Biochimica et Biophysica Acta 1771 (2007) 613–622

Pro-inflammatory action of LDL(−) on mononuclear cells is counteracted by increased IL10 production

Sònia Benítez [a,*], Cristina Bancells [a], Jordi Ordóñez-Llanos [b,c], Jose Luis Sánchez-Quesada [a,b]

[a] *Servei Bioquímica, Institut de Recerca, Hospital de la Santa Creu i Sant Pau, C/Antoni Maria Claret 167, 08025 Barcelona, Spain*

[b] *Servei de Bioquímica, Hospital de la Santa Creu i Sant Pau, Spain*

[c] *Departament de Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, Spain*

* Corresponding author. Tel.: +34 932919261; fax: +34 932919196. *E-mail address:* sbenitez@hsp.santpau.es (S. Benítez).

Abstract

Objective: LDL(−) is a minor LDL subfraction that induces inflammatory factor release by endothelial cells. Since LDL(−) is present in plasma, its interaction with leucocytes, a cell type involved in atherosclerosis phenomena, is feasible; therefore, the aim of the current study was to evaluate LDL(−) effect on lymphocytes and monocytes isolated from human plasma. *Methods and Results:* Mononuclear cells were incubated with LDL(+) and LDL(−) and expression and release of several inflammatory mediators were analyzed by protein membrane assay, ELISA and real-time RT-PCR. LDL(−) induced a significantly increased production versus LDL(+) in MCP1, GROβ, GROγ, IL6, IL8 and IL10 in monocytes as well as in lymphocytes. These induced molecules are inflammatory, except for IL10 which is considered an anti-inflammatory cytokine. Therefore, the role of IL10 was evaluated in experiments where exogenous IL10 or antibodies anti-IL10 or anti-IL10 receptor were added. IL10 addition diminished the release of the other factors induced by LDL(−) near to basal production both at protein and RNA level. In contrast, the antibody anti-IL10 increased inflammatory cytokine release around two-fold, whereas the antibody anti-IL10 receptor produced a lower effect. *Conclusions:* LDL(−) promoted inflammatory cytokine production in leucocytes; however, it also induced IL10 that minimized this effect. Therefore, IL10 developed a significant role in counteracting the LDL(−) inflammatory action.

Keywords: LDL(−); Cytokine; Monocyte; Lymphocyte; IL10

Introduction

Modified LDL is a key factor in the initiation and development of atherosclerosis and it is widely accepted that LDL modification takes place when the lipoprotein is trapped in the arterial intima where it can exert its inflammatory action [1]. The existence of a modified LDL in the circulation is controversial; however, several reports have described the presence of an electronegative LDL (LDL(−)), a minor LDL subfraction with increased electronegative charge, which presents atherosclerotic and pro-inflammatory properties [2]. LDL(−) proportion is increased in diseases associated with atherosclerosis, such as diabetes mellitus or familial hypercholesterolemia [3–5]. Furthermore, in endothelial cells LDL(−) promotes inflammatory molecule release [6–9], cytotoxicity [10,11] and apoptosis [12].

Apart from endothelial cells, leucocytes are also closely involved in atherosclerosis, since recruitment of monocytes and lymphocytes from peripheral blood to the intima of the vessel wall is a primordial event in atherogenesis [13]. In fact, recruitment of mononuclear leucocytes is essential for the development and progression of atherosclerotic lesions and is an event that appears to depend on the local presence of LDL. On the other hand, lymphocytes, and especially monocytes, synthesize several inflammatory factors, such as different interleukins, growth factors, chemokines or cytokines, some of which are also shared with endothelial cells. Furthermore, these cell types can interact with LDL; since LDL(−) is present in plasma, it is feasible that this modified LDL could act on leucocytes, thereby promoting an inflammatory response. The aim of the current work was to evaluate LDL(−) effect on monocytes and lymphocytes isolated from human blood. In both cell types, LDL(−) induced two-fold versus native non- electronegative LDL (LDL(+)), the release and transcription of different inflammatory molecules, including monocyte che moattractant protein (MCP1), growth-related oncogene β and γ (GROβ and GROγ), interleukin 6 (IL6) and interleukin 8 (IL8). All these molecules are also released by endothelial cells in response to LDL(−) and are related to inflammatory processes. However, in monocytes and lymphocytes, LDL(−) also induced synthesis and release of interleukin 10 (IL10), a cytokine considered as anti-inflammatory. Several studies show a protective action of IL10 in vivo, such as those with mice overexpressing or lacking IL10, the consequence of which is a diminution in atherosclerotic lesion size [14] or lesion development [15], respectively. Regarding the effect of IL10 in culture cells, it exerts different immunoregulatory actions depending on cell type [16], with the decrease in inflammatory cytokine production in monocytes [17], lymphocytes [18], macrophages [19] and neutrophils [20] being one of the most important.

Owing to these properties ascribed to IL10, its effect on the inflammatory response induced by LDL(−) on leucocytes was evaluated. Taken together, data indicated that IL10 promoted a decrease in the release of inflammatory cytokines induced by LDL(−); hence, IL10 appears to develop a pivotal modulatory role in LDL(−)

action.

Materials and methods

1. Lipoprotein isolation

Plasma samples from healthy normolipemic subjects (total cholesterol < 5.2 mmol/L, triglyceride < 1 mmol/L) were obtained in EDTA-containing Vacutainer tubes. Total LDL (1.019–1.050 kg/L) was isolated by sequential flotation ultracentrifugation at 4 °C in presence of 1 mmol/L EDTA, and LDL was dialyzed against buffer A (Tris 10 mmol/L, EDTA 1 mmol/L, pH 7.4). LDL(+) and LDL(−) were separated by preparative anion-exchange chromatography in an ÄKTA– FPLC system (Amersham Pharmacia, Uppsala, Sweden) and characterized as described [8]. In all experiments, LDL(−) proportion of the subjects ranged from 4 to 6% of total LDL and main characteristics of both LDL subfractions did not differ from previously reported.

2. Cell isolation and incubation with LDLs

Peripheral blood of human volunteers was collected and mononuclear cells were isolated from it the same day. The study was approved by the institutional Ethics Committee and subjects gave their written informed consent. Cells were isolated according to density [21] using density gradient centrifugation at 1.077 kg/L with Linfosep (Biomedics, Madrid, Spain). Differentiation between monocytes and lymphocytes from total mononuclear cells was made depending on their adhesive properties after 20 h of culture to allow adhesion. Differential cell count was assessed by flow-cytometry with 2 laser FACScalibur (Becton Dickinson, Madrid, Spain) with a four-marker combination, and by May Grünwald/Giemsa staining (monocyte purity 80–85%, lymphocyte purity 90–95%). Cell viability was verified by LDH assay (Roche Diagnostics, Basel, Switzerland) and ethidium bromide/acridine orange staining (Sigma, Madrid, Spain).

Cells were cultured in 6-well or 12-well plates with RPMI 1640 medium (Biowhittaker, Walkersville, MD, USA) supplemented with 10% fetal calf serum at 37 °C and 5% CO_2. Before incubation, plates with the appropriate number of cells for each experiment (10^6 cells for protein membrane assay and ELISA and 3×10^6 cells for real-time RT-PCR studies) were incubated in deficient medium (1% fetal calf serum); this medium was used to avoid the presence of other lipoproteins apart from LDL subfractions. LDL(+) and LDL(−) subfractions were dialyzed against RPMI 1640 medium in Sepharose G25M columns (Amersham, Pharmacia, Uppsala, Sweden) and filtered in sterile conditions prior to incubation. After testing different concentrations in preliminary experiments, it was determined that 150 mg apoB/L LDLs was the appropriate concentration to add to the wells. Lipopolysaccharide (LPS) (Sigma, Madrid, Spain) at 0.1 mg/L was used as a positive control, and cells without added stimulus were considered as basal production.

