Group of Cardiovascular Biochemistry
Sant Pau Institute of Biomedical Research

Division of Biochemistry and Molecular Biology
Autonomous University of Barcelona

DOCTORAL THESIS

MECHANISMS OF CYTOKINE RELEASE INDUCED BY ELECTRONEGATIVE LDL IN MONOCYTES

THE ROLE OF CERAMIDE AND THE RECEPTORS CD14-TLR4

Montserrat Estruch Alrich

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For your courage, enthusiasm and joy of life

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ABBREVIATIONS

12-LO: 12-lipoxygenase

ABCA1: ATP binding cassette 1 transporter

acLDL: acetylated LDL

agLDL: aggregated LDL

AP1: transcription factor activator protein 1

apo: apolipoproteins

BDP: boron-dipyromethene

BHT: butylated hydroxytoluene

BSA: bovine serum albumin

C1P: ceramide-1-phosphate

CD14: cluster of differenciation 14

CER: ceramide

CETP: cholesteryl esters transporter protein

CMs: chylomicrons

CVD: cardiovascular diseases

DAG: diacylglicerol

DAMPs: damage-associated molecular patterns

DGAT: diacylglycerol acyltransferase

DiI: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate

EC: esterified cholesterol or cholesteryl esters

EDTA: ethylenediaminetetraacetic acid

E-LDL: multienzymatic modification of LDL

ELISA: Enzyme-linked immunosorbent assay

ENA-78: epithelial-derived neutrophil-activating peptide 78

ERK: endothelial cell signal-regulated kinase

FC: free cholesterol

FCS: fetal calf serum

FGF-2: fibroblast growth factor 2

fLDL: fast migrating LDL

FPLC: fast protein liquid chromatography

glyLDL: glycated LDL

GM-CSF: granulocyte macrophage colony-stimulating factor

GRO: growth regulatory oncogene

HDL: high density lipoproteins

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HL: hepatic lipase

HMG-CoA: 3-hydroxy-3-methyl-glutaryl-coenzyme A

HMGCoAR: 3-hydroxy-3-methyl-glutaryl- coenzyme A reductase

HPLC: high-performance liquid chromatography

HRP: horse reddish peroxidase

HUAEC: human arterial endothelial cells

HUVEC: human venous endothelial cells

ICAM-1: intercellular adhesion molecule-1

IDL: intermediate-density lipoproteins

IL: interleukin

IRF-3: interferon regulatory factor-3

LBP: LPS-binding protein

LCAT: lecithin-cholesterol acetyl-transferase

LDH: lactate dehydrogenase

LDL(-): electronegative LDL

LDLR: LDL receptor

LOX-1: lectin-like oxidized LDL receptor

Lp(a): lipoprotein a

LPC: lysophosphatidylcholine

LPL: lipoprotein lipase

LPS: lipopolysaccharide

LRP1: LDLR-related protein 1

LRR: leucine-rich repeat

MAG: monoacylglicerol

MCP-1: monocyte chemoattractant protein-1

MD2: myeloid differentiation protein-2

mmLDL: minimally modified LDL

MMPs: metalloproteinases

NEFA: non-esterified fatty acids

NEFA-LDL: NEFA-enriched LDL

NRF-2: nuclear factor-erythroid 2-related factor 2

oxLDL: extensively oxidized LDL

PAF: platelet associated factor

PAF-AH: platelet-associated factor acetyl-hydrolase

PAMPs: pathogen-associated molecular pattern molecules

PC: phosphatidylcholine

P-chol: phosphorylcholine

PDGF-β: platelet derived growth factor β

PE: phosphatidylethanolamine

PG: proteoglycans

PI3K: phosphatidyl-inositol-3 kinase

PL: phospholipids

PLA_2: phospholipase A2

PON1: paraoxonase 1

PRR: pattern recognition receptors

RCT: reverse cholesterol transport

rLPS: rough lypopolysacharide

ROS: reactive oxygen species

S1P: sphingosine-1-phosphate

S1PR2: sphingosine-1-phosphate receptor 2

SEAP: secreted embryonic alkaline phosphatase

sLPS: smooth lypopolysacharide

SMase: sphingomyelinase

SMC: smooth muscle cells

SPH: sphingosine

$sPLA_2$: secreted PLA_2

SR: scavenger receptors

SREC-1: SRs expressed by endothelial cells

sSMase: secretory SMase

TBARS: thiobarbituric acid reactive substances

TBS: tris buffered sulphate

TC: total cholesterol

TG: triglycerides

TIR: toll interleukin-1 receptor domain

TLC: thin layer chromatography

TLR: toll-like receptor

TMB: tetrametilbenzidine

TNF: tumor necrosis factor

TRAM: Trif-related adaptor molecule

TRIF: TIR-domain-containing adaptor protein inducing interferon

TTBS: TBS containing 0.1% Tween

VCAM-1: vascular cell adhesion molecule-1

VEGF: vascular endothelial growth factor

XTT: 2,3-bis(2methoxy-4-nitro-5sulfophenil)-2H-tetrazolium-5-carboxyanilide inner salt

ABSTRACT

Electronegative LDL (LDL(-)) is a minor modified LDL present in circulation with pro-inflammatory effects, including the induction of cytokine release in cells involved in atherosclerosis, such as endothelial and mononuclear cells. However, the cellular pathways activated by LDL(-) are scarcely understood, particularly in monocytes. In this thesis, we aimed to determine the first steps of the mechanisms by which LDL(-) induces IL-6, IL-10 and MCP-1 release in human monocytes. We focussed the study on the inflammatory components of LDL(-) and their interaction with cell receptors in monocytes.

Regarding the inflammatory components, we found that ceramide (CER) is, in part, responsible for the inflammation promoted by LDL(-). CER has been related to the increased LDL(-)-susceptibility to aggregation and the induction of cytokine release in monocytes. These properties of LDL(-) are enhanced at 37ºC and diminished by the incubation of this lipoprotein with HDL. CER levels increase in LDL(-) because of the LDL(-)-intrinsic PLC-like activity, which is also counteracted by HDL. This suggests that PLC-like activity of LDL(-) participates in the modifications undertaken in native LDL (LDL(+)) to form LDL(-). The enrichment of LDL with CER (CER-LDL) and its treatment with PLC-like activity mimics the cytokine secretion of LDL(-), although the effect of CER-LDL does not reach that of LDL(-). This suggests that other components linked to LDL(-) could contribute to its inflammatory effects.

The study of the receptors involved in the IL-6, IL-10 and MCP-1 release induced by LDL(-) in monocytes revealed that CD14 and TLR4 were pivotal. The addition of antiTLR4 and antiCD14 antibodies decreased cytokine release by 70%-80%, whereas that of TLR2 inhibited it by 15%-25%. The cytokine release was also diminished when cells were treated with a specific TLR4 inhibitor, but this release increased in a monocytic THP1 cell line overexpressing CD14 (THP1-CD14). These results were confirmed by TLR4 and CD14 gene silencing studies. Binding studies showed that LDL(-) presents high affinity to CD14, and in a lesser extent to TLR4; the neutralisation of these receptors decreased the amount of LDL(-) bound to monocytes. Immunoassay techniques elucidated that CD14 is the main receptor involved in LDL(-) binding. LDL(-) binds to CD14 and, then, form a complex with TLR4 to activate the intracellular

signalling leading to cytokine release in these cells. However, LDL(-) can alternatively interact with TLR4 to activate the intracellular signalling.

CD14 and TLR4 are receptors that mediate the cytokine release induced by the lipopolysaccharide (LPS) present in the bacterial membrane. The results showed a competition between LDL(-) and LPS for both the inflammatory effect and the binding to CD14 receptor in monocytes and in CD14-coated microtiter wells. This competition could represent an LDL(-)-counteracting role in cases of overwhelming inflammation.

The increased content of CER present in LDL(-) seems to be responsible for the activation of the CD14-TLR4 system which leads to cytokine secretion in monocytes. The cytokine release induced by CER-LDL in monocytes decreased with the addition of the TLR4 inhibitor. The CER-LDL-induced cytokine secretion in THP1-CD14 cells was much greater than in THP1 cells, which showed almost null cytokine release. This behaviour of CER-LDL is similar to LDL(-), excepting the fact that CER-LDL is not able to induce its cytokine release directly through TLR4 and needs CD14 for this effect.

To sum up, the increased content of CER present in LDL(-) plays a key role in the induction of IL-6, IL-10 and MCP-1 release through CD14-TLR4 in monocytes.

RESUM

L'LDL electronegativa (LDL(-)) és una LDL modificada present en circulació amb propietats inflamatòries incloent la inducció de l'alliberament de citoquines en cèl·lules relacionades amb l'arteriosclerosi, tals com monòcits i cèl·lules endotelials. Es coneix poc sobre les vies intracel·lulars activades per l'LDL(-), en especial en monòcits. En aquesta tesi s'han estudiat els primers passos a través dels quals l'LDL(-) indueix l'alliberament d'IL-6, IL-10 i MCP-1 en monòcits humans. Concretament, s'han estudiat els components inflamatoris de l'LDL(-) i la seva interacció amb receptors cel·lulars de monòcits.

En relació amb els components inflamatoris, es va observar que la ceramida (CER) és, en part, responsable dels efectes inflamatoris de l'LDL(-). La CER es relaciona amb un augment en la susceptibilitat a l'agregació de l'LDL(-) i a la seva inducció de citoquines en monòcits. Aquestes propietats de l'LDL(-) augmenten a 37ºC i disminueixen per la incubació d'aquesta lipoproteina amb l'HDL. El contingut en CER en l'LDL(-) augmenta per acció de l'activitat tipus fosfolipasa C (PLC), intrínseca en l'LDL(-) i que també es veu inhibida per l'HDL. Aquest fet suggereix que l'activitat PLC de l'LDL(-) participa en les modificacions que tenen lloc en l'LDL nativa (LDL(+)) per a la formació de LDL(-). L'enriquiment d'LDL en CER (CER-LDL)) i el tractament d'LDL amb PLC promouen una LDL que mimetitza l'alliberament de citoquines de l'LDL(-). Tanmateix, l'efecte de la CER-LDL és menor al de l'LDL(-), proposant la possible acció d'altres components en l'LDL(-) que podrien contribuir al seus efectes inflamatoris.

En l'estudi dels receptors involucrats en l'alliberament d'IL-6, IL-10 i MCP-1 induït per l'LDL(-) en monòcits es va determinar que CD14 i TLR4 juguen un paper central. L'adició dels anticossos antiTLR4 i antiCD14 va disminuir un 70%-80% l'alliberament de citoquines, mentre que antiTLR2 va disminuir l'alliberament sols en un 15-25%. L'alliberament de citoquines també va disminuir al tractar les cèl·lules amb un inhibidor específic de TLR4. Aquest alliberament de citoquines va augmentar en la línia de monòcits THP1 que sobreexpressen CD14 (THP1-CD14). Aquests resultats es van confirmar per silenciament gènic de TLR4 i CD14. Experiments d'unió van mostrar que l'LDL(-) presenta una gran afinitat per CD14 i TLR4; la neutralització d'aquests

receptors va disminuir la quantitat d'LDL(-) unida a monòcits. Per immunoassaig es va demostrar que CD14 és el principal receptor involucrat en la unió de LDL(-). L'LDL(-) uneix CD14, es forma un complex amb TLR4 i d'aquesta manera s'activa la senyalització intracel·lular que donarà lloc a l'alliberament de citoquines per aquestes cèl·lules. L'LDL(-) pot, d'altrabanda, interaccionar directament amb TLR4 per activar la senyal intracel·lular.

CD14 i TLR4 són receptors que mitjancen l'alliberament de citoquines induït pel lipopolisacàrid (LPS) present en la membrana bacteriana. Els resultats mostren una competència entre LDL(-) i LPS tant per l'efecte inflamatori com per la unió al receptor CD14 de monòcits i de plaques multipou recobertes de CD14. Aquesta competència podria representar una acció compensatòria de l'LDL(-) en casos d'excessiva inflamació.

El contingut augmentat en CER present en l'LDL(-) sembla ser el responsable de l'activació del sistema CD14-TLR4, el qual provoca l'alliberament de citoquines en monòcits. L'alliberament de citoquines induït per la CER-LDL en monòcits va disminuir per l'adició de l'inhibidor de TLR4. La secreció de citoquines induïda per la CER-LDL és similar a la de l'LDL(-), excepte pel fet que la CER-LDL no pot activar l'alliberament de citoquines directament per TLR4 i necessita CD14 per a aquest efecte.

En conclusió, l'augment del contingut en CER per l'LDL(-) exerceix un paper central en l'alliberament d'IL-6, IL-10 i MCP-1 a través de CD14- TLR4 en monòcits.

Chapter I

INTRODUCTION

1. ATHEROSCLEROSIS

1.1 A CARDIOVASCULAR DISEASE

The American Heart Association classifies cardiovascular diseases (CVD) as those affecting heart and blood vessels. The World Health Organization determined CVD as the *main cause of death worldwide* (WHO 2011) (Samson and Garber 2014), not only in rich countries, but also in developing areas (Andre Pascal Kengne and Jean-Claude Mbanyaf 2013). Atherosclerosis is the main cause of CVD, consisting of the accumulation of lipids and cells into the artery wall. Atherosclerosis is a silent inflammatory process that culminates in a heart attack or an ischemic stroke (Lusis 2000). Heart attacks occur when the blood flow to the heart, generally in the aorta, is blocked by a blood clot. A stroke can be originated by haemorrhage, when a blood vessel within the brain bursts, or by ischemia (the most common type) when a blood vessel that feeds the brain, generally in the carotid, gets blocked making brain cells die (Hammond EC 1969).

Among the main risk factors assigned to induce the development of atherosclerosis (Ross 1999) are high total cholesterol (TC) levels (Stokes 1988, Rudolf and Lewandrowski 2014), the presence of modified lipids and lipoproteins (Stokes 1988, Ovbiagele, Goldstein et al. 2014), hypertension (Hermann, Flammer et al. 2006), high plasma homocystein levels (Steed and Tyagi 2011), high levels of C-Reactive Protein (Yousuf, Mohanty et al. 2013) some bacterial infections (Mendy, Vieira et al. 2013), cigarette smoking (Abbott, Yin et al. 1986), older age and the male sex (Rudolf and Lewandrowski 2014).

1.2 ORIGIN AND EVOLUTION OF THE ATHEROMATOUS PLAQUE

The earliest changes that precede the formation of atherosclerotic lesions take place in endothelium (Haust 1971). Endothelial cells are known to be aligned regarding blood flow shear stress. Blood flow in contact with endothelial cells are relevant to wall physiology and define its vulnerability to atherosclerosis (Dewey, Bussolari et al. 1981). In this regard, different endothelial phenotypes have recently been described (Davies,

Civelek et al. 2013). Endothelial regions in specific arterial sites such as branches, bifurcations and curvatures, where there is turbulence and increasing shear stress, are susceptible to suffer from lesions.

Apart from shear stress, atherogenic lipoproteins in blood induce changes in gene expression of adhesion molecules and chemokines, thereby facilitating the migration of cells into endothelium. The accumulation of cells, lipoproteins and extracellular matrix is the key factor for the development of atherosclerosis (Ross 1999, Haustein KO 2010).

Atherosclerotic lesions have been classified in five different stages by the American Heart Association (Stary, Chandler et al. 1995, Ross 1999, Stary 2000) and recently updated by the group of Sakakura (Sakakura, Nakano et al. 2013) and explained below (Figure 1). The main inflammatory processes of each stage will be described, since atherosclerosis is an inflammatory disease (Libby 2002).

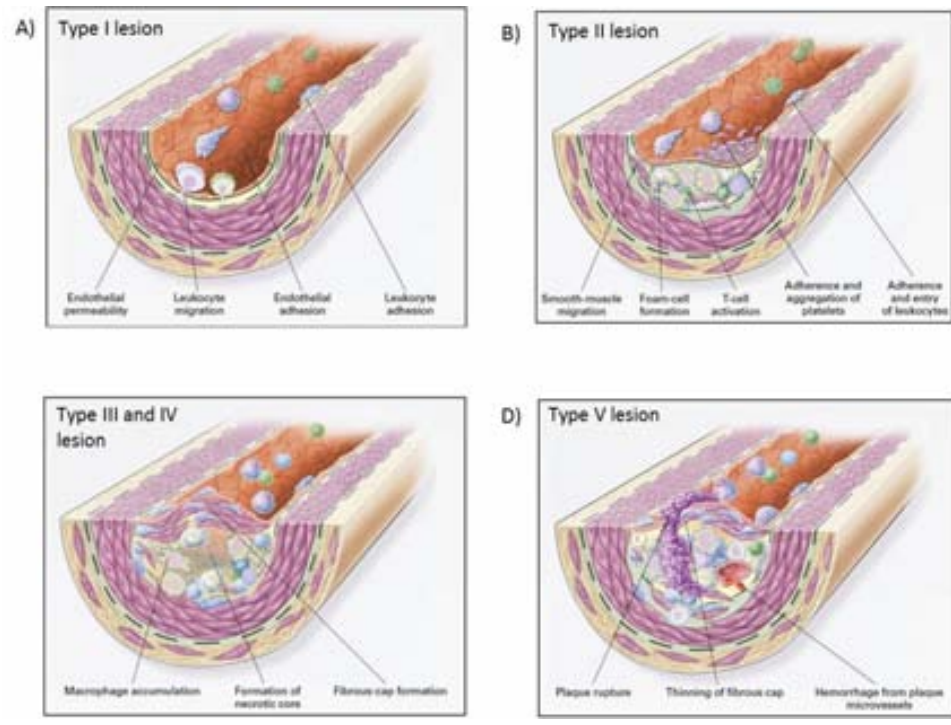


Figure 1: Progression of the atheromatous lesion. A) Type I lesion: First changes in the endothelium; B) Type II lesion: formation of the fatty streak; C) Type III and IV lesion: intimal thickening and fibroatheroma; D) Type V lesion: rupture of the vulnerable plaque.

- **Type I lesion or intima thickening** (Figure 1A): Endothelial cells compensate erosion produced by alterations in blood flow by inducing the proliferation of smooth muscle cells (SMC) and extracellular matrix that reduce the size of the arterial lumen (Sakakura, Nakano et al. 2013).

The disturbed flow also provokes an increased expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), P-selectin and E-selectin in the endothelium. These molecules together with chemokines promote the rolling and attachment of monocytes and other cells to the endothelium.

Low Density Lipoprotein (LDL) levels in the blood flow are a main cause of injury and inflammation (Navab, Berliner et al. 1996), particularly in areas predisposed to lesion formation (Cybulsky and Gimbrone 1991).

- **Type II lesion or fatty streak** (Figure 1B): Once adherent to the activated endothelial layer, the monocyte diapedeses between endothelial cells and penetrates in the intima (Libby 2002). In the intima, the monocyte acquires characteristics of the tissue macrophage, that upregulates both toll-like receptors (TLR), involved in macrophage activation, and scavenger receptors (SR) (Clinton, Underwood et al. 1992, Sugiyama, Okada et al. 2001, Hansson, Robertson et al. 2006) enrolled in foam cell formation.

Endothelial cells sensitize to local inflammation and increase permeability, which permits LDL and other atherogenic lipoproteins to pass through the endothelium by a concentration-dependent manner that does not require receptor-mediated endocytosis (Hoff, Heideman et al. 1977). LDL can then intimately associate with the proteoglycans (PG) of the extracellular matrix of the subendothelial space and become trapped (Camejo, Olofsson et al. 1988). Retained lipoproteins can be modified and then internalized by macrophages, by means of SR, forming the foam cells, in which cholesteryl esters or esterified cholesterol (EC) accumulate in cytoplasmic droplets (Navab, Berliner et al. 1996). Foam cells secrete pro-inflammatory cytokines and reactive oxygen species (ROS) that amplify the local inflammatory response.

There is also infiltration of T lymphocytes in the intima, where they become activated and induce the release of inflammatory mediators, producing an amplification loop of inflammation (Libby 2002). Mast cells in the intima degranulate and release proteases and other factors that will help the progression of the fatty streak (Libby 2002). SMCs are also present in the lumen, placed together with foam cells and proteoglycans in the enlarged intima. Adherence and aggregation of platelets are also mechanisms undergone at this stage (Ross 1999).

It has been shown that foam cells can leave the arterial wall (Ley, Laudanna et al. 2007). Therefore, lesions at this stage, which are mainly silent, are capable to regress.

- **Type III lesion or pathological intimal thickening** (Figure 1C)**:** This stage is characterized by the presence of a big lipid pool in the intima, specially aggregated LDL (agLDL) retained by PG. PG and cells are placed at the periphery of this lipid pool; and this is what is called an atheromatous plaque, that develop at an advanced stage with an strong accumulation of macrophages to the lumen (Nakashima, Wight et al. 2008). Varying degrees of small free TC clefts that may come from dying SMCs, as well as micro-calcification, can be found (Sakakura, Nakano et al. 2013).

- **Type IV lesion, fibroatheroma or advanced lesion** (Figure 1C): The necrotic core is the result of apoptosis and necrosis of cells of the plaque, including SMCs, increased proteolytic activity and high amounts of extracellular matrix, collagen and lipids. Macrophages accumulate and die at this stage producing the formation of large TC clefts. At this stage, advanced lesions tend to form a fibrous cap as a response to the injury. The fibrous cap is a mixture of leukocytes that continue adhering to endothelium, lipid, collagen fibers and cellular debris that cover a necrotic core. When the fibrous cap is calcified, it is critical for the maintenance of the lesion integrity (Sakakura, Nakano et al. 2013).

- **Type V lesion, thin cap fibroatheroma or vulnerable plaque** (Figure 1D)**:** The advanced lesion can develop to a thrombotic event by three different mechanisms: rupture, erosion, microvessel formation or calcification (Virmani, Kolodgie et al. 2000, Libby 2002).

The rupture of the fibrous cap is mediated by the activation of proteolytic enzymes, such as metalloproteinases (MMPs) which degrade the extracellular matrix (Rajavashisth, Liao et al. 1999), and the release of huge amounts of platelets. These processes finish with the formation of the thrombus and the occlusion of the artery.

Plaque **erosion** is defined as an acute thrombus that is in direct contact with the intima (Rajavashisth, Liao et al. 1999). The eroded plaque produces thrombosis in a lower frequency than the ruptured plaque (Libby 2002).

Microvessel formation is an event that represents nutritive function for the atheroma that promotes plaque growth (de Boer, van der Wal et al. 1999) and could yield to a silent microvascular haemorrhage. The development of microvascular channels in the atheroma are produced by angiogenic mediators (Ramos, Kuzuya et al. 1998).

Finally, **calcified nodules** form a lesion that occurs in highly calcified arteries.; but this lesion is the least frequent cause of coronary thrombi (Sakakura, Nakano et al. 2013).

As it has been shown, many factors contribute to the progression of the atheromatous plaque. However, over the past 50 years, the understanding of this silent pathology has been focused on lipids. Lipids not only accumulate in the intima and lead to the formation of the fatty streak, but also are one of the key causes of inflammation. In the next section, we will explain the metabolism and the characteristics of the main lipoproteins involved in atherosclerosis, with a deeper consideration to LDL and its several modifications.

2. LIPOPROTEINS IN ATHEROSCLEROSIS

2.1 LIPIDS AND LIPOPROTEINS

Lipids are hydrophobic small molecules and essential components for all biological entities (Fahy, Subramaniam et al. 2009). Their biological functions include energy resource: triglycerides (TG); structural components of cellular membranes: phospholipids (PL) and TC; precursors of hormones such as sterols; molecules of intra or extracellular signaling: free-fatty or non-esterified fatty acids (NEFA), PL, prostaglandins, eicosanoids; as well as they are essential to solubilize some vitamins (Fahy, Subramaniam et al. 2005).

In circulation, lipids and proteins are held together by non-covalent forces forming macromolecular structures called lipoproteins. They are constituted by an internal hydrophobic nucleus with lipids mainly EC and TG, and an external layer of amphipathic lipids such as PL and free cholesterol (FC). Lipoproteins contain proteins, including enzymes, with a role in lipid transfer or in lipoprotein metabolism and apolipoproteins (apo) placed at the surface (Segrest, Jones et al. 2001). Apolipoproteins have not only a structural function but also determine the destination, interaction with receptor molecules and the metabolism of lipoproteins.

Lipoproteins are distinguished from each other by size, density, electrophoretic mobility, composition and function. The classification of lipoproteins is based on their density, which permit their isolation by ultracentrifugation (Havel, Eder et al. 1955), They are mainly classified in: chylomicrons (CMs), very low density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), LDL and high density lipoproteins (HDL). Lipoprotein a (Lp(a)) is a lipoprotein situated in-between LDL and HDL. LDL can be further subdivided into light LDL and heavy LDL, and HDL into HDL1, HDL2 and HDL3. There is an inverse relationship between size and density, so that the biggest and lightest lipoproteins are chylomicrons, and the smallest and densest are HDL subtypes. HDL is the only lipoprotein with no apoB-100. In general, lipoproteins containing apoB are potentially atherogenic, whereas lipoproteins with no apoB are antiatherogenic. The main characteristics and composition of lipoproteins are summarised in Table 1.

	CM	VLDL	IDL	LDL	Lp(a)	HDL
Density	< 0.096 Kg/l	0.096-1.006 Kg/l	1.006-1.019 Kg/l	1.019-1.063 Kg/l	1.050-1.100 Kg/l	1.063-1.210 Kg/l
Diameter	100-1000 nm	35-80 nm	30-40 nm	18-28 nm	28-32 nm	5-12 nm
Origin	lipidic diet	hepatic	from VLDL lipolisis	from IDL and VLD lipolisis	hepatic	periferal tissues
Function	lipid transport	transport of triglycerydes synthesised in liver	increased in pathologies	Cholesterol transport from peripheral tissues	Increased in pathologies	reverse cholesterol transport
Life time	1 hour	1-3 hours		1.5 - 3.5 days		5-6 days
COMPOSITION						
Triglycerydes	81%	52%	15%	8%	6%	8%
Cholesterol	9%	22%	48%	47%	44%	19%
Phospholipids	8%	19%	22%	23%	20%	27%
Protein	2%	7%	15%	22%	30%	46%
Main apolipoproteins	B-48, E, A-I, A-II, C-I, C-II, C-III	B-100, E, C-I, C-II, C-III	B-100, E, C-III	B-100	B-100 (a)	A-I, A-II, D, E

Table 1. Main characteristics, function and composition of lipoproteins.

2.2 LIPOPROTEIN METABOLISM AND LIPID TRANSPORT

A description of the lipoprotein metabolism and its main function is represented in Figure 2 and summarised afterwards (Hegele 2009).

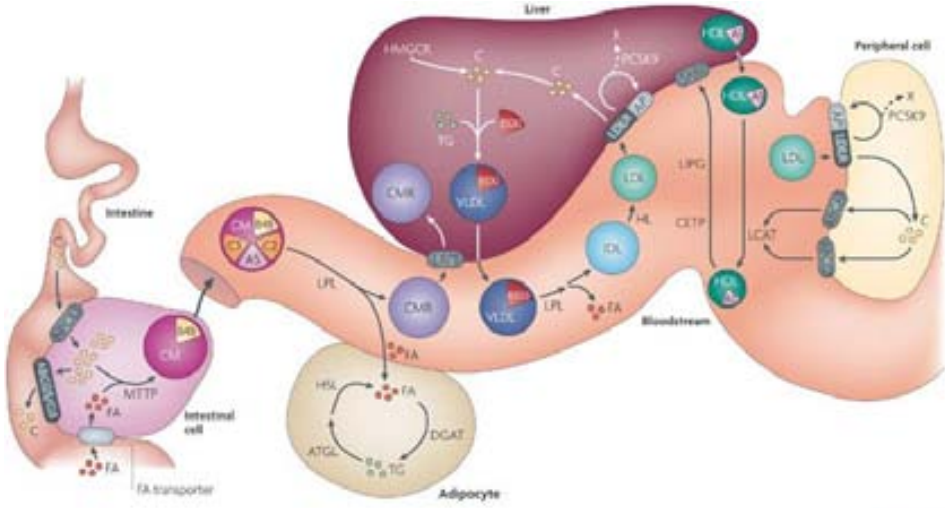


Figure 2. An overview of lipoprotein metabolism (Hegele 2009).

