19 (66%)	10 (38%)	NS
Smoke	5 (18%)	8 (28%)	12 (46%)	NS
Hypercholesterolemia	11 (41%)*	23 (79%)	18 (69%)	<0.05
Hypertension	3 (48%)	23 (79%)	16 (61%)	NS
Diabetes	2 (7%)	4 (14%)	4 (15%)	NS
Current therapy:				
Ace-inhibitors	17 (63%)	17 (59%)	9 (35%)	NS
Statins	3 (11%)	7 (24%)	3 (11%)	NS
Aspirin	8 (30%)	22 (76%)†	13 (50%)	<0.05
Clinical history:				
Previous ACS	-	19 (65%)‡	8 (31%)	<0.01
Previous PCI	-	3 (11%)‡	2 (8%)	<0.01

Data are expressed as numbers and percent. * = C vs SA and UA, † = SA vs C, ‡ = SA vs UA. C = controls; SA = stable angina; UA = unstable angina; ACS = acute coronary syndrome; PCI = percutaneous coronary intervention.

Fratta Pasini et al., Data supplement 2

Table II. Laboratory data of the three groups of patients.

	C (n=27)	SA (n=29)	UA (n=26)	p
Total cholesterol (mg/dl)	206.1±25.9	206.4±23.4	215.0±27.1	NS
LDL cholesterol (mg/dl)	133.0±26.4	136.5±4.5	139.9±27.4	NS
HDL cholesterol (mg/dl)	49.9±9.42	46.8±10.9	44.4±8.50	NS
Triglycerides (mg/dl)	135.5±69.4	139.9±49,6	155.0±66.2	NS
Glucose (mg/dl)	106.3±25.6	97.3±18.0	100.8±16.2	NS
White blood cells (10^9/L)	6.75±1.57	6.51±1.81	9.07±1.63*	<0.001
CRP (mg/dl)	0.43 (0.08-0.83)	0.51 (0.08-1.21)	0.98 (0.25-2.01)*	<0.001

Data are expressed as mean±SD or median (ranges).
* UA vs SA and C
C = controls; SA = stable angina; UA = unstable angina.

