Oxidized low-density lipoprotein promotes mature dendritic cell transition from differentiating monocyte.

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Abstract

Pro-inflammatory oxidized phospholipids are generated during oxidative modification of low density lipoproteins (LDL). The production of these pro-inflammatory oxidized phospholipids is controlled by secreted enzymes which circulate as proteins complexed with LDL and HDL (high density lipoprotein). During the acute phase response to tissue injury profound changes occur in lipoprotein enzymatic composition that alter their anti-inflammatory function. Monocytes may encounter oxidized phospholipids in vivo during their differentiation to macrophages or dendritic cells (DC). Here we show that the presence of oxidized LDL at the first day of monocyte differentiation to DC in vitro yielded phenotypically atypical cells with some functional characteristics of mature dendritic cells. Addition of oxidized LDL during the late stage of monocyte differentiation gave rise directly to phenotypically mature dendritic cells with reduced uptake capacity, secreting IL-12 but not IL-10 and supporting both syngeneic and allogeneic T cell stimulation. In contrast to known mediators of DC activation, oxidized LDL did not trigger maturation of immature DC. An intriguing possibility is that a burst of oxidized phospholipids is an endogenous activation signal for the immune system which is tightly controlled by lipoproteins during the acute phase response.

Introduction

Monocytes exit blood and enter tissues where they can differentiate to macrophages or dendritic cells (DC)[3]. This differentiation occurs when monocytes cross the endothelium but regulatory mechanisms controlling macrophage or DC generation *in vivo* are poorly understood. Some monocytes may only transiently traverse tissues and, under local inflammatory conditions, migrate to draining lymph node while acquiring DC phenotype and functions. Randolph *et al.* showed that transendothelial transport and phagocytosis are critical events that promote monocyte to DC transition (1). Monocytes that reverse transmigrate, a process mimicking cellular migration from tissue to lymph, become DC while the others become macrophages. Monocyte to DC transition is greatly enhanced by a phagocytic stimulus provided by particulate material. Thus, endothelial tissues initiate differentiation of monocytes to DC but efficient differentiation requires an additional stimulus provided in this study by foreign particulate material. In support of this is the *in vivo* injection of microspheres in the skin that induces phagocytic monocytes to migrate to T cell area of draining lymph nodes where they express costimulatory and DC-restricted markers (2).

Upon entry in the tissues, monocytes also interact with environmental factors influencing their differentiation. For instance, IL-6 from fibroblasts promotes monocytes differentiation to macrophages (3). Of the many environmental factors that deserve to be analyzed, oxidized LDL (oxLDL) is particularly relevant because it is generated from native LDL trapped in the subendothelial space, stimulates the expression of monocyte chemoattractant protein-1 (MCP-1), macrophage-colony stimulating factor (M-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) by endothelial cells and induces increased monocyte adhesion to and transmigration through the endothelial cell layer (4-8).

Lipoproteins are water-soluble spherical particles which transport nonpolar lipids. In humans, LDL is the major cholesterol transporter and consists of a hydrophobic core containing

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cholesteryl ester molecules and a surface monolayer of polar lipids (primarily phospholipids) and apolipoprotein B. Plasma LDL is transported across the intact endothelium and become trapped in the extracellular matrix of the subendothelial space where it can be subjected to oxidative modifications (9, 10). Various cellular and biochemical mediators have been proposed to initiate or regulate LDL oxidation but which enzymatic or nonenzymatic oxidative mechanisms are implicated *in vivo* is still the subject of debate and intensive study (11-16). OxLDL is a potent inducer of inflammatory molecules but the substances within the oxLDL that are responsible for this effect are only partially known. This is mainly due to the complex mixture of various components in oxLDL including lipid hydroperoxides, oxysterols, lysophosphatidylcholine and aldehydes (17, 18).