Hydrolysed dietary fats and TC enter intestinal cells or enterocytes. Reconstituted TGs are packaged with EC and the apoB isoform B48 (apoB48) into CMs. Unlike apoB100,

which is synthesised in the liver, apoB48 is synthesised in the intestine where its transcription is truncated generating a shorter protein. CMs, secreted via the lymphatic system, enter vena cava and circulate until they interact with lipoprotein lipase (LPL), releasing NEFA that incompletely enter peripheral cells. In adipocytes, enzymes including acyl CoA such as Diacylglycerol Acyltransferase (DGAT) resynthesize TGs, which may be hydrolysed. CM remnants are taken up by hepatic LDL receptor (LDLR) or LDLR-related protein 1 (LRP1). In hepatocytes, TC is recycled or synthesized *de novo*, with 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCR), before being packed together with TG and the apoB isoform B100 into VLDL.

In circulation, TG contained in VLDL are hydrolysed by LPL, releasing NEFA. VLDL is metabolised leading to IDL. TG in the newly IDL formed is hydrolysed by hepatic lipase (HL), thereby yielding LDL. LDL is finally endocytosed by peripheral cells mainly by LDLR. By LDL, TC can arrive to all cells of apoA-Itissues in need (Hegele 2009).

Excess TC in tissues may return to the liver. This role is developed by HDL, whose synthesis requires the secretion of apoA-I by intestine and liver cells and a gradual incorporation of lipids. ApoA-I of HDL binds ATP binding cassette 1 (ABCA1) transporter, not only in hepatocytes but also in macrophages from peripheral tissues, and incorporates FC, which will be esterified to EC by lecithin-cholesterol acetyl-transferase (LCAT) to form mature HDL. EC transporter protein (CETP), which exchanges EC and TG, also participates in HDL maduration. This TC is subsequently taken up by the liver via the SR subtype B1, where TC is further excreted into the bile. This mechanism permits HDL to exert probably its most important function: the reverse cholesterol transport (RCT) from peripheral tissues to the liver (Navab, Reddy et al. 2011).

2.3 HDL IN ATHEROSCLEROSIS

There is an inverse relationship between levels of plasma HDL and CVD (Gordon, Castelli et al. 1977). HDL is known to play two main atheroprotective roles: firstly it

exerts RCT (Glomset 1968), summarised in the previous section, and secondly it modulates inflammation (Navab, Reddy et al. 2011).

The anti-inflammatory effect of HDL can be exerted by preventing LDL oxidation, which is known to have inflammatory effects. LDL can be oxidized mainly in the arterial intima, where arterial wall cells release 12-lipoxygenase (12-LO) protein, as well as oxidised PL that promote the oxidation of LDL. HDL can directly prevent the oxidation of LDL by the transfer of oxidation products from LDL to HDL, so that HDL serves as a "sink" for oxidized lipids (Navab, Hama et al. 2000, Navab, Hama et al. 2000). Moreover, HDL possesses antioxidant activity itself, which avoids any of the steps of the oxidation of LDL (Navab, Hama et al. 2000).

The antioxidant effect of HDL is also attributed to some of the HDL activities, including apoA-I, paraoxonase (PON1), platelet-associated factor acetyl-hydrolase (PAF-AH), LCAT, and glutathione peroxidase (Jaouad, Milochevitch et al. 2003). PON1 activity catalyses the hydrolysis of the oxidized polyunsaturated fatty acids at the sn-2 position of oxidized PL and inhibits the formation of both minimally modified LDL and extensively oxidized LDL. PON1 also decreases the transition metal ions and free radical generator-induced LDL and HDL oxidation (Watson, Navab et al. 1995). Another activity which is noteworthy in HDL is PAF-AH. Although PAF-AH enzyme is not present in HDL, PAF-AH activity has been associated with other different enzymes present in this lipoprotein (Tselepis and John Chapman 2002). PAF-AH, equally to PON1 (Rodrigo, Mackness et al. 2001) can hydrolyse oxidized PL to generate lysophosphatidylcholine (LPC) and oxidized fatty acids but also hydrolyses short chain PL such as platelet associated factor (PAF). Furthermore, another enzyme that could potentially contribute to HDL-associated PAF-AH activity is LCAT. LCAT can also hydrolyse PAF and oxidized species of phosphatidylcholine (PC) (Liu and Subbaiah 1994). PAF is known to be a strong pro-inflammatory molecule, thus its hydrolysis is atheroprotective (Tselepis and John Chapman 2002).

HDL also exerts direct anti-inflammatory properties by inhibiting the endothelial expression of adhesion molecules and preventing monocyte recruitment into the artery wall (Ley, Laudanna et al. 2007, Tabet and Rye 2009). Worth noting are the

studies of Navab and co-workers (Navab, Imes et al. 1991) who reported that HDL added into cell culture not only inhibits the release of MCP-1 induced by modified LDL, but also HDL could directly avoid the transmigration of monocytes to the intima.

Furthermore, HDL possess an anti-atherothrombotic role by inhibiting the aggregation of platelets by inactivating the synthesis of molecules such as thrombin, collagen, adenosine diphosphate, as well as triggering the inhibition of nitric oxide (NO) synthase (Nofer, Kehrel et al. 2002). It is important to highlight that many of these anti-atherogenic properties can be triggered by lipid-free apoA-1 (Murphy, Woollard et al. 2008), yielding obvious implications for the design of anti-inflammatory apoA-1 mimetic peptides.

In summary, HDL has an important role as an antiatherogenic particle, since it is known to be an antioxidant, anti-inflammatory and antithrombotic lipoprotein (Berrougui, Momo et al. 2012), which counteracts the effects of LDL. The following section is focussed on describing the role of LDL in atherosclerosis, which is a key factor in the development of cardiovascular events.

3. LDL IN ATHEROSCLEROSIS

3.1 LDL STRUCTURE AND BINDING TO LDLR

As it has been shown, LDL is the main transporter of TC, which is an essential source for cell membrane structure, hormones, vitamins and bile acid. LDL levels in blood must be maintained to guarantee its proper function. In this line, a proper LDL structure is essential to be recognised by the LDLR, which is responsible of the LDL clearance.

LDL particles are not homogeneous, but they are in a dynamic state in which their structure and physical properties depend on their lipid composition and on the apoB-100 conformation (Hevonoja, Pentikainen et al. 2000). Their density in plasma is from 1.019-1.063 Kg/L and the diameter varies from 18 to 28 nm. The particles possess a hydrophobic nucleus core that contains non-polar lipids such as EC (1600 molecules/LDL), TG (170 molecules/LDL) and some FC (180 molecules/LDL). This nucleus is surrounded by an amphipathic bilayer of PLs (700 molecules/LDL), FC (420 molecules/LDL) and one single apoB-100 copy (Esterbauer, Gebicki et al. 1992) (Figure 3).

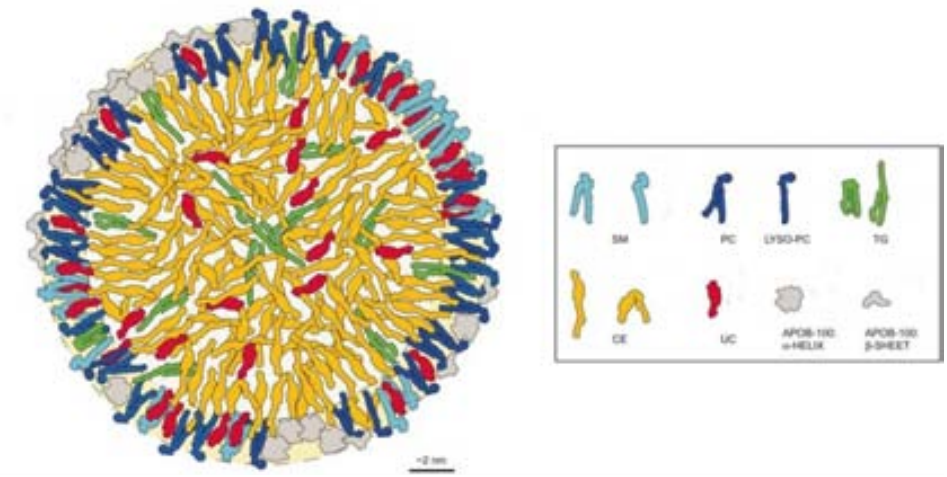


Figure 3: Molecular model of lipids contained in LDL and their location (Hevonoja, Pentikainen et al. 2000). SM: esphingomyelin, PC: phosphatidilcholine, Lyso-PC: lysophosphatidilcholine, TG: triglycerides, CE: cholesterol esters, UC: unesterified or free cholesterol (FC in the manusript).

The main phospholipidic components are PC and sphingomyelin (SM) (Lund-Katz and Phillips 1986), although LDL also contains traces of LPC (Esterbauer, Gebicki et al. 1992) and phosphatidylethanolamine (PE) (Sommer, Prenner et al. 1992), diacylglicerol (DAG) (Lalanne, Pruneta et al. 1999), ceramide (CER) (Schissel, Tweedie-Hardman et al. 1996) and some phosphatidylinositol (Ravandi, Kuksis et al. 1999). LDL also carries lipophilic antioxidants such as α-tocopherol, γ-tocopherol, carotenoids, oxycarotenoids and ubiquinol-10 (Esterbauer, Gebicki et al. 1992).

These lipid constituents of LDL particles are heterogeneously distributed and interact with each other to form local molecular microenvironments. Any molecular or physical stress such as temperature oscillations or the presence of certain enzymes may influence this lipid arrangement. These sites enriched in a combination of particles might be detrimental on the activation of some membrane proteins or enzymes, as well as on the recognition of cell receptors (Hevonoja, Pentikainen et al. 2000).

ApoB-100 is the principal protein in LDL particles; it constitutes approximately 20% of the LDL and is placed on the surface although a moderate hydrophobicity. It is a huge monomer of 4,536 residues with a molecular weight of 513 KDa. Studies of Segrest and colleagues revealed a pentapartite structure composed of amphipathic α-helical domains and amphipatic β-stranded domains (Segrest, Jones et al. 2001). It also has a particular role in maintaining the structural integrity and controlling the interactions of LDL particles. In the recent years, numerous studies of nuclear magnetic resonance have described the interactions of some domains of LDL particles with cell receptors, enzymes, heparin, as well as PG of the arterial wall (Tannock 2014).

An apoB-100 domain enriched in basic amino-acid residues has been identified as the LDL binding and uptake site of the LDLR (Knott, Pease et al. 1986), a region near the residue 3,500. This region has much in common with the receptor-binding domain of apoE (Weisgraber 1994), the principal apoprotein of VLDL and IDL, the other well-characterised ligands of the LDLR. These binding sites are rich in positively charged lysines (Boren, Lee et al. 1998) that interact with negatively charged cysteines in LDLR (Hobbs, Brown et al. 1986).

The main LDLR function is the regulation of LDL levels in blood. It was discovered by Goldstein and Brown in the 1980's (Goldstein, Brown et al. 1985); since then, it has been extensively studied (Goldstein and Brown 2009). The LDLR is a glycoprotein that contains 839 aminoacides forming 5 well-preserved domains. It is highly expressed in liver but it can also be found in endothelial cells, monocytes and macrophages (Hiltunen and Yla-Herttuala 1998).

The LDLR is synthesised in the endoplasmic reticulum in cells of all tissues (Hiltunen and Yla-Herttuala 1998); the protein is further matured in golgi complex. Afterwards, it is transported to the cell membrane, where it is exposed in the extracellular space. When it binds LDL, the LDL-LDLR complex is internalised by endocytosis through clathrine vesicles, and further dissociated in acidic endosomes. LDL is degraded in the lysosome as EC, NEFA and aminoacids, whereas LDLR is recycled and back to the cell surface.

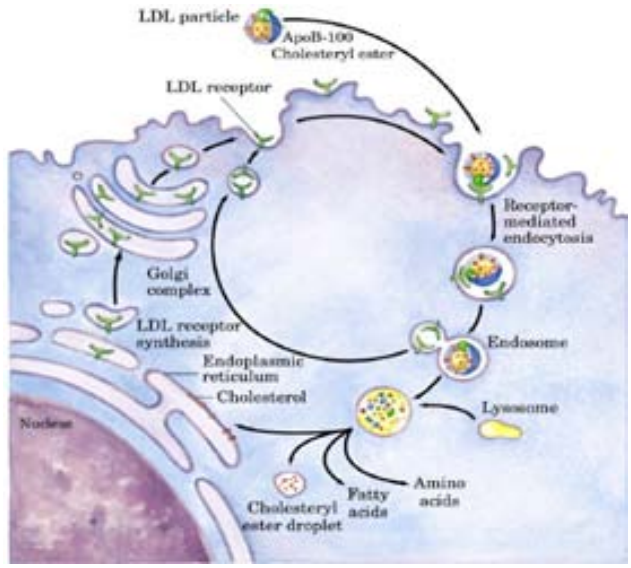


Figure 4: The cycle of LDL receptor.

Intracellular TC levels are tightly regulated partly through the LDLR expression. When the concentration of intracellular TC falls, not only the synthesis of LDLR increases, but the endogenous synthesis of TC also rises; this is triggered by the enzyme hydroximethil-glutaryl-coenzyme A (HMG-CoA) reductase. Otherwise, when the

intracellular CT levels are high enough to supply all the cell requirements, the two mechanisms are inhibited. In this situation, the enzyme LCAT is activated to form EC that can be stored in the cytoplasm (Brown and Goldstein 1985).

3.2 ATHEROSCLEROTIC ROLE OF LDL

An imbalance of plasma TC levels is accepted to be the most dealing cause of atherosclerosis (Navab, Berliner et al. 1996). An elevation of lipoproteins containing apoB-100, TG, LDL as well as low levels of HDL is known as dyslipidemia. Numerous epidemiological studies have demonstrated an extensive correlation of dyslipidemia with cardiovascular risk (Stokes 1988, Kaur 2014). Since LDL is the lipoprotein in circulation that transports 70% of TC to all cells, its involvement on atherosclerosis is of great importance. The LDL lowering therapies are still the most used treatments for atherosclerosis (Tonkin and Byrnes 2014).

A proof of the atherogenic characteristics of LDL is the familial hypercholesterolemia (FH), which is an autosomal dominant disease. The increase in TC in FH is caused by mutations on LDLR that lead to its partial or total deficiency. In homozygotes, TC levels are from six to ten times higher than in normolipemics, which yields to heart attack in young people (Goldstein and Brown 1983, Brown 1987).

Although intracellular TC levels are regulated in cells under physiological conditions, foam cells in the atheromatous plaque accumulate LDL with no limitation. Goldstein and colleagues described that patients of FH, despite having a mutated LDLR, contain foam cells in the atherosclerotic plaque (Goldstein, Ho et al. 1979). This is what is called “the TC paradox” and explains an alternative pathway for the internalisation of LDLs that had been previously modified. Modified LDLs could be recognised and further accumulated in macrophages by SRs (Goldstein, Ho et al. 1979). SRs internalise apoptotic cell fragments, bacterial endotoxins, as well as different forms of modified LDL. SRs possess no regulation, so that TC contained in these altered LDL can be accumulated and form atheromatous lesions.

Native LDL is scarcely proatherogenic (Lim and Ryoo 2011), but it presents clear atherogenic properties once it gets modified (Steinberg, Parthasarathy et al. 1989). When LDL levels in blood are increased, the internalisation of LDL in the intima is higher. LDL may be retained on the extracellular matrix, get modified and captured by SRs leading to foam cell formation. However, modifications that affect LDL are not only produced in the intima, but also in circulation (Sevanian, Bittolo-Bon et al. 1997, Kovanen and Pentikainen 2003). Because of its altered structure, modified LDL in circulation leads to a lower rate of clearance by the LDLR, accumulating in the intima and getting further modified. In general, modification of LDL leads to an increase on the negative charge that provokes an impaired recognition by LDLR. However, SRs usually present affinity to this electronegativity (Apostolov, Shah et al. 2009).

Modified LDL affect cells involved in atherosclerosis by different mechanisms, but all modified LDL forms are involved in inflammation. The principal mechanisms of modification of LDL that yield to inflammatory effects, as well as the receptors known to mediate recognition and internalisation are explained in the next and the following section, respectively.

3.3 MODIFICATIONS OF LDL AND ITS INFLAMMATORY EFFECTS

3.3.1 Acetylated LDL (acLDL)

AcLDL is an *in vitro* modified LDL generated by the addition of acetic anhydride. This was the first modified LDL form studied *in vitro*, and was used as a model in studies with SR (Basu, Goldstein et al. 1976, Goldstein, Ho et al. 1979). acLDL cannot be found in circulation, hence its implications in inflammation are scarce. acLDL is known to induce the release of monocyte chomoattractant protein-1 (MCP-1) and interleukin (IL)-6 in macrophages (Wang, Tabas et al. 1996).

3.3.2 Minimally modified (mmLDL) and extensively oxidized LDL (oxLDL)

In circulation, oxLDL levels are really scarce (less than 0.1%) (Holvoet, Perez et al. 1995), but LDL can be further oxidized in the intima. Oxidation of LDL is the most studied modification of LDL. The oxLDL oxidation is a gradual process that starts in

circulation and continues in the arterial intima (Navab, Hama et al. 2002). It is promoted mainly by enzymes such as myeloperoxidase and lypoxygenase, as well as ROS and thiol groups from sulphur compounds such as homocystein, secreted by cells of the artery wall into the subendothelial space or in circulation (Steinberg 1997, Hansson, Robertson et al. 2006).

The oxidation of LDL includes both modification of lipids, which is known as lipoperoxidation, and oxidation of apoB-100. The oxidation of lipids begins with modification of polyunsaturated NEFA yielding lipoperoxides, which are very instable molecules that can be transformed in hydroxides, ketones or aldehyde groups (Parthasarathy, Litvinov et al. 2008). Moreover, oxysterols produced by the enzymatic TC catabolism, by the oxidizing ability of lipid peroxides or even directly absorbed from diet, are also formed. Finally, in an advanced state of oxidation, apoB-100 can break down (Fong, Parthasarathy et al. 1987). The oxLDL found *in vivo* can be imitated *in vitro* by the incubation of native LDL with copper sulphate (Fox, Mazumder et al. 2000).

LDL oxidation can be counteracted by antioxidants, such as ubiquinol Q10, tocopherols and vitamin E or C (Kamal-Eldin and Appelqvist 1996, Lankin, Tikhaze et al. 2007), or HDL (Parthasarathy, Barnett et al. 1990). This balancing provokes different grades of oxidation in LDL, which permits the differentiation between mmLDL and oxLDL. Any grade of oxidation in LDL, has been correlated to atherosclerosis (Bae, Lee et al. 2009). In contrast to oxLDL, mmLDL is still recognised by LDLR but not by SR and therefore does not present enhanced uptake by macrophages (Miller, Viriyakosol et al. 2003).

OxLDL is involved in atherosclerosis by different mechanisms which includes cytotoxicity (Li, Li et al. 2013), originated by certain oxidant molecules that alter cell function and integrity yielding to cell death, apoptosis or programmed cell death (Al-Banna and Lehmann 2013), release of MMPs (Paim, Schreiber et al. 2013), inhibition of NO (Bao, Zhang et al. 2014) as well as induction of cytokine release and other inflammatory mediators.

Regarding inflammation, mmLDL induces the release of MCP-1 and MCP-8 in endothelial cells; IL-1, IL-6 and IL-10 in monocytes; tumor necrosis factor (TNF)-α, MCP-

1, IL-1, IL-6 and IL-10 in macrophages and MCP-1 in SMC (Cushing, Berliner et al. 1990, Yeh, Leitinger et al. 2001, Subbanagounder, Wong et al. 2002, Miller, Viriyakosol et al. 2005, Chavez-Sanchez, Chavez-Rueda et al. 2010). Otherwise, oxLDL can also trigger the release of some inflammatory mediators such as IL-8 in monocytes, macrophages, endothelial cells and SMC (Terkeltaub, Banka et al. 1994, Claise, Edeas et al. 1996, Wang, Tabas et al. 1996, Lee, Shi et al. 2000, Ryoo, Kim et al. 2004); IL-10, IL-12 and interferon (IFN)-γ in leukocytes (Frostegard, Huang et al. 1997, Fei, Huang et al. 2003); MCP-1 in endothelial cells (Wang, Deng et al. 1996); and IL-1 in macrophages and SMC (Lin, Yen et al. 2003).

Moreover, it has been shown that mmLDLs have a tendency to aggregate, whereas extensive oxidation such as in oxLDL renders LDL aggregation and fusion (Hoff, Whitaker et al. 1992, Pentikainen, Lehtonen et al. 1996). However, in comparison to native LDL, oxLDL is poorly attached to PG of the arterial wall (Oorni, Pentikainen et al. 1997) (see LDL aggregation). In fact, Öörni and colleagues (Oorni, Pentikainen et al. 1997) found that the higher the degree of oxidation LDL has, the fewer lysine residues are exposed in LDL, in order to bind to PGs.

3.3.3 Glycated LDL (glyLDL)

Glycation of LDL occurs chiefly because of the nonenzymatic reaction of glucose and its metabolites with the free amino groups of lysine in apoB-100. Higher concentrations of glyLDL are present in diabetes than in nondiabetic individuals and metabolic syndrome (Soran and Durrington 2011). Even in nondiabetic individuals, there is 3.6% circulating glyLDL (Younis, Charlton-Menys et al. 2009), which is more than the content of oxidatively modified LDL. It is included in what is known as LDL phenotype B, characterised by a small and dense LDL subfraction, which correlates to a higher cardiovascular risk (Sanchez-Quesada, Vinagre et al. 2013) and even the initiation of atherosclerosis in diabetic patients (Ahmad, Akhter et al. 2013). GlyLDL also contributes to inflammation by increasing the expression of CCR2 and the release of its ligand MCP-1 in monocytes (Isoda, Folco et al. 2008). In SMCs, glyLDL has been shown to have a high proatherogenic role, since it not only induces chemotaxis through the release of MCP-1, but also lipid loading, cell proliferation and oxidation (Sima, Botez et

al. 2010). Moreover, low doses of glyLDL induce proliferation of fibroblasts but high glyLDL levels decrease fibroblast viability (Zhao, Xie et al. 2013). The promotion of monocyte adhesion to the endothelium surface was also a property described of glyLDL (Zhao, Ren et al. 2014). For studies of glycation in LDL, glyLDL can be generated in the laboratory by the incubation of LDL with glucose (Sobenin, Tertov et al. 1996).

3.3.4 Carbamylated LDL

It is a recently discovered LDL modification present in patients with renal disease as well as smokers (Wang, Nicholls et al. 2007). Cyanate, a reactive compound in equilibrium with urea, carbamylates protein lysine residues to form homocitrulline, and thereby alters protein structure and function. More importantly, it has been determined as the most abundant modified LDL isoform in human blood, especially in chronic kidney disease patients and its presence is an indicator of cardiovascular risk. Recently, new insights into its biological effects have been reported (Speer, Owala et al. 2014). Carbamylation of LDL induces endothelial dysfunction via LOX-1 activation and increased ROS production.

3.3.5 NEFA-enriched LDL (NEFA-LDL)

NEFA are bioactive products that accumulate in the atheromatous plaque and contribute to inflammation and the progression of atherosclerosis (MacPhee, Moores et al. 1999). They are formed in the catalysis of TG-rich lipoproteins in the lipoprotein metabolism (Hegele 2009). However, NEFA are also generated from the degradation of lipoproteins by enzymes with phospholipolytic activities, mainly phospholipase A2 (PLA_2) (Ghesquiere, Hofker et al. 2005).

In this regard, LDL treated with PLA_2 or the incubation of LDL with a mixture of NEFA has been used *in vitro* to modify LDL by the enrichment in NEFA (Jayaraman, Gantz et al. 2011). In plasma, LDL can be enriched in NEFA when the levels of NEFA increase, such as in stress situations like physical exercise (Sondergaard, Poulsen et al. 2014) and altered metabolism for example in patients of diabetes (Carmena 2005). Moreover, since albumin is the transporter of NEFA in plasma (Olson 1998), low albumin levels

produce an increase on NEFA, a characteristic of patients with nephritic syndrome (Hong, Jeong et al. 2011, Xu, Dai et al. 2014).

NEFA-LDL presents a higher negative charge that leads to a decrease on the binding affinity to LDLR (Benitez, Villegas et al. 2004). The enrichment on NEFA has also been related to a higher aggregation of LDL (Hakala, Oorni et al. 1999, Bancells, Benitez et al. 2008). It also induces the release of IL-8 and MCP-1 in endothelial cells (Benitez, Camacho et al. 2004) and IL-6, IL-8, IL-10 and MCP-1 in monocytes (Bancells, Sanchez-Quesada et al. 2010).

3.3.6 Aggregated LDL (agLDL)

LDL particles can suffer from aggregation when their composition and structure vary. It has been postulated that agLDL can be found in vessel sites with an increased turbulence (Steinberg, Parthasarathy et al. 1989), where haemodynamic stress might induce modification of LDL structure. Modification of the structure of an LDL particle can result in loss of particle stability, and this can further affect the interactions with other particles.

The presence of enlarged LDL particles in the subendothelial space is mainly due to aggregation and subsequent fusion of LDL. The cleavage of the lipid packing on the surface of LDL, results in an increased penetration of hydrophobic core molecules toward the particle surface and increases its hydrophobicity (Ala-Korpela, Pentikainen et al. 1998). The contact of hydrophobic domains of different LDL particles brings LDL to aggregation, which does not change the size of individual particles. However, when the particle modification is sufficient extensive, the collision provokes an energetic stabilisation that will result in an irreversible attachment of particles, called fusion (Oorni, Pentikainen et al. 2000).

LDL particles are known to aggregate due to changes in apoB-100 and its lipid composition by many processes including proteolysis, lipolysis and oxidation (Oorni, Pentikainen et al. 2000).

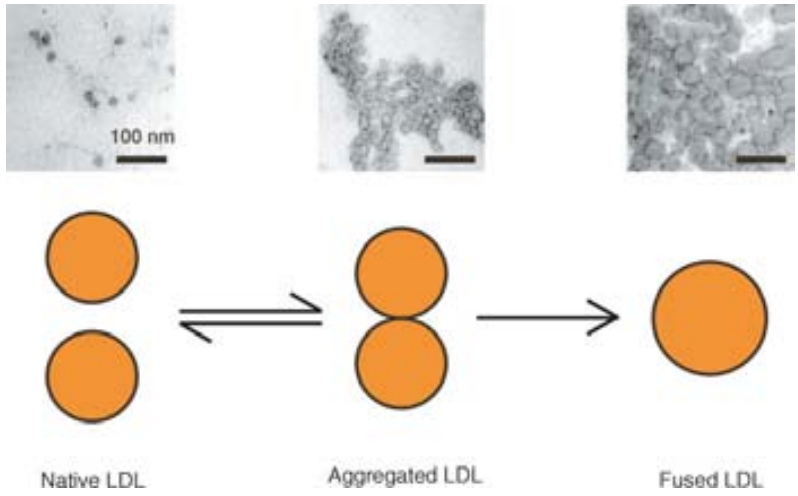


Figure 5. Schematic representation of LDL aggregation and fusion (Oorni, Hakala et al. 1998)

The **oxidation** of lipids produce lipid peroxides that discompose further into aldehydes capable of reacting with apoB-100, leading to degradation of its aminoacids and cleavage of apoB-100 (Esterbauer, Gebicki et al. 1992). This cleavage, and the degradation and relocation of hydrophobic regions in apoB-100, have been shown to cause LDL aggregation and fusion (Singh, Feix et al. 1995, Oorni, Pentikainen et al. 2000).

The effect of several biological **proteases** on the structure and function of apoB-100 has been deeply studied. ApoB-100 can be catalysed by chymotrypsin, trypsin, thrombine, kallikrein, methaloproteinases and lisosomal proteases, among others (Leroy, Castro et al. 1992, Piha, Lindstedt et al. 1995, Chait and Wight 2000, Oorni, Sneck et al. 2004, Panasenko, Aksenov et al. 2005). Particularly, proteases such as plasmin, kallikrein and thrombin induce fragmentation, whereas trypsin and pronase, a commercial mixture of proteases, can degrade apoB-100 extensively (Piha, Lindstedt et al. 1995). This distinction is of great importance, since evaluating the effect of the apoB-100 structure on the incubation of LDL with different proteases, Kovanen and co-workers (Kovanen and Kokkonen 1991) found that exocytosed mast cells granules containing proteases induced the formation of lipid droplets with a higher diameter and a lower density than native LDL (Nievelstein, Fogelman et al. 1991). This observation was attributed to the fusion of aggregated LDL particles.