Fratta Pasini et al., Data supplement 3 and 4

Figure I
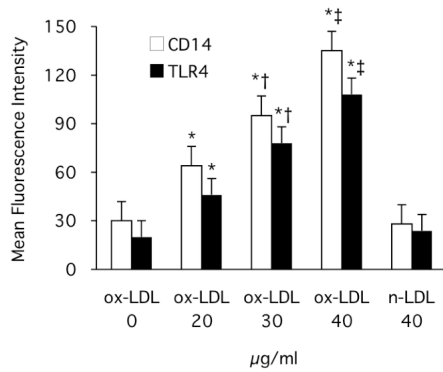

CD14 (MFI)
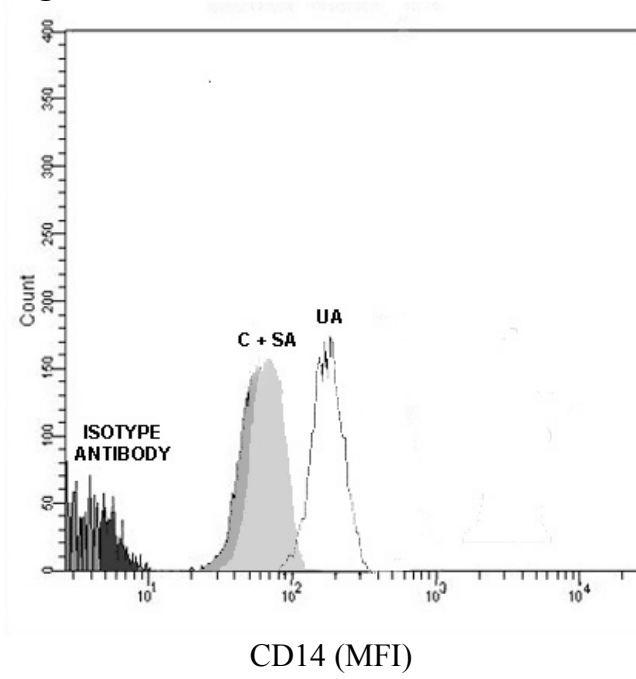
Figure II

TLR-4 (MFI)
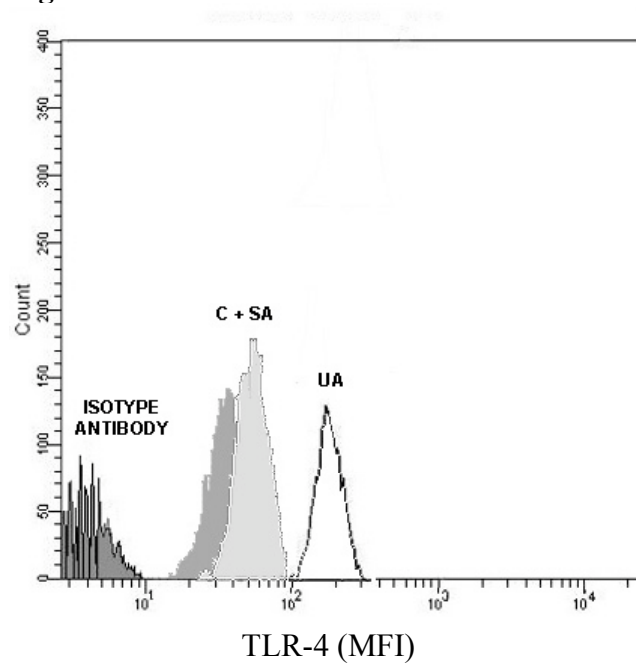
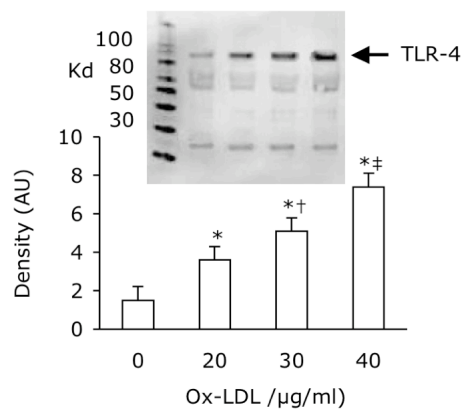# Fratta Pasini et al., Data supplement 5

Figure III A
Real-time PCR
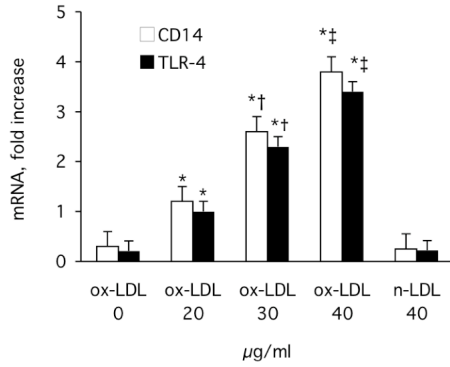
Figure III B
Flow cytometry: mean values

Figure III C
Western blotting analysis
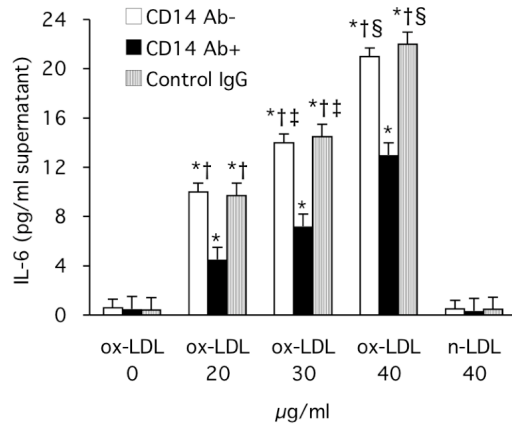
Fratta Pasini et al., Data supplement 6