Pro-inflammatory oxidative modification of LDL is under strict control by native LDL and HDL. Paraoxonase (PON1) and platelet-activating factor acetylhydrolase (PAF-AH) are two enzymes complexed with lipoproteins that prevent the accumulation of oxLDL (19-21). PON1 can inhibit LDL oxidation and destroy various bio-active oxidized phospholipids as well as hydrogen peroxide (22-24). In the model proposed by Watson *et al.*, oxidized phospholipids are first substrate for PON1 in HDL (20). If PON1 cannot stop the process, oxidized phospholipids undergo further modifications and become substrate for PAF-AH. PAF-AH is a phospholipase A2 that hydrolyzes short chain acyl groups and longer chain aldehyde esterified to the sn-2 position of phospholipids (21). Failure to inactivate all active compounds results in highly oxidized LDL production similar to those found in the necrotic core of atherosclerotic lesions. Under normal condition, the two lines of control maintain the constant production of oxLDL below a critical threshold. During the acute phase response, HDL exhibits decreases in PON1 and PAF-AH and increases in the copper carrier ceruloplasmin (25). These changes alter HDL ability to inhibit LDL oxidation and anti-inflammatory HDL becomes pro-inflammatory during the acute phase response.

As the control of LDL oxidation is transiently lost during the acute phase response to tissue injury, we asked whether oxLDL could be an internal signal favoring monocytes to DC transition initiated by endothelial tissue. Using GM-CSF and IL-4 to initiate monocyte differentiation, we investigated whether oxLDL could alter the phenotype and functional abilities of the cells at different stages in culture.

Materials and methods

LDL preparation

LDL (1.025≤ d ≤1.055 g/ml) was isolated from human plasma of normolipidemic healthy individuals by ultracentrifugation. The density of the plasma was raised to 1.025 g/ml using NaBr. After ultracentrifugation at 100 000 rpm, 4°C for 4 h using a TL 100.4 rotor, the light fraction containing chylomycron, VLDL (very low density lipoprotein) and IDL (intermediate density lipoprotein) was removed. The density was adjusted at 1.055 g/ml with NaBr and after ultracentrifugation in the same conditions, the light fraction containing LDL was collected, dialyzed extensively against NaCl 150 mM / EDTA 2.4 mM pH 7.2 at 4°C, filtered at 0.45 µm and stored under nitrogen. The protein content of the fraction was estimated by Coomassie Protein Micro-Assay procedure (Pierce, Rockford, IL, USA) and its lipid composition was determined using Cholesterol RTU, Triglycerides enzymatic PAP 150 and Phospholipids enzymatic PAP 150 kits from bioMérieux (Marcy l'Etoile, France). LDL was composed of 23 ± 1 % protein (exclusively Apo B), 41 ± 4 % cholesterol, 24 ± 1 % phospholipids and 13 ± 4 % triglycerides. Endotoxins in LDL or oxLDL were less than 0.6 pg/ml in final concentration as assessed by E-toxate test (Sigma, St Quentin-Fallavier, France).

LDL oxidation

LDL concentration was adjusted at 1 mg/ml of protein by dilution in PBS and dialyzed at 4°C against PBS to eliminate EDTA. Cu^{2+}-mediated oxidation was carried out at 37 °C for 24 h by dialysis against 5 µM $CuSO_4$ / PBS. The reaction was stopped by addition of 40 µM

Butylated-Hydroxy-Toluene and extensive dialysis at 4 °C against PBS containing 100 µM diethylendiamine pentaacetic acid (DTPA). The oxidation degree was assessed by malonyl-dialdehyde (MDA) production measured by the LPO-586 assay (Oxis, Portland, OR, USA), and hydrogen peroxide content was measured by PeroxOQuant™ quantitative peroxide assay (Pierce). Typically, native LDL preparations contained < 1.5 ± 0.3 nmol TBARS /mg total protein and 230 ± 82 nmol peroxides / mg of total protein. After 24 h oxidation, oxLDL contained 16.7 ± 6.6 nmol TBARS /mg total protein and 896 ± 295 nmol peroxides / mg of total protein.