In some experiments, actinomycin D (Sigma, Madrid, Spain) was used at 1 mg/L to inhibit transcription. In other experiments, exogenous IL10, antibodies anti-IL10 or anti-IL10R were also added under the conditions explained below. Control experiments in the presence of polymyxin B (50 mg/L) (Sigma, Madrid, Spain) in the medium were performed to rule out the possibility that trace amounts of LPS present in LDL samples were responsible for the LDL effect.

3. Protein membrane assay and ELISA assay

In all studies in which protein release was evaluated, 10^6 cells were incubated in 12-well plates (1 mL) under each condition. After incubation of cells with samples, culture medium supernatants were collected and frozen at −80 °C.

For protein membrane assay, supernatants were used without dilution in RayBio Human Cytokine Array III (RayBiotech, Norcross, GA, USA). This system is a membrane array based on detection with antibodies and chemoluminescent measurement, and permits the determination of 42 cytokines, chemokines and related molecules simultaneously. Protein membrane assays were incubated with samples for 20 h and developed according to the manufacturer's instructions as described [7]. A differential induction by LDL(−) was considered when the ratio in spot intensity between LDL(−)/LDL(+) was higher than 1.5, spots appeared in the 2 independent experiments performed and spot intensity was greater than 5 pixels. Proteins released to a greater degree by LDL(−) compared to LDL(+), according to the former criteria, were later evaluated and quantified by ELISA.

ELISA used was Module Set antibody pairs (Bender MedSystems, Burlingame, CA, USA) and antibodies for GROβ and GROγ (Peprotech, Rocky Hill, NJ, USA) following the protocols described [7]. In ELISA assays, several incubation times, in time-course experiments, and LDL concentrations were tested to establish the more suitable conditions, and following ELISA studies were performed under those conditions.

4. Real-time RT-PCR

RNA was extracted from 3×10^6 cells cultured in 6-well plates (2 mL). After incubation with LDLs for 4 and 20 h, cells were scraped and collected in RNase- free conditions and pellets frozen after centrifugation. RNA extraction was performed with the Qiagen extraction kit RNAeasy minikit (Qiagen, Hilden, Germany) from cell pellets. Reverse transcription was performed with 1 µg of each RNA using Moloney murine leukemia virus reverse transcriptase H- (Promega, Madrid, Spain). Quantitative RNA analysis was developed by real-time RT-PCR (AbiPrism 7000; Applied Biosystems, Foster City, CA, USA). The mRNA expression of the selected genes was studied using predesigned validated assays (assays-on-demand; Applied Biosystems,

Foster city, CA, USA) and human β-actin (Applied Biosystems, Foster City, CA, USA) was used as internal control.

5. Effect of IL10

IL10 effect on mononuclear cells was evaluated by comparing cytokine release induced by LDLs alone or co-incubated with different concentrations of the following compounds: IL10 (2, 5 and 10 μg/L) (Sigma, Madrid, Spain), antibody anti-IL10R subunit α (IL10R) (5 and 15 mg/L) (Sigma, Madrid, Spain) or antibody anti-IL10 (5 and 15 mg/L) (Peprotech, Rocky Hill, NJ, USA). After incubation under the different conditions, supernatants were collected and frozen to assess cytokine release by ELISA. Real-time RT-PCR was performed following the method described above to evaluate IL10 effect on transcription of the cytokines induced by LDL(−).

6. Statistical analysis

Results are expressed as mean±SD. A Sigma Stat 2.0 statistical package was used. Differences between groups were tested with Wilcoxon's *t*-test (for paired data). A value of $p < 0.05$ was considered significant.

Results

1. Protein membrane assay

Up to 42 inflammatory molecules, indicated in the template (Fig. 1), can be detected by RayBiotech III protein array in which factors reported to be produced by mononuclear cells are included. Monocyte and lymphocyte supernatants were assessed in the array after 20 h of incubation with LDLs or LPS; this time was chosen according to our previous experience with endothelial cells [7]. A representative example of the results ($n = 2$) is shown in Fig. 1.

LDL(−) induced MCP1, GRO, IL6, IL8 and IL10 to a greater degree than LDL(+) in both cell types. The cytokine release by LDL(−) action was even greater than by LPS effect for MCP1, IL8 and GRO. Position in the template for GRO included GROα, GROβ and GROγ; however, GROα, which is present alone in the next position, was not induced by LDL(−).

MCP1, IL8 and GRO are chemokines and IL6 a pleiotropic cytokine associated with inflammation; hence, these factors released by LDL(−) are considered pro-inflammatory. However, IL10, which is also induced by LDL(−), develops functions that are considered mainly immunoregulatory. LDL(−) induced the same molecules in both monocytes and lymphocytes; however, spots were more intense in monocytes than in lymphocytes, and this

greater release by monocytes was also observed in control cells or when cells were incubated with LDL(+) or LPS.

Array from control cells, not incubated with LDLs or LPS, showed only the presence of IL-8 in monocytes. In contrast, cells stimulated with LPS released several inflammatory factors in monocytes as well as in lymphocytes.

2. ELISA assay

As the protein membrane assay method lacks standard curves and only permits semi-quantitative assessment, ELISA assays were performed. MCP1, GROβ, GROγ, IL6, IL8 and IL10 were evaluated, since their release was increased by LDL(−), following the criteria of differential induction versus LDL(+) as described in Materials and methods. First of all, optimal incubation conditions of maximal difference between LDL(+) and LDL(−) were found, at 20 h of incubation and 150 mg apoB/ L LDL, after testing several LDL concentrations and time-course of cytokine release. Regarding concentration, LDL(−) effect increases with concentration, whereas LDL(+) increases at a much lower rate. It is noteworthy that when LDL(+) concentration was increased to 900 mg/L apoB, the cytokine release promoted on monocytes was only half that induced by LDL(−) at 150 mg/L apoB (data not shown). These concentrations would be more similar to those found in the circulation. In relation to time-course, a representative IL6 and IL10 time-course experiment is shown in Fig. 2. The other cytokine release time-courses showed the same pattern as IL6, since protein release rose with increasing time, at least up to 48 h. However, IL10 was the only cytokine that presented different behavior, since its protein level peaked at 20 h and decreased thereafter.

At 20 h, the maximum difference between LDL(−) and LDL(+) was practically reached, with cell viability over 95% being preserved whereas, at longer times, cell viability decreased (86–91% viability at 48 h). Therefore, cytokine release in the supernatant was measured by ELISA (n = 15) at 20 h (Table 1). LDL(−)/LDL(+) ratio expressed after subtracting basal production is also shown in Table 1. In general, LDL(−) promoted a two-fold cytokine release compared to LDL(+), and similar to or even greater than that induced by LPS. LDL(−)/LDL(+) ratio was similar in both cell types, although production was greater in monocytes, thereby corroborating the protein membrane assay data.

3. Transcription induction

Transcription of cytokine genes was analyzed to establish whether the inflammatory factor release by LDL(−) was induced by transcriptional mechanisms. An initial approach was to incubate cells with actinomycin D (1 mg/L) simultaneously with 150 mg apoB/L LDL(+) or LDL(−). In both cases, actinomycin D promoted inhibition

in cytokine release between 60 and 100% depending on the cytokine evaluated ($n = 3$ experiments) whereas, in control cells, cytokine levels remained constant.