Among the **lipases** found to induce aggregation, there are PLA_2, phospholipase C (PLC), sphingomyelinase (SMase), carboxyl ester lipase and lysosomal acid lipase (Hevonoja, Pentikäinen et al. 2000, Hui and Howles 2002, Fouchier and Defesche 2013). As reviewed by Öörni and collaborators (Oorni, Pentikainen et al. 2000), PLA_2 produces only LDL aggregation but not fusion, whereas SMase yields particle aggregation and fusion. The differences between the modifications induced by both phospholipases will be summarised in 3.3.7. Nevertheless, a significant property ascribed to aggregated or fusioned LDL is its increased binding to PG of the arterial wall, as detailed hereafter (Oorni, Pentikainen et al. 2000).

A common feature of all these type of modifications triggering LDL aggregation is the binding affinity to PG. It is known that the apoB-100 of LDL has specific sequences which contain clusters of positively charged amino acids (Hurt-Camejo, Camejo et al. 1990), also known as active lysine and arginine residues of apoB-100 (Paananen, Saarinen et al. 1995). They interact with the negatively charged carboxyl and sulphate groups of the glycosaminoglycan chains of the PG. It then leads to the retention of LDL particles inside the arterial intima.

Moreover, the binding to PGs can be enhanced by the presence of many apoB-100 molecules due to the aggregation of several LDL particles (Oorni, Posio et al. 2005). In fact, aggregated and fused lipolysed particles are known to bind to PGs more tightly than native LDL, which has been attributed to a higher number of lysine residues. It can then yield to the formation of new PG-binding sites in apoB-100 (Oorni, Hakala et al. 1998).

The binding of LDL to PGs of the arterial wall promotes its retention in circulation and increase the chance of being modified by proteases, enzymes, etc. (Williams and Tabas 1995), as explained in this section 3.3.

To mimic the aggregation of LDL that could be found *in vivo*, a rapid and useful method described is the mechanic agitation of LDL samples by vortex (Zhang, Gaynor et al. 1997). AgLDL is known to induce the release of TNF-α, IL-6 and IL-1β in macrophages (Sabeva, McPhaul et al. 2011).

3.3.7 Enzymatically modified LDL

Enzymes such as phospholipases have been deeply studied since many of them have been related to the onset and progression of atherosclerosis. Not only because of the generation of products derived from their activity, but also due to the ability to modify lipoproteins such as LDL (Ghesquiere, Hofker et al. 2005). The principal phospholipolytic activities that produce modified LDL in plasma and are related to atherosclerosis are commented below and the catalytic sites in PLs are represented in Figure 6.

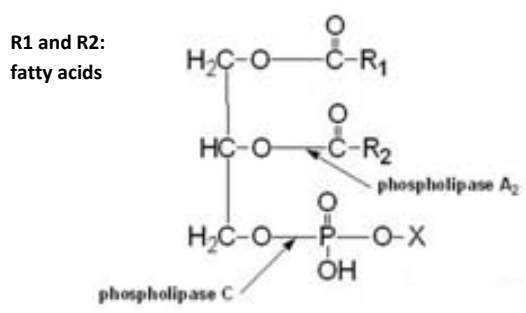


Figure 6: Degradation sites of phospholipase A2 and phospholipase C in a common PL.

Phospholipase A_2 (PLA_2)

The PLA_2 family include several unrelated proteins with a common enzymatic activity, which is the recognition and cleavage of the sn-2 acyl bond of glycophospholipids leading to the production of NEFA and a lysophospholipid. If LDL is treated with PLA_2 in the absence of lipid-binding proteins, such as albumin, the lipolytic products, LPC and NEFA accumulate in LDL particles. However, in the presence of physiologic albumin concentrations, most of the fatty acid and some of the LPC molecules are transferred from LDL to albumin (Hakala, Oorni et al. 1999). Lipolysis of LDL with the secretory form of PLA_2 ($sPLA_2$) in the presence of albumin leads to conformational changes in the apoB-100 component and reorganization of lipids (Kleinman, Krul et al. 1988), producing LDL aggregates but not LDL fusion (Oorni, Hakala et al. 1998). Although the surface hydrophobicity of such modified LDL particles increase their tendency to aggregate, the enhanced structural rigidity of the particles may stabilize the aggregates

and preclude particle fusion. However, LDL modified with the $sPLA_2$ leads to a reduction on size, and an increase in strength and aggregability of LDL particles. It is also important to highlight the enhanced affinity of LDL treated with $sPLA_2$ to PG present in the arterial wall (Flood, Gustafsson et al. 2004)(Hakala, Oorni et al. 1999).

The sPLA2 and the lipoprotein-associated PLA_2 (Lp-PLA_2) are the main enzymatic forms associated with atherosclerosis and could be potentially predictors of cardiovascular risk as well as therapeutic targets (Tselepis and John Chapman 2002, Rosenson and Stafforini 2012, Murakami and Lambeau 2013). $sPLA_2$, a calcium dependent PLA_2, is an acute phase reactant whose production is upregulated by inflammatory cytokines such as IL-1β, IL-6 and TNFα (Hurt-Camejo, Camejo et al. 2000). More than 10 isoforms of $sPLA_2$ have been described (Gelb, Valentin et al. 2000). The altered lipid content in LDL treated with $sPLA_2$, such as the increase in the products NEFA and LPC, have been shown to induce the release of IL-6, IL-8 and MCP-1 in endothelial cells (Benitez, Camacho et al. 2004), IL-8 in monocytes (Terkeltaub, Banka et al. 1994, Benitez, Camacho et al. 2004), and IL-6 and MCP-1 in SMCs (Viedt, Vogel et al. 2002).

In blood, Lp-PLA_2 a calcium independent PLA_2 is more closely associated with LDL (24%) than with HDL (20%) (Tselepis, Dentan et al. 1995). As it can degrade highly potent inflammatory mediators such as PAF lipids, it is alternatively called PAF-AH. PAF-AH leads to the production of NEFA and LPC (Aggerbeck, Kezdy et al. 1976). They are bioactive lipid mediators generated in lesion-prone vasculature and to a lesser extent in the circulation (Zalewski and Macphee 2005).

Phospholipase C (PLC) and SMase

PLC is a lipolytic activity that hydrolyses glycerophospholipids, such as PC or LPC in LDL, yielding to the production of dyacylglicerol (DAG) or monoacylglicerol (MAG), and phosphorylcholine (P-chol). P-chol is soluble in water and leaves LDL, but DAG and MAG are retained in the particle (Polacek, Byrne et al. 1988). When the PLC-like activity degrades sphingolipids such as SM, it is called SMase and produces CER and P-chol.

In regard to the lipid rearrangements that are susceptible to induce aggregation and fusion (Oorni, Pentikainen et al. 2000), the hydrophobicity of DAG produced by PLC-like activity provokes aggregation but has been shown to be insufficient to produce fusion, since it requires at least an 18% of neutral phospholipids in the LDL surface. Otherwise, when PLC-like activity degrades SM (SMase activity), the produced CER induce changes in the lipids of the cell bilayer, lipid fractioning and LDL fusion (Liu, Scraba et al. 1993, Oorni, Hakala et al. 1998). The role of LDL treated with SMase is important to increase the affinity of LDL to the PG of the arterial wall, by means of increasing aggregation on LDL.

There are many types of SMase, but the most related form known to induce modifications in LDL is the secretory SMase (sSMase). sSMase is secreted by macrophages (Schissel, Schuchman et al. 1996) and endothelial cells (Marathe, Schissel et al. 1998) residing in the intima, thereby modifying LDL by increasing its content on CER. sSMase can also act on cellular phospholipids. Although apoB-100 could, by similarity of some domains, exert this action (Kinnunen and Holopainen 2002), there is still no evidence of a specific protein in LDL with such effect. CER is hydrophobic and would remain retained in the LDL particle and modify the surface structure (Oorni, Pentikainen et al. 2000). Particularly, it has been shown that the increase on CER is the cause of not only LDL aggregation but also LDL fusion induced by SMase activity (Schissel, Tweedie-Hardman et al. 1996). CER-enriched domains may act as nonpolar spots at the surface of the particles and lead initially to particle aggregation through hydrophobic associations (Fenske, Chana et al. 1990).

CER, or alternatively called N-acetylsphingosine, is present in the cell membrane and regulates a variety of signal transduction processes such as apoptosis, autophagy, cell-differentiation, survival and inflammatory responses (Arana, Gangoiti et al. 2010), for instance atherosclerosis (Steinbrecher, Gomez-Munoz et al. 2004). Lightle *et al* described an increase of secreted SMase in serum and CER in LDL and VLDL particles after administration of lipopolysacharide (LPS) in humans (Lightle, Tosheva et al. 2003). As shown in Figure 7, CER is the precursor of important bioactive sphingolipids, such as sphingosine (SPH) and sphingosine-1-phosphate (S1P) (Arana, Gangoiti et al. 2010).

Moreover, CER, glycosphingolipids and S1P have been shown to accumulate in atherosclerotic lesions and participate in signal transduction pathways. Particularly, they can enhance the expression of adhesion molecules and promote migration and adhesion of monocytes to the injured endothelium (Kockx, De Meyer et al. 1998).

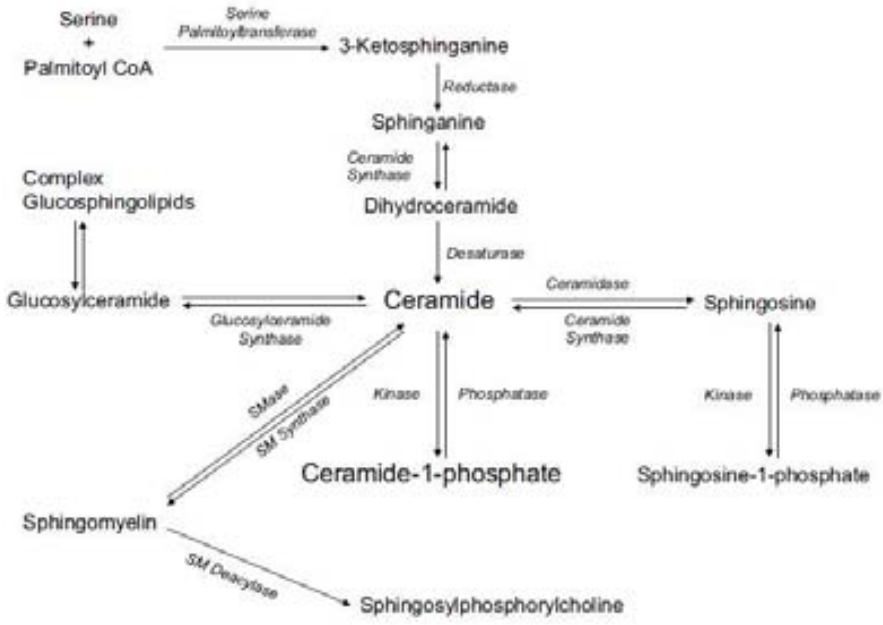


Figure 7. The ceramide metabolism (Arana, Gangoiti et al. 2010)

Multienzymatic modification of LDL (E-LDL)

The incubation of LDL with several phospholipases, such as trypsin and TC esterase, leads to an enzymatic modification of LDL (E-LDL) used in some studies. Its properties mimic the lipid drops observed in the atherosclerotic lesions (Bhakdi, Dorweiler et al. 1995). E-LDL can exert a strong inflammatory effect, since the release of MCP-1 in macrophages and IL-6 in SMC has been reported to be by far higher than that of oxLDL (Klouche, Gottschling et al. 1998, Klouche, Rose-John et al. 2000). Moreover, in endothelial cells, E-LDL has been reported to induce IL-8 due to the increased NEFA content in this modified LDL (Suriyaphol, Fenske et al. 2002).

3.4 RECEPTORS INVOLVED ON THE INFLAMMATORY RESPONSE OF MODIFIED LDL

A common treat of all the modifications on LDLs, both in apoB-100 or in their lipid components, is a low or absent recognition by the LDLR. This leads to impaired LDL clearance in circulation, the accumulation of LDLs in the arterial wall, formation of foam cells and consequently the atheroma plaque. In this section, there is a description of the main receptors described to be involved in the recognition of modified LDLs.

3.4.1 Scavenger Receptors (SR)

SRs were identified by Goldstein and Brown because of their ability to bind modified LDLs (Goldstein, Ho et al. 1979). Modified LDLs present negatively charged forms of apoB-100 in LDL, hence they cannot be captured by LDLR, but then by SR. They are mainly expressed in macrophages and are involved in foam cell formation (Brown and Goldstein 1983), although they are also found in SMC, endothelial cells and fibroblasts (Pitas 1990).

Among the SR superfamily, the members SR-A (I and II), SCARA5, CD36, SRBI, CD163, CD68, lectin-like oxidized LDL receptor (LOX-1), SREC-1 and SRPSOX (Figure 7) have been involved in atherosclerosis (Stephen, Freestone et al. 2010).

SR-A is the classic SR described by Goldstein and Brown (Brown and Goldstein 1983). SR-AI and II would be the main receptors in macrophages for oxLDL that have been aggregated (Asmis, Begley et al. 2005), whereas CD36 would be the one for non-aggregated oxLDL (Endemann, Stanton et al. 1993). SR-A and CD36 are as well the receptors that internalise glycated LDL in monocyte–derived macrophages (Brown, Rashid et al. 2007). SRBI can bind not only modified LDL but also native LDL, HDL and VLDL (Acton, Rigotti et al. 1996, Calvo, Gomez-Coronado et al. 1998). It has the ability to extract TC from cells to be incorporated in liporpoteins, also known as CT efflux. Thus, SRB1 possess atheroprotective properties.

LOX is the major oxLDL receptor in endothelial cells, although it is also found in macrophages and SMCs (Levitan, Volkov et al. 2010). CD68 is another possible receptor for oxLDL, as well as the SR that binds to phosphatidylserine and oxidized LDL (SR-

PSOX). Carbamylated LDL can also be recognized by LOX-1 (Apostolov, Shah et al. 2007, Apostolov, Shah et al. 2009, Speer, Owala et al. 2014). In the case of acLDL, it can be recognised by SRs expressed by endothelial cells (SREC-1) (Stephen, Freestone et al. 2010).

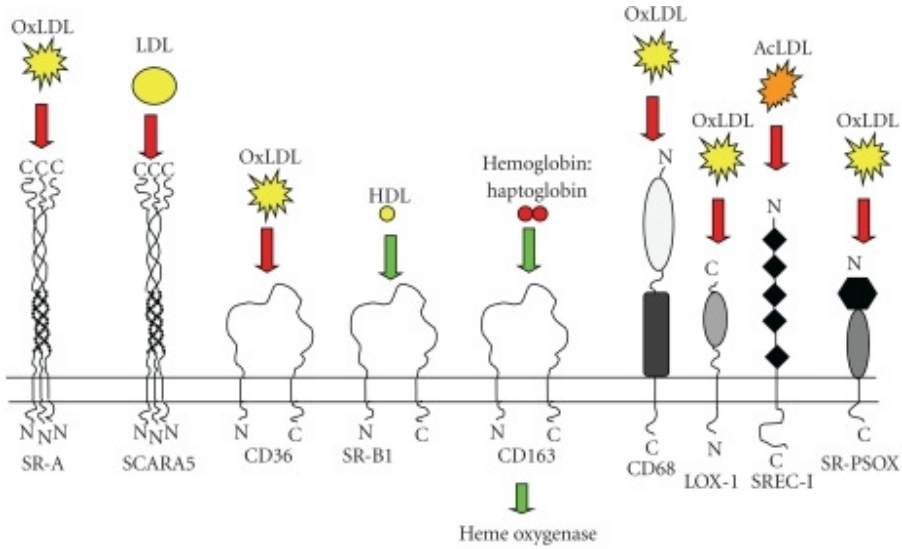


Figure 10. Major SR that bind modified LDLs in atherosclerosis (Stephen, Freestone et al. 2010). Red arrows indicate proatherogenic effects; green arrows indicate antiatherogenic or protective effects.

3.4.2 LDL receptor protein 1 (LRP1)

LRP1 is known to be essential in the development and its absence provokes lethality (Herz, Couthier et al. 1993). LRP1 is involved in many biological processes including cancer and injury to the nervous system, apart from atherosclerosis (Gonias and Campana 2014). It is a transmembrane receptor of the LDLR family. The extracellular binding domains of the LRP1 are similar to the ectodomains of the LDLR (Figure 8) (Pieper-Furst and Lammert 2013). However, LRP1 has a mechanism of regulation opposite to which of the LDLR (Llorente-Cortes, Otero-Vinas et al. 2002).

Through its extracellular domain, LRP1 interacts with at least 40 different ligands ranging from lipoproteins, extracellular matrix glycoproteins, proteases, viruses,

cytokines and growth factors (Boucher and Herz 2011). In atherosclerosis, LRP1 is exerting many protective actions including the control of either vascular SMC proliferation (Boucher, Gotthardt et al. 2003), and the RCT through the regulation of $cPLA_2$ activation and ABCA1 expression (Zhou, Choi et al. 2009). It also mediates clearance of TC remnants in the liver such as CM and VLDL through its interaction with apoE (Cal, Castellano et al. 2012). Nevertheless, the presence of LRP1 in macrophages not only facilitates the removal of apoptotic cells (Yancey, Blakemore et al. 2010), but also accelerates progression of atherosclerosis by facilitating uptake of atherogenic lipoproteins and foam cell formation (Llorente-Cortes and Badimon 2005). LRP1 is the most important receptor for agLDL recognition and uptake in vascular SMC through the recognition of CE present in this modified LDL (Llorente-Cortes and Badimon 2005, Llorente-Cortes, Otero-Vinas et al. 2006).

It has been observed that agLDL are internalised in diffuse and large vesicles, which are clearly different from the smaller, well-defined vesicles involved in normal LDL uptake (Llorente-Cortes, Martinez-Gonzalez et al. 1998). Interestingly, agLDL and increased LDL, such as in hypercholesterolemia, upregulate the expression of LRP1 in macrophages (Costales, Castellano et al. 2013).

3.4.3 Toll like receptors (TLRs)

TLRs and Toll, described in *Drosophila melanogaster*, have a central role in innate immunity and are also required for the development of an adaptive immune response (Akira and Sato 2003, Gay, Gangloff et al. 2006). TLRs are class I receptors, with a single α-helix that spans the cell membrane and can be divided into two groups according to their cellular location, either in the surface or localized in vesicles from the endoplasmic reticulum and golgi. TLRs are expressed by various cells of the immune system, mainly monocytes but also macrophages and dendritic cells, and respond to molecules derived from bacterial, viral or fungal origin. TLRs are pattern recognition receptors (PRR) that recognise pathogen-associated molecular pattern molecules (PAMPs) that are derived from microorganisms. PAMPs are antigens such as lipopolysaccharide (LPS) from the outer membrane of gramnegative bacteria (Iwasaki

and Medzhitov 2004) or several components from gram-positive bacteria (Schwandner, Dziarski et al. 1999).

The ectodomains of these receptors contain blocks of repeats called a leucine-rich repeat (LRR) (Figure 8). LRRs are found in diverse groups of proteins, involved in RNA processing and transcriptional regulation to cell adhesion, bacterial pathogenesis and signal transduction. The characteristics of LRRs explain the ability of different members of the TLR family to signal in response to stimuli with very different properties (Figure 9). The C-terminal capping structure of TLRs is connected to the single transmembrane α-helix and the cytoplasmic domain, known as the Toll interleukin-1 receptor (TIR) domain, which couples downstream signal transduction (Gay, Gangloff et al. 2006).

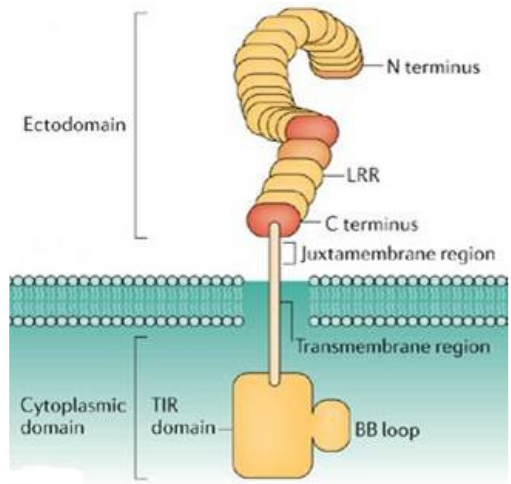


Figure 8. General schematic structure for TLRs. Adapted from Gay and Gangloff et al. (Gay, Gangloff et al. 2006).

LPS constitutes a model to study TLRs (Figure 9). The recognition of LPS by TLR4 requires the myeloid differentiation factor 2 (MD2), which is a lipid-binding protein (Nagai, Akashi et al. 2002). Initially, LPS is bound by the LPS-binding protein (LBP) and then transferred to a cluster of differentiation 14 (CD14), an extrinsic membrane protein that is found on the surface of various cells of the immune system. It is a

glycosylphosphatidylinositol-anchored receptor that lacks a transmembrane domain and then needs the membrane proteins TLRs to mediate the intracellular signalling of CD14 ligands. From CD14, LPS will move to MD2, thereby activating TLR4 (Hailman, Lichenstein et al. 1994) or TLR2 and inducing the intracellular signal transduction (Krishnan, Selvarajoo et al. 2007, Cole, Georgiou et al. 2010). TLR4 signalling encompasses both the MyD88-dependent and the MyD88-independent pathway. The MyD88-independent pathway depends on the TIR-domain-containing adaptor protein-inducing interferon (TRIF). TRIF interact with Trif-related adaptor molecule (TRAM) to mediate interferon regulatory factor (IRF)-3 and NF-kB activation. TLR4 can activate the MyD88-dependent pathway, in which MyD88 associates with toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP) to activate NF-kB (O'Neill and Bowie 2007).

CD14 is not always required for TLR4 signalling, TLR4-MD2 can bind LPS in a CD14-independent process (Godowski 2005) (Figure 9). CD14 facilitates the activation of TLRs to mediate the inflammatory signalling (Segura, Vadeboncoeur et al. 2002). CD14 has been shown to internalise in monocytes, only when LPS has previously activated a TLR, mainly TLR4 (Rabehi, Irinopoulou et al. 2001). CD14 is expressed in the 90%-95% of all monocytes and for this reason it has become a monocyte marker (Maiwald, Zwetsloot et al. 2013).

Another TLR that uses MyD88-dependent pathway is TLR2. TLR2 was found to be expressed in all lymphoid tissues with the highest expression in peripheral blood leukocytes. It is mainly mediating gram-positive bacteria signalling, although it has also been reported to transduce LPS signals from gram-negative bacteria (Yang, Mark et al. 1998).

Nevertheless, TLRs not only recognise membrane compounds from pathogens or PAMPs, but also endogenous molecules that are recognised as foreigners, also called damage-associated molecular patterns (DAMPs) (Piccinini and Midwood 2010). Moreover, several members of the TLR family, have been found of cells in the atheromatous plaque and can elicit proinflammatory cytokine release, lipid uptake and foam cell formation, as well as activating cells of the adaptive immune response (Cole,

Georgiou et al. 2010). The specific combination of TLRs in each cell type would determine the ability to respond to exogenous or endogenous ligands.

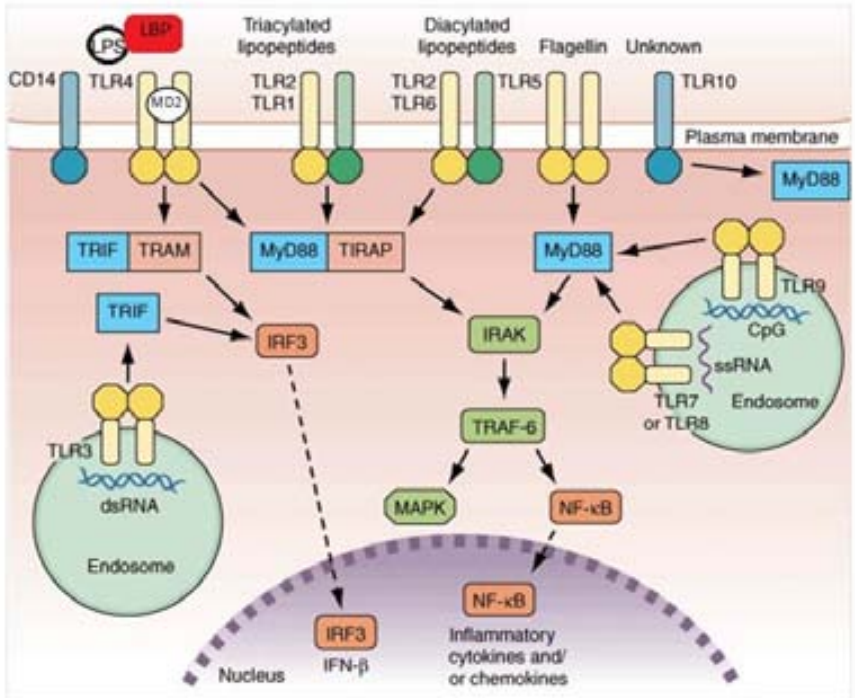


Figure 9. TLR-family activation of intracellular pathways leading to changes in gene expression and cytokine release. Adapted from van Duin (van Duin, Medzhitov et al. 2006).

Supporting the idea that modified LDLs are endogenous pro-inflammatory entities, it has been shown that TLRs and CD14 are also enrolled in mediating the effect of some proatherogenic LDL forms (Cole, Georgiou et al. 2010). In macrophages, mmLDL induces the release of IL-6 and MCP-1 in a TLR4-dependent or independent manner (Miller, Viriyakosol et al. 2005). However, not only TLR4 but also TLR2 and CD14 are implicated in IL-1, IL-6, IL-10 and TNF-α induced by mmLDL in monocytes and macrophages (Chavez-Sanchez, Chavez-Rueda et al. 2010, Chavez-Sanchez, Madrid-Miller et al. 2010).

Miller and coworkers (Miller, Viriyakosol et al. 2003) found that CD14 binds mmLDL in macrophages but through a different binding site than LPS. This binding promotes CD14 and TLR4 association yielding to phagocytosis, macropinacytosis and TC accumulation (Choi, Harkewicz et al. 2009). Interestingly, other groups have described that both mmLDL and oxLDL upregulates the expression of TLR2 and TLR4 in monocytes and in macrophages (Chavez-Sanchez, Madrid-Miller et al. 2010, Geng, Wang et al. 2010, Mogilenko, Kudriavtsev et al. 2012). Indeed, this induced upregulation by oxidized forms of LDL has been correlated to a higher release of IL-8, MCP-1 and TNFα in monocytes and macrophages (Chavez-Sanchez, Madrid-Miller et al. 2010, Geng, Wang et al. 2010). Very recent results show that oxLDL induce cytokine release in macrophages not only by TLR2 and TLR4 but also through CD36, and this modified lipoprotein uses all these receptors to mediate cytokine release in these cells (Chavez-Sanchez, Garza-Reyes et al. 2014).

4. ELECTRONEGATIVE LDL (LDL(-))

4.1 WHAT IS LDL(-)?

In 1988, studies of Avogaro and colleagues revealed the presence in plasma of a minor subfraction of LDL, which was called electronegative LDL (LDL(-)) due to its increased electronegative charge (Avogaro, Bon et al. 1988). From that time forward, it has been studied by several groups and alternatively called: minor LDL (Shimano, Yamada et al. 1991), LDLB (Vedie, Myara et al. 1991), L5 (Chen, Jiang et al. 2003) or fast migration LDL (fLDL) (Zhang, Maeda et al. 2006). In this manuscript, all these forms will be called LDL(-), since only slight differences due to the isolation method have been appreciated (Ke, Stancel et al. 2014).

LDL(-) is a heterogeneous pool of modified LDLs, including particles that differ in size, density, and protein and lipid-composition (Sanchez-Quesada, Benitez et al. 2002). The common treat of this pool of modified LDL particles is the increased negative charge, which permits its isolation from electropositive or native LDL (hereafter referred to LDL(+)) by anion exchange chromatography by fast-protein liquid chromatography (FPLC) (Sanchez-Quesada, Benitez et al. 2004) or by high-performance liquid chromatography (HPLC) (Vedie, Myara et al. 1991). It can also be isolated by capillary electrophoresis (Zhang, Kaneshi et al. 2005). The methodology used in isolation, determines the percentage of LDL(-) found in plasma, ranging from 1%-10% of total LDL in subjects with a normal lipid profile.