Monocyte-derived dendritic cells

Mononuclear cells were isolated from human peripheral blood by density gradient centrifugation using Ficoll-Hypaque at 400 g for 20 min and then by centrifugation on a 50 % Percoll solution at 400 g for 20 min. Monocytes were purified by immunomagnetic depletion (Dynal, Oslo, Norway), using a cocktail of mAbs anti-CD19 (4G7 hybridoma, provided by Dr Ron Levy), anti-CD3 (OKT3, American Type Culture Collection, Rockville, MD) and anti-CD56 (NKH1, Beckman Coulter, Fullerton, CA, USA). Recovered monocytes were around 90 % pure as assessed by CD14 labeling. Monocyte differentiation to immature DC was initiated with 40 ng/ml human recombinant GM-CSF and 250 U/ml human recombinant IL-4. After 6 days, more than 95 % of the cells cultured in the absence of oxLDL were immature DC as assessed by CD1a labeling. Culture were performed in RPMI 1640 (Life Technologies, Rockville, MD, USA) supplemented with 2 mM Glutamin (Life Technologies), 10 mM Hepes (Life Technologies), 40 ng/ml Gentamycin (Life Technologies) and 10 % fetal calf serum (FCS) or 10% lipoprotein-deficient fetal calf serum (LPDS) (Sigma). OxLDL (from 2.5 to 10 µg/ml) was added at different days of monocyte differentiation. Competition with native LDL

was performed by concomitant adjunction of 10 µg/ml oxLDL and 50 µg/ml native LDL. At the end of differentiation, the viability of cells treated or not with oxLDL or native LDL was superior to 90 %.

Phenotype

Phenotype was analyzed by flow cytometry on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) using FITC-conjugated anti-CD14, -HLA-DR, -CD80 and PE-conjugated anti-CD1a, -CD83, -CD86, -CD40 all from Beckman Coulter.

Endocytosis

Cells were resuspended in 10 % FCS medium and incubated at 37°C for 30 min with 1 mg/ml FITC-T70-Dextran (Sigma) or 1 mg/ml Lucifer Yellow (LY) (Sigma) or for 3 h with carboxylate-modified yellow-green FluoSpheres of 0.45 µm (Beads) (Molecular Probes, Leiden, The Netherlands) for macropinocytosis. Internalization was stopped on ice with cold PBS containing 1 % BSA and 0.05 % NaN$_3$. Cells were washed three times at 4 °C in this buffer and analyzed on a FACScalibur (Becton Dickinson).

Mixed Lymphocyte Reaction

Naïve T lymphocytes were isolated from human peripheral blood. PBMC were isolated by density gradient centrifugation on Ficoll-Hypaque. After depletion of monocytes on Percoll gradient, PBL were recovered in the dense fraction. T lymphocytes were purified by immunomagnetic depletion using a cocktail of mAbs anti-CD19 (4G7), anti-CD16 (3G8), anti-CD56 (NKH1), anti-glycophorin A (11E4B7.6) and anti-CD14 (RMO52) all from

Beckman Coulter. T lymphocytes were more than 95 % pure as assessed by CD3 labeling. Primary MLRs were conducted in 96-well flat-bottomed culture plates. Antigen presenting cells (APC) were treated or not with oxLDL, collected at d6, extensively washed and resuspended in RPMI / 10 % FCS. These cells were then co-cultured with 2×10^5 allogeneic or syngeneic T cells in 200 µl complete culture medium at 1/5, 1/10 or 1/20 APC / responder T cells ratio. After 4 days, 50 µl of culture supernatant was frozen for IL-2 secretion assay and replaced by 50 µl of fresh medium containing 1 µCi of $[^3H]$-Thymidine. After 16 h of culture, the cells were harvested onto filter paper and thymidine incorporation was measured using a Matrix 9600 Direct Beta counter (Packard, Meriden, CT, USA).

Cytokine assay

Culture supernatants were aliquoted and stored at −80°C until they were analyzed for the presence of cytokines. IL-1β, IL-2, IL-10, IL-12 p70 and TNFα levels were determined using cytokine-specific ELISA kits purchased from Endogen (Woburn, MA, USA).