Real-time RT-PCR studies were also conducted to evaluate the factors induced by LDL(−) as target genes. Monocytes and lymphocytes were incubated for 4 or 20 h with stimulus prior to RNA extraction and real-time RT-PCR assays ($n = 4$). More RNA copies, between 3-fold and 10-fold depending on the cytokine evaluated, were observed at 4 h compared to 20 h; however, differences in induction mediated by LDL(−) versus LDL(+) did not change and concurred with protein results. Hence, at transcriptional level there was a decrease in the number of copies with time whereas, by protein time-course, the effect was opposite (except for IL10 at long incubation times). Data at 4 h of incubation are presented in Table 2 as the number of relative RNA copies versus β-actin, which presents constitutive expression. In summary, these results indicate that LDL(−) induces cytokine release in leucocytes at transcriptional level.

4. Modulatory IL10 effect

The most striking result of this study was the increased IL10 production by LDL(−), since only inflammatory properties had been attributed to LDL(−), whereas IL10 is considered an anti-inflammatory molecule. Therefore, additional experiments evaluating IL10 effect on monocytes and lymphocytes were developed. Cells were incubated with LDLs, alone or with the addition of exogenous IL10, and the antibodies anti-IL10 or anti-IL10R; supernatant cytokine release was then quantified by ELISA ($n = 6$). The number of RNA copies was evaluated by real-time RT-PCR assays when exogenous IL10 was added ($n = 3$).

A diminished cytokine release when exogenous IL10 was added to LDL(−) compared to LDL(−) alone at 20 h of incubation is shown in Fig. 3. Concentrations of exogenous IL10 were only slightly higher than those released by cells incubated with LDL(−) alone; hence, the results obtained can be considered quite representative. The inhibition after IL10 addition was concentration-dependent, reaching almost 100% inhibition at 10 μg/L; however, at 2 μg/L, inhibition was already over 50%, except for GROγ in monocytes (31% inhibition).

On the other hand, the inhibitory effect of exogenous IL10 was observed not only in protein release but also at transcriptional level, as shown by real-time RT-PCR. The inhibition in RNA copies in cells co-incubated with LDL(−) and IL10 (5 μg/L) versus incubation with LDL(−) alone ($n = 3$) at 4 h of incubation is shown in Fig. 4. The inhibitory effect ranged from 50 to 80% versus LDL(−) alone and, in addition to decreasing the number of RNA copies of the inflammatory factors induced by LDL(−), IL10 also diminished the number of copies of IL10 itself, pointing to a self-regulatory role. The transcriptional inhibition of IL10 remained similar when compared between 4 and 20 h of incubation, being between 50 and 75% depending on the cytokine evaluated at 20 h.

The effect of the antibodies anti-IL10 and anti-IL10R added to LDL(−) is shown in Fig. 5. All cytokines induced

by LDL(−) increased, in a dose-dependent manner, when IL10 was inhibited by the addition of anti-IL10. Nevertheless, using anti-IL10R, the effect, in general, was lower, increasing cytokine release only slightly, and in some cases no significant inhibition at the concentrations of antibody used was observed. On the other hand, it was proved that the antibody anti-IL10 worked correctly since it inhibited IL10 quantity in the supernatant (70–85%), whereas the antibody anti-IL10R promoted an increase in IL10. LDL(+) produced similar results compared to LDL(−) when both antibodies were used or exogenous IL10 was added (data not shown).

Discussion

LDL(−) is a plasma LDL subfraction whose interaction with mononuclear cells is feasible. These cells play a significant role in atherosclerosis, a disease characterized by infiltration of monocytes and lymphocytes from peripheral blood to the intima. Furthermore, lymphocytes, and particularly monocytes, synthesize several inflammatory factors, some of which are also shared with endothelial cells [13]. For all these reasons, the LDL(−) effect on leucocytes is an interesting topic that has not been previously described.

The results of the current study show that LDL(−) induced in mononuclear cells the production of several factors related to inflammatory processes. LDL(−) promoted around two-fold versus LDL(+) protein release of chemokines MCP1, IL8, GROβ and GROγ and interleukins IL6 and IL10. The role of LDL(−) was also confirmed at transcriptional level and the number of RNA copies was greater at 4 h versus 20 h, a logical response since RNA-encoding cytokines are usually unstable to ensure that cytokine synthesis is transient.

All the cytokines induced by LDL(−) action followed the same pattern in monocytes and lymphocytes; however, cytokine release by monocytes was always greater than by lymphocytes, regardless of the stimulus: LDL(+), LDL(−) or LPS. Monocytes are cells whose involvement in atherosclerosis is evident, since they develop a significant role in the early phases of atherogenesis and synthesize several inflammatory factors[13]. In contrast, the role of lymphocytes is not so widely understood, although they also appear to be implicated in atherosclerotic lesion progression [22].

It is noteworthy that LDL(−), besides inducing cytokine release by endothelial cells, also exerts an inflammatory effect on mononuclear cells which could interact with LDL(−) in plasma circulation or when they are retained in the endothelium. The fact that molecules induced by LDL(−) in leucocytes, except IL10, are also stimulated in HUVEC [7] is biologically significant since the same stimulus can act on different cell types. In turn, cytokines released by the effect of LDL(−) in one cell type can activate these cells or others, triggering an amplified response mediated by LDL(−). Therefore, the effects promoted by LDL(−) should not be considered isolated actions but rather as physiologically interrelated in the context of an inflammatory process.

On the other hand, LDL(−) isolated from diabetic and hypercholesterolemic patients, at the same concentration as in normolipemic subjects, did not present a greater inflammatory effect in endothelial cells compared to normolipemic subjects [8,23]. For this reason, the current study was focused on normolipemic subjects. Nevertheless, it is noteworthy that the inflammatory effect promoted by LDL(−) would be greater in diabetic and hypercholesterolemic patients than in normolipemic subjects, since these patients present increased LDL(−) proportion [3–5].

Besides promoting cytokines with an inflammatory action, LDL(−) also induced in monocytes and lymphocytes the synthesis and release of IL10, a cytokine considered as anti- inflammatory. The relationship between IL10 and atherosclerosis is widely accepted, since clinical studies in humans reported increased levels of IL10 in acute coronary syndromes [24,25], atherosclerotic plaques [26], hypercholesterolemia [25,27] and diabetes [28]. The biological function of IL10 in these situations would be to counteract an excessive inflammatory response. This protective role was corroborated by experiments with mice in which animals overexpressing IL10 presented a smaller atherosclerotic lesion [14,29], whereas mice lacking IL10 presented increased atherosclerosis [30,31] and autoimmune diseases [32]. This evidence supports a physiological function of IL10 as a controller of inflammatory response and, in fact, IL10 has even been used with therapeutic aims in autoimmune diseases such as psoriasis [33].

Concerning the anti-inflammatory properties described for IL10 on cells, IL10 activates Th2 anti-inflammatory phenotype [34], promotes cholesterol efflux [35], and inhibits monocyte–endothelium interaction [14], apoptosis [36,37], and phagocytosis [38]. Furthermore, IL10 plays an important role in decreasing the production of inflammatory cytokines in several stimulated cell types [17], and even inhibits the autocrine effect of MCP1 on monocytes [39] and of IL8 on lymphocytes [40]. In this respect, the aim was to evaluate the possible role of IL10 released by LDL(−) on the other cytokines induced in mononuclear cells. Our results showed that IL10 decreased inflammatory cytokine release by LDL(−) and this effect was exerted at transcriptional level, even promoting inhibition of IL10 itself. In this regard, it has been previously reported that IL10 can induce degradation of RNA codifying for specific genes [41] and that IL10 expression is self-regulated [17,42].