Noteworthy is the increased LDL(-) percentage in pathologies linked to a high cardiovascular risk, cases in which it can reach 20%. It includes hypercholesterolemia (Sanchez-Quesada, Otal-Entraigas et al. 1999), hypertriglyceridemia (Sanchez-Quesada, Benitez et al. 2002), type I diabetes mellitus (Sanchez-Quesada, Perez et al. 1996), type II diabetes mellitus (Moro, Zambon et al. 1998) insulin resistance (Zhang, Kaneshi et al. 2005), acute renal disease (Ziouzenkova, Asatryan et al. 2002), previous myocardial infarction (Chan, Ke et al. 2013) and coronary artery disease (Oliveira, Sevanian et al. 2006). Moreover, an increase on LDL(-) has also been detected in high-stressed

conditions such as intense long-duration aerobic exercise (Sanchez-Quesada, Homs-Serradesanferm et al. 1995).

The putative implications of LDL(-) in such many diseases related to atherosclerosis, leads to wonder whether it could be a useful biomarker of cardiovascular risk and considered for application in clinical practice (Sánchez-Quesada, Estruch et al. 2012, Ke, Stancel et al. 2014).

On the other hand, hypolipemiant or hypoglycemic drugs such as statines (Benitez, Ordonez-Llanos et al. 2004, Zhang, Matsunaga et al. 2009), ezetimibe (Park, Shima et al. 2011) or insulin (Sanchez-Quesada, Perez et al. 2001), have been shown to reduce this percentage in circulation. Moreover, studies with known atheroprotective compounds show to reduce LDL(-). It includes dietary products such as soy protein (Damasceno, Goto et al. 2000), selenium (Natella, Fidale et al. 2007), coffee (Natella, Nardini et al. 2007) or α-tocopherol (Mafra, Santos et al. 2009), but also physiological molecules such as estrogens (Lee, Chen et al. 2014).

4.2 PHYSICOCHEMICAL CHARACTERISTICS

The lipid and protein composition of LDL(-) differs extensively from LDL(+). LDL(-) contains increased levels of TG, FC (De Castellarnau, Sanchez-Quesada et al. 2000), NEFA, LPC and CER (Benitez, Camacho et al. 2004, Bancells, Villegas et al. 2010). NEFA, LPC and CER are products of phospholipolytic activities increased in LDL(-), such as PAF-AH and PLC-like activity (Benitez, Sanchez-Quesada et al. 2003, Bancells, Benitez et al. 2008), both nearly absent in LDL(+). Noteworthy is the presence of 5-fold higher PAF-AH, by means of total protein and activity, in LDL(-) than in LDL(+) (Benitez, Sanchez-Quesada et al. 2003). Moreover, LDL(-) contains nearly 600-fold increased PLC-like activity than LDL(+). This activity, hydrolyses the choline-containing polar head of major PLs in the LDL(-) surface, which are in order of substrate preference: LPC, SM and PC (Bancells, Benitez et al. 2008). The products of this hydrolysis are CER, MAG, DAG and P-chol. P-chol is water-soluble and preferentially leaves the particle, but the

other products are hydrophobic and remain retained. The higher proportion on phospholipolytic products has been associated to an impaired recognition by the LDLR (Benitez, Villegas et al. 2004), a higher susceptibility to aggregation and its inflammatory actions, as will be explained afterwards.

Apart from PAF-AH, LDL(-) possesses an increased content on other proteins such as apoA-I, apoA-II, apoE, apoC-III, apoD, apoF, apoJ and haemoglobin (Sanchez-Quesada, Benitez et al. 2004, Bancells, Canals et al. 2010), providing higher density to the particle. LDL(-) possess a higher density because of the increased protein than lipid content, which yields to a lower-sized particle. Moreover, LDL(-) is heterogeneous in density depending on its origin. In normolipemic subjects, LDL(-) particles are small and dense, whereas in FH patients there are bigger and lighter particles. Particularly, in hypertriglyceridemia there are two main LDL populations, one containing small and dense particles and another with big and light LDLs.

LDL(-) presents an altered apoB-100 conformation vs LDL(+). LDL(-) presents a partial loss of secondary structure in apoB-100 that results in a relatively higher content of β-structure and a lower content of α-helix than that in LDL(+) (Parasassi, Bittolo-Bon et al. 2001). Studies of Blanco *et al.* revealed different populations of exposed lysine residues in the apoB-100 protein of the LDL(-) and that of LDL(+) (Blanco, Villegas et al. 2010), which would explain the lesser recognition by the LDLR (Benitez, Villegas et al. 2004). Thereby avoiding its clearance and promoting the LDL(-) accumulation in circulation.

This different structure in apoB-100, as well as the lipid destabilization promoted by the phospholipolytic activitites acting on LDL(-) provides to LDL(-) an enhanced susceptibility to aggregation (Bancells, Benitez et al. 2008). In fact, it was notably to observe a spontaneous self-aggregation in LDL(-), already described by Avogaro *et al*. (Avogaro, Bon et al. 1988), but studied in-depth afterwards (Parasassi, De Spirito et al. 2008). Parassassi and collaborators found aggregation in LDL(-) present in plasma, since they noticed LDL(-) protein misfolding and increased NEFA and LPC content, which attributed to the $sPLA_2$ activity released by cells in the circulation (Greco, Balogh

et al. 2009). However, Bancells and coworkers reported that this behaviour was the consequence of the intrinsic PLC-like activity present in the particle (Bancells, Benitez et al. 2008), which also induces changes in LDL(-) in the apoB-100 structure and PL composition. These two LDL(-) aggregation theories could both coexist, either by the exogenous $sPLA_2$ present in plasma or by the intrinsic PLC-like activity. Nevertheless, the aggregation in LDL(-) in plasma induces an increased affinity and binding to PG on the arterial wall. Recently, the use of several antibodies against different apoB-100 residues has suggested that binding of fused LDL(-) to PGs is due to conformational changes in the N-terminal of the apoB-100, specifically between the residues 84 and 94 (Bancells, Benitez et al. 2011).

Moreover, a highly aggregated subfraction of LDL(-), also known as agLDL(-), has been recently described. AgLDL(-), compared to LDL(-), presented a higher affinity to proteoglycans (Bancells, Villegas et al. 2010). agLDL(-) showed increased CER, DAG, MAG and P-chol that concurred with a 6-fold increase on the PLC-like activity. Since an association of PLC-like activity with the induction of particle fusion was described (Oorni, Hakala et al. 1998), it is not surprising that agLDL(-) aggregates, fuses and gets trapped by PGs of the arterial wall (Bancells, Villegas et al. 2010).

Furthermore, LDL(-) may also act as a seeding factor, since its aggregation induces aggregation of other LDL particles (Bancells, Villegas et al. 2010). In this line, the heterogeneous physicochemical characteristics of LDL(-) and its interaction with components of the arterial wall yield to controversy regarding its formation.

4.3 POTENTIAL ORIGINS

The different methodologies used to LDL(-) isolation and the heterogeneity on its physicochemical characteristics of the particle yields to discrepancy regarding the origin of LDL(-). However, LDL(-) is such a heterogeneous modified LDL form that a combination of different mechanisms could occur in parallel. Four different origins of LDL(-) have been proposed and summarized below.

4.3.1 Oxidation

As stated above, there is controversy regarding the oxidized origin of LDL(-). Some groups point that LDL(-) may be produced by oxidation, since they can find an increased content on thiobarbituric acid reactive substances (TBARS), which is an oxidation marker, hydroperoxides and dienes and lower levels of antioxidants, such as tocopherol (Cazzolato, Avogaro et al. 1991, Bittolo-Bon, Cazzolato et al. 1994, Sevanian, Hwang et al. 1996). Moreover, the addition of copper in LDL(-) is described to greater oxidize LDL(-) than LDL(+) (Shimano, Yamada et al. 1991). These researchers sustain that these oxidized components would explain the majority of the atherogenic properties attributable to LDL(-).

However, other groups discard oxidation as the mechanism to originate LDL(-) (Chappey, Myara et al. 1995, Sanchez-Quesada, Camacho et al. 2003). They do not detect the presence of TBARS nor a decrease on antioxidants regarding LDL(+). Moreover, compared to LDL(+), they found even more resistance to copper-induced oxidation. The cause of this discrepancy could be the methodology used to isolate LDL(-). Methodological variables such as the concentration of ethylenediaminetetraacetic acid (EDTA) used could interfere the composition of LDL, since oxidized lipids are very instable molecules. Moreover, a more restrictive range of density used to isolate LDL would avoid the presence of Lp(a) and oxLDL (Sanchez-Quesada, Camacho et al. 2003), which is the main transporter of oxidized PLs in plasma. Finally, the fact that oxLDL is around 0,1% of total LDL in plasma (Nishi, Itabe et al. 2002) and that LDL(-) in blood ranges 1%-10% (Sanchez-Quesada, Benitez et al. 2004), also suggests that oxLDL would be a mere component of the heterogeneously pooled LDL(-).

4.3.2 PLA_2 and PAF-AH

PLA_2 hydrolyses PL of LDL yielding LPC and NEFA, both playing a role as pro-inflammatory molecules (MacPhee, Moores et al. 1999). These components have been found increased in LDL(-) (Benitez, Camacho et al. 2004) and are known to contribute to the inflammatory action of LDL(-). Moreover, PLA_2 modifies the conformation of

apoB-100 promoting in LDL a lower recognition by LDLR (Benitez, Sanchez-Quesada et al. 2003, Asatryan, Hamilton et al. 2005, Bancells, Benitez et al. 2008). The apoB-100 misfolding because of PLA_2 activity in LDL(-) has also been suggested to lead to particle aggregation (Brunelli, De Spirito et al. 2014).

Cells present in the atheromatous lesion such as SMC, monocytes and macrophages release $sPLA_2$ not only in the arterial wall but also in circulation, especially in inflammatory situations (Nevalainen 1993, Hurt-Camejo, Camejo et al. 2001). $sPLA_2$ could modify LDL in circulation yielding LDL(-). In a similar way, the PLA_2 associated to lipoproteins, known as Lp-PLA_2 or PAF-AH, could also produce this kind of modifications on LDL. PAF-AH degrades the highly pro-inflammatory and oxidized PAF, as well as other products containing fragmented PLs in the sn-2 position (PAF-like) (Hurt-Camejo, Camejo et al. 2001). The PAF-AH activity is controversial, because the hydrolysis of PAF-like is considered to be an anti-inflammatory mechanism, but it also yields to the formation of LPC and NEFA, which are inflammatory entities (Suriyaphol, Fenske et al. 2002, Sonoki, Iwase et al. 2003, Benitez, Camacho et al. 2004).

It has been hypothesized that PAF-AH could be involved in the formation of LDL(-) in circulation (Figure 11). In fact, the increased inflammation partly attributed to high PAF-AH activity in diabetic patients has been hypothesized as a putative mechanism of LDL(-) formation in this pathology (Sanchez-Quesada, Benitez et al. 2005). As explained, PAF-AH can degrade oxidized PAF-like lipids in mmLDL thereby increasing LPC and NEFA content in LDL, generating LDL(-) and avoiding the generation of oxLDL (Sanchez-Quesada, Benitez et al. 2004) (Figure 9). This suggestion concurs with the lesser inflammatory action of LDL(-) compared to oxLDL, since the most inflammatory product PAF is degraded in LDL(-) (Benitez, Sanchez-Quesada et al. 2003).

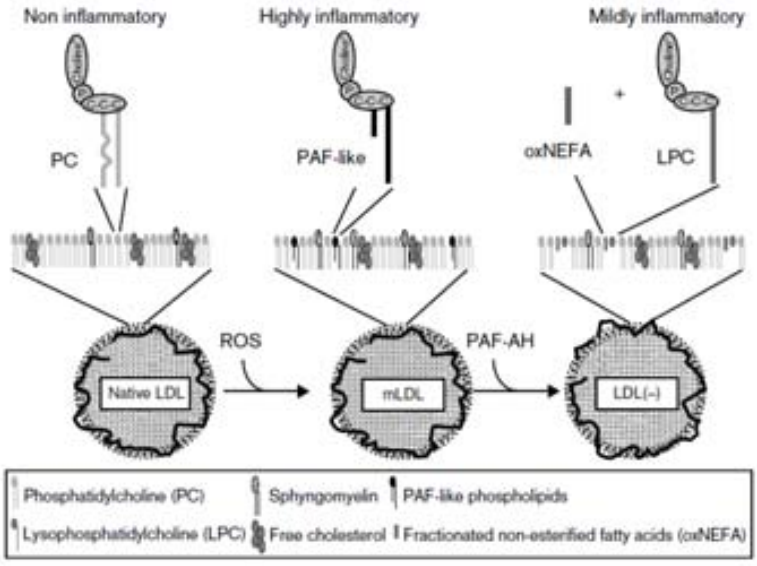


Figure 11. Hypothesis of the degradation of mmLDL for the origin of LDL(-) (Sanchez-Quesada, Benitez et al. 2004).

4.3.3 SMase

SMase induces changes in the composition and structure as well as in the aggregability of LDL (Oorni, Hakala et al. 1998). The degradation of SM by this activity increases the content of CER, which in turn can be degraded yielding NEFA, both components increased in LDL(-). It is known that CER, retained in the LDL surface after its production by the SMase activity, can form CER-enriched hydrophobic domains promoting massive aggregation of LDL particles (Bancells, Benitez et al. 2008, Sanchez-Quesada, Villegas et al. 2012), which is also a property of LDL(-). Moreover, the treatment with SMase yields to LDL an increased affinity to aggregation and proteoglycan binding (Oorni, Posio et al. 2005), which are in turn properties of LDL(-) (Bancells, Benitez et al. 2011).

Therefore, SMase could be in part the origin of LDL(-). The secretory form of SMase released by macrophages and endothelial cells (Schissel, Tweedie-Hardman et al. 1996) or/and the SMase activity present in LDL (Hakala, Oorni et al. 1999), are putative

sources of such activity. This activity is increased in LDL(-) although this action has not yet been attributed to any protein, so the origin of this increase is still unknown (Sanchez-Quesada, Villegas et al. 2012).

4.3.4 Other LDL modifications leading to an increased negative charge

Situations of imbalanced metabolism would yield to modifications in LDL that would increase its negativity, thereby yielding to LDL(-) in blood (Mello, da Silva et al. 2011). Chronic inflammation is accompanied by increased NEFA levels (Nevalainen 1993), as well as in diabetic patients, where there is an increase of NEFA together with TG (Lam, Carpentier et al. 2003). These compounds are increased in LDL(-) and renders a higher electronegativity to the particle. Alterations on lipid metabolism yield to changes in the content of apolipoproteins; in this regard, LDL(-) has an increased apoC-III and apoE content that could be the cause of the atherogenic properties of LDL(-) (De Castellarnau, Sanchez-Quesada et al. 2000). Other mechanisms that increase electronegative charge in LDL and that could generate the particle are: carbamylation (Apostolov, Shah et al. 2007), enrichment with haemoglobin, such as in patients in haemodialysis (Ziouzenkova, Asatryan et al. 1999), glycosylation (Younis, Sharma et al. 2008) and other modifications such as the exerted by methylglyoxal (Rabbani, Godfrey et al. 2011) in diabetic patients.

In summary, LDL(-) results to be a pool of several plasmatic modifications on LDLs. These mechanisms involved in the generation of LDL(-) would have a greater or lesser role depending on the factors of each individual and the presence of an underlying pathology (Mello, da Silva et al. 2011). LDL(-) could also be formed in the subendothelial space and back to plasma circulation. LDL, mainly in aggregates, would be trapped with PG and further modified, such as by enzymatic modification or oxidation, yielding to LDL(-). At that point, it has been postulated that an enzymatic activity such as myeloperoxidase could provoke the release of modified LDLs, such as LDL(-), from PG and return them to the blood (Pentikainen, Oorni et al. 2001).

4.4 BIOLOGICAL EFFECTS OF LDL(-)

The combination of the physicochemical properties described make LDL(-) be a proatherogenic molecule and possess several biological effects that will be detailed in next section.

4.4.1 Retention by proteoglycans

On the hypothesis of response-to-retention, Williams and Tabas (Williams and Tabas 1995, Williams and Tabas 1998) declared that lipoprotein retention is a key factor on the atheroma formation. To highlight is the presence of modified LDL in the intima (Steinberg, Parthasarathy et al. 1989, Pentikainen, Oorni et al. 2000).

Since LDL(-) presents impaired affinity to LDLR, there is a decreased clearance of LDL(-) from blood to tissues (Benitez, Villegas et al. 2004). This decreased clearance has important consequences: first, LDL(-) increases its time in circulation thereby rising the probabilities to become further modified. Second, since LDL(-) presents changes in lipid composition as well as in the apoB-100 structure, it aggregates and increases its affinity to PG present in the arterial wall. Third, the accumulation of LDL(-) in circulation strengthens its LDLR-independent infiltration rate through the endothelium and LDL(-) particles can enter the intima. Once in the intima, several proteases released by cells in the atherosclerotic plaque induce aggregation in LDL(-). In a similar manner than in circulation, LDL(-) then binds to PG present in the intima, thereby being retained (Bancells, Benitez et al. 2009). Since the aggregation of LDL(-) can induce aggregation of other LDL, not only LDL(-) but also native LDL (Parasassi, De Spirito et al. 2008, Bancells, Villegas et al. 2010), LDL(-) particles can fuse in the intima and increase its retention when binding to PG. At this site, LDL(-) can exert its effect such as cytotoxicity, apoptosis and inflammation on cells present in the atheromatous lesion (Figure 12) (Sanchez-Quesada, Villegas et al. 2012).

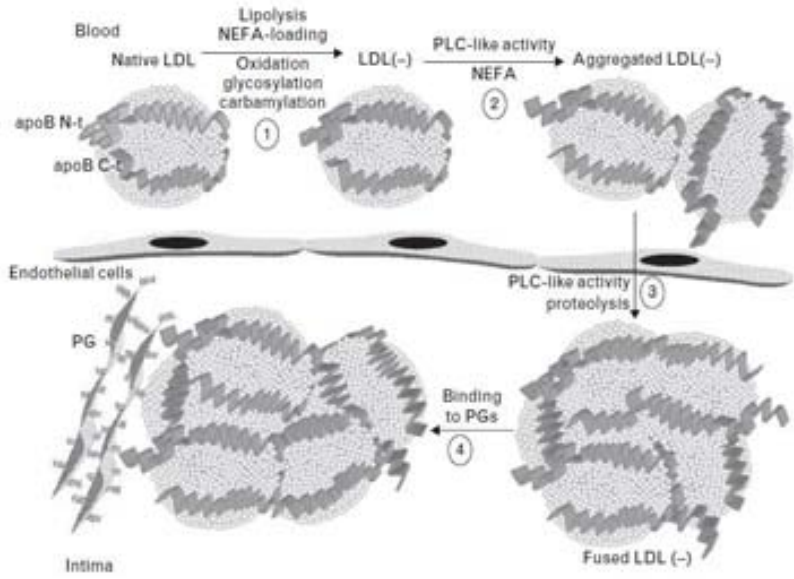


Figure 12. Hypothesis of the mechanism by which LDL(-) can get inside the intima and bind to PG (Sanchez-Quesada, Villegas et al. 2012).

4.4.2 Cytotoxicity and apoptosis

The exposure of cells to an agent that produces damage to them is what is known as cytotoxicity. The agreement to attribute a **cytotoxic effect** to LDL(-) has not been possible, maybe due to the heterogenicity of LDL(-) as a consequence of the methodology used. Some researchers found that the cause of cytotoxicity in cultured endothelial cells was induced by high levels of oxidized products in LDL(-) (Hodis, Kramsch et al. 1994, Sevanian, Hodis et al. 1995). However, for other investigators, the effects of LDL(-) should be unlike those of oxLDL, since they do not detect oxidation markers on LDL(-) compared to LDL(+). In this line, these researchers could not find a cytotoxic effect induced by LDL(-) in endothelial cells (Demuth, Myara et al. 1996, De Castellarnau, Sanchez-Quesada et al. 2000). Nevertheless, they detected some LDL(-)-induced morphological changes, such as gaps explained by endothelial cell detachment (Demuth, Myara et al. 1996). They hypothesized that only at elevated LDL(-) doses, an

increased production of atherogenic oxysterols could be toxic for the endothelium (De Castellarnau, Sanchez-Quesada et al. 2000).

In spite of the controversial cytotoxic effect, there is agreement on attributing to LDL(-) a **proapoptotic effect** on different cells involved in atherogenesis. Apoptosis is a controlled cell death that involves the activation of cellular pathways causing growth and dive failure and triggering cell death.

In mononuclear cells, LDL(-) has been reported to induce the expression of Fas protein (Bancells, Sánchez-Quesada et al. 2010) which, through the activation of its ligand, mediates stimulation of the apoptotic cascade (Itoh, Yonehara et al. 1991). LDL(-) is also known to induce, in endothelial cells, the release of IL-1β and TNF-α, both capable of potentiating the apoptosis of SMCs (Lee, Wang et al. 2012). Nevertheless, some compensatory mechanisms of the apoptosis induced by LDL(-) have also been described. LDL(-) induces the release of IL-10 (Benitez, Bancells et al. 2007), which has antiapoptotic effects (Halvorsen, Waehre et al. 2005), and the protective nuclear factor-erythroid 2-related factor-2 (NRF-2) in monocyte-derived macrophages (Pedrosa, Faine et al. 2010). However, the number of studies supporting a proapoptotic role for LDL(-) are higher than those suggesting the contrary.

The apoptotic pathway induced by LDL(-) in endothelial cells has been described. This signaling involves the downregulation of the fibroblast growth factor 2 (FGF-2), which is a potent and prosurvival anti-apoptotic protein, controlling the activation of caspase-3 (Lu, Jiang et al. 2008). LDL(-) induces this apoptosis in endothelial cells through LOX-1 (Lu, Yang et al. 2009). LDL(-) induces this effect in the endothelium of normolipemic subjects (Lu, Yang et al. 2009), patients of FH (Chen, Jiang et al. 2003, Tai, Kuo et al. 2006) and diabetes mellitus subjects (Yang, Chen et al. 2007, Lu, Jiang et al. 2008). Moreover, LDL(-) produces protein misfolding through FGF-2 (Lu, Yang et al. 2009) and avoids the regeneration of endothelium (Lu, Jiang et al. 2008). LDL(-) also induces apoptosis of vascular SMCs (Kataoka, Kume et al. 2001). The cardioprotective aspirin, that inhibits inflammation by suppressing the eicosanoid pathway has been

described to inhibit the LDL(-)-induced downregulation of FGF-2 (Chang, Chen et al. 2013).

A recent work of Chen *et al.* (Chen, Hsu et al. 2012) studies the effects of the LDL(-)-induced release of ROS on endothelial cells. They report that LDL(-) provokes mitochondrial dysfunction and thus destruction of endothelial cells through dysregulation of the endoplasmic reticulum antiapoptotic proteins. LDL(-) also induces senescence of SMCs (Tang, Lu et al. 2008).

However, some of the molecules of the proapoptotic downstream signalling are also involved in inflammation. Apart from oxidation, LDL(-) could also induce apoptosis through CER (Hannun and Obeid 1995), a bioactive lipid involved in inflammation (Pettus, Chalfant et al. 2004).

4.4.3 Inflammation

Inflammation is the mechanism by which the innate immune system responds through some strange molecules that it detects as pathogens. This is a rapid non-specific response that triggers the recruitment of cells and the production of chemical mediators such as cytokines and chemokines.

Circulating modified LDLs are known to play a role in inflammation (Kovanen and Pentikainen 2003) (Figure 13). In fact, patients of FH share a high proportion of LDL(-) and high plasma levels of MCP-1 and IL-8 and statin therapy decreases both LDL(-) (Sanchez-Quesada, Otal-Entraigas et al. 1999) and chemokine plasma concentration (Rezaie-Majd, Maca et al. 2002). In this line, one of the most important biological effects of LDL(-) is triggering an inflammatory response by means of cytokine induction on many cells involved in the atheromatous lesion. Below there is an explanation of the LDL(-)-induced inflammatory effects on endothelial and mononuclear cells.

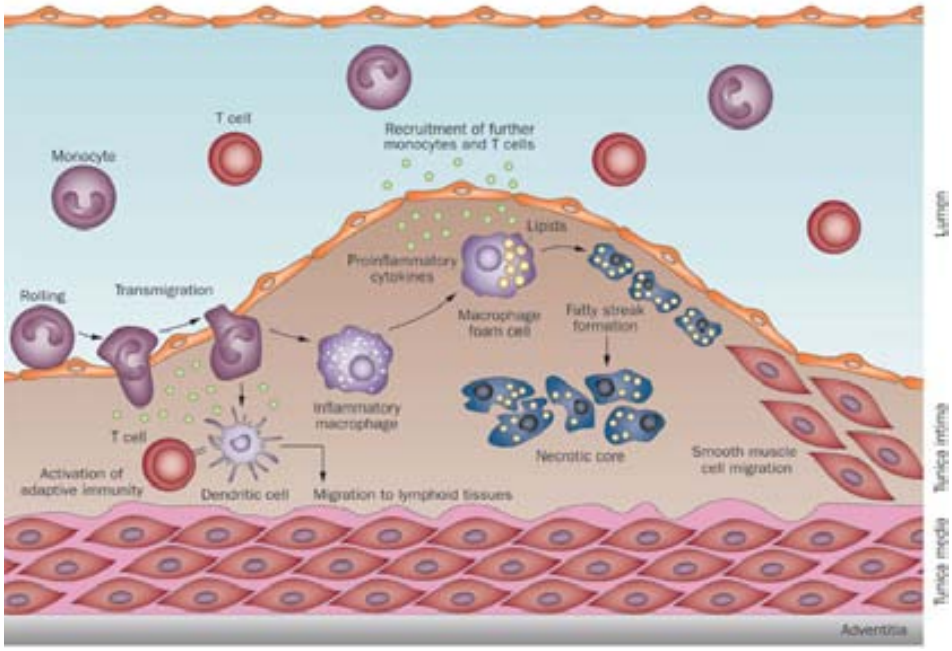


Figure 13. Summary of the immune responses activated by modified lipoproteins such as LDL(-) (Heine, Ortiz et al. 2012).

4.4.3.1 Endothelial cells

The endothelium plays a pivotal role in atherosclerosis. It is the barrier through which both molecules and cells can access the intima to form the atheromatous plaque. However, the endothelium also responds to inflammatory molecules. LDL(-) has been described to induce the expression of adhesion molecules and the release of cytokines and chemokines in endothelial cells.

In regard to the expression of adhesion molecules, LDL(-) is known to induce VCAM-1 in human venous endothelial cells (HUVEC) (Abe, Fornage et al. 2007). This molecule is involved in the capture and stable adhesion of monocytes to endothelium. In relation to the release of inflammatory mediators, De Castellarnau and colleagues (De Castellarnau, Sanchez-Quesada et al. 2000) were the first who described the release of IL-8 and MCP1 by LDL(-) isolated from healthy normolipemics, in HUVEC. Both IL-8 and MCP1 are chemokines known to mediate recruitment, extravasation and migration of mononuclear cells, specifically IL-8 for neutrophils and MCP-1 for monocytes (De

Castellarnau, Sanchez-Quesada et al. 2000). Moreover, studies in which these cells were treated with LDL(-) isolated from patients with FH (Sanchez-Quesada, Camacho et al. 2003) and type I and type II diabetes also showed release of both chemokines (Sanchez-Quesada, Benitez et al. 2005, Benitez, Perez et al. 2006). In these patients, the inflammatory effect of LDL(-) is similar to that of normolipemics. However, as their LDL(-) levels are increased, the cytokine release would be higher. Further studies on this cell-type described the induction of other chemokines such as the growth regulatory oncogene (GRO) subtypes α, β, γ, and the epithelial-derived neutrophil-activating peptide 78 (ENA-78). LDL(-) also activates IL-6, a first wave cytokine, and the granulocyte macrophage colony-stimulating factor (GM-CSF), which is involved in the maturation of monocytes to macrophages (Benitez, Camacho et al. 2006, Abe, Fornage et al. 2007). Furthermore, in bovine arterial endothelial cells, LDL(-) has been shown to enhance the expression of the vascular endothelial growth factor (VEGF) and decrease metalloproteinases (Tai, Kuo et al. 2006), thus demonstrating the role of LDL(-) in the control of vasculogenesis and angiogenesis.