Statistical analysis

Data are expressed as mean±S.D. Differences between means were examined by paired Student's *t* test and were considered significant when $p < 0.05$. All experiments were repeated at least 3 times with different cell and lipoprotein preparations.

Results

OxLDL induces phenotypical and morphological changes during monocyte differentiation to DC

The most common procedure to generate monocyte-derived DC is to expose $CD14^+ CD1a^-$ monocytes to GM-CSF and IL-4 for 6-7 days to obtain $CD14^- CD1a^+$ immature DC (26, 27). DC express many accessory molecules that interact with receptors on T cell to enhance adhesion and signaling. These molecules are regulated upon exposure to various stimuli including stress and microbial products. Maturation level of DC can thus be followed by several typical surface markers (here, CD14, CD1a, MHC class II, CD80, CD83, CD86, CD40). To determine how the presence of oxLDL would affect monocyte differentiation, 10 µg/ml oxLDL was added at d0 corresponding to the first day of culture with GM-CSF, IL-4 and FCS. Phenotype was monitored at d6 and compared to phenotype of immature DC generated without oxLDL. Figure 1A shows that oxLDL did not interfere with the differentiation of $CD14^+ CD1a^-$ into $CD14^- CD1a^+$ DC although CD1a labeling appeared consistently slightly weaker. As expected, control immature DC were $CD86^-$ but monocytes differentiated in the presence of oxLDL were mostly $CD86^+$. Other usual differentiation markers were not affected by oxLDL (data not shown). The addition of native LDL at d0 of differentiation did not induce any phenotypic modification of the cells (Fig. 1A).

Native lipoproteins contain enzymes that can destroy bioactive oxidized phospholipids. In addition, OxLDL are recognized by scavenger receptors (28-31) which bind a wide variety of ligands, some of them being able to bind native lipoproteins. To test the inhibitory action of native lipoproteins on oxLDL, monocytes were then differentiated in lipoprotein deficient serum (LPDS). Control culture without oxLDL showed that LPDS did not affect differentiation of monocytes into typical $CD14^- CD1a^+ CD86^-$ immature DC (Fig. 1B). Other

markers showed the same labeling whether DC were generated in LPDS or FCS (data not shown). The effect of oxLDL was striking in LPDS. Cells obtained after a 6 day culture in LPDS supplemented with GM-CSF, IL-4 and oxLDL were all CD14$^-$ CD1a$^-$ and expressed high level of CD86 (Fig. 1B). Thus, CD1a and CD86 appeared to be two differentiation markers affected by oxLDL during the monocyte differentiation into DC. Under the same experimental conditions, native LDL did not induce phenotypical modifications (Fig.1B). However, CD1a down-regulation and CD86 up-regulation induced by oxLDL was inhibited by an excess of native LDL (Fig.1C).

Morphological observation indicated that 30 to 40 % of monocyte-derived DC obtained in LPDS and oxLDL had a bipolar elongated appearance at d6 (Fig.1D). Ten to 50 µg/ml of native LDL alone did not induce any morphological modification (data not shown). As expected from the FACS analysis shown in figure 1C, DC obtained in LPDS with oxLDL and a 5 fold excess of native LDL (50 µg/ml) have a nearly normal morphology as compared to control LPDS (Fig.1D) or FCS culture without oxLDL (data not shown). In addition, CD1a was down-regulated and CD86 was up-regulated by oxLDL in a dose-dependent manner (Fig.1E). Weak induction of CD86 expression in FCS, dose response activity of oxLDL and competition with native LDL indicate that the effect of oxLDL can be weakened by endogenous competitors including native lipoproteins.