IL10 inhibition of inflammatory cytokines points to a modulatory role, which concurs with the theory that, against an external aggression, the immune system activates both inflammatory and anti-inflammatory pathways to counteract collateral damage [43]. In our case, LDL(−) would be the external stimulus that activates not only inflammatory but also anti-inflammatory responses. In this way, anti-inflammatory IL10 would counteract inflammatory cytokine expression induced by LDL(−). On the other hand, the specific pathway activated by IL10 to promote cytokine inhibition is not clear, since knowledge of IL10 intracellular

signal transduction pathways is limited and several possible mechanisms, such as ERK, p38MAPK, STAT or NFkB, have been suggested [44–48]. In experiments using the antibody anti-IL10R, a lower effect than anti-IL10 on raising inflammatory cytokines was observed. This suggests that IL10R could play some role in the modulatory action of IL10; however, the effect varied depending on the cytokine evaluated. In this context, different pathways could be responsible for differential cytokine expression, and there could be alternative entries, apart from IL10R, such as binding to another integral membrane protein, as suggested previously [38]. On the other hand, the addition of anti-IL10R could inhibit endogen IL10 entry into the cell, since IL10 levels increased in the supernatant. In this respect, it was suggested that IL10 had an autocrine regulation by a negative feed-back effect which was inhibited by anti-IL10R [49]. These results, together with IL10 time-course experiments and real- time RT-PCR data adding exogenous IL10 to LDL(−), appear to suggest a self-regulatory role of IL10. This effect could have a biological explanation, with IL10 self-regulation being important to achieve an efficient immune response, since longer IL10 inhibition could cause inflammatory problems [43].

In summary, the fact that LDL(−) promotes the release of inflammatory cytokines in leucocytes, besides inducing a response in endothelium, is significant since it supports its inflammatory role. However, LDL(−) also induces IL10 expression which decreases the release of inflammatory cytokines, thereby developing an important control system. If LDL(−) did not induce IL10, the inflammatory response would be still greater and maybe excessive; hence, in this respect, IL10 could develop a pivotal immunoregulatory function.

Acknowledgments

This work was supported by research grants FIS CP04/0110 and FIS PI03/0885 from the Fondo de Investigación Sanitaria (FIS) and 2004 Basic Research from the Spanish Society of Atherosclerosis (SEA). Sonia Benítez is the recipient of a post- doctoral FIS CP04/110 contract and Cristina Bancells is a recipient of a pre-doctoral fellowship FPU from the Ministry of Education and Science (MEC). The authors are grateful to the Hematology Department of Hospital de Sant Pau for flow- cytometry analysis and to Christine O'Hara for editorial assistance.

References

[1] M.O. Pentikainen, K. Oorni, M. Ala-Korpela, P.T. Kovanen, Modified LDL — Trigger of atherosclerosis and inflammation in the arterial intima, J. Intern. Med. 247 (2000) 359–370.

[2] J.L. Sanchez-Quesada, S. Benitez, J. Ordonez-Llanos, Electronegative low-density lipoprotein, Curr. Opin. Lipidol. 15 (2004) 329–335.

[3] J.L. Sanchez-Quesada, A. Perez, A. Caixas, J. Ordonmez-Llanos, G. Carreras, A. Payes, F. Gonzalez-Sastre, A. de Leiva, Electronegative low density lipoprotein subform is increased in patients with short-duration IDDM and is closely related to glycaemic control, Diabetologia 39 (1996) 1469–1476.

[4] J.L. Sanchez-Quesada, C. Otal-Entraigas, M. Franco, O. Jorba, F. Gonzalez-Sastre, F. Blanco-Vaca, J. Ordonez-Llanos, Effect of simvastatin treatment on the electronegative low-density lipoprotein present in patients with heterozygous familial hypercholesterolemia, Am. J. Cardiol. 84 (1999) 655–659.

[5] J.L. Sanchez-Quesada, A. Perez, A. Caixas, M. Rigla, A. Payes, S. Benitez, J. Ordonez-Llanos, Effect of glycemic optimization on electro- negative low-density lipoprotein in diabetes: relation to nonenzymatic glycosylation and oxidative modification, J. Clin. Endocrinol. Metab. 86 (2001) 3243–3249.

[6] C. De Castellarnau, J.L. Sanchez-Quesada, S. Benitez, R. Rosa, L. Caveda, L. Vila, J. Ordonez-Llanos, Electronegative LDL from normolipemic subjects induces IL-8 and monocyte chemotactic protein secretion by human endothelial cells, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 2281–2287.

[7] S. Benitez, M. Camacho, C. Bancells, L. Vila, J.L. Sanchez-Quesada, J. Ordonez-Llanos, Wide proinflammatory effect of electronegative low- density lipoprotein on human endothelial cells assayed by a protein membrane assay, Biochim. Biophys. Acta 1761 (2006) 1014–1021.

[8] J.L. Sanchez-Quesada, M. Camacho, R. Anton, S. Benitez, L. Vila, J. Ordonez-Llanos, Electronegative LDL of FH subjects: chemical char- acterization and induction of chemokine release from human endothelial cells, Atherosclerosis 166 (2003) 261–270.

[9] C. Castellarnau, C. Bancells, S. Benitez, J. Ordoñez-Llanos, J.L. Sanchez- Quesada, Atherogenic and inflammatory profile of human arterial endothelial cells (HUAEC) in response to LDL fractions, Clim. Chem. Acta 376 (2007) 233–236.

[10] K. Demuth, I. Myara, B. Chappey, B. Vedie, M.A. Pech-Amsellem, M.E. Haberland, N. Moatti, A cytotoxic electronegative LDL subfraction is present in human plasma, Arterioscler. Thromb. Vasc. Biol. 16 (1996) 773–783.

[11] H.N. Hodis, D.M. Kramsch, P. Avogaro, G. Bittolo-Bon, G. Cazzolato, J. Hwang, H. Peterson, A. Sevanian, Biochemical and cytotoxic character- istics of an in vivo circulating oxidized low density lipoprotein (LDL−), J. Lipid. Res. 35 (1994) 669–677.

[12] C.H. Chen, T. Jiang, J.H. Yang, W. Jiang, J. Lu, G.K. Marathe, H.J. Pownall, C.M. Ballantyne, T.M. McIntyre, P.D. Henry, C.Y. Yang, Low- density lipoprotein in hypercholesterolemic human plasma induces vascular endothelial cell apoptosis by inhibiting fibroblast growth factor 2 transcription, Circulation 107 (2003) 2102–2108.

[13] B. Osterud, E. Bjorklid, Role of monocytes in atherogenesis, Physiol. Rev. 83 (2003) 1069–1112.

[14] L.J. Pinderski Oslund, C.C. Hedrick, T. Olvera, A. Hagenbaugh, M. Territo, J.A. Berliner, A.I. Fyfe, Interleukin-10 blocks atherosclerotic events in vitro and in vivo, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 2847–2853.

[15] Z. Mallat, S. Besnard, M. Duriez, V. Deleuze, F. Emmanuel, M.F. Bureau, F. Soubrier, B. Esposito, H. Duez, C. Fievet, B. Staels, N. Duverger, D. Scherman, A. Tedgui, Protective role of interleukin-10 in atherosclerosis, Circ. Res. 85 (1999) e17–e24.