LDL(-) also shows a pro-inflammatory action in human arterial endothelial cells (HUAEC) by the induction of firstly IL-6 and then IL-8, MCP-1, GRO, GM-CSF and platelet derived growth factor β (PDGF-β) (de Castellarnau, Bancells et al. 2007). The cytokine release induced by LDL(-) in endothelial cells would exert several effects in other cells, such as the described angiogenesis and chemotaxis. Moreover, a recent work of Lee et al (Lee, Wang et al. 2012) reports that LDL(-) induces the release of IL-1β and TNF-α in endothelial cells and this produces apoptosis in cardiomyocytes. Indeed, LDL(-) induces tissue factor and P-selectin in HUAEC in patients suffering from thrombosis. Thus, it activates the endothelium and induces platelet activation, aggregation and adhesion to endothelium, adscribing prothrombotic effects to LDL(-) (Chan, Ke et al. 2013).

4.4.3.2 Monocytes and lymphocytes

LDL(-) not only plays a role in endothelium activation, but also triggers the release of inflammatory mediators in cells of the immune system such as leukocytes, mainly

mononuclear cells, monocytes and lymphocytes. Monocytes circulate in the bloodstream for about one to three days and then move into tissues where they differentiate into macrophages or dendritic cells (See section 4.4.4). In circulation, monocytes are activated by microbial products and this leads to the production of inflammatory cytokines and chemokines (Swirski, Nahrendorf et al. 2009). Similarly, lymphocytes, including class B and T, are a big family of cells of the immune system that participate mainly in the adaptive but also in the innate immune response triggered in atherosclerosis (Chistiakov, Sobenin et al. 2013). This is particularly the case of some classes of T lymphocytes that possess the capability to induce cytokine release.

Since monocytes and lymphocytes are present in blood, as well as LDL(-), it is feasible that these cells and LDL(-) interact. The first studies of cytokine release induced by LDL(-) in mononuclear cells showed an increased production of MCP-1, GROβ, GROγ, IL-6, IL-8 and IL-10 in both monocytes and lymphocytes (Benitez, Bancells et al. 2007). In spite of IL-10, the other molecules are known to have a pro-inflammatory role, whose production is always higher in monocytes compared to lymphocytes. It is important to note that this pro-inflammatory cytokine release in mononuclear cells mimic the response of LDL(-) in endothelial cells (Benitez, Camacho et al. 2006). This result is biologically significant since the same stimulus, LDL(-), can act on different cell types and promote an amplified inflammatory response. However, the effects of LDL(-) are not exacerbated, since the release of the anti-inflammatory IL-10 in monocytes and lymphocytes would act as a control. In this study (Benitez, Bancells et al. 2007), the release of IL-10 in monocytes and lymphocytes produced a decrease of the pro-inflammatory cytokines levels induced in the same cells, thereby counteracting a putative exacerbated inflammatory response.

Regarding the inflammation of LDL(-) in monocytes, noteworthy is the study of Bancells *et al.* (Bancells, Sanchez-Quesada et al. 2010), in which HDL is reported to counteract inflammation induced by LDL(-). In these experiments, the incubation of LDL(-) with HDL decreases the cytokine release, the PLC-like activity, the NEFA content as well as the susceptibility to aggregation of LDL(-). These attenuated characteristics

also provoke a decrease on the electronegative charge of the particle. Moreover, this study correlates the cytokine release induced by LDL(-) with the NEFA content of this particle, which will be discussed in Section 4.5.1.

LDL(-) not only produced the cytokine induction at protein but also at RNA level in mononuclear cells (Benitez, Bancells et al. 2007) and in endothelial cells (De Castellarnau, Sanchez-Quesada et al. 2000). This fact implies that LDL(-) has the ability to modulate gene expression, as it was demonstrated further on. A microarray study showed differences in gene expression between LDL(-) and LDL(+). Fas ligand expression was not increased in LDL(-), but a high Fas level merely would lead to activation of this pathway (Bancells 2010). Fas is not only a proapoptotic molecule, but its levels also rise in inflammatory states such as atherosclerosis (Blanco-Colio, Martin-Ventura et al. 2007). It concurs with other studies in that Fas induces cytokine release in monocytes and monocyte-derived macrophages (Park, Thomsen et al. 2003).

However, LDL(-) downregulates colony stimulating factor 1 receptor (CSF1R) and CD36 (Bancells, Sanchez-Quesada et al. 2010) suggesting that it can act as a macrophage differentiation inhibitor. By the transcription inhibition of the monocyte receptors CSF1R and CD36, LDL(-) could inhibit the CSF-1-mediated activation of macrophages (Sester, Trieu et al. 2005) and the CD36-induced foam cell formation (Febbraio, Hajjar et al. 2001). This pattern concurs with the pro-inflammatory actions of LDL(-), since monocytes are stronger cytokine release inducers compared to macrophages.

LDL(-) would therefore play a role in the early events of atherosclerosis, since it induces the secretion of chemokines for mononuclear cell attraction, as well as pro- and anti-inflammatory cytokines to signal a controlled inflammatory state. However, LDL(-) not only activates the innate immune, but also the adaptive immune response, which is explained below.

4.4.4 Activation of the adaptive immune response

LDL(-) has also been described to activate a long-term adaptive immune response, as is the production of antibodies and immune complexes (Santo Faulin Tdo, de Sena et al.

2008). As stated above, some subtypes of T lymphocytes produce a fast inflammatory cytokine release by the LDL(-) effect, but LDL(-) also activates the humoral response of B lymphocytes. B cells, after antigen presentation by macrophages or dendritic cells, produce antibodies specific to neutralize the effect of exogenous entities such as pathogen components or even parts of endogenous strange molecules like modified LDLs. By this manner, B cells are specialized antibody producers capable to "remember" these molecule portions and mount a strong and rapid response if they recognize them again.

In this regard, autoantibodies and immunocomplexes against LDL(-) have been detected in blood (Faulin Tdo, de Sena-Evangelista et al. 2012) and their levels are increased in diabetes (Apolinario, Ferderbar et al. 2006) as well as in acute coronary syndrome (Oliveira, Sevanian et al. 2006). It concurs with results suggesting that LDL(-) could be a biomarker of inflammation and a predictor of cardiovascular risk (Lobo, Mafra et al. 2011). In the recent years, enzyme-linked immunosorbent assay (ELISA) kits have been developed in order to rapid detect immunocomplexes of antibodies against LDL(-) (Faulin Tdo, de Sena-Evangelista et al. 2012), although its introduction in clinical practices is still controversial and more basic research on the LDL(-) effects in atherosclerosis should be performed.

In the next section, there is a review of the principal cellular mechanisms of signal transduction known to be activated by LDL(-).

4.5 CELL MECHANISMS ACTIVATED BY LDL(-)

LDL(-) can exert many biological effects on both endothelial and mononuclear cells. However, the components of LDL(-) that are in charge of exerting such effects, the cells receptors involved and the intracellular pathways activated by this LDL subfraction still need further studies. In this section there is a revision of the information known to date in this regard.

4.5.1 Inflammatory components

The investigators who suggested that the origin of the cytotoxicity induced by LDL(-) were their oxidative components contained on it, also sustained that this oxidation was the cause of the inflammation promoted by this LDL subfraction (Hodis, Kramsch et al. 1994, Demuth, Myara et al. 1996). Alternatively, as described, other groups do not find oxidation in LDL(-) and point other causes of inflammation (Sanchez-Quesada, Camacho et al. 2003, Benitez, Perez et al. 2007).

Benitez *et al.* (Benitez, Camacho et al. 2004) described that the non-oxidative products NEFA and LPC, derived from the phospholipolytic activities contained in LDL(-), are involved in the cytokine release induced in endothelial cells. Both NEFA and LPC are the products of the PAF-AH activity in LDL(-) and are two-fold higher than in LDL(+). In this study, the modification of LDL(+) with $sPLA_2$ and the enrichment of LDL(+) with NEFA, produced a concentration-dependent increase on the IL-8 and MCP-1 release (Benitez, Camacho et al. 2004). However, this inflammation exerted had a lesser level, than of LDL(-). Besides, in monocytes, NEFA are also involved in IL-6, IL-8, IL-10 and MCP-1 release (Bancells, Sanchez-Quesada et al. 2010). Noteworthy is the fact that incubating LDL(-) with the antiatherogenic HDL produces a decrease in NEFA in LDL(-) and an increase in HDL, and this content correlates with the capability, at least in part, to induce cytokine release. These results show that HDL can counteract the inflammatory effect of LDL(-), and that it is not caused by oxidation, since peroxide content in LDL(-) did not change after incubation with HDL.

Moreover, the incubation of LDL(-) with both HDL or apoA-I produced a fall of the PLC-like activity in LDL(-) (Bancells, Sanchez-Quesada et al. 2010). To date, no enzymatic protein has been described with such activity (Sanchez-Quesada, Villegas et al. 2012), thus a protein transfer would not be feasible and the products resulting from this activity are potential candidates to be transferred from LDL(-) to HDL. PLC-like activity in LDL(-) degrades with more efficiency LPC than other PLs, but this PL is really scarce on LDL (Bancells, Benitez et al. 2008). Although this activity degrades with lower

efficiency SM and PC, its higher content in LDL produce an increased amount of CER and DAG derived from this activity in LDL(-).

The content on CER has been described to increase in the cell membrane of macrophages by the stimulation with oxLDL and acLDL (Kinscherf, Claus et al. 1997, Grandl, Bared et al. 2006). Then, CER-lipid rafts form, which represents a dynamic association of signaling molecules and cell receptors that permit the activation of intracellular pathways. Moreover, inflammatory states such as host acute phase response or LPS, course with an increase of CER synthesis in liver and further incorporation in LDL or VLDL (Lightle, Tosheva et al. 2003, Schenck, Carpinteiro et al. 2007). CER and specially its phosphorylated form are known to mediate intracellular pro-inflammatory pathways (Pettus, Chalfant et al. 2004, Chalfant and Spiegel 2005). Moreover, CER has been described to induce cell cycle arrest and apoptosis (Hannun and Obeid 1995). Sphingolipids such as CER are implicated in many physiopathological processes and become putative drug targets in inflammatory diseases (Arana, Gangoiti et al. 2010). Taken together, these data prompt to CER as a putative molecule mediating the effect of modified LDLs (Kinscherf, Claus et al. 1997) and probably that of LDL(-).

DAG can be originated by the degradation of members of the eicosanoid family of all cells of the vascular wall. It has been described that the permeability of endothelium to lipids and cells, leads to an inflammatory state including cell proliferation, increased cytokines, growth factors and eicosanoid release by activated endothelium (Hajjar and Pomerantz 1992). For this reason, DAG could also contribute to the cytokine induction of LDL(-).

4.5.2 Cell receptors

The first investigations trying to figure out the receptors that could bind and mediate the inflammatory response of LDL(-) where the known receptors for native or other modified LDLs.

The role of LDLR in binding LDL(-) has been controversial. On the one hand, LDLR was discarded to bind LDL(-) since the early beginning, by Avogaro and collaborators (Avogaro, Bon et al. 1988), because of the physicochemical characteristics of LDL(-). The impaired binding of LDL(-) for LDLR was further corroborated by Benitez *et al.* (Benitez, Villegas et al. 2004), who showed an important decrease on affinity of LDL(-) by LDLR compared to LDL(+). In this study, LDL(-) from normolipemic subjects presented 3-fold lower affinity to LDLR compared to LDL(+) whereas LDL(-) from hypercholesterolemic patients had only 1,5-fold lower affinity than that of LDL(+).

The decreased binding to LDLR can be originated by several causes: firstly, the increased NEFA content in LDL(-) would negatively charge the apoB-100 binding site to LDLR, thereby avoiding the interaction with cysteine residues in LDLR (Benitez, Camacho et al. 2004). Secondly, the higher aggregation level of LDL(-) due to its PLC-like activity (Bancells, Villegas et al. 2010), which is involved in the abnormal conformation of apoB-100 in LDL(-) (Bancells, Benitez et al. 2011) might impair its binding to LDLR. The loss of affinity of LDL(-) for LDLR could trigger a lower LDL clearance rate, thereby increasing the time of LDL(-) in circulation and enhancing its probability to get modified. On the other hand, other authors suggests that the presence of increased apoE in LDL(-) could be the cause of a similar affinity of LDL(-) and LDL(+) to LDLR (Shimano, Yamada et al. 1991, Demuth, Myara et al. 1996).

SR was considered a putative receptor for LDL(-), since they recognize other modified LDLs with high electronegativity such as oxLDL and acLDL (Kodama, Reddy et al. 1988). There is also divergence on the results probably due to the method of isolation, since some groups do not detect any different uptake of LDL(+) or LDL(-) for the type A SR (Cazzolato, Avogaro et al. 1991, Benitez, Villegas et al. 2004). However, other investigators sustained that LDL(-) could be internalized by SRs (Holvoet, Perez et al. 1995, Tertov, Bittolo-Bon et al. 1995). In fact, a specific SR highly expressed in endothelial cells, LOX-1, known to internalise oxLDL, has been described to bind LDL(-), which can thereby exert its biological effects (Tang, Lu et al. 2008, Lu, Yang et al. 2009). Worth noting, LDL(-) can induce platelet activation and aggregation through LOX-1 and PAFR (Chan, Ke et al. 2013). LOX-1 and PAFR were proposed to mediate the LDL(-)

effects in monocytes (Chen, Jiang et al. 2003). However, it was further discarded since LOX-1 is poorly expressed in monocytes (Moheimani, Tan et al. 2011) and there is an small amount of PAF found in LDL(-) due to PAF-AH activity (Benitez, Sanchez-Quesada et al. 2003).

To date, no receptor has been described to bind LDL(-) in monocytes. However, the similarities of LDL(-) with mmLDL may address the investigations to figure out whether LDL(-) could bind to the same receptors of mmLDL. In monocytes and macrophages, not only mmLDL but also oxLDL have been described to activate the CD14-TLR4 system (Chavez-Sanchez, Chavez-Rueda et al. 2010, Chavez-Sanchez, Garza-Reyes et al. 2014), as explained in section 3.4.1. More investigations are needed to ascertain the role of these receptors in mediating LDL(-) inflammatory effect.

4.5.3 Intracellular mechanisms activated

There is not much information about the signaling mechanisms activated by LDL(-) and, the pathways seem to be different regarding the cell type. In cardiomyocytes, LDL(-) has been suggested to activate the phosphatidyl-inositol-3 kinase (PI3K) and the nuclear factor-kB (NF-kB), thereby inducing apoptosis (Lee, Wang et al. 2012). However, opposite is the effect of LDL(-) in endothelial cells, where it inhibits PI3K-Akt pathway by enhancing the expression of LOX-1 (Lu, Jiang et al. 2008, Tang, Lu et al. 2008, Lu, Yang et al. 2009).

Regarding the transcription factors, it has been described that LDL(-) signals through the transcription factor activator protein 1 (AP1) and NF-kB to induce VCAM-1 expression in endothelial cells (Ziouzenkova, Asatryan et al. 2003, Sanchez-Quesada, Benitez et al. 2005). In monocytes, some data point to LDL(-) could also mediate its inflammatory effects by the activation of NF-kB and AP1. In fact, a gene expression study in monocytes and lymphocytes showed activation of NF-kB and inhibition of the peroxisome proliferator-activated receptor γ (PPARγ) (Bancells, Sanchez-Quesada et al. 2010).

Furthermore, some results describe the activation of the endothelial cell signal-regulated kinase (ERK) and AP1 by mmLDL (Choi, Wiesner et al. 2012), as well as the induction of ERK and p38 mitogen activated protein kinase by oxLDL (Su, Ao et al. 2011). Therefore, the similitudes of mmLDL and LDL(-) leads to also address these pathways in order to ascertain the intracellular mechanisms activated by LDL(-). Nevertheless, there is a need for further investigation in this regard.

Chapter II

BACKGROUND AND OBJECTIVES

LDL(-) is a minor modified LDL present in circulation with inflammatory properties such as the induction of cytokine and chemokine release in cells involved in atherosclerosis, including endothelial cells, macrophages, lymphocytes and monocytes.

In endothelial cells, it has been shown that LPC and NEFA, are the responsible agents of the cytokine release induced by LDL(-). In these cells, the receptor accepted to be involved in this action is LOX-1.

However, there is lacking information about the inflammatory components of LDL(-) and the cellular mechanisms leading to cytokine release in mononuclear cells. Regarding the inflammatory components of LDL(-), it has been reported that NEFA participate in LDL(-)-induced cytokine release in monocytes. However, it has been suggested that other products, such as the derived from the PLC-like activity ascribed to LDL(-), could also be involved. Since the products CER and DAG, are increased in LDL(-) and are known proinflammatory agents, their involvement in the induction of cytokines promoted by LDL(-) is feasible.

The other subject to be elucidated is the putative receptors responsible of mediating the inflammatory signaling by means of cytokine release induced by LDL(-) on monocytes. Since these cells are poor in LOX-1, this receptor would not be a candidate. However, LDL(-) shares some characteristics with mmLDL and even with oxLDL. Both oxidatively modified LDLs have been recently reported to induce cytokine release through TLR2, TLR4 and CD14. These receptors are highly expressed in monocytes and are known to signal the LPS inflammatory response, which is commonly used as a positive control of cytokine release in these cells.

In this regard, with the current knowledge of the physicochemical characteristics and the inflammatory behaviour of LDL(-) compared to LDL(+), the main aims of this thesis were the following:

1. Get inside on the components of LDL(-) responsible of its inflammatory effects on monocytes. Specifically, to evaluate the PLC-like activity products such as CER and DAG on the cytokine release induced by this modified LDL.

2. To study whether the receptors TLR2, TLR4 and CD14 are mediating the cytokine release induced by LDL(-) in monocytes and test the preference on binding of these receptors for LDL(-).

3. Deepen on the relationship between the inflammatory components found in LDL(-) and their interaction with the receptors mediating LDL(-)-induced cytokine release in monocytes.

Specifically and in this order, each of these objectives has been treated in the publications of this thesis.

Chapter III

MATERIALS AND METHODS

The methods used in this thesis are summarised in Table 2 and detailed afterwards.

METHOD	P 1	P 2	P 3
1. Lipoprotein isolation and separation of LDL subfractions	X	X	X
2. Characterisation of LDLs			
2.1 Apoprotein and lipid composition	X	X	
2.2 Minor lipid content evaluation by TLC	X		
2.2.1 Lipid extraction			
2.3 Electrophoretic characterisation	X	X	
2.4 Oxidation test	X		
2.5 Aggregation test	X		
3. Enzymatic activity tests			
3.1 PLC-like activity	X		
3.2 PAF-AH activity	X		
4. Modification of LDL *in vitro*			
4.1 LDL induced oxidation		X	
4.2 LDL enrichment in CER or other minor lipids	X		X
4.3 LDL modification with PLC and PLA_2	X		
4.4 LDL induced aggregation	X		
5. LDL incubation with HDL	X		
6. LPS neutralisation		X	
7. Culture of primary monocytes	X	X	X
8. THP1-XBlue and THP1-XBlue-MD2-CD14 cell culture		X	X
9. Cell viability assessment			
9.1 Cytotoxicity XTT Assay	X	X	
9.2 Lactate Dehydrogenase Assay			X
10. Incubation of cells with Stimuli	X	X	X
11. Incubation conditions of cells for cytokine release			
11.1 LPS inhibition		X	
11.2 Receptor neutralisation		X	
11.3 Incubation with soluble CD14 (sCD14)		X	
11.4 Inhibition of TLR pathways in monocytes, THP1 and THP1-CD14 cells		X	
11.5 CD14 and TLR4 gene silencing		X	
11.5.1 siRNA transfection		X	
11.5.2 Gene knockdown testing		X	
11.5.2.1 RNA extraction and qRT-PCR			
11.5.2.2 Protein extraction and WB analysis		X	
12. Induction of NF-kB/AP-1 and cytokine release in THP1 cells		X	
13. Cytokine Release evaluation by ELISA	X	X	X
14. Binding assays			
14.1 Binding to human monocytes			

14.1.1 Total binding		X	
14.1.2 Binding Displacement Studies		X	
14.2 Binding to CD14-coated microtiter wells		X	
15. Statistical analysis	X	X	X

Table 2: Methodology used in each of the publications presented. P: publication.

1. LIPOPROTEIN ISOLATION AND SEPARATION OF LDL SUBFRACTIONS

Plasma in EDTA-2K containing *Vacutainer* tubes were obtained from a pool of healthy normolipemic donors who gave their written informed consent. Only samples with a total cholesterol <5.2 mmol/L and triglycerides <1 mmol/L were accepted, pooled and frozen. Lipoproteins were isolated by sequential flotation ultracentrifugation for 20h at 36,000 rpm 4ºC (Havel, Eder et al. 1955) in accordance with their density as in Table 3.

Lipoprotein	Density
Quilomicrons	< 0.96 Kg/L
VLDL	0.96-1.006 Kg/L
IDL	1.006-1.019 Kg/L
LDL	1.019-1.063 Kg/L
Lp(a)	1.050-1.100 Kg/L
HDL	1.063-1.210 Kg/L

Table 3: Lipoprotein density distribution.

Assuming that plasma density is 1.006 kg/L, the proper density to isolate each fraction was reached by adding KBr addition, following the equation of Radding and Steinberg (Radding and Steinberg 1960):

$$KBr\ (g) = \frac{Volum\ (ml) * (final\ density - initial\ density)}{1 - (0.312 * final\ density)}$$

When isolating LDL, the first isolation step at a density of 1.019 Kg/L was performed to discard VLDL and IDL. LDL was then isolated at 1.050 Kg/L to avoid sample contamination with Lp(a). When isolating HDL, the first step with 1.100 Kg/L density solution was performed to remove Lp(a) and oxidized LDL. HDL was then obtained at 1.210 kg/L.

A proper density solution was gently overloaded to plasma at a ratio of density solution to plasma volumes of 1:4 in order to optimise isolation. They were prepared by adding KBr to a basal 1.006 kg/L density solution (See the equation of Radding and Steinberg).

- 1.006 Kg/L Density Solution: 0.15 mM chloramphenicol, 0.15 M NaCl, 1 mM EDTA, 0.08g gentamycin.

Isolated LDL was dialysed against Buffer A to eliminate KBr and filtered in 0.45 µM filter to avoid aggregates. The total LDL was subfractioned depending on its electric charge in LDL(+) and LDL(-) by preparative anion exchange chromatography using a HiLoad 26/10 Q-Sepharose High Performance Column with 53 mL of volume (Amersham Biosciences) (Sanchez-Quesada, Camacho et al. 2003). The method used was a step salt gradient (Table 4) using A Buffer as a binding buffer and B Buffer as an elution buffer.

- A Buffer: Tris-HCl 10 mM, EDTA 1 mM, pH 7.4
- B Buffer: Tris-HCl 10 mM, EDTA 1 mM, NaCl 1M, pH 7.4

Buffer volumes	B Buffer (%)
0 - 108 mL (2 column volumes)	0 %
108 - 161 mL (1 column volume)	0 - 10 %
161 - 267 mL (2 column volumes)	24.5 %
267 - 373 mL (2 column volumes)	60 %
373 – 479 mL (2 column volumes)	100 %
479 – 506 mL (0.5 column volume)	0 %

Table 4. Step salt gradient for the isolation of LDL(+) and LDL(-) by the FPLC methodology. B buffer is the salted buffer (see composition above).

LDL(+) eluted at 0.25M NaCl (26% B Buffer) and LDL(−) eluted at 0.6M NaCl (60% B Buffer). This procedure was made in an ÄKTA-Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare). Chromatograms were performed by monitoring absorbance at 280 nm (Figure 14).

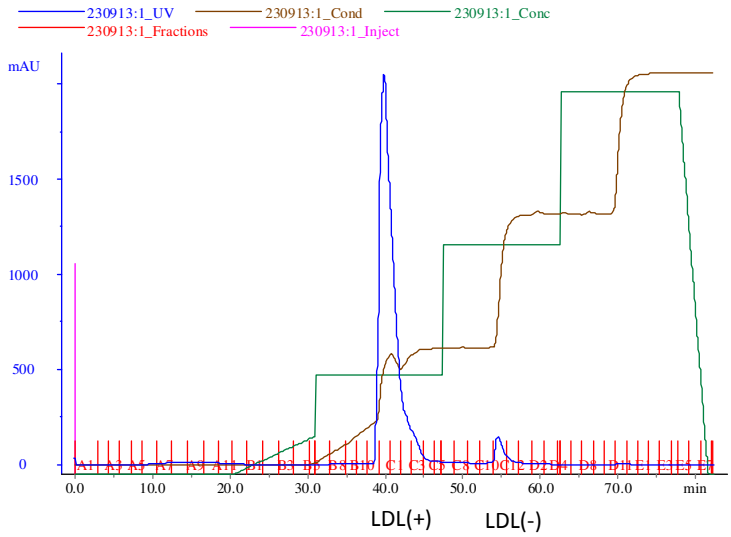


Figure 14. Chromatograph of the LDL(+) and LDL(-) isolation by anion exchange chromatography.

The percentage of LDL(-) was calculated by integration of chromatographic curves. LDL(+) and LDL(-) compositions were then analysed as explained in the following section.

2. CHARACTERISATION OF LDLS

Prior to studying the effect of the different LDL subfractions, as well as modified LDL, samples were characterised with the proper following tests.

2.1 APOPROTEIN AND LIPID COMPOSITION OF LDLS

The composition in TC, TG, apoB or apoA (Roche Diagnostics), phospholipid and NEFA (Wako chemicals) content were systematically tested in a Hitachi 509 autoanalyser.

An enzyme-colorimetric assay is used for the characterisation of TC, TG, PL and NEFA. In all cases, the reactions form H_2O_2, which is the substrate of peroxidase.

Total cholesterol

Cholesterol esters $\xrightarrow{\text{Cholesterol esterase}}$ free cholesterol + R-COOH

Free cholesterol $\xrightarrow[\text{oxidase}]{\text{Cholesterol}}$ H_2O_2 + cholestenone

Triglycerides

Triglyceride $\xrightarrow[\text{lipase}]{\text{Lipoprotein}}$ glycerol + fatty acids

Glycerol + ATP $\xrightarrow[\text{kinase}]{\text{Glycerol}}$ dihydroxyacetone phosphate + H_2O_2 (Glycerol-3P-oxidase)

Phospholipids

Phosphatidylcholine + H_2O $\xrightarrow{\text{PLD}}$ choline + phosphatidic acid

Choline + H2O+ O2 $\xrightarrow[\text{oxidase}]{\text{Choline}}$ H_2O_2 + betaïne

NEFA

NEFA + coenzim A + ATP $\xrightarrow{\text{Coenzim A}}$ acyl-CoA + AMP + PPi

acyl-CoA + O_2 $\xrightarrow[\text{reductasa}]{\text{AcilCoA}}$ H_2O_2

Peroxidase then u perform an oxidative condensation giving a product readable at 505 nm:

H_2O_2 + 4-aminoantipyrine/4-chlorophenol $\xrightarrow{\text{Peroxidase}}$ H_2O + dye

The determination of apoB and apoA was performed in the same autoanalyser by an immunoturbidimetric assay using specific antibodies anti apoB and apoA, which

produce insoluble aggregates that higher the turbidimetry of the sample, measurable at 700-340 nm.

The coefficient of variation (CV) on different days was under 5% in all tests. Results were expressed as the percentage of lipoprotein mass for all the components except from NEFA, expressed as NEFA/mol apoB.

2.2 MINOR LIPID CONTENT EVALUATION BY THIN LAYER CHROMATHOGRAPHY (TLC)

The various phospholipids in LDL samples, separated regarding their polarity, were quantified by thin layer chromatography (TLC) of lipid extracts. Lipid extraction was performed following the Bligh and Dyer method (Bligh and Dyer 1959) as follows.

2.2.1 Lipid extraction

Firstly, native LDL (0.5 mL at 1g apoB/L) was treated with 3-Bromo-4-hydroxitoluene (BHT) 20 µM to avoid oxidation and mixed with 1.88 mL chloroform/methanol (1:2), vortexed and incubated at room temperature (RT) for 30 minutes. Afterwards, 0.625 mL chloroform was added and vortexed, followed by the addition of 0.625 mL water. Samples were centrifuged at 2000 rpm for 10 minutes to separate the different phases. The upper chloroform phase contained the lipid extracts.

Lipid extracts were evaporated in a nitrogen stream and further reconstituted in 20 µL chloroform to be partly applied in 4-rail silica gel plates of 17 cm long (Whatman). The compounds used to enrich LDLs were also added as controls in different rails of the plate.