In the above experiments cells were analyzed at d6 and oxLDL was added concomitantly to GM-CSF and IL-4 at the first day of culture (d0) without subsequent supplementation. We next asked whether the degree of monocyte differentiation changes its ability to respond to oxLDL. 10 µg/ml oxLDL was added once at d0, d3, d4, d5 or d6 (corresponding to immature DC) and CD86 expression was analyzed by FACS at d6 or d7 when oxLDL was added at d6. Figure 2A shows that CD86 expression was always increased by oxLDL. However, CD86 expression was more important when oxLDL was added at d4 or d5. As a control, addition of

native LDL under the same timing conditions never induced CD86 expression. Extended phenotypes are shown in figure 2B and compare the consequences of oxLDL addition at d0 and d5 of monocyte differentiation. Day 0 treated monocytes appeared to be CD14$^-$ CD1a$^-$ CD86$^+$ but remained negative for other co-stimulatory molecules. In contrast, d5 treated-monocytes exhibited a fully mature DC phenotype except that these cells remained CD1a$^+$. OxLDL added to immature DC (d6 treatment) induced CD86 expression (Fig.2A) but phenotype analysis revealed no induction of other co-stimulatory molecules or maturation markers after 24 or 48 h (data not shown). Therefore, phenotypic analysis indicated that oxLDL has a rapid and maximal effect during the late stage of monocyte differentiation and skews this differentiation directly towards mature DC. In contrast, immature DC have lost the ability to fully mature under the action of oxLDL.

OxLDL treated cells display limited endocytic activity

Immature DC express a potent ability to uptake large amount of external molecules essentially by two main mechanisms: receptor mediated-endocytosis and macropinocytosis (32, 33). This ability is lost when immature DC receive a maturation-inducing stimulus. Both pathways have been studied by flow cytometry to test whether oxLDL can downregulate the capturing machinery like other classical maturation-inducing signals. FITC-dextran has been used to measure mannose receptor-mediated endocytosis, fluorescent beads for macropinocytosis and LY as nonspecific fluid phase marker. Figure 3A shows that although oxLDL does not induce a fully mature DC phenotype when added early on monocytes, it still inhibits mannose receptor pathway and LY uptake. Inhibition of macropinocytosis is less potent. OxLDL added at d5 of monocyte differentiation, a condition that rapidly induces fully mature DC phenotype, results in a strong inhibition of both pathways. Cells treated with 10

µg/ml native LDL display the same endocytic activity than control cells differentiated in LPDS.

OxLDL regulates IL-12p70 production

We next analyzed whether or not oxLDL might induce release of cytokines by treated cells. Culture supernatants of control immature dendritic cells and monocytes treated at d0 or d5 during the differentiation process were analyzed by ELISA to detect TNFα, IL-1β, IL-10 and bioactive IL-12p70. TNFα and IL-1β could not be detected in any of the culture condition (data not shown). Very low quantity of IL-10 could be detected in supernatants of d0 or d5 treated monocytes and immature DC (Fig.3B). A major difference was observed for IL-12p70 production. Control immature DC and d0-treated monocytes did not secrete significant amount of IL-12p70. In contrast, d5-treated monocytes were induced to produce substantial amount of IL-12p70 by oxLDL but not by native LDL (Fig.3B). Thus, maturation of these cells promoted by oxLDL is associated with the production of the dominant cytokine in directing the development of Th1 cells.

OxLDL treated cells activate allogeneic and syngeneic T cells

To further study the maturation level of these differentiating monocytes, we tested their capacity to stimulate allogeneic and syngeneic T cells, a functional characteristic of mature DC. As figure 4A shows, only oxLDL treated monocytes, but not LDL or untreated cells (immature DC), stimulated IL-2 secretion by allogeneic naïve T cells. Cells treated at d5 of differentiation, which display a mature DC phenotype, were better stimulators than cells treated at d0 which express CD86 but no other activation markers. Allostimulatory capacity of these monocytes derived cells was inhibited when an excess of native LDL was added concomitantly to oxLDL, an experimental procedure that also inhibits phenotypical changes.

Figure 4B confirmed the inhibitory effect of native LDL and showed that the action of oxLDL on the acquisition of allostimulation capacity was dose dependent. Monocyte derived cells that have been treated with oxLDL at d5 for 24 h also induced the proliferation of syngeneic T cells indicating that these cells have the ability to present exogenous antigens from the medium (Fig.4C). Induction of autologous T cell proliferation by monocytes treated at d0 of differentiation varied from experiment to experiment.