[16] K.W. Moore, R. de Waal Malefyt, R.L. Coffman, A. O'Garra, Interleukin-10 and the interleukin-10 receptor, Annu. Rev. Immunol. 19 (2001) 683–765.

[17] R. de Waal Malefyt, J. Abrams, B. Bennett, C.G. Figdor, J.E. de Vries, Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes, J. Exp. Med. 174 (1991) 1209–1220.

[18] R. de Waal Malefyt, H. Yssel, J.E. de Vries, Direct effects of IL-10 on subsets of human CD4+ T cell clones and resting T cells. Specific inhibition of IL-2 production and proliferation, J. Immunol. 150 (1993) 4754–4765.

[19] D.F. Fiorentino, A. Zlotnik, T.R. Mosmann, M. Howard, A. O'Garra, IL- 10 inhibits cytokine production by activated macrophages, J. Immunol. 147 (1991) 3815–3822.

[20] P. Wang, P. Wu, J.C. Anthes, M.I. Siegel, R.W. Egan, M.M. Billah, Interleukin-10 inhibits interleukin-8 production in human neutrophils, Blood 83 (1994) 2678–2683.

[21] A. Boyum, Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granul- ocytes by combining centrifugation and sedimentation at 1 g, Scand. J. Clin. Lab. Invest., Suppl. 97 (1968) 77–89.

[22] P.A. Vanderlaan, C.A. Reardon, Thematic review series: the immune system and atherogenesis. The unusual suspects: an overview of the minor leukocyte populations in atherosclerosis, J. Lipid. Res. 46 (2005) 829–838.

[23] S. Benitez, A. Perez, J.L. Sanchez-Quesada, A.M. Wagner, M. Rigla, R. Arcelus, O. Jorba, J. Ordonez-Llanos, Electronegative low-density lipoprotein subfraction from type 2 diabetic subjects is proatherogenic and unrelated to glycemic control, Diabetes Metab. Res. Rev. 23 (2007) 26–34.

[24] C. Heeschen, S. Dimmeler, C.W. Hamm, S. Fichtlscherer, E. Boersma,
M.L. Simoons, A.M. Zeiher, Serum level of the antiinflammatory cytokine interleukin-10 is an important prognostic determinant in patients with acute coronary syndromes, Circulation 107 (2003) 2109–2114.

[25] K. Mizia-Stec, Z. Gasior, B. Zahorska-Markiewicz, J. Janowska, A. Szulc,

E. Jastrzebska-Maj, I. Kobielusz-Gembala, Serum tumour necrosis factor-alpha, interleukin-2 and interleukin-10 activation in stable angina and acute coronary syndromes, Coron. Artery Dis. 14 (2003) 431–438.

[26] Z. Mallat, C. Heymes, J. Ohan, E. Faggin, G. Leseche, A. Tedgui, Expression of interleukin-10 in advanced human atherosclerotic plaques: relation to inducible nitric oxide synthase expression and cell death, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 611–616.

[27] K. Mizia-Stec, B. Zahorska-Markiewicz, T. Mandecki, J. Janowska, A. Szulc, E. Jastrzekbska-Maj, Z. Gasior, Hyperlipidaemias and serum cytokines in patients with coronary artery disease, Acta Cardiol. 58 (2003) 9–15.

[28] J. Mysliwska, K. Zorena, E. Semetkowska-Jurkiewicz, D. Rachon, H. Suchanek, A. Mysliwski, High levels of circulating interleukin-10 in diabetic nephropathy patients, Eur. Cytokine Netw. 16 (2005) 117–122.

[29] Y. Liu, D. Li, J. Chen, J. Xie, S. Bandyopadhyay, D. Zhang, A.R. Nemarkommula, H. Liu, J.L. Mehta, P.L. Hermonat, Inhibition of atherogenesis in LDLR knockout mice by systemic delivery of adeno- associated virus type 2-hIL-10, Atherosclerosis (2005).

[30] M.N. Ajuebor, A.M. Das, L. Virag, C. Szabo, M. Perretti, Regulation of macrophage inflammatory protein-1 alpha expression and function by endogenous interleukin-10 in a model of acute inflammation, Biochem. Biophys. Res. Commun. 255 (1999) 279–282.

[31] S. Potteaux, B. Esposito, O. van Oostrom, V. Brun, P. Ardouin, H. Groux, A. Tedgui, Z. Mallat, Leukocyte-derived interleukin 10 is required for protection against atherosclerosis in low-density lipoprotein receptor knockout mice, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 1474–1478.

[32] R. Kuhn, J. Lohler, D. Rennick, K. Rajewsky, W. Muller, Interleukin-10- deficient mice develop chronic enterocolitis, Cell 75 (1993) 263–274.

[33] K. Asadullah, W. Sterry, H.D. Volk, Interleukin-10 therapy—review of a new approach, Pharmacol. Rev. 55 (2003) 241–269.

[34] K. Uyemura, L.L. Demer, S.C. Castle, D. Jullien, J.A. Berliner, M.K. Gately, R.R. Warrier, N. Pham, A.M. Fogelman, R.L. Modlin, Cross- regulatory roles of interleukin (IL)-12 and IL-10 in atherosclerosis, J. Clin. Invest. 97 (1996) 2130–2138.

[35] T. Rubic, R.L. Lorenz, Downregulated CD36 and oxLDL uptake and stimulated ABCA1/G1 and cholesterol efflux as anti-atherosclerotic mechanisms of interleukin-10, Cardiovasc. Res. 69 (2006) 527–535.

[36] T. Arai, K. Hiromatsu, H. Nishimura, Y. Kimura, N. Kobayashi, H. Ishida, Y. Nimura, Y. Yoshikai, Endogenous interleukin 10 prevents apoptosis in macrophages during Salmonella infection, Biochem. Biophys. Res. Commun. 213 (1995) 600–607.

[37] S.B. Cohen, J.B. Crawley, M.C. Kahan, M. Feldmann, B.M. Foxwell, Interleukin-10 rescues T cells from apoptotic cell death: association with an upregulation of Bcl-2, Immunology 92 (1997) 1–5.

[38] F. Capsoni, F. Minonzio, C. Mariani, A.M. Ongari, P. Bonara, G. Fiorelli, Development of phagocytic function of cultured human monocytes is regulated by cell surface IL-10, Cell. Immunol. 189 (1998) 51–59.

[39] C. Vestergaard, B. Gesser, N. Lohse, S.L. Jensen, S. Sindet-Pedersen, K. Thestrup-Pedersen, K. Matsushima, C.G. Larsen, Monocyte chemotactic and activating factor (MCAF/MCP-1) has an autoinductive effect in monocytes, a process regulated by IL-10, J. Dermatol. Sci. 15 (1997) 14–22.

[40] B. Gesser, B. Deleuran, M. Lund, C. Vestergard, N. Lohse, M. Deleuran, S.L. Jensen, S.S. Pedersen, K. Thestrup-Pedersen, C.G. Larsen, Inter- leukin-8 induces its own production in CD4+ T lymphocytes: a process regulated by interleukin 10, Biochem. Biophys. Res. Commun. 210 (1995) 660–669.

[41] P. Wang, P. Wu, M.I. Siegel, R.W. Egan, M.M. Billah, IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells, J. Immunol. 153 (1994) 811–816.

[42] P.A. Knolle, A. Uhrig, U. Protzer, M. Trippler, R. Duchmann, K.H. Meyer zum Buschenfelde, G. Gerken, Interleukin-10 expression is autoregulated at the transcriptional level in human and murine Kupffer cells, Hepatology 27 (1998) 93–99.