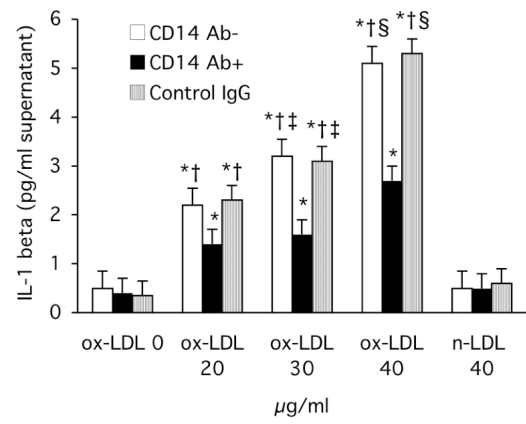**Figure IV A**

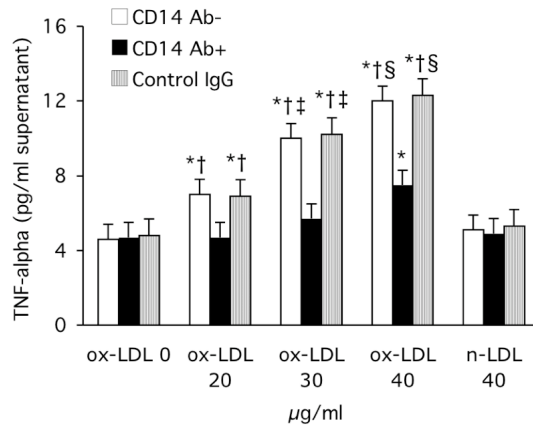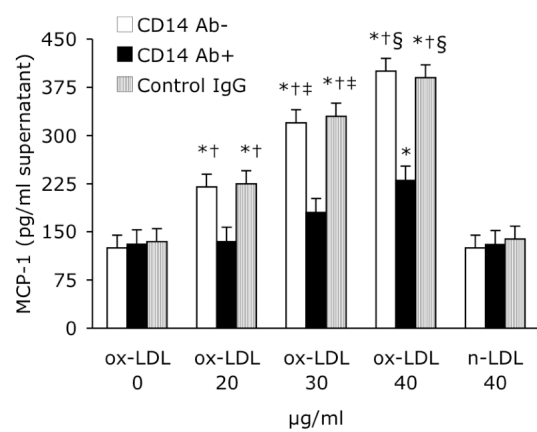**Figure IV B**

Figure IV C

Figure IV D

FIGURE LEGENDS

Figure I and II. Representative analysis of the expression of CD14 and toll-like receptor-4 (TLR-4) in peripheral blood monocytes from control subjects (C), stable angina patients (SA) and unstable angina patients (UA). As controls, isotype antibodies for CD14 and TLR-4 were used (ISOTYPE ANTIBODY). Data are expressed as mean fluorescence intensity (MFI).

Figure III. Oxidized (Ox)- and native (n)-LDL-dependent CD14 and toll-like receptor-4 (TLR-4) mRNA and protein expression in monocytes derived from healthy donors.
Increasing amounts of ox-LDL and a fixed amount of native (n)-LDL were incubated with monocytes for 20 hours. CD14 and TLR-4 mRNA (A) and protein expression as evaluated by flow cytometry (B) and Western analysis (data on CD14 and on n-LDL not shown) (C) were then measured. mRNA was quantified as Ct value, where Ct is defined as the threshold cycle of the polymerase chain reaction at which the amplified product is first detected, and expressed as the ratio of target to control (ß-actin). Results of flow cytometry are expressed as mean fluorescence intensity. Data on Western analysis are expressed as density in arbitrary units (AU). Results are the means±SD of experiments performed in triplicate in six separated occasions. *$p<0.001$ versus control (ox-LDL= 0 µg/ml); †$p<0.001$ versus ox-LDL 20 µg/ml; ‡$p<0.001$ versus ox-LDL 30 µg/ml.

Figure IV. Interleukin (IL)-6, IL-1 beta, tumor necrosis factor-alpha (TNF-alpha) and monocyte chemoattractant protein-1 (MCP-1) production induced by increasing amounts of oxidized (ox)-LDL and a fixed amount of native (n)-LDL in monocytes derived from healthy volunteers.
Increasing amounts of ox-LDL and a fixed amount of n-LDL were incubated with monocytes for 20 hours. IL-6 (A), IL-1 beta (B), TNF-alpha (C) and MCP-1 were then measured in the supernatant. In some experiments, anti CD14 antibody (Ab) (20µg/ml) or control IgG (50µg/ml) were also added to cell culture. Results are the

means±SD of experiments performed in triplicate in six separate occasions.

*$p<0.001$ versus none; †$p<0.001$ versus anti CD14 Ab-; ‡$p<0.001$ versus ox-LDL 20; §$p<0.001$ versus ox-LDL 30.