Discussion

When crossing the endothelial barrier, monocytes differentiate in an environment containing unknown internal signals that contribute to determine whether monocytes progress along the DC or macrophage differentiation pathway. One such signal could be oxLDL which accumulates in the subendothelial space under specific acute or chronic conditions (34, 35). We therefore examined how oxLDL could affect monocyte differentiation polarized toward DC by GM-CSF and IL-4.

OxLDL added at the first day of differentiation has a striking effect in LPDS while only weak CD86 expression was induced in FCS, suggesting that native lipoproteins are endogenous inhibitors of oxLDL. In LPDS, all oxLDL-treated monocytes became CD14$^-$ CD1a$^-$ CD86$^+$. Although full maturation phenotype was not achieved under these conditions, these cells display some functional characteristics of mature DC. They have a reduced uptake capacity and can activate allogeneic T cells. In contrast, they do not secrete cytokines that are usually secreted by mature DC. Cells were more reactive when oxLDL was added at the late stages of differentiation. In 24 hours, these cells acquired a fully mature DC phenotype with a reduced uptake capacity and can stimulate allogeneic and syngeneic T cells. Importantly, these cells secreted bioactive IL-12 but very little IL-10. At day 6, once monocytes had become typical immature DC, oxLDL still induces CD86 expression but does not promote full maturation, indicating that oxLDL is not a classical activator of immature DC. Rather, our data suggest that oxLDL could be an internal stimulus inducing a polarized Th1 response by acting directly on monocytes during their differentiation to DC.

If oxLDL is an endogenous activator signal of the immune response, its delivery must be tightly controlled to allow reactivity when necessary and prevent immunopathology. We showed that FCS containing native lipoproteins and LPDS supplemented with native LDL are efficient inhibitors of oxLDL activity. This strongly suggests that under normal conditions

oxLDL signals are not active because of endogenous native lipoproteins bearing appropriate enzymes hydrolyzing bioactive phospholipids. It has been reported that phospholipid liposomes prevent the biological activity of oxLDL by serving as a sink for the biologically active lipids (8). These two mechanisms might apply to our competition experiments where native LDL inhibited oxLDL activity. Thus, oxLDL may only express its biological activity when oxLDL level increases above a critical threshold. This would only happen under specific conditions, like acute phase response or atherosclerosis.

Although biological activity of oxLDL is attributable to oxidized phospholipids and their derivatives, only a small fraction of these molecules has been identified. OxLDL and apoptotic cells share common oxidatively modified moieties that are recognized by macrophage receptors and mediate their phagocytosis (36, 37). As oxLDL activate antigen presenting cells but removal of apoptotic cells usually does not, It is likely that the common structures of oxLDL and apoptotic cells are not essential for the generation of mature DC from differentiating monocytes (38). OxLDL interacts with scavenger receptors on phagocytic cells (28-31). These receptors belong to a large family and bind ligands that have domains with high negative charge like polyinosinic acid (39). This suggests that ligand binding is mediated by ionic interactions and that oxidized phospholipids are not likely to express their biological activity through a single receptor. The mechanism of action of oxLDL is further complicated by the fact that structurally similar oxidized phospholipids differentially regulate endothelial binding of monocytes (40).

Factors that govern the balance between immunity and tolerance remain largely unknown. So far, two types of signals that can activate DC have been identified. The first type of signal consists of conserved molecules of infectious agents that are recognized by pattern recognition receptors (41, 42). The second type of signal consists of endogenous activation signals released during infectious or ill-defined stressful events. DC can be activated by

endogenous signals released by cells that are stressed, virally infected or necrotic (38). The emerging consensus, pioneered by P. Matzinger, is that the immune system is reacting to a dangerous situation by sensing internal signals (43, 44). The observation that CD91 could sensor necrotic or tumoral cells is arguing in that direction (45). DC might themselves be able to sense these signals which provide the context of antigen presentation.