[43] A. O'Garra, P.L. Vieira, P. Vieira, A.E. Goldfeld, IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage, J. Clin. Invest. 114 (2004) 1372–1378.

[44] J. Suttles, D.M. Milhorn, R.W. Miller, J.C. Poe, L.M. Wahl, R.D. Stout, CD40 signaling of monocyte inflammatory cytokine synthesis through an ERK1/2-dependent pathway. A target of interleukin (il)-4 and il-10 anti- inflammatory action, J. Biol. Chem. 274 (1999) 5835–5842.

[45] K. Sato, H. Nagayama, K. Tadokoro, T. Juji, T.A. Takahashi, Extracellular signal-regulated kinase, stress-activated protein kinase/c-Jun N-terminal kinase, and p38mapk are involved in IL-10-mediated selective repression of TNF-alpha-induced activation and maturation of human peripheral blood monocyte-derived dendritic cells, J. Immunol. 162 (1999) 3865–3872.

[46] G.A. Ricchetti, L.M. Williams, B.M. Foxwell, Heme oxygenase 1 expression induced by IL-10 requires STAT-3 and phosphoinositol-3 kinase and is inhibited by lipopolysaccharide, J. Leukoc. Biol. 76 (2004) 719–726.

[47] F. Driessler, K. Venstrom, R. Sabat, K. Asadullah, A.J. Schottelius, Molecular mechanisms of interleukin-10-mediated inhibition of NF- kappaB activity: a role for p50, Clin. Exp. Immunol. 135 (2004) 64–73.

[48] A.A. Rahimi, K. Gee, S. Mishra, W. Lim, A. Kumar, STAT-1 mediates the stimulatory effect of IL-10 on CD14 expression in human monocytic cells, J. Immunol. 174 (2005) 7823–7832.

[49] G.H. Giambartolomei, V.A. Dennis, B.L. Lasater, P.K. Murthy, M.T. Philipp, Autocrine and exocrine

regulation of interleukin-10 production in THP-1 cells stimulated with Borrelia burgdorferi lipoproteins, Infect. Immun. 70 (2002) 1881–1888.

Table 1

Cytokine release evaluated by ELISA in the supernatant of monocytes and lymphocytes incubated with LDL(+), LDL(−) and LPS

Protein release	20 h	MCP1	IL6	IL8	IL10	GROβ	GROγ
Monocytes	Control	0.40 ± 0.39	0.02 ± 0.03	0.37 ± 0.49	0.05 ± 0.07	1.25 ± 1.81	8.58 ± 4.08
	LDL(+)	1.98 ± 1.37	0.40 ± 0.30	13.3 ± 4.8	0.62 ± 0.40	6.71 ± 3.48	19.8 ± 13.8
	LDL(−)	4.94 ± 4.15*	0.73 ± 0.41*	18.2 ± 5.8*	1.25 ± 0.50*	12.5 ± 7.2*	25.6 ± 16.5*
	LPS	4.61 ± 3.63*	1.63 ± 0.85*	17.6 ± 5.0*	1.43 ± 0.50*	14.2 ± 9.4*	27.9 ± 18.3*
	(−)/(+)[†]	3.0 ± 1.4	2.2 ± 1.1	1.4 ± 0.4	2.6 ± 1.0	2.3 ± 0.7	2.0 ± 0.9
Lymphocytes	Control	0.52 ± 0.36	0.01 ± 0.01	0.07 ± 0.10	0.04 ± 0.04	1.80 ± 3.71	7.38 ± 7.13
	LDL(+)	1.01 ± 0.65	0.22 ± 0.19	5.72 ± 3.60	0.14 ± 0.09	6.05 ± 3.30	10.7 ± 4.7
	LDL(−)	2.05 ± 1.44*	0.42 ± 0.25*	9.68 ± 3.65*	0.40 ± 0.33*	11.1 ± 5.4*	16.2 ± 5.3*
	LPS	3.57 ± 2.82*	0.93 ± 0.43*	9.80 ± 3.12*	0.52 ± 0.51*	11.6 ± 4.6*	15.5 ± 3.7*
	(−)/(+)[†]	3.3 ± 0.8	2.1 ± 0.5	2.0 ± 0.7	3.7 ± 0.4	2.3 ± 0.8	2.7 ± 0.8

Results are expressed as ng/10^6 cells (cell density 10^6 cells/mL). Data are the mean±SD of 15 independent experiments. *$p < 0.05$ versus LDL(+). [†]LDL(−)/LDL(+) ratio showing the difference in induction between both fractions subtracting control from each assay value.

Table 2

Number of RNA copies evaluated by real-time RT-PCR of the genes encoding for the cytokines differentially induced by LDL(+) and LDL(−) in monocytes and lymphocytes

RNA copies	4 h	MCP1	IL6	IL8	IL10	GROβ	GROγ
Monocytes	Control	2.36 ± 0.67	0.08 ± 0.05	59.6 ± 29.9	0.19 ± 0.06	3.28 ± 1.68	3.73 ± 1.88
	LDL(+)	12.2 ± 10.1	2.05 ± 1.21	190.8 ± 115.9	0.64 ± 0.28	9.92 ± 8.29	23.3 ± 20.4
	LDL(−)	18.8 ± 16.6*	5.34 ± 4.11*	263.4 ± 158.7*	1.12 ± 0.59*	17.5 ± 22.1*	53.6 ± 59.3*
	(−)/(+)[†]	1.8 ± 0.5	2.5 ± 0.9	1.6 ± 0.2	2.3 ± 1.4	1.9 ± 0.6	2.2 ± 0.8
Lymphocytes	Control	2.11 ± 1.07	0.19 ± 0.19	22.6 ± 14.1	0.14 ± 0.03	1.27 ± 0.61	1.57 ± 0.83
	LDL(+)	4.50 ± 1.56	0.94 ± 0.48	57.5 ± 14.8	0.31 ± 0.08	3.26 ± 1.89	7.6 ± 4.4
	LDL(−)	7.49 ± 5.25*	3.13 ± 2.86*	142.8 ± 88.5*	0.45 ± 0.08*	13.5 ± 19.3*	27.3 ± 35.3*
	(−)/(+)[†]	2.6 ± 1.9	3.5 ± 2.0	4.0 ± 3.7	2.7 ± 2.2	4.3 ± 3.7	3.2 ± 2.5

Results are expressed as number of relative RNA copies after incubation of cells (3×10^6 cells) with LDL(+) or LDL(−) for 4 h. Data are the mean±SD of 4 independent experiments. *$p < 0.05$ versus LDL(+). [†]LDL(−)/LDL(+) ratio showing the difference in induction between both fractions subtracting control from each assay.