Three sequential mobile phases were used to develop the plates to make the different lipids run according to their capillarity. Plates were vertically placed in cuvettes where each mobile phase was changed as described.

Phase 1: chlorophorm/methanol/water (v/v/v 65:40:5) to 5 cm.
Phase 2: toluene/diethilether/ethanol (v/v/v 60:40:3) to 13 cm.
Phase 3: heptane to 17 cm.

Lipids were stained in ethanol containing 5% phosphomolibdic acid and 5% sulphuric acid and the plate was dried for 10 minutes at 100ºC and further scanned.

2.3 ELECTROPHORETIC CHARACTERISATION

Electrophoresis in agarose gels was performed to determine lipoprotein mobility. Commercial gels *Midigel lipo* (Biomidi) were used to differentiate lipoproteins because of its negative charge. In spite of the lipid and protein composition, this assay corroborated the difference between native LDL, LDL(-), modified LDLs, LDL incubated or not with HDL (or *vice versa*) and the correct isolation of HDL.

Samples (15 µL at 0.3g apoB or apoAI/L) were preincubated with 5 µL 50% sacarose and 5 µL 0.1% Negro Sudan in ethanol to lipid staining for 15 minutes. Samples were loaded into agarose gel and electrophoresis was run at 100V for 1h.

2.4 OXIDATION TEST

Oxidation was determined by measuring the peroxide content of lipoproteins following the method described by Auerbach (Auerbach, Kiely et al. 1992) using leucomethylenblue reagent (LMB) (TCI).

A standard curve was done with 0.1 to 20 µg 13-S-hydroperoxide (HPODE) (Biomol) in ethanol. For this purpose, 100 µL of HPODE was removed, dried down under nitrogen gas, and further resuspended with 16 µL 5X cholate/ethanol/PBS solution.

Samples (1,5 mL at 0.7 g/L) were submitted to lipid extraction by the method of Bligh and Dyer. LMB reagent was prepared by mixing 2 different solutions (Solution 2 was added to Solution 1). Finally, pH was adjusted to 5 and hemoglobine (5.5 mg) was added.

- Solution 1 LMB: 5 mg LMB + 8 mL N,N-dimetilformamide
- Solution 2 LMB: 80 mL H_2O + 1.4 mL X-100 triton, 0.68g KH_2PO_4

Lipid extracts from samples (40 µL) were incubated with the Cholate Solution (10 µL) and 100 µL LMB reagent for 30 minutes and absorbance was further read at 650 nm in a 96-well plate.

- Cholate Solution: 1% sodium cholate + 25% ethanol + 74 % PBS.

2.5 AGGREGATION TEST

Basal aggregation of samples was determined by testing turbidimetry in terms of absorbance at 450 nm (0.5 g/L apoB or ApoA-I).

The susceptibility to aggregation was evaluated in samples at 0.2 g/L that were submitted to aggregation by vortexing for increasing times up to 1 minute (0, 2.5, 5, 15, 20, 30, 40, 50 and 60 seconds). Absorbance was then read at 450 nm.

3. ENZYMATIC ACTIVITY TESTS

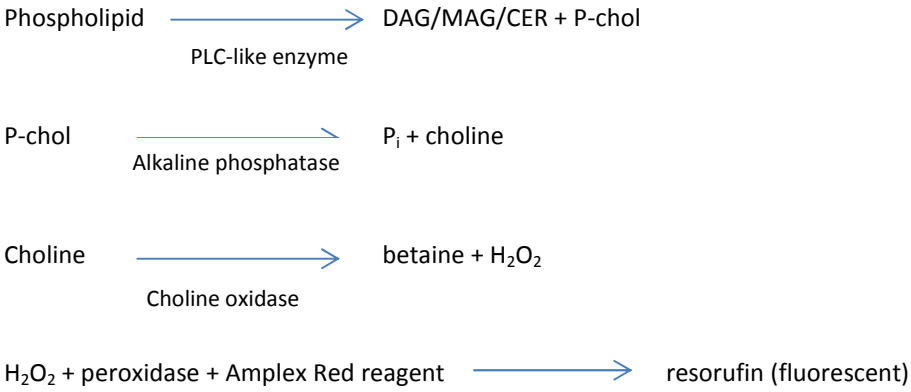
In this study, the PLC-like and the PAF-AH enzyme activity were determined in some LDL and HDL samples.

3.1 PLC-LIKE ACTIVITY

PLC-like activity was determined by two different methods: Amplex Red and Fluorescent TLC.

3.1.1 PLC-like activity by Amplex Red

PLC-like activity in LDLs was measured by the commercial fluorimetric method Amplex Red (Molecular Probes). This method detects PLC-like or SMase activities present in samples, which degrade LPC, SM or PC as substrates. If samples possess these enzymatic activities, the phospholipid P-chol is generated as a product of the substrate degradation. P-chol can be degraded and further produce a fluorescent reagent by the following reactions:



LDL samples were firstly dialyzed against the following buffer.

- PLC-like Enzyme Buffer: 10 mM Tris, 2 mM $CaCl_2$, 10 mM $MgCl_2$, 140 mM NaCl, pH 7.4.

The procedure starts by the addition of 100 µL of LDL samples at 0.3 g/L into black specific 96-well plates (Greiner Bio-One). Working reaction had been recently prepared by adding all the components. The mixture was added into wells at a final volume of 100 µL. A standard curve was made with PLC-like activity from *Staphilococcus sp*. The composition of the working solution is described in Table 5.

Final concentration per sample (200 µl final vol)	**Volume and concentration to add**
0.5 mM substrate (SM/PC/LPC) in 2% triton X-100	10 µL at 5mM
8 U/mL alkaline Phosphatase	2 µL at 400 U/mL
0.2 U/mL choline oxidase	1 µL at 20 U/mL
2 U/ml peroxidase (HRP)	1 µL at 200 U/mL
0.1 mM Amplex Red in DMSO	1 µL at 10 mM
Reaction Buffer (85 mM Tris-HCl, 8.5 mM $MgCl_2$, pH 7.4)	85 µL (100 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4)

Table 5. Composition of the Working Solution in PLC-like activity method.

The assay is a kinetic reaction in which we assay the detection of fluorescence (530 nm excitation and 590 nm emission) for 3h at 37ºC. PLC-like activity was calculated from each sample interpolating the results of the reference line of *Staphilococcus sp.*

3.1.2 Fluorescent TLC

The degradation of SM, one of the substrates of PLC-like activity was also measured by a more specific method by incubating samples with SM labelled with the fluorescent probe boron-dipyrromethene (bodipy, BDP) as described (Holopainen, Medina et al. 2000). Briefly, LDLs and HDLs (0.3 g/L apoB or apoA-I) were incubated with Bodipy-FL-C12-SM (Molecular Probes) at 0.025 mM for 3h at 37ºC in the PLC-like enzyme buffer (See *Enzymatic Activity tests*).

Samples were then submitted to lipid extraction as described in *Minor lipid content evaluation by TLC*. Afterwards, samples were evaporated under nitrogen gas and further reconstituted in 40 µL chloroform to be applied in the 4-rail silica gel plates.

Lipids were separated in the mobile phase regarding its polarity. The composition of the lonely mobile phase used was Dichloroethane:Methanol:H2O (90:20:0.5), which was added in a cuvette to make samples run thanks to capillarity. The enriching SM-BDP and CER-BDP compounds were added as controls in the TLC plate to be run in parallel.

When mobile phase reached the top of the silica gel plate, samples are ready to fluorescent exposure in ChemiDoc 2000 (BioRad).

3.2 PAF-AH ACTIVITY

PAF-AH activity was measured by a commercial colorimetric assay using 2-tio-PAF as substrate (Cayman Europe). This substrate was evaporated under nitrogen gas and further resuspended in 12 mL PAF-AH assay buffer.

- PAF-AH Assay Buffer: 100 mM Tris-HCl, 1 mM EGTA, pH 7.2

96-well plates (Nunc) were used to proceed with the assay. LDL was added (10 µL at 0,25g apoB/L) in A buffer, followed by the addition of 5 µL PAF-AH Assay Buffer and 10 µL of 10 mM 5,5'-ditio-bis-(acid 2-nitrobenzoic) (DTNB) in DTNB buffer.

- DTNB Buffer: Tris-HCl 0.4 M pH 8,0

Absorbance was measured at 414 nm, each 30 seconds for 15 minutes. The slope of the standard curve was used to calculate the PAF-AH enzymatic activity.

4. MODIFICATION OF LDL *IN VITRO*

Four different LDL modifications were performed *in vitro*, in which compare the LDL(-) behaviour: oxidation, enrichment with PLC-like activity products (CER, DAG, SM), incubation with PLC and aggregation. The tests evaluated on these samples are summarised in Table 6.

LDL modifications	Tests evaluated
oxLDL	- Lipid and apoprotein composition - Binding displacement to human monocytes
CER-LDL, SM-LDL, DAG-LDL	- Lipid and apoprotein Composition - Minor lipid composition evaluation by TLC - Susceptibility to aggregation - IL-6, IL-10 and MCP-1 release in primary monocytes. - Cytokine release in THP1 and THP1-CD14 (CER-LDL)
PLC-LDL and PLA_2-LDL	- Lipid and apoprotein composition - Minor lipid composition evaluation by TLC (Control pattern) - IL-6, IL-10 and MCP-1 release in primary monocytes (PLC-LDL)
agLDL	- Lipid and apoprotein Composition - IL-6, IL-10 and MCP-1 release in primary monocytes

Table 6. List of tests evaluated on each modified LDL.

4.1 LDL INDUCED OXIDATION

LDL was firstly dialysed against phosphate saline buffer (PBS). Dialysis was performed until the amount of the chelating agent Ethylenediaminetetraacetic Acid (EDTA) in samples prepared to oxidation was less than 10 mM.

- PBS: 137 mM NaCl, 2,7 mM KCl, 6.5 mM Na_2HPO_4 ($2H_2O$), 1.5 mM KH2PO_4 at pH 7.4.

Once dialised, $CuSO_4$ at 5-10 µM was used to oxidise LDL (0.2 g/L apoB) in PBS. LDL was incubated at RT for 15h. The more time with and/or concentration of $CuSO_4$, the more oxidized LDL gets. Oxidation is stopped by dialysis against A Buffer supplemented with 20 µM β-hydroxytoluene (BHT).

4.2 LDL ENRICHMENT IN CERAMIDE OR OTHER MINOR PHOSPHOLIPIDS

LDL was enriched in the products derived from phospholipolytic activities by incubating LDL with liposomes selectively enriched in these compounds as described (Boyanovsky, Karakashian et al. 2003). The steps were enrichment of liposomes with compounds and incubation of these liposomes with LDL.

- Liposome formation

  Lipid extracts, performed following the Bligh and Dyer Method (Bligh and Dyer 1959) and explained in *Minor Lipid Content evaluation by TLC* were incubated with the desired compounds: N-acetylsphingosine or CER, diacylglycerol (DAG) or sphingomyelin (SM) (all from sigma) at 5, 10 and 20 µM. As a control, one sample with no compound was processed in parallel to form non-enriched liposomes. Samples were dried down under nitrogen gas and further resuspended in 0.2 mL KBr solution with a density of 1.019 Kg/L (See 1.006 Kg/L density solution). Samples were sonicated in a water bath for 75 minutes, time in which the suspension got translucent and liposomes were formed.

- Incubation of liposomes with LDL

Native LDL (0.5 mL at 1 mg apoB/L) was used as an acceptor molecule, whereas liposomes enriched with compounds represented the donor molecules. Native LDL was incubated with liposomes at 0.5 mg at 37ºC for 45 minutes. Non-enriched liposomes were also incubated in parallel with LDL. Samples were preserved from oxidation with 20 µM BHT. Enriched LDLs were then re-isolated with KBr solution at a density of 1.019 Kg/L from liposomes by overnight ultracentrifugation at 36,000 rpm. Liposomes had lower density than 1.019 Kg/L and were found on the surface. After removing liposomes, the overnight ultracentrifugation was repeated. The resultant LDL samples were: CER-LDL, DAG-LDL, SM-LDL (all 5 or 10 µM) and non-enriched LDL.

4.3 LDL TREATMENT WITH PLC AND PLA_2

The enzyme PLC was used at 50 and 100 U/L in the incubation with LDL (0.5 g/L) for 1h at 37ºC. This enzyme needs salt and neutral pH to develop a proper activity. For that reason, the PLC-like enzyme buffer was used. The reaction was stopped with 10 mM EDTA. The resultant LDL was called PLC-LDL 50 and 100 U/L. LDL with no PLC-like enzyme treatment was performed in parallel.

The modification with PLA_2 was performed as described in previous studies of our group (Benitez, Camacho et al. 2004) and here summarised. Native LDL at 0.5g apoB/L diluted in PLA_2 buffer was incubated with increasing concentrations (5 - 20 µg/mL) of soluble PLA_2 ($sPLA_2$) (Sigma Aldrich), 45 g/L bovine serum albumin (BSA) free of fatty acids and 2µM BHT. After 2h at 37ºC, the reaction was stopped with 10 mM EDTA. Ultracentrifugation at 100000 rpm for 12h was performed to clean samples from excessive PLA_2 and albumin. LDL treated with PLA_2 (PLA_2-LDL) is dialysed in A Buffer and filtered.

- PLA_2 buffer: 5 mM HEPES, 5 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4

The modification of LDL by PLC raises the content of CER and DAG, whereas LDL modification by PLA_2 increases the amount of NEFA and LPC of the sample.

4.4 LDL INDUCED AGGREGATION

Aggregation was induced in LDL by intense agitation (vortex) of samples for increasing times (0, 2.5, 5, 15, 30 and 60 seconds). The lipoprotein formed was called agLDL.

5. LDL INCUBATION WITH HDL

LDL(+) and LDL(-) at 0.5 g/L apoB were incubated with HDL at 0.5 g/L apoA for 2h at 37ºC in PBS in the presence of 20 µM BHT.

Afterwards, LDLs and HDLs were properly separated by ultracentrifugation regarding its density (See LDL isolation and separation of LDL subfractions) using 1.050 kg/L density solution. Half of LDLs and HDLs samples were kept at 4ºC (called 0h at 37ºC) and the other half were kept at 37ºC for 20h (called 20h) to mimic the incubation conditions of LDLs with cells. The resulting samples were classified as follows (Table 7):

LDLs at 0h or 20h at 37ºC	HDLs at 0h or 20h at 37ºC
LDL(+)	HDL
LDL(+)/HDL	HDL/LDL(+)
LDL(-)	HDL/LDL(-)
LDL(-)/HDL	

Table 7. LDL and HDL samples after preincubation at 37ºC for 20h. Half of each sample was also kept at 4ºC for 20h (also known as 37ºC for 0h).

LDL(+)/HDL indicates LDL(+) that had been pre-incubated with HDL for 2h and re-isolated. Inversely, HDL/LDL(+) indicates HDL that had been preincubated with LDL(+) for 2h and re-isolated. In a similar manner, all samples were classified as “0h” or “20h” regarding the time kept at 37ºC (previously explained), so LDL(+)/HDL 0 h was kept at 4ºC for 20h whereas LDL(+)/HDL 20h was kept at 37ºC for 20h. All sample nomenclature follows the same pattern. The tests evaluated in these samples are summarised in Table 8.

Tests performed in LDL/HDLs and HDL/LDLs
Characterisation of samples by apoprotein and lípid composition
Minor lipid content evaluation by TLC
Basal and susceptibility to aggregation
Oxidation
PLC-like activity (Amplex Red and fluorescent TLC)

Table 8. List of tests evaluated in LDL and HDL samples. LDL/HDL (LDL that had been preincubated with HDL) and HDL/LDL (HDL that had been preincubated with LDL).

6. LPS NEUTRALISATION

The ability of LDL(-) to inactivate LPS was evaluated by the Limulus Amebocyte Lysate (LAL) test kit (Lonza) following the manufacturer's instructions. Briefly, the reactions are summarised in Figure 15 and detailed afterwards.

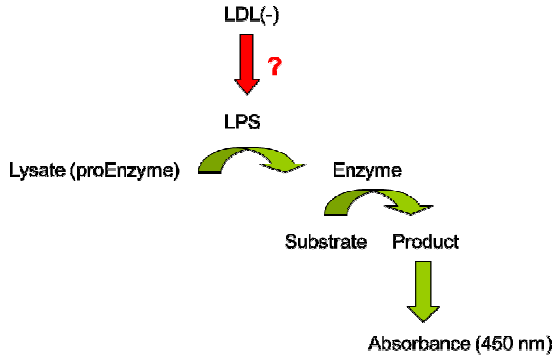


Figure 15. Reactions that take place in the LAL test.

LDL(+) and LDL(-) (50 µL at 0.15 and 0.5 g apoB/L) were preincubated with the endotoxin or LPS from *Escherichia coli* 0111:B4 (Sigma) (0.1 mg apoB/L) for 2 and 20h at 37ºC. Samples were placed in a sterile 96-well plate. LDLs alone and LPS alone were used as negative and positive controls, respectively. A standard curve was prepared with LPS diluted in sterile water.

Lysate (50 µL) containing the proenzyme was added into wells and incubated for 7 minutes at 37ºC. Substrate (100 µL) was further incubated for 4 more minutes in the

same conditions. The reaction was stopped with 100 µL 20% Acetic acid. Absorbance was then read at a wavelength of 405 nm and the standard curve was used to calculate LPS activity.

7. CULTURE OF PRIMARY MONOCYTES

Primary monocytes were isolated from peripheral blood of human volunteers, who gave their written informed consent. Each experiment was an independent preparation of monocytes from one donor. Subjects between 20 - 45 years old, 55% females and 45% males were included, whereas those with dyslipemia, hyperglycaemia, hypertension, chronic diseases or diseases associated with an inflammatory state and smokers were discarded. This procedure was approved by the Institutional Ethics Committee.

Cells were isolated according to their density (Boyum 1968) using density gradient centrifugation at 1.077 kg/L with Lympholyte (Cedarlane). After plasma centrifugation at 2500 rpm for 15 minutes, white cell layer was collected and diluted 1:2 with PBS. Diluted cells were gently layered over the Lympholyte Solution at a final ratio of 2:1 (diluted cells:Lympholyte Solution).

Cells were centrifuged at 2400 rpm for 20 minutes. Afterwards, the defined mononuclear cell layer at the interface was collected and transferred to a new centrifuge tube. Cells were washed twice with PBS and further resuspended with Complete RPMI Medium, which is RPMI 1640 medium (Sigma Aldrich) supplemented as detailed below.

- Complete RPMI Medium: RPMI 1640 10% Fetal Calf Serum (FCS), 1% non-essential aminoacids, 1% sodium pyruvate, 1% Penicillin-Streptomycin.

The density of cells seeded was 2 million cells/well, which is the density used in all monocyte experiments in this study. Differentiation between monocytes and

lymphocytes was made the day after, depending on their adhesive properties, since monocytes can attach to plate. In this regard, the lymphocytes contained in supernatant were discarded. An overview of this method is summarised in Figure 16.

The purity of monocytes was 80-85%, which was assessed by flow cytometry with 2-laser FACScalibur (Becton Dickinson) with a four-marker combination, and by May Grünwald/Giemsa staining. Monocytes were approximately 20% of total mononuclear cells and were cultured overnight prior to stimulation.

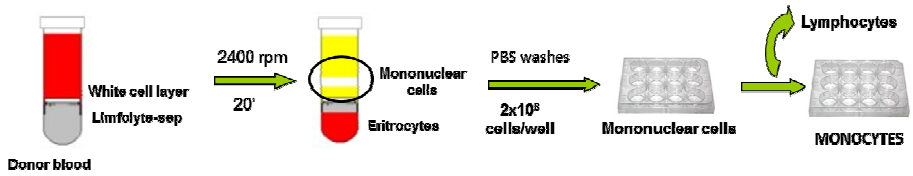


Figure 16. The mononuclear cell layer isolation process in fresh blood.

Deficient Medium was added into monocytes before the incubation of cells with the stimuli to avoid interference with lipoproteins from FCS (See *Incubation of cells with stimuli*).

- Deficient Medium: RPMI 1% Fetal Calf Serum (FCS), 1% non-essential aminoacids, 1% sodium pyruvate, 1% Penicillin-Streptomycin.

8. THP1-XBLUE AND THP1-XBLUE-MD2-CD14 CELL CULTURE

THP1 is a human monocytic cell line deriving from an acute monocytic leukemia patient, which is extensively used for in vitro studies of monocytes (Tsuchiya, Yamabe et al. 1980). The two THP1 cell lines used in this thesis were THP1-Xblue™ (THP1) and THP1-XBlue™-MD2-CD14 (THP1-CD14) (Invivogen).

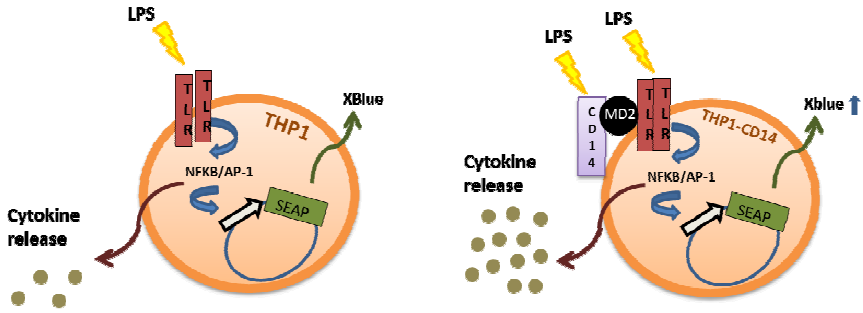


Figure 17. Representation of the genetic modification induced in THP1 to obtain THP1 and THP1-CD14 human monocytic cell lines.

They derived from THP1 and naturally expressed TLRs, but THP1-CD14 overexpressed MD2 and CD14 to increase the response to TLR ligands. Both cell lines stably expressed an inducible Secreted Embryonic Alkaline Phosphatase (SEAP) reporter system to monitor the activation of TLR-induced NF-κB/AP-1 (Figure 17).

RPMI 1640 containing L-glutamine was used as growth medium supplemented with 1% Penicillin-Streptomycin as in the case of human monocytes plus Normocin (100 mg/L) and Zeocin (200 mg/L) for THP1 cells, as well as Zeocin (200 mg/L) and G418 (250 mg/L) for THP1- CD14. The antibiotic Normocin is used to avoid cell contamination with activators of TLR ligands, and Zeocin as well as G418 are essential for the maintenance of a single cell type culture, regarding the presence or absence of CD14-MD2 induced expression. Cells were subcultured for every other day to guarantee proper nutrient levels and cell density to maintain an exponential cell proliferation rate.

9. CELL VIABILITY ASSESSMENT

The good status of cells in culture was corroborated in all the experiments by one of the following assays to discard putative basal stress.

9.1 CYTOTOXICITY XTT ASSAY

Cell viability was assessed by the in vitro 2,3-bis(2methoxy-4-nitro-5sulfophenil)-2H-tetrazolium-5-carboxyanilide inner salt (XTT)-based toxicology assay kit (Sigma), as described by the manufacturer. The mitochondrial dehydrogenases of viable cells reduce the tetrazolium ring of XTT yielding an orange formazan derivative. Cell supernatant was collected and XTT stock solution was added at the ratio of 5:1 (5 supernatant:1 XTT solution). Absorbance was read at 450 nm after 2h of incubation at 37ºC.

9.2 LACTATE DEHYDROGENASE ASSAY

Cell viability was measured faster by testing mortality in terms of Lactate Dehydrogenase (LDH) released from mitochondria to the cell media. For this purpose, we used Roche Diagnostics assay kit as described by the manufacturer. As high control, LDH in cell supernatant was measured from cells lysed with Triton-X 100; besides, as low control, LDH released in untreated cells was tested. Cytotoxicity was expressed as a percentage using the following equation.

$$\%\ cytotoxicity = \frac{experimental\ value - low\ control}{high\ control - low\ control} * 100$$

10. INCUBATION OF CELLS WITH STIMULI

LDL subfractions, modified LDLs and other molecules were studied in this thesis as cell stimulators. Deficient Medium was added previously to stimuli addition both into primary monocytes and THP1 cells. The concentration of the stimuli used and the time of incubation was previously established in our group (Benitez, Bancells et al. 2007) and conserved throughout the thesis. Stimuli were dialysed against RPMI 1640 to avoid interference with lipoproteins present in FCS.

All cell stimuli used were filtered in sterility prior addition into cells. The incubation time was 20h, although 4h incubation was performed in some cases as a comparing situation. Cells were kept during the incubation time at 37ºC, 5% CO_2 and 100% humidity. The stimuli used on each experiment are summarised in Table 9.

Stimuli	Concentration added to cells	Cell type	P1	P2	P3
LDL(+) and LDL(-)	0.15g apoB/L	Primary monocytes, THP1 and THP1-CD14	X	X	X
LPS	0.1 g/L	Primary monocytes, THP1 and THP1-CD14	X		
LDL(+) and LPS LDL(-) and LPS	LDL: 0.15 g/L LPS: 0.1 g/L	Primary monocytes	X		
CER-LDL (0, 5, 10 µM)	0.15g apoB/L	Primary monocytes, THP1 and THP1-CD14		X	X
SM-LDL, DAG-LDL (0, 5, 10 and 20 µM)	0.15g apoB/L	Primary monocytes		X	
Aggregated LDL (0, 2.5, 5, 15, 30 and 60 seconds)	0.15g apoB/L	Primary monocytes		X	
PLC-LDL (0, 50 and 100 U/L)	0.15g apoB/L	Primary monocytes		X	

Table 9. Summary of the stimuli and their concentrations as well as the cell type used in the cell culture experiments of this thesis. P: Publication.

The role of LDL(+), LDL(-) and LPS in cytokine release after 20h was studied when they were added separately or in combination into cell culture. The stimuli alone were compared with the effect of LDL(+) or LDL(-) added into cells at the same time of LPS ("coincubation conditions"). The addition of LDL(+) or LDL(-) that had been incubated with LPS for 2h previous to cell addition ("preincubation conditions") was also studied.

After incubation, cell supernatants were collected and centrifuged at 1,200 rpm for 10 minutes. Supernatant was transferred to a new tube to eliminate remaining cells and

samples were kept at -80ºC until protein determination by Enzyme Linked Immunoassay (ELISA) (See Section 13 of this chapter).

11. INCUBATION CONDITIONS OF CELLS FOR CYTOKINE RELEASE

The effect of LDL(+), LDL(-) and CER-LDL on cytokine release in primary monocytes was evaluated in the following diverse conditions.

11.1. LPS INHIBITION

The putative contamination of LDL(+) and LDL(-) samples with LPS was discarded by the assessment of cytokine release induced in primary monocytes when the LPS inhibitor Polymyxin B (50 mg/L) was added into cells together with the addition of stimuli.

11.2 RECEPTOR NEUTRALISATION

Neutralisation of the putative receptors involved in mediating LDL(-) effect was evaluated. Before the stimuli addition, cells were preincubated for 1h at 37ºC with neutralising antibodies: TLR2 antibody (antiTLR2), TLR4 antibody (antiTLR4) (HighCult Biotech) and CD14 antibody (antiCD14) (Anaspec) at 0.5, 2 and 5 mg/L, and LDL receptor antibody (antiLDLr) (R&D) at 0.1 and 0.5 mg/L. Control IgG was also assayed at 5 and 10 mg/L (Sigma).

11.3 INCUBATION WITH SOLUBLE CD14 (sCD14)

The addition of sCD14 at 10 and 50 µg/L 1h prior to the addition of stimuli was performed to evaluate its effect in combination with LDLs or LPS on cytokine release.

11.4 INHIBITION OF TLR PATHWAYS IN MONOCYTES, THP1 AND THP1-CD14 CELLS

Some TLR pathway inhibitors were added to cells to further compare the inflammatory effect of LDL(+) and LDL(-) when the pathway was not blocked. The TIRAP inhibitor

blocks the interaction between the adapter protein TIRAP and TLR2 or TLR4. The MyD88 homodimerisation inhibitory peptide (MyD88i) blocks the formation of MyD88 homodimer. The VIPER blocks the interaction between the adapter proteins Mal and TRAM with TLR4. The TIRAP inhibitor and MyD88i (both at 100 μM and 200 μM), and VIPER (15 μM and 30 μM) were incubated for 24h (TIRAP and MyD88) or 2h (VIPER) prior to stimuli addition.