In reaction to infectious or non-infectious tissue injury, the host immediately executes wide ranging physiological changes known as the acute phase response (46, 47). It is in the context of the acute phase response that antigen presentation first takes place. The acute phase response is a dynamic homeostatic reaction that involves the major biological systems, in addition to the immune system, cardiovascular and central nervous system . It is characterized by a series of highly coordinated physiological reactions and by rapid and transient changes in the level of a large number of plasma proteins. These changes are predominantly the result of alterations in the pattern of protein synthesis in the liver. Serum amyloid A proteins (SAA) circulate primarily with HDL and appears to be major acute phase reactants after a variety of stimuli such as surgery, infection and chronic arthritis (48). During the acute phase response, SAA in plasma is increased by up to a 1000 fold. As a consequence, HDL exhibits a marked increase in SAA and a loss in apolipoprotein A (25, 49). Concomitantly, PAF-AH and PON1 are displaced from HDL which looses its protective effect against LDL oxidation. HDL also exhibits increases in ceruloplasmin which alters its ability to inhibit LDL oxidation. Therefore, one consequence of the acute phase response is that control of LDL oxidation by HDL is transiently lost which results in enhanced MCP-1 production by endothelial cells and increased monocyte transmigration in an environment that contains elevated level of oxLDL. This would favor a rapid and transient generation of mature DC from monocytes.

An aberrant continuation of some aspects of the acute phase response can lead to chronic inflammation. Atherosclerosis is a chronic inflammatory disease developing in response to

injury in the vessel wall. Most of the clinical events have been attributed to the arterial inflammatory responses initiated by the oxidation of LDL trapped in the extracellular space. Ludewig *et al.* have shown that hypercholesterolemia, high level of LDL associated with elevated oxLDL production, is a predisposing factor for inflammation and immunity in the vascular wall and contributes to the establishment of a chronic inflammatory state (50).

Environmental signals inducing co-stimulatory molecules on DC appear to be critical factors to discriminate between tolerance and reactivity. Our data suggest that, by controlling oxidative modification of LDL, the interplay between HDL and LDL might be one factor regulating the balance between immune activation and tolerance induction. It can reasonably be speculated that the immune system does not react to an on/off signal provided by oxLDL but rather that the immune status would be influenced by the degree and level of LDL oxidation. Besides the basic research observation that oxLDL promotes development of mature DC, the data presented here may have practical implications in the field of cell therapy. Treatment of differentiating monocytes with oxLDL or derivatives may facilitate and improve the production of DC in vitro for various therapeutic approaches.

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Footnotes

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[3] Abbreviations used in this paper: APC, Antigen presenting cells; DC, Dendritic cells; FCS, Fetal calf serum; GM-CSF, Granulocyte macrophage-stimulating factor; HDL, High density lipoprotein; LDL, Low density lipoprotein; LPDS, Lipoprotein deficient serum; MCP-1, Monocyte chemoattractant protein-1; M-CSF, Macrophage-colony stimulating factor; oxLDL, Oxidized low density lipoprotein; PAF-AH, Platelet activating factor-acetylhydrolase; PON1, Paraoxonase.

Figure legends

Figure 1. Flow cytometric and morphological evaluation of oxLDL treated cells. (A,B)-
Monocytes were cultured for 6 days in either FCS (A) or LPDS (B) medium containing GM-
CSF and IL-4. FCS or LPDS control conditions: thin line; FCS or LPDS with 10 µg/ml native
LDL: dotted line; FCS or LPDS with 10 µg/ml oxLDL: filled profiles. Phenotype was
analyzed at day 6. (C)- Competition of oxLDL by native LDL. Monocytes were cultured for 6
days in LPDS medium in control conditions (thin line), with 50 µg/ml LDL (dotted line), with
10 µg/ml oxLDL (filled profiles) or with 10 µg/ml oxLDL plus 50 µg/ml native LDL (bold
line). Data are representative of more than five independent experiments. (D)- Morphological
changes induced by oxLDL. Monocytes were cultured in the presence of GM-CSF and IL-4
in LPDS (LPDS), with 10 µg/ml oxLDL (oxLDL) or with 10 µg/ml oxLDL plus 50 µg/ml
native LDL (oxLDL+LDL). OxLDL with or without LDL was added on monocytes at d0 and
photographs were taken at d6. (E)- Dose dependence of oxLDL on CD1a and CD86 markers.
Cells were harvested at day 6 of differentiation in LPDS medium containing 10 µg/ml native
LDL or varying doses of oxLDL: 0 (control LPDS), 2.5, 5 or 10 µg/ml. Mean fluorescence
intensities of CD1a and CD86 for 3 independent experiments were normalized at 100 for the
control LPDS. Mean ± S.D. is shown.