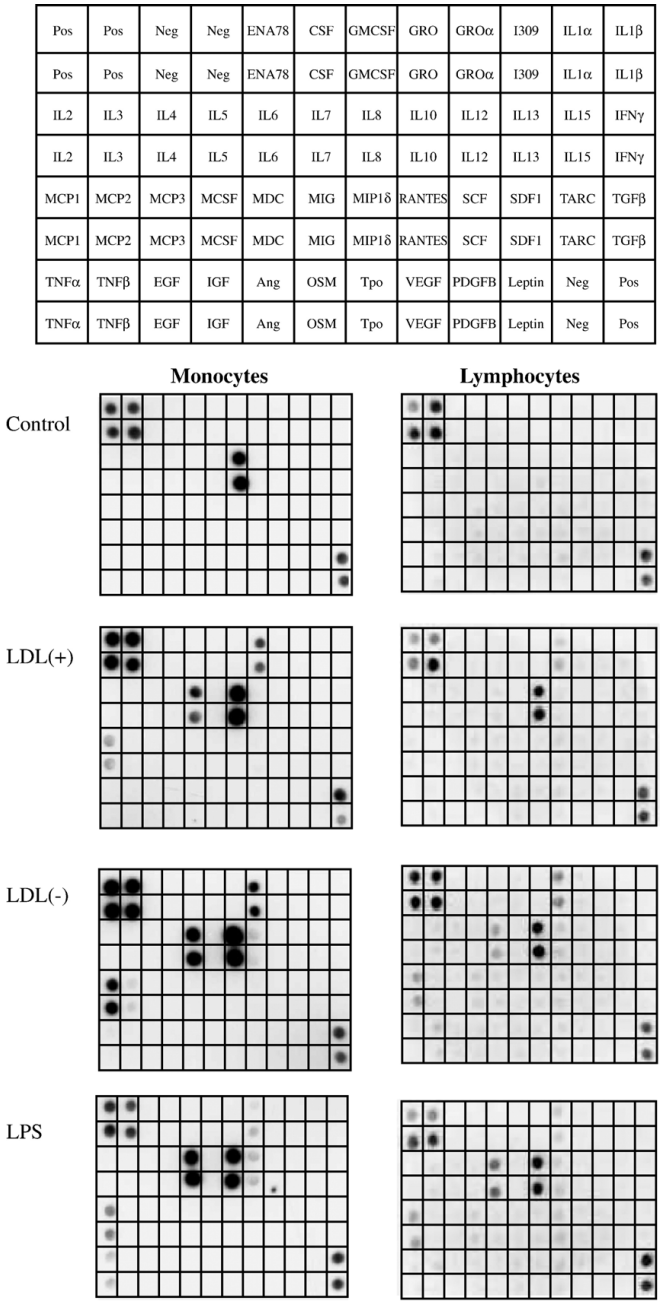

Fig. 1. Representative protein membrane assay showing results from supernatants of monocytes (left side) and lymphocytes (right side). Cells (1×10^6 cells) were incubated with LDL(+), LDL(−) or LPS for 20 h, as described in Materials and methods. Each protein membrane assay was incubated with 1 mL of each supernatant without dilution and was revealed following the manufacturer's instructions. Upper figure shows a template with the antibody distribution in the protein membrane assay (Pos: positive control, Neg: negative control). Note that the sensitivity of the array varies for the different molecules assayed; therefore, the intensity of the spot cannot be used as representative of the amount of cytokine induced.

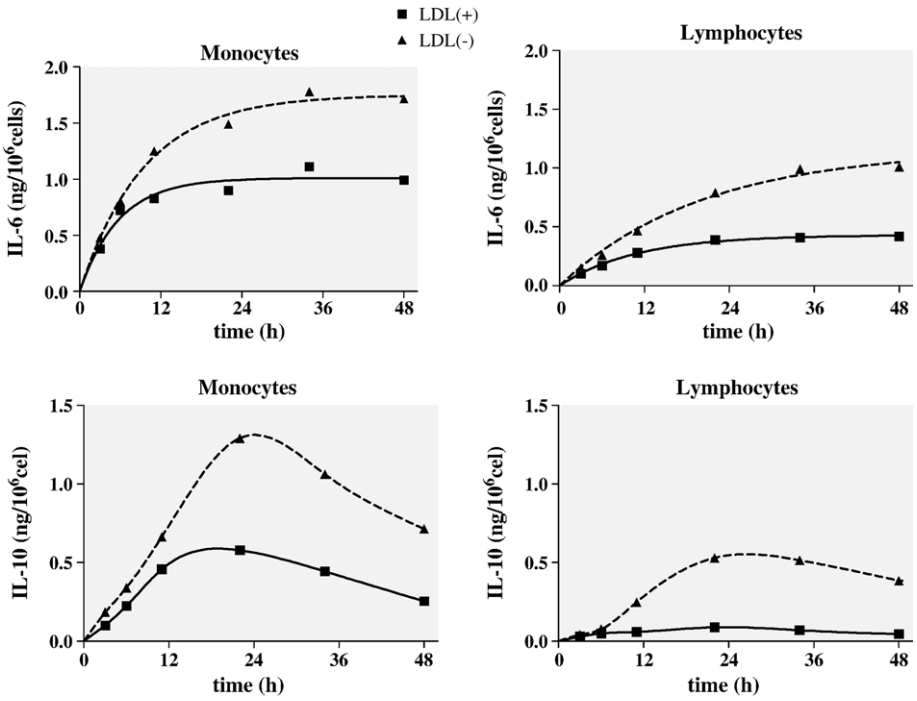

Fig. 2. Time-course release of IL6 and IL10 evaluated by ELISA. Monocytes and lymphocytes (1×10^6 cells) were incubated with LDL(+) or LDL(−) (150 mg/L apoB), and cytokines induced by LDL(−) were quantified at different times of incubation. IL6 time-course is an example of the other cytokines induced by LDL(−), MCP1, IL8, GROβ and GROγ and, in contrast, IL10 presented a different behavior time-course from the other cytokines. Data are the results of a representative experiment.