VIPER (30 μM) was also used to evaluate the effect of CER-LDL on cytokine release. In all cases, the inert control peptide, CP7 peptide (Imgenex) was also used.

11.5 CD14 AND TLR4 GENE SILENCING

CD14 and TLR4 gene expression was downregulated in primary monocytes in order to study the change on cytokine release induced by LDL(-) in comparison to LDL(+).

11.5.1 siRNA transfection

To knock down the expression of CD14 and TLR4 in primary monocytes, specific small interference RNA (siRNA) was used (Santa Cruz Biotechnologies). Cell culture and culture conditions were adapted according to the manufacturer's instructions.

On the first day, primary monocytes were seeded at $2 \cdot 10^6$ cells/well in 6-well plates in Complete RPMI Medium.

On the second day, medium was changed to Complete DMEM (Sigma Aldrich) and plates were incubated for 30 minutes at 37ºC.

- Complete DMEM Medium: 4.5 g/L glucose, 10% SBF, 1% Penicillin-Streptomycin.

A Stock Solution for each siRNA was prepared, just before the Transfection Mix, with Powerfect Transfection Buffer (SignaGen Laboratories) diluted in water.

- Stock Solutions:

  - TLR4 siRNA: 100 uM (4 μL + 36 μL PowerFect Transfection Buffer)

- CD14 siRNA: 200 uM (4µL + 16 µL PowerFect Transfection Buffer)

- Transfection Mix: 1 µL Stock siRNA, 4µL GenMute siRNA Transfection Reagent for Primary Macrophages (SignaGen Laboratories) and 15µL PowerFect Transfection Buffer 1X (SignaGen Laboratories). A negative control of gene silencing without any specific siRNA was also performed.

The transfection Mix was kept for 15 minutes at RT and added into cells (20 µL/well). Cell plates were intensively rocked and incubated for 5 hours at 37ºC. Cell medium was then replaced by Deficient RPMI Medium to avoid high levels of cytotoxicity and incubated at 37ºC for 2 days.

On the fourth day, cells were incubated with LDLs and LPS for 20h at with the same conditions described (see *Incubation of cells with stimuli*). Afterwards, cell supernatant was collected for cytokine release assessment.

The effect of TLR4 and CD14 silencing was tested in primary monocytes at RNA level by quantitative real-time polymerase chain reaction (qRT-PCR) and at protein level by Western Blot (WB). RNA and protein were extracted from cells, cultured as in cytokine release experiments but in different wells.

11.5.2 Gene knockdown testing

The efficiency of TLR4 and CD14 gene silencing was evaluated both at RNA and protein level.

11.5.2.1 RNA extraction and qRT-PCR

Cells were scraped from plates and RNA was isolated using the commercial TRIZOL reagent (Ambion, Life Technologies) as described by the manufacturer. RNA pellet was dried and resuspended in 200 µL Diethylpyrocarbonate (DEPC) treated H_2O. RNA concentration was evaluated using the equivalence of 1 $UAbs_{260}$=40 µg/mL in a nanodrop (Thermo Scientific). Samples were kept at -80ºC until reverse transcription to generate cDNA.

cDNA was generated from RNA samples equalled at the same concentration (13 µL, up to 1 µg), which were incubated with 1 µL thymine oligonucleotides (oligodT) (Sigma Aldrich) for 5 minutes at 70ºC. Samples were cooled on ice and the Reverse Transcription Mix was added and incubated at 42ºC for 1h, followed by 15 minutes of incubation at 70ºC. cDNA samples were kept at -80ºC until qRT-PCR analysis.

- Reverse Transcription Mix (amount per sample): 5 µL 5X *Moloney murine leukemia virus H* (MLV)-Buffer, 1 µL reverse transcriptase MLV (Promega), 1.25 µL dNTPs, 3.75 µL PCR-H_2O (Sigma Aldrich).

Designed probes for the selected genes TLR4 and CD14 (assay-on-demand, Applied Biosystems) as for the housekeeping gene B-actin (control) were used to perform qRT-PCR. Samples and the proper Reverse Transcription Mix specific for each gene were dispensed into a 96-well PCR-plate. Per well, 2 µL of sample was added to 18 µL of Transcription Mix and the plate was further placed in an AbiPrism 7,000 to perform qRT-PCR. Plate was submitted to 40 cycles at 95ºC for 10 minutes, 95ºC for 15 seconds and 60ºC for 1 minute.

- qRT-PCR Mix (per sample): 1 µL cDNA (40 ng), 12.5 µL Taqman enzyme (Universal PCR Master Mix), 1.25 µL probe *assay-on-demand(Applied Biosystems),* 10.25 µL PCR-water (Sigma Aldrich).

Cycle threshold (Ct) is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold. It is inversely proportional to the relative expression level of the gene studied and it is calculated by the following equation.

$$\textbf{\textit{Gene expression} = 2}^{\textbf{\textit{-(Ct studied gene – Ct housekeeping)}}}$$

11.5.2.2 Membrane protein extraction and WB analysis

After scraping, cells were collected and washed with PBS. PBS was discarded and cells were resuspended in 0.3 mL Denaturing Cell Lysis Buffer (containing 6M urea) for each 20 million cells, and incubated for 20 minutes.

- Denaturing cell lysis buffer: 10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% (v/v) X-100 Triton, 10 µg/mL Leupeptin, 10 µg/mL Pepstatin, 3 µg/mL Aproptotin,

100 µM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 2mM $Na_4P_2O_7$ and 5 mM NaF.

Disrupted cells were submitted to ultracentrifugation at 40,000 rpm for 30 minutes at 4ºC to precipitate membrane extracts. Membrane pellets were collected and further disrupted with 2% sodium dodecylsulphate (SDS) solution containing protease inhibitor (Roche Diagnostics), pH 8.42. Membrane protein extracts were concentrated in 10-kDa centrifugal filters (Amicon) and the total protein amount was evaluated by the BCA method (Thermo Scientific). Samples were frozen at -80ºC until evaluation by WB. Protein extracts (50 µg) were mixed with 15 µL Laemmli Sample Buffer (BioRad) in non-reducting conditions, and subjected to SDS-Polyacrilamide gel electrophoresis (PAGE). SDS-PAGE was prepared with 10% acrylamide in the Separation gel and 5% in the Stacking gel, which were the proper percentages to isolate TLR4 (110 KDa) and CD14 (56 KDa). Electrophoresis was run for 15 minutes at 30V followed by 100V for 2h.

- Electrophoresis Buffer: Tris 25 mM, Glicina 192 mM, 0.1 % SDS.

Protein bands from electrophoresis were then transferred to a nitrocellulose membrane, by using a specific sandwich support, for 2h at 30V in cool conditions with Transference Buffer.

- Transference Buffer: 400 mL Electrophoresis Buffer, 400 mL H_2O, 200 mL ethanol.

Proteins were then blocked in Tris Buffered Saline (TBS) buffer containing 0.1% casein (TBS-0.1% casein) for 30 minutes at RT. The same buffer was used to dilute these human primary antibodies at the following dilutions: 1/200 antiTLR4 (Rockland), 1/1000 antiCD14 (Novus Biologicals), 1/250 antiβ-actin (Sigma Aldrich). After overnight incubation with these primary antibodies, the nitrocellulose membrane was washed 3 times in TBS containing 0.1% Tween (TTBS). An IgG secondary antibody (Jackson Immunoresearch) was used in a dilution of 1/1000 in TBS-0.1% casein, to incubate the membrane for 2h at RT. Membranes were washed again in TTBS before the chemoluminiscent developing in a ChemiDoc system. Blots were then relativised in regard to the internal control β-actin.

- TBS Buffer: 200 mM Tris, 5 M NaCl, pH 7.5.

12. INDUCTION OF NF-KB/AP-1 AND CYTOKINE RELEASE IN THP1 AND THP1-CD14

Two monocytic THP1 cell lines (THP1 and THP1-CD14) were used to evaluate the effect of LDL(-) in the putative activation of the TLR-induced NF-kB/AP-1, as well as the role of CD14 when inducing cytokine release. Both cell lines contained a SEAP reporter system and were incubated in a 96-well plate with LDL(+), LDL(-), LPS and CER-LDL for 20h in Complete RPMI Medium (see Sections 8 and 10 of this chapter).

Firstly, the activation of induced NFkB/AP-1 with the SEAP reporter system was evaluated by seeding THP1 and THP1-CD14 at 200,000 cells/well. Cells were resuspended in complete medium and incubated with the stimuli. After 20h incubation with the LDL subfractions, 15 µL of cell supernatant was incubated (1:10) with QUANTI-Blue™. SEAP was measured at 620 nm at different times beginning at 30 minutes to 3 hours.

Cytokine release was evaluated from cell supernatant collected from triplicated wells and pooled. However, control wells at 10^6cells/well in Deficient RPMI medium in 12-well plates were performed in parallel, since Complete RPMI medium (containing 10% FCS) required in SEAP test could be interfering with the cytokine release induced by the lipoprotein samples.

13. CYTOKINE RELEASE EVALUATION BY ELISA

The cytokine release promoted by stimuli in primary monocytes and THP1 cell lines was evaluated by ELISA. The cytokines IL-6 and IL-10, and the chemokine MCP-1 were elected in our group among others (Benitez, Bancells et al. 2007) as the representative mediators induced by LDL(-) in monocytes.

IL-6 is a proinflammatory cytokine secreted in the acute phase of the pathogen response, IL-10 has anti-inflammatory properties since diminish the release of

inflammatory cytokines and MCP-1 regulates the migration of monocytes/macrophages to the injured place.

Commercial ELISA Module Set antibody pairs were used to quantify IL-6 (BenderMedsystems), IL-10 and MCP-1 (eBioscience) following the manufacturer's instructions. In general, plates were coated with 100 µL Capture Antibody diluted in PBS and incubated overnight at 37ºC. Wells were washed twice with 300 µL Washing Buffer and the remaining protein binding sites were blocked with 250 µL Assay Buffer containing BSA from 1 up to 7 days until assessment.

- Washing Buffer: PBS 0.5%-Tween
- Assay Buffer: PBS 0.5%-Tween, 0.5% BSA

Samples (100 µL) were added to wells at the proper dilution with Assay Buffer and incubated for 2h at RT. A standard curve was prepared and processed in parallel to samples. Wells were 3 times washed and incubated with biotin-conjugated secondary antibody specific for IL-6, IL-10 or MCP-1. In IL-6 method, biotinilated primary antibody was incubated together with samples for 2h. Table 10 summarises the dilution factor of samples and antibodies.

Procedure-steps containing dilutions	**IL-6**	**IL-10**	**MCP-1**
Sample	1:6	1:2	1:4
Coating antibody dilution	1:40	1:250	1:250
Biotinilated antibody dilution	1:1000	1:250	1:250
HRP	1:200	1:250	1:250
Highest Standard concentration in plate	200 pg/mL	300 pg/mL	1000 pg/mL

Table 10. Dilutions used in the ELISA methodology

Cells were washed 3 times and incubated with the detection enzyme Avidin-linked Horse Reddish Peroxidase (HRP), a luminescent probe that amplifies the signal. Finally, the substrate tetrametilbenzidine (TMB) (Sigma) was added to wells until a coloured product was formed. The reaction was stopped with sulphuric acid and plate was read at 450 nm. An overview of the ELISA method is represented in Figure 18.

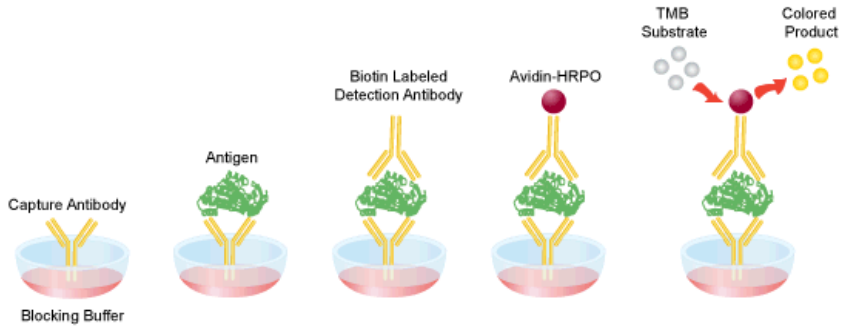


Figure 18. Representation by steps of the ELISA procedure

14. BINDING ASSAYS

The putative binding of LDL subfractions into the candidate receptors was analysed not only in primary monocytes but also in CD14-coated microtiter wells and summarised in Tables 11 and 12, respectively. Primary monocytes were submitted to TLR2, TLR4 and CD14 receptor neutralisation before the addition of bound LDL, whereas more specific binding to CD14 was assessed in CD14-coated microtiter wells, since there was no interference of any other receptor. Both binding to human monocytes and binding to CD14-coated microtiter wells were assessed with one single stimulus (*Total Binding*) or when two stimuli were competing for the receptor (*Binding Displacement Studies*).

14.1 BINDING TO PRIMARY MONOCYTES

14.1.1 Total Binding

Total binding of LDL(+) and LDL(-) to human monocytes was performed essentially as described by Innerarity *et al* (Innerarity, Pitas et al. 1986). LDL subfractions were labelled with the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) following Stephan and Yurachek method (Stephan and Yurachek 1993). Briefly, LDLs were previously dialysed against A Buffer and incubated with DiI in a proportion 1:10,000 (DiI:LDL) for 18h at 37ºC. LDL

was re-isolated by ultracentrifugation and extensively dialysed in osmotic sack membranes to eliminate excess of the fluorescent probe. DiI-LDL(+) and DiI-LDL(-) were dialysed in PD-10 columns (Amersham Bioscience) against 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) Medium and filtered to avoid the formation of aggregates.

- HEPES Medium: RPMI + 1% SBF + 10 mM HEPES, pH 7.4

Prior to DiI-LDLs addition, the complete medium from cells was changed to HEPES Medium. Labelled LDLs were incubated with cells at 0, 25, 50, 100 and 150 mg apoB/L in HEPES buffer for 3h at 37ºC. Samples that had been processed in parallel were alternatively kept at 4ºC. Binding at 37ºC represents a physiological situation but incubation at 4ºC provides a better estimation of the affinity of a ligand to its receptor, since internalisation or lateral movements of receptors and other membrane components cannot occur. Non-specific binding was determined by incubating labelled LDLs with 10-fold excess non-labelled LDLs. Non-specific binding was substracted from total binding to calculate specific binding.

After incubation, supernatant was discarded and cells were washed once in PBS containing 2 g/L BSA, followed by two more washes in PBS alone. Putative LDLs labelled to cells were then submitted to lipid extraction with isopropanol for 15 minutes with gentle agitation. Supernatant was then separated from cells by centrifugation. Fluorescence from DiI-LDL(+) or DiI-LDL(-) bound to cells was measured in a spectrophotometer (excitation at 528 nm and emission at 578 nm).

In some experiments, primary monocytes were preincubated with the same neutralising antibodies used in receptor neutralisation conditions for cytokine release experiments. Cells were pretreated with antiTLR2, antiTLR4, antiCD14 (2 and 5 mg/L) and antiLDLr (0.1 and 0.5 mg/L) to assess the change of binding on labelled LDLs when epitopes are blocked. Labelled LDLs (150 mg apoB/L) were then added to cells and incubated at 4ºC or 37ºC for 3h in HEPES Medium.

14.1.2 Binding Displacement Studies

Primary monocytes were incubated for 3h at 37ºC with a fix concentration of DiI-LDL(+) or DiI-LDL(-) (50 mg apoB/L) and increasing concentrations of LPS (0-1 mg apoB/L) or oxLDL (0-150 mg apoB/L). The concentration of DiI-LDLs in this assay was lower than used in total binding experiments, since we have checked that the fluorescent signal at this concentration was enough. Moreover, the addition of LPS and oxLDL in high concentrations competed and displaced binding of DiI-LDLs. After incubation, fluorescence was extracted from cells and measured as in *Total binding*. A schematic overview of this procedure is summarised in Table 11.

BINDING TO PRIMARY MONOCYTES

Type of experiment	Stimuli and concentration	Incubation temperature	Quantification
Total binding (absence of antibodies)	DiI-LDL(+) or DiI-LDL(-) (0 - 150 mg apoB/L).	4ºC and 37ºC	Fluorescence bound to cells
Total binding with antiTLR2, antiTLR4, antiCD14 and antiLDLr antibodies	DiI-LDL(+) or DiI-LDL(-) (150 mg apoB/L)	4ºC and 37ºC	Fluorescence bound to cells
Binding displacement studies	Coincubation of DiI-LDL(+) or DiI-LDL(-) (50 mg apoB/L) and LPS (0 - 1 mg) or oxLDL (0 - 100 mg apoB/L).	37ºC	Fluorescence bound to cells.

Table 11. Representation of the conditions used and the analysis made in experiments of total binding and binding displacement studies in primary monocytes.

14.2 BINDING TO CD14-COATED MICROTITER WELLS

To specifically study LDL(-) binding to CD14, LDL(+) and LDL(-) binding to CD14-coated microtiter wells was evaluated according to the procedure described by Dziarski

(Dziarski, Tapping et al. 1998). Total binding and binding displacement tests were also performed in these assays, which are summarised in Table 12.

BINDING TO CD14-COATED MICROTITER WELLS

Type of experiment	Stimuli and concentration	Incubation temperature	Quantification
Total binding	LDL(+) or LDL(-) (150 mg apoB/L)	37ºC	LDL bound to plate (as total cholesterol)
Binding displacement studies	Coincubation of LDL(+) or LDL(-) (150 mg apoB/L), and LPS (0 - 0.5 mg) or sCD14 (10 and 50 μg/L)	37ºC	LDL bound to plate (as total cholesterol)

Table 12. Representation of the conditions used and the analysis made in binding to CD14-coated microtiter wells.

The Polystyrene High-binding 96-well plates were coated with antiCD14 at 10 mg/L (Abnova) for 18h at 4ºC. Wells were blocked for 1h at 37ºC with PBS supplemented with 3% BSA and 1% Fat-free milk powder. Washes (5) were then made with TBS and recombinant human CD14 (Prospec) was added at 2 mg/L for 2h at RT. Some wells were kept free of CD14 and used as control wells.

- TBS: 200 mM Tris, 5 M NaCl, pH 7.5

The stimuli were added after washing wells again. In Total Binding assays in CD14 coated microtiter, LDL(+) or LDL(-) (150 mg apoB/L) were added alone, whereas in Binding Displacement Studies, LDLs were added in the presence of LPS at increasing concentrations (0, 10, 100 and 500 μg/L) or sCD14 (10 and 50 μg/L). In all cases, the stimuli were incubated for 4h at 37ºC. Wells were washed again to remove unbound LDL Amplex Red method for cholesterol assessment was performed to determine the amount of LDL that was bound to wells.

14.2.1 Cholesterol determination by Amplex Red

Amplex Red Cholesterol assay Kit (Molecular Probes) is a fluorimetric assay based on enzyme coupled reactions that detects both free cholesterol and cholesteryl esters. This method is the same used by the Hitachi 902 autoanalyser, as described above. Briefly, cholesterol esterase hydrolyses cholesterol esters into cholesterol, which is in turn oxidized by cholesterol oxidase into H_2O_2. In this method, H_2O_2 is then detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent) and Horseradish Peroxidase (HRP) to produce *resorufin*, a product having an absorbance and fluorescent emission of approximately 571 and 585 nm, respectively.

Samples were diluted, and a standard curve (cholesterol reference standard concentrations 0-8 µg/mL) and a positive control were prepared (10 µM H_2O_2) in 1X Reaction Buffer. A working solution was also prepared as in Table 13.

WORKING SOLUTION

Final concentration per sample (5 mL final vol)	Volume and concentration to add
300 µM Amplex Red reagent	75 µL reagent stock solution (20mM)
2 U/mL HRP	50 µL HRP stock solution (200 U/mL)
2 U/mL cholesterol oxidase	50 µL cholesterol oxidase stock solution (200 U/mL)
0.2 U/mL cholesterol esterase	5 µL cholesterol esterase stock solution (200 U/mL) 4.82 mL 1x Reaction Buffer

Table 13. Composition of the Working Solution used in the determination of cholesterol by the method of Amplex Red.

The assessment starts by adding 50 µL Working solution to wells from a 96-well plate containing samples and controls. Multiple time point measurements were performed in the dark during the 30 minutes incubation at 37ºC.

17. STATISTICAL ANALYSIS

The results in this study were expressed as mean ± SD. The statistic program SPSS and Sigma Stat 2.0 statistical package for Windows were used to test the differences between groups. Wilcoxon t-test was performed for paired data and U-Mann Whitney for non-paired data. Results were considered significant when $p<0.05$.

Chapter IV

ORIGINAL PUBLICATIONS

PUBLICATION 1:

The Induction of Cytokine Release in Monocytes by Electronegative Low Density Lipoprotein (LDL) is Related to its Higher Ceramide Content than Native LDL.

Montserrat Estruch, Jose Luis Sanchez-Quesada, Lorea Beloki, Jordi Ordoñez-Llanos, Sonia Benitez.

International Journal of Molecular Sciences (2013) 14: 2601-2616

PUBLICATION 2:

CD14 and TLR4 mediate cytokine release promoted by electronegative LDL in monocytes.

Montserrat Estruch, Cristina Bancells, Lorea Beloki, Jose Luis Sanchez-Quesada, Jordi Ordóñez-Llanos, Sonia Benitez.

Atherosclerosis (2013) 229: 356-362

PUBLICATION 3:

Ceramide-enriched LDL induces cytokine release through TLR4 and CD14 in monocytes. Similarities with electronegative LDL.

Montserrat Estruch, Jose Luis Sanchez-Quesada, Jordi Ordoñez-Llanos, Sonia Benitez.

Clínica e Investigación en Atherosclerosis (2014) [Epub ahead of print].

Chapter V

GLOBAL DISCUSSION

As reviewed in the introduction, LDL(-) is a minor modified LDL in circulation with pro-inflammatory effects. LDL(-) induces chemokine and cytokine release in cells involved in atherosclerosis, such as endothelial and mononuclear cells. However, the cellular pathways activated by LDL(-) are scarcely understood, particularly in monocytes, cells that could interact easily with LDL(-) in circulation. For this reason, the present thesis aimed to determine the first steps involved in inducing MCP-1, IL-6 and IL-10 release in human monocytes. First, we have studied the LDL(-)-inflammatory components, second the cell receptors mediating the inflammatory effect and lastly the interaction of these inflammatory components with cell receptors.

1. PUBLICATION 1: LDL(-) COMPONENTS INVOLVED ON INFLAMMATION

1.1 BACKGROUND OF LDL(-) COMPONENTS REPORTED TO MEDIATE CYTOKINE RELEASE

In contrast with LDL(+), LDL(-) has greater intrinsic PAF-AH and PLC-like activities. These activities can degrade exogenous and endogenous substrates (Benitez, Sanchez-Quesada et al. 2003, Bancells, Benitez et al. 2008). Degradation of PAF-like lipids by the PAF-AH enzyme increases the content of LPC and NEFA in LDL(-). (Benitez, Camacho et al. 2004). In parallel, PLC-like activity in LDL(-) degrades -in order of substrate preference- LPC>> SM>> PC (Bancells, Benitez et al. 2008) yielding MAG, CER and DAG, respectively, and also P-chol. Although LPC is preferentially degraded by the PLC-like activity, MAG content in LDL(-) is scarce, since the amount of LPC is much lower (3.5% of total phospholipids), than SM (20%) and PC (70%) (Benitez, Camacho et al. 2004). Hence, DAG and CER are increased products in LDL(-). Since LPC, NEFA, CER and DAG are all known pro-inflammatory lipids derived from phospholipase activity and increased in LDL(-), they become putative mediators for cytokine release in LDL(-) (MacPhee, Moores et al. 1999, Pettus, Chalfant et al. 2004).

In endothelial cells, the increased content of NEFA and LPC in LDL(-) has been described to lead to the cytokine release induced by this modified lipoprotein (Benitez, Camacho et al. 2004, Benitez, Camacho et al. 2006). The NEFA-loading of LDL(+) and

the treatment of LDL(+) with $sPLA_2$ enabled the induction of cytokine release, but only when the levels of LPC and NEFA were equal to or higher than those of LDL(-).

The role of NEFA in LDL(-)-induced cytokine release has also been established in mononuclear cells (Bancells, Sanchez-Quesada et al. 2010). These authors evaluated the role of HDL and apoA-I in the regulation of the inflammatory effects of the LDL(-). They found that incubating LDL(-) with HDL or apoA-I decreased the release of cytokines induced by LDL(-), whereas HDL preincubated with LDL(-) promoted an increased cytokine release compared to that of HDL that was not previously incubated with LDL(-). This finding led us to hypothesize that some inflammatory components on LDL(-) could be transferred to HDL. The PAF-AH and PLC-like activities ascribed to LDL(-) and the resulting products were evaluated in both LDL(-) and HDL fractions. A decrease in the PLC-like activity in LDL(-) was found by its incubation with HDL, which gained in this activity, thereby suggesting the transfer of PLC-like activity from LDL(-) to HDL. It is of note that NEFA were also decreased in LDL(-) and increased in HDL. Next, the role of NEFA content in the cytokine release induced by LDL(-) was evaluated. The enrichment of LDL(+) in NEFA promoted cytokine release in monocytes. However, this release was lower than that of LDL(-), even if the content of NEFA reached that of LDL(-). This suggested that other components such as the PLC-like activity products, increased in LDL(-), could be involved in the cytokine release promoted by LDL(-). In this line, the first aim of this thesis was to assay the involvement of CER and DAG in the cytokine induction in monocytes. Since both components are increased in LDL(-) and involved in inflammatory processes, they could exert an effect on cytokine and maybe on other properties ascribed to LDL(-).

1.2 INCREASED CER CONTENT AND INDUCTION OF CYTOKINE RELEASE

The results of this thesis show that the increased content of CER contained in LDL(-) contributes to the release of specific inflammatory mediators induced by this lipoprotein in monocytes, particularly IL-6, IL-10 and MCP-1.

The hypothesis followed in this study was that the PLC-like activity and its derivative products would increase at 37ºC, which is the temperature of incubation of LDL(-) in the experiments with monocytes. We compared the differences between the incubation of LDL(-) at 37ºC and 4ºC, as well as the effect of HDL in these two conditions.

The incubation of LDL(-) at 37ºC induced changes in LDL(-) composition. First, NEFA content increased, and this was not explained by oxidation. The increased NEFA content could be produced, among other causes, by the degradation of PLC-like activity products such as CER, which still has to be elucidated. Second, there were an increase of CER and DAG in LDL(-), which correlates with an increase on PLC-like activity that would degrade SM and PC, respectively. Because of the low amounts of LPC in LDL, the MAG produced by this activity was not significant, as explained above. Third, the incubation of LDL(-) at 37ºC increased its aggregation. Nevertheless, the preincubation of LDL(-) with HDL counteracted all the effects of incubation at 37ºC. After incubation with HDL, the content in NEFA, CER and DAG and in PLC-like activity was reduced in LDL(-). HDL resulted in an increase of both PL and PLC-like activity, demonstrating a transfer from LDL(-) to HDL. As explained above, the transfer of NEFA to HDL and its role in LDL(-) cytokine release had been previously reported (Bancells, Sanchez-Quesada et al. 2010). The involvement of CER and DAG in the LDL(-)-induced cytokine release had not yet been studied, although it could be feasible since HDL decreases the content of these products as well as the cytokine release promoted by LDL(-).

The current results show that when LDL(+) is treated with commercial PLC-like activity it can induce cytokine release. We studied the role of each product derived from the PLC-like activity by enriching LDL(+) with CER or with DAG. We found that CER-LDL induced cytokine release but DAG-LDL and the CER compound did not. The cytokine secretion promoted by CER-LDL was lower than that promoted by LDL(-). This fact suggests that other components such as NEFA could be involved, although other candidates should not be discarded.

CER is a bioactive component of cell membranes that acts as a signalling molecule in many pathophysiological processes (Arana, Gangoiti et al. 2010), including atherosclerosis (Steinbrecher, Gomez-Munoz et al. 2004). CER accumulates in atheromatous plaques (Schissel, Tweedie-Hardman et al. 1996), not only in cells but also into lipoproteins.

In cells, CER is mainly formed by the action of intracellular SMases degrading SM from membranes. Depending on the cellular CER levels, this secondary messenger can mediate immune actions activating intracellular pathways leading to changes in cell cycle and apoptosis, or inducing inflammatory molecules (Mathias, Pena et al. 1998). CER induces not only cytokine release (Ballou, Laulederkind et al. 1996), but also the expression of endothelial adhesion molecules. As a result, neutrophils are incorporated into the intima and the atheromatous plaque is formed (Chatterjee 1998).

This is not the first time that CER is reported to increase naturally in modified lipoproteins under inflammatory states. In atherosclerotic lesions, CER is increased in modified lipoproteins forming aggregates of LDLs (Schissel, Tweedie-Hardman et al. 1996). Moreover, Kinscherf et al. (Kinscherf, Claus et al. 1997) showed that the inflammation produced by either acLDL or oxLDL was partly due to CER. The CER content has been shown to increase in VLDL and LDL after the activation of serine-palmitoyl transferase, which synthesizes CER in the liver for further incorporation into lipoproteins (Lightle, Tosheva et al. 2003). Serine palmitoyl-transferase (Nikolova-Karakashian, Russell et al. 1997) and liver SMases (Deaciuc, Nikolova-Karakashian et al. 2000) can be activated by cytokines or even by LPS (Lightle, Tosheva et al. 2003). Interestingly, these inflammatory stimuli can also activate the secretory form of SMase in macrophages and endothelial cells, thereby inducing its release in plasma. This activity in the plasma degrades SM in both native LDLs and modified LDLs, though to a greater extent in the latter. This degradation increases CER in these lipoproteins (Schissel, Jiang et al. 1998). However, the increased CER content in LDL(-) is not feasibly caused by the sSMAse released from cells, since the results here presented show that

LDL(-) kept at 37ºC increases the CER levels, in spite of the absence of any cells and external PLC-like activity.

The increased CER content would therefore be a consequence of the intrinsic SMase activity in LDL(-). This activity acts both on its own phospholipids but also to external products (Bancells, Benitez et al. 2008). Incubation of LDL(-) with cells could promote the degradation of the SM present in lipid rafts of the cell membrane by the LDL(-)-intrinsic sphingomyelinase and thereby increasing the content of intracellular CER. Moreover, the CER transported in LDL(-) could also be transferred to the cell. The content of CER in the cell could also increase through the induction of Fas expression by LDL(-) (Bancells 2010). Fas induction has been described to activate intracellular SMases, thereby enhancing CER content and activating the inflammatory signalling cascade (Cifone, De Maria et al. 1994).

However, other products are involved in the inflammatory response of LDL(-) in monocytes. In the case that LDL(-) possessed ceramidase action, the products derived from the degradation of CER, SP1 and SPH, could also play a role in the LDL(-)-induced cytokine release, since both agents are involved in inflammation (Mao and Obeid 2008). SPH induces growth arrest, differentiation and apoptosis by acting on several intracellular targets (Ruvolo 2003). SPH can be phosphorylated to form S1P. It is involved in angiogenesis, vascular maturation, cardiac development and immunity (Maceyka, Harikumar et al. 2012). It can interact with TLR2 (Duenas, Aceves et al. 2008) and it mediates cytokine release and adhesion molecule expression through NF-kB activation (Daum, Grabski et al. 2009). It also enhances cell proliferation, attachment to the endothelium, cell migration and accumulates in atherosclerotic lesions (Arana, Gangoiti et al. 2010). Nevertheless, it has been reported that S1P can exert anti-inflammatory actions (Okajima 2002, Rodriguez, Gonzalez-Diez et al. 2009, Sato and Okajima 2010).

Apart from its involvement in cytokine release, our results also suggest a role of CER in the aggregation level of LDL(-), since LDL(-)-susceptibility to aggregation as well as the CER content in LDL(-) increase at 37ºC. In this regard, CER-LDL showed a higher susceptibility to aggregation than native LDL.

Therefore, as both LDL(-) and CER-LDL showed a tendency to aggregate and the ability to induce cytokine release in monocytes, we hypothesized that aggregation could be involved in cytokine release promoted by LDL(-). However, the in vitro-induced aggregation of LDL by vortex did not induce cytokine release. It should be kept in mind, however, that this is a mechanical method that might not reproduce the aggregation found *in vivo*. However, aggregation induced by CER could contribute to the increased affinity of LDL(-) to PG present in the arterial wall. This affinity has been related to the increased content of CER and PLC-like activity in LDL(-) (Bancells, Benitez et al. 2009). It concurs with previous studies in which high levels of CER in lipoproteins modify LDLs by changing their physicochemical properties and leading to their retention in lesion sites (Lightle, Tosheva et al. 2003).

Some of the properties of LDL(-) on monocytes could be partly attributed to CER. On one hand CER is involved in cytokine release and, on the other hand, it contributes to the physicochemical changes on its surface leading to aggregation. However, apart from the findings described in this thesis, other reported effects of CER have also been ascribed to LDL(-). It includes apoptosis (Hannun and Obeid 1995, Birbes, El Bawab et al. 2001, Hannun, Luberto et al. 2001, Chen, Hosken et al. 2007). LDL(-) activates the extrinsic apoptotic pathway by enhancing gene expression of the membrane-bound protein Fas (Bancells, Sanchez-Quesada et al. 2010). As intracellular CER can also signal apoptosis (Hannun and Obeid 1995), and as part of the LDL(-) inflammatory properties are attributed to CER, LDL(-) could also induce apoptosis through CER.

To summarise, the increase of CER in LDL(-) is partly responsible for the inflammatory effects of LDL(-). However, the cell mechanisms by which CER in LDL(-) can trigger cytokine release in monocytes are unknown. The first step in finding the mechanisms that lead to LDL(-)-induced cytokine release in monocytes would be to elucidate the cell receptors involved in LDL(-) recognition. In the publications 2 and 3 discussed below, we determined the receptors in monocytes that are responsible for cytokine release induced by LDL(-) and the relationship with the increased CER content in LDL(-), respectively.

2. PUBLICATION 2: LDL(-) RECEPTORS INVOLVED ON INFLAMMATION

2.1 BACKGROUND OF LDL(-) RECEPTORS

Previous studies about the interaction between LDL(-) and cell receptors have allowed some of them to be ruled out as mediators of cytokine release induced by LDL(-), in particular LDLR and the macrophage SR-AII. LDL(-) shows impaired binding affinity to LDLR and it is not uptaken by monocyte-derived macrophages in a higher degree than LDL(+) (Benitez, Villegas et al. 2004), cells that mediate less cytokine release (Kavanagh, Symes et al. 2003). PAFR, is another receptor suggested to recognise LDL(-), although its involvement is controversial. Whereas some authors support its role in mediating LDL(-) effects (Chen, Jiang et al. 2003), others consider that PAF ligand levels in LDL(-) are too low -because of PAF-AH activity in LDL(-)- to mediate these effects (Bancells, Benitez et al. 2008).

In endothelial cells, the receptor activated by LDL(-) and involved on mediating its biological effects is LOX-1 (Tang, Lu et al. 2008, Lu, Yang et al. 2009). Nevertheless, the expression of LOX-1 in monocytes is low and therefore it is not feasible that this receptor mediates cytokine release in this cell type (Moheimani, Tan et al. 2011).

Monocytes possess TLRs and CD14 (Kadowaki, Ho et al. 2001), which are known LPS receptors involved in mediating the cytokine release induced by this entity (Dentener, Bazil et al. 1993, Sabroe, Jones et al. 2002). As reviewed, these receptors have also been related to atherosclerosis mainly by the recognition of modified LDLs (Cole, Georgiou et al. 2010). Specifically, TLR2, TLR4 and CD14 have been described to mediate the inflammatory effects of oxidized forms of LDL not only in macrophages (Miller, Viriyakosol et al. 2003, Su, Ao et al. 2011, Chavez-Sanchez, Garza-Reyes et al. 2014), but also in monocytes (Chavez-Sanchez, Chavez-Rueda et al. 2010). Noteworthy is the study of Chavez-Sanchez and collaborators showing that the blockage of TLR2, TLR4 and CD14 with antibodies inhibits the release of the pro-inflammatory IL-1β and IL-6 and the antinflammatory IL-10 induced by mmLDL in both cell types (Chavez-Sanchez, Chavez-Rueda et al. 2010).

In this context, TLR2, TLR4 and CD14 were the putative receptors studied in this thesis as mediators of cytokine release induced by LDL(-) in monocytes.

2.2 CD14-TLR4 AS RECEPTORS MEDIATING THE INFLAMMATORY EFFECT OF LDL(-)

The binding experiments performed show that CD14 is the main receptor of LDL(-) in monocytes. It is demonstrated by a 67%-decrease of LDL(-) binding when it is neutralised by anti-CD14 antibody at 4ºC, the temperature at which there were no lateral movements and no other receptor could interfere. Moreover, the binding of LDL(-) to specific CD14-coated microtitter wells was 5-fold higher than in the case of LDL(+). The addition of a soluble form of CD14 (sCD14) diminished LDL(-) binding, as similarly described for LPS in co-incubation with sCD14 (Kitchens, Thompson et al. 2001). AntiTLR4 antibody could also impair LDL(-)-binding, although to a lesser extent (36%), and antiTLR2 did not produce significant effects. In a similar way to LPS, CD14 is the main receptor, although TLR4 may also recognize LDL(-), with no need for CD14 (Sweet and Hume 1996).

The next aim was to assess whether the increased binding to CD14 and TLR4 translated into the biological inflammatory effect of LDL(-). Therefore, the role of these receptors on mediating the cytokine release promoted by LDL(-) was studied. The LDL(-)-induced cytokine release was strongly inhibited with the neutralization of CD14 and TLR4, producing a decrease of 75%-80% and 70%-75%, respectively. It is important to note that the sum of the inhibition on cytokine release promoted by antiTLR4 and antiCD14 was over 100%, thereby suggesting that both receptors are working as a complex, where the inhibition of one of these entities affects the other. In these experiments, LPS was used as a positive control of cytokine release and had a parallel behaviour, since LPS mediates its inflammatory effect through CD14 and TLR4. Thus, similarly to LPS, after binding LDL(-), CD14 would associate with TLR4, thereby activating the intracellular signalling cascade leading to cytokine release. The role of CD14 and TLR4 in this regard was confirmed by gene silencing studies, in which cytokine release promoted by LDL(-) was also inhibited.

The involvement of CD14 in mediating LDL(-)-induced cytokine release was also evidenced by the addition of sCD14. This addition diminished the cytokine secretion promoted by LDL(-), which would be explained by a competition between sCD14 and CD14 present in the cell membrane for the binding of LDL(-). Moreover, THP1 cells overexpressing MD2-CD14 released significantly higher cytokine release than THP1 cells not expressing CD14. However, there was a small amount of cytokine release in THP1 and not THP1-CD14 monocytes could be attributed to TLR4 or TLR2, that are normally expressed in this cell line.

The activation of TLR4 by LDL(-) was corroborated by studying the initial steps of the intracellular pathways activated by this receptor. MyD88-dependent and independent pathways were studied. It was found that LDL(-) preferentially mediates IL-6, IL-10 and MCP-1 release through the TRAM-TRIF pathway, which is specific for TLR4, although it also induces TIRAP-MyD88 pathway. The LDL(-) pattern is parallel to that observed by Miller and coworkers for mmLDL (Miller, Viriyakosol et al. 2005). However, the levels of mmLDL-induced cytokine release found by these authors were lower than those of LPS, whereas the effects of LDL(-) were more similar to LPS.

The TLR-dependent signalling cascade leads to cytokine release mainly through the activation of NF-kB/AP-1. In this thesis, the use of THP1 cells possessing a reporter system for NF-kB/AP-1 highlights the activation of this transcription factor by LDL(-), and concurs with previous studies of the group (Bancells, Sanchez-Quesada et al. 2010).

In summary, LDL(-) thus induces IL-6, IL-10 and MCP-1 release in monocytes through the activation of CD14-TLR4 pathways, similarly to LPS.

2.3 COMPETITION BETWEEN LDL(-) AND LPS

The fact that LDL(-) and LPS use the same receptors and present similar inflammatory behaviour lead to hypothesize a putative competition between the two entities for CD14 and TLR4. Interestingly, the coincubation of both stimuli produced cytokine release levels that resembled those of LDL(-) alone. Thus, LDL(-) was to some extent

promoting the reduction of the LPS inflammatory effect. This could be explained by a competition between the two stimuli on binding for the complex CD14-TLR4. Indeed, LDL(-) showed a decreased binding either to monocytes or to CD14-coated wells when LPS was added, and this occurred in an LPS concentration-dependent manner.

Native LDL and other lipoproteins such as HDL are reported to block LPS in certain infections (Weinstock, Ullrich et al. 1992, Park, Kim et al. 2007). However, the competition between LPS and LDL(-) was not due to a higher inactivation of LPS by LDL(-) than by LDL(+), as occurred in the LAL test here performed.

In this thesis, no competition on binding has been observed between oxLDL and LPS. However, controversial results have been reported elsewhere. Oxidized PL present in atheromatous lesions inhibits LPS-induced TLR signalling although with no direct binding of oxLDL to the receptors (Hamilton, Ma et al. 1990, Bochkov 2007, Kannan, Sundaram et al. 2012). However, other authors found a synergic inflammatory effect between oxLDL and LPS (Wiesner, Choi et al. 2010).

In contrast to lipoproteins modified in vitro, LDL(-) is present in the blood in all individuals. The plasma inflammatory cytokine release level could not only depend on the LPS but also on the LDL(-) concentration. In this regard, in the absence of bacterial infection, CD14-TLR4 activation mediated through LDL(-) would be deleterious in case of high LDL(-) levels. However, this effect would be lower than that induced by LPS at high concentrations such as in infection situations, and normal levels of LDL(-). In summary, the global inflammation would depend, among other factors, on the relative concentrations of LDL(-) and LPS. Although the counteracting action of LDL(-) on the inflammatory LPS effect is found *in vitro*, it does not discard that this effect may also occur in a physiopathologic context *in vivo*.

The concentration of LDL(-) used in all the assessments was 150 μg/L, the highest concentration found in normolipemic subjects (Sanchez-Quesada, Otal-Entraigas et al. 1999). The experiments of cytokine release were performed at LPS concentrations of 100 μg/L (100,000 pg/mL), levels reported to be useful for a positive inflammatory control in in vitro studies (Benitez, Bancells et al. 2007). When both agents coexist at

these concentrations, a competition for binding to CD14-TLR4 receptors is observed. The plasma LPS level in septic patients and in low-grade but sustained chronic infections is around 800 pg/mL (Opal, Scannon et al. 1999, Wiesner, Choi et al. 2010). This concentration is lower than that used in this thesis, but preliminary data derived from this study show that the cytokine release of LPS at 1000 pg/ml can be neutralized even at lower LDL(-) concentrations (50 µg/L) (Tomás-Hernández 2012). It cannot therefore be ruled out that LDL(-) could neutralize LPS when it is present in blood at this or even at lower concentrations.

Low concentrations of LPS in circulation -endotoxemia- are found in healthy subjects. These individual can suffer the silent attack of the organism vasculature, allowing LPS to enter circulation. This entrance of LPS is associated not only to recurrent bacterial infections (Hasday, Dubin et al. 1996, Vassallo, Mercie et al. 2012) but also to high-fat meal ingestion (Amar, Burcelin et al. 2008). In the case of diabetes mellitus patients, LPS levels rise to 600 pg/mL (Wiesner, Choi et al. 2010). Because diabetes mellitus is a high cardiovascular risk pathology, an increase in LPS in plasma has been related to a higher risk of atherosclerosis (Wiedermann, Kiechl et al. 1999, Clemente-Postigo, Queipo-Ortuno et al. 2012). The inflammatory action of LPS in these situations could be diminished by the presence of LDL(-), which could exert a protective role because of its competition with LPS.

This role of LDL(-) in counteracting the cytokine release of LPS concurs with other anti-inflammatory properties already ascribed to this modified LDL form. LDL(-) induces the release of IL-10 in monocytes and lymphocytes (Benitez, Bancells et al. 2007). IL-10 is a cytokine that not only exerts anti-apoptotic effects on macrophages (Halvorsen, Waehre et al. 2005) but also regulates the production of other anti-inflammatory cytokines (Terkeltaub 1999). The release of IL-10 by LDL(-) is thereby a mechanism to control its own inflammatory response (Benitez, Bancells et al. 2007). Another anti-inflammatory mechanism of LDL(-) is the activation of Nrf-2, an antiapoptotic agent (Pedrosa, Faine et al. 2010). LDL(-) can also degrade oxidized phospholipids to minor inflammatory components, thanks to the PAF-AH (Benitez, Sanchez-Quesada et al. 2003) and PLC-like intrinsic activities (Bancells, Benitez et al. 2009). LDL(-) could thus

exert either an inflammatory or an anti-inflammatory role, depending on different conditions.

3. PUBLICATION 3: CER IS RESPONSIBLE FOR THE INFLAMMATORY EFFECT OF LDL(-) THROUGH CD14-TLR4

As we have seen, the cytokine release induced by LDL(-) in monocytes is mediated through the receptors CD14-TLR4. The content of CER is increased in LDL(-), and CER-LDL reproduces some of the main properties of LDL(-). We thus hypothesized that CER-LDL could also induce cytokine release through the activation of the CD14-TLR4 pathway. The role of TLR4 on CER-LDL-induced cytokine release was highlighted when the TLR4 receptor was blocked with a specific inhibitor. This inhibitor decreased cytokine release induced by CER-LDL by up to 90%. Moreover, the involvement of CD14 in the cytokine release induced by CER-LDL was corroborated in a monocytic THP1 cell line overexpressing CD14 (THP1-CD14). In this cell line, IL-6, IL-10 and MCP-1 release by CER-LDL was much higher than in common THP1 cells, whose cytokine release was almost undetectable even though they possessed TLRs. Therefore, CER-LDL needs the presence of CD14 to mediate the secretion of IL-6, IL-10 and MCP-1 in human monocytes, and no direct effect of CER to TLR4 was found. CD14 would detect CER from CER-LDL and then form a complex with TLR4 to activate the intracellular signalling cascade, coinciding with the described CER docking to CD14 in lipid rafts formed *in vivo* (Pfeiffer, Bottcher et al. 2001). As described, and in contrast to CER-LDL, LDL(-) can also bind directly to TLR4 in a similar way to LPS.

Lightle and collaborators (Lightle, Tosheva et al. 2003) proposed that cells present in the atheromatous plaque accumulate CER delivered from LDLs, probably through cell receptors. In this line, CER in lipoproteins have specifically been described to bind the LPS inflammatory receptor CD14 and induce its clustering to other receptors in cell lipid rafts (Pfeiffer, Bottcher et al. 2001). CER present in cell lipid rafts tend to accumulate closed to CD14 (Wang, Kitchens et al. 1995, Simons and Ikonen 1997),

possibly because both LPS and CER share some structural similarities (Joseph, Wright et al. 1994).

LPS and CER induce a differential recruitment of receptors into lipid rafts despite their binding to the same receptor CD14 (Pfeiffer, Bottcher et al. 2001). The involvement of CER in TLR4 signalling has been reported (Fischer, Ellstrom et al. 2007). Specific cellular microdomains enriched in CER also possess the ability to regulate TLR4 activation through protein kinase C (PKC) (Cuschieri, Billigren et al. 2006).

Fischer and collaborators reported that CER can activate TLR4 signalling (Fischer, Ellstrom et al. 2007). However, the fact that CER-LDL activates cytokine release through CD14, and only slightly through TLR4, agrees with other previous studies (Jozefowski, Czerkies et al. 2010, Hankins, Fox et al. 2011). In this study it is shown that the products derived from the CER metabolism could exert a regulatory effect on the LPS-induced TLR4-dependent cytokine release. They thereby concur with the counteracting action of LDL(-) on LPS-induced cytokine release.

The fact that the enrichment of LDL in CER is essential for its recognition by CD14 concurs with studies showing that high levels of CER modify the surface structure of LDL (Oorni, Pentikainen et al. 2000). These modifications would enhance its recognition by CD14 and then promote the induction of cytokine secretion, not only in CER-LDL but also in LDL(-).

Apart from CER, other components increased in LDL(-) could participate in CD14-TLR4 activation. It has been described that NEFA work as ligands of TLR4 capable to activate NF-kB and induce TNF-α release in macrophages (Suganami, Tanimoto-Koyama et al. 2007). Therefore, NEFA should be evaluated as putative components of LDL(-) responsible for inducing cytokine release through the CD14-TLR4 system.

In conclusion, although other components are also involved, this work shows that CER plays a pivotal role in mediating the inflammatory effects of LDL(-) through CD14-TLR4**.**

4. IMPORTANCE OF THE RESULTS

The main findings in this thesis are, on one hand, the description of CD14 and TLR4 as the receptors involved in mediating the cytokine release induced by LDL(-) in monocytes and, on the other hand, the elucidation of the role of the increased content in CER present in LDL(-) in the activation of this pathway.

CD14-TLR4 are the receptors shown to bind LDL(-) and mediate its inflammatory effect in monocytes. These data not only elucidate the beginning of the cellular response but also suggest the next steps in the putative intracellular signalling pathway activated by LDL(-). Since these receptors commonly recognise LPS, the competition between LDL(-) and LPS in binding leads to a competition on cytokine release, possibly being a compensatory mechanism in cases of overwhelming inflammation. LDL(-) would then reduce the high-grade systemic inflammation induced by LPS to yield a chronic but sustained inflammatory state. However, a possible protective role for LDL(-) in cases of a lower LPS concentration should not be overlooked.

The fact that both LPS and LDL(-) activate a very similar and coordinated inflammatory pathway suggests that our organism recognises LDL(-) as a danger signal and thereby responds by triggering inflammation in atherosclerosis as if it was an infectious state. The activation of TLRs by LDL(-) implies that this lipoprotein activates the innate immune response but it has been reported that LDL(-) can also activate the adaptive immunity (Oliveira, Sevanian et al. 2006). Interestingly, in the postprandial state, both an increase in the LDL(-) proportion (Ursini, Zamburlini et al. 1998) and in the levels of endotoxemia (Clemente-Postigo, Queipo-Ortuno et al. 2012) have been described. However, as stated above, the increase in LDL(-) in this situation could be a mechanism to counteract excessive inflammation.

Among the putative inflammatory components forming LDL(-), the increase in CER due to the presence of the PLC-like activity in LDL(-) is a key inducer of the cytokine release in monocytes. These data allow us to hypothesize that LDL(-) is formed by the modification of LDL(+) with PLC-like and/or PAF-AH activities. This concurs with an increase in these activities in aged LDLs (Bancells, Benitez et al. 2008) and LDLs kept at

37ºC, and a decrease in the phospholipolytic products derived from the treatment with HDL.

Our data also show that CER is the main compound in LDL(-) that mediates the activation of the CD14-TLR4 pathway. However, the cytokine release induced by CER-LDL is lower than that induced by LDL(-), suggesting other components present in LDL(-) are involved in this effect. This includes NEFA and CER metabolites, which could be generated from PAF-AH activity and CER degradation, respectively.

CER is not always present as an active form able to induce cytokine release. CER in lipoproteins or its content in cell lipid rafts serve as a storage of this bioactive lipid ready to be released in case of any inflammatory input (Mathias, Pena et al. 1998). Thus, some CER in CER-LDL and LDL(-) could also be transferred to cell lipid rafts and kept on a transient state instead of directly triggering cytokine release. Thereby, CER forming part of LDLs such as LDL(-) could mediate but also avoid an excessive inflammatory response in cells. Similarly to LDL(-), CER also regulates the LPS inflammatory signalling, since the CER-metabolite ceramide-1-phosphate (C1P) has structural similarities to those of LPS (Joseph, Wright et al. 1994) and both are described to compete in the cytokine induction through TLR4 (Hankins, Fox et al. 2011).

Apart from promoting cytokine release in monocytes, the increased CER content in LDL(-) induces a high susceptibility to aggregation in the particle. This aggregation is not the origin of the inflammatory effect, but it could facilitate the aggregation of other lipoproteins and the binding to proteoglycans present in the arterial wall.

Besides its role in atherosclerosis, LDL(-) is involved in diseases associated with an increased cardiovascular risk. Levels of LDL(-) increase in pathologies like FH, diabetes mellitus and renal disease, whereas they decrease with the drugs used to treat these diseases. The action of statins (Sanchez-Quesada, Otal-Entraigas et al. 1999), aspirin (Chang, Chen et al. 2013) and insulin (Sanchez-Quesada, Perez et al. 2001) decrease the percentage of LDL(-) in plasma. It has thus been hypothesized that LDL(-) could serve as a biomarker of cardiovascular risk (Oliveira, Sevanian et al. 2006, Sánchez-

Quesada, Estruch et al. 2012).. However, the relative contribution of each modification in the total pool of LDL(-) varies in each associated pathology, but should be elucidated for a more specific diagnosis (Sánchez-Quesada, Estruch et al. 2012). ELISA assay kits have been developed to detect LDL(-) autoantibodies and immune complexes (Faulin Tdo, de Sena-Evangelista et al. 2012). Apart from being a putative marker of cardiovascular risk, LDL(-) could have a role as a marker for coronary events, since it has been described that LDL(-) and antiLDL(-) autoantibodies are three times higher in unstable angina than in stable angina (Oliveira, Sevanian et al. 2006). Nevertheless, the fact that LDL(-) could act as a compensatory mechanism of inflammation in certain circumstances, such as in cases of infection, would add controversy to the use of LDL(-) as a biomarker molecule.

LDL(-) could be involved in the therapeutics of inflammatory diseases. As in the case of LDL(-), the inflammation induced by compounds such as CER and/or by the activation of CD14-TLR4 pathway is a common process. In pathologies with excessive inflammation, therapies not totally suppressing the immune system, such as the inhibition of TLR ligands, may be useful (Piccinini and Midwood 2010). CER inhibitors also serve as antineoplastic drugs (Ruvolo 2003, Seki, Nakashima et al. 2011), suggesting that LDL(-) could play a role in cancer. Interestingly, modified LDLs have been suggested to play a role in brain pathologies since they can pass through the brain barrier and localize in the cerebrospinal fluid (Danik, Champagne et al. 1999). In demential pathologies such as Alzheimer disease, lipoprotein aggregation, CD14 expression and cytokine release occur to a higher extent than in healthy subjects (Maccioni, Farias et al. 2010, Torres, Lima et al. 2014). Thus, it is feasible that LDL(-) could be involved in diseases associated with the formation of senile plaques, in which accumulation of CER and SMases have been found (Panchal, Gaudin et al. 2014). CER inhibitors have been developed as a therapeutic strategy in Alzheimer disease (Cervia, Perrotta et al. 2013).

In conclusion, this thesis provides further data regarding the cellular mechanisms activated by LDL(-) and suggests the involvement of LDL(-) in inflammation and in

related diseases. Deeper knowledge of the mechanisms surrounding LDL(-) biology and its effects is of great importance and further studies should be performed.

5. FUTURE STUDIES

The results from this thesis show some of the mechanisms that are activated by LDL(-) in the inflammatory response in monocytes. This topic is currently being continued by our group to amplify our knowledge of these LDL(-) actions, in atherosclerosis and in other inflammatory diseases. The lines on which we are working are the following.

First, we aim to study the complete range of components in LDL(-) that participate in cytokine release in cells. LDL(-) could have a ceramidase activity, able to degrade CER into inflammatory products such as SPH and S1P. Such products could play a role in the cytokine release induced by this lipoprotein. Our preliminary results show that LDL(-) degrades CER, and that both SPH and S1P play a role in the MCP-1 release induced by LDL(-) (Estruch, Sanchez-Quesada et al. 2014). We will also study the putative activation of CD14-TLR4 by the SPH and S1P metabolites, as well as by NEFA.

Second, we hope to elucidate the complete downstream signalling that leads to cytokine release induced by LDL(-) in monocytes. Some results derived from this thesis show that after docking CD14-TLR4, LDL(-) triggers cytokine release through the activation of p38-MAPK and NF-KB in these cells (Estruch, Sanchez-Quesada et al. 2014).

Third, we are also working to determine the release of other cytokines activated by LDL(-) in monocytes. The fact that LDL(-) shares CD14 and TLR4 receptors with LPS to induce cytokine release suggests that other cytokines secreted by LPS can also be activated by LDL(-). We are studying the LDL(-)-induced IL-1β release in monocytes. For the IL-1β secretion, the NLRP3 inflammasome and caspase-1 activation are required. Thus, the secretion of IL-1β by LDL(-) would imply the activation of the inflammasome pathway and increase our knowledge about the role of LDL(-) in inflammation. Some preliminary results are already available (Estruch, Rajamäki et al. 2014).

Fourth