Figure 2. Effect of oxLDL is dependent on the differentiation stage of the monocytes. (A)-
Control is monocytes cultured in LPDS differentiation medium with or without LDL (10
µg/ml). OxLDL (10 µg/ml) was added at day 0, 3, 4, 5 or 6 of the differentiation. Cells were
harvested at day 6 or at day 7 when oxLDL was added at d6. CD86 expression was quantified
by flow cytometry. Mean ± S.D. of three independent experiments is shown (B)- Comparison
of phenotypes of the cells obtained at day 6 of differentiation when oxLDL (10 µg/ml) was
added at d0 or d5.

Figure 3. Endocytic activity and cytokine production of oxLDL treated cells. (A)- Inhibition of endocytosis by oxLDL. Monocytes were differentiated in LPDS medium with or without 10 µg/ml LDL (control) or with oxLDL (10 µg/ml) added at d0 (d0 + oxLDL) or d5 (d5 + oxLDL). Cells were harvested at d6. Cells were incubated at 37°C with 1 mg/ml FITC-Dextran or LY for 30 min or with fluorescent beads for 3 h. The amount of probe internalized was measured by flow cytometry. The mean fluorescent intensities of a representative experiment were normalized at 100 % uptake in the control. (B)- Induction of IL-12p70 secretion by oxLDL. Monocytes were differentiated in LPDS medium in the presence or not of LDL (10 µg/ml) or oxLDL (10 µg/ml) added at d0 (d0+LDL or d0+oxLDL) or d5 (d5+LDL or d5+oxLDL). Culture supernatants were collected at day 6 and the amount of IL-10 and IL-12p70 was measured by sandwich ELISA. Mean values of four independent experiments for 10^6 cells.

Figure 4. Allogeneic and syngeneic T cell stimulation by oxLDL treated cells. (A)- Control immature dendritic cells (LPDS) or monocytes treated at d0 or d5 with 50 µg/ml native LDL (d0 + LDL or d5 + LDL) or with 10 µg/ml oxLDL (d0 + oxLDL or d5 + oxLDL) or with 10 µg/ml oxLDL plus 50 µg/ml native LDL (d0 + oxLDL + LDL or d5 + oxLDL + LDL) were washed and cultured with allogeneic purified T cells (2×10^5/well) at ratios ranging between 1/5 and 1/20 (DC/T) for 4 days. The amount of IL-2 in the supernatants of the co-culture was measured by sandwich ELISA. Statistical significance was calculated with immature DC (LPDS) as control. * p < 0.05 ** p < 0.01 (B)- Control immature dendritic cells (LPDS) or monocytes treated at d5 with 50 µg/ml native LDL, 2.5, 5 or 10 µg/ml oxLDL or with 10 µg/ml oxLDL + 50 µg/ml native LDL were cultured in triplicate with allogeneic purified T cells (2×10^5/well) at the 1/10 DC/T cells ratio for 5 days. * p < 0.005, compared with control

LPDS cells. (C)- Control immature dendritic cells (LPDS) or monocytes treated at d5 with 50 µg/ml native LDL, 10 µg/ml oxLDL or with 10 µg/ml oxLDL + 50 µg/ml native LDL were cultured in triplicate with syngeneic purified T cells ($2x10^5$/well) at the 1/10 DC/T cells ratio for 5 days. [^{3}H]-Thymidine (1 µCi/well) was added for the last 16 h of culture and incorporation was measured by a β-plate counter. * $p < 0.01$, compared with control LPDS cells.