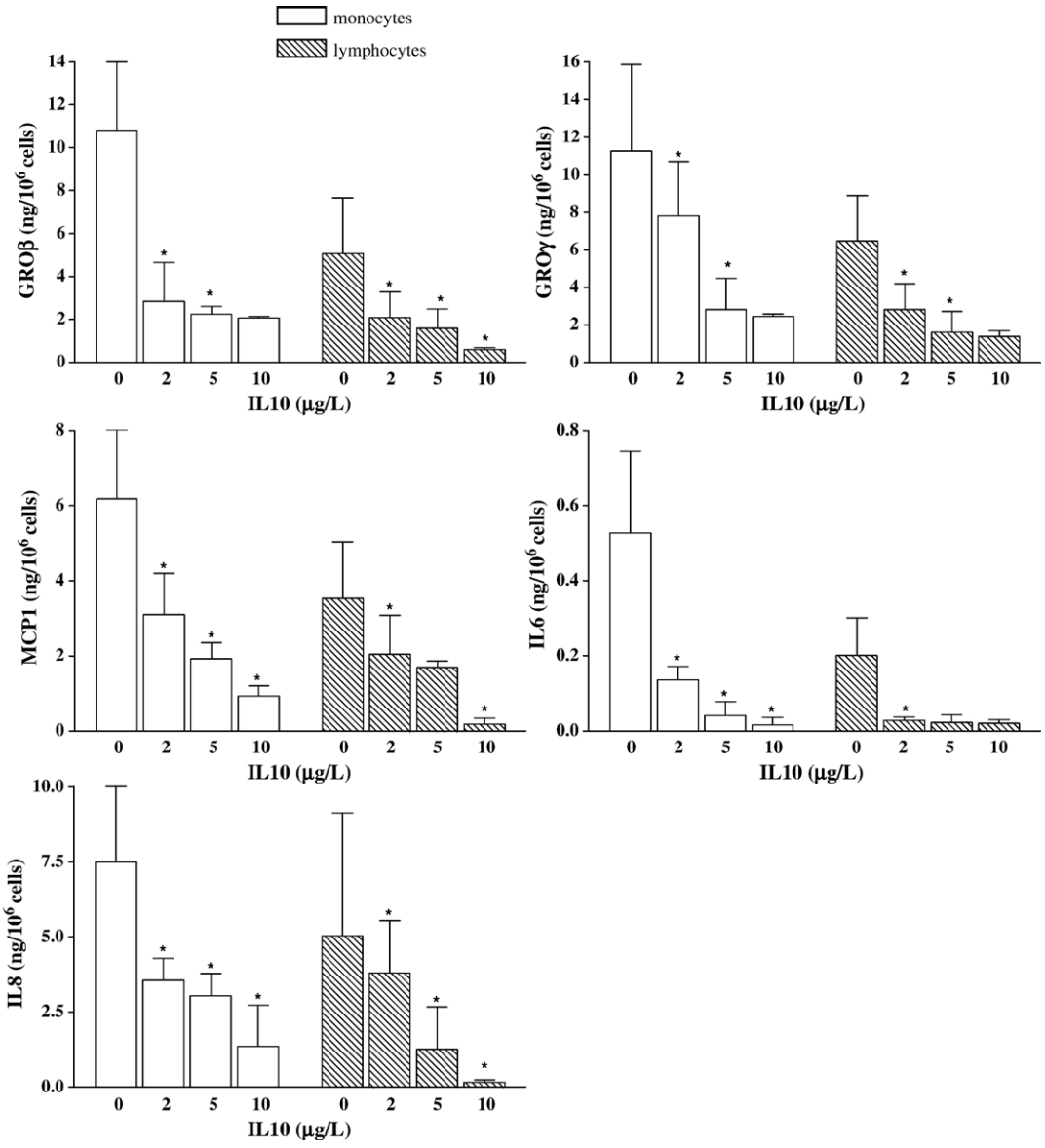

Fig. 3. Effect of exogenous IL10 on cytokine release by monocytes and lymphocytes. Cells (1×10^6 cells) were incubated with LDL(−) (150 mg/L) alone or co- incubated with different concentrations of IL10 (2, 5 and 10 µg/L). After incubation for 20 h in the different conditions, supernatants were collected and frozen until quantification of MCP1, GROβ, GROγ, IL6 and IL8 by ELISA. Data are the mean±SD of 6 independent experiments. * Indicates statistical differences versus lower IL10 concentration, $p < 0.05$.

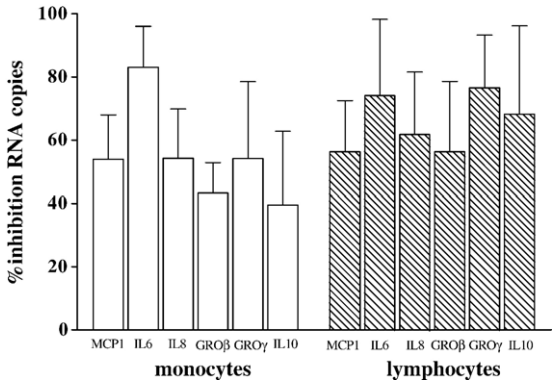

Fig. 4. Inhibition in RNA copies of cytokines by exogenous IL10 in monocytes and lymphocytes. Cells (3×10^6 cells) were incubated for 4 h with exogenous IL10 (5 μg/L) plus LDL(−) (150 mg/L). Number of RNA copies was evaluated by real-time RT-PCR, as described in Materials and methods, and results are expressed as % of inhibition versus LDL(−) alone. Data are the mean±SD of 3 independent experiments.

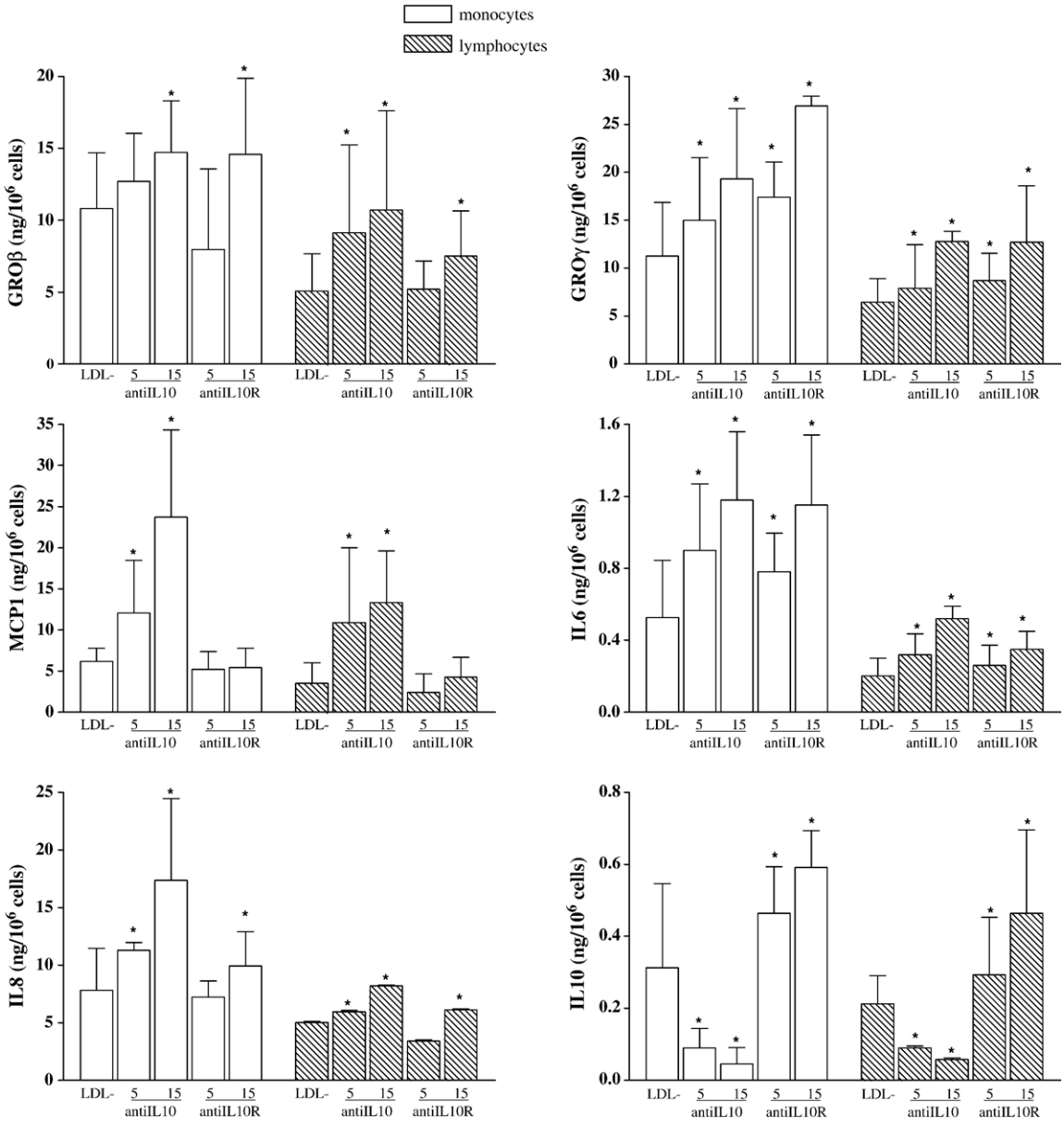

Fig. 5. Effect of antibodies anti-IL10 and anti-subunit α of IL10R on cytokine release induced by LDL(−) on monocytes and lymphocytes. Anti-IL10 and anti-IL10R (5, 15 mg/L) were added to cells (1×10^6 cells) simultaneously with LDL(−) (150 mg/L). After incubation for 20 h, cytokine release was evaluated in the supernatant by ELISA. Data are the mean±SD of 6 independent experiments. * Indicates statistical differences versus LDL(−) alone, $p < 0.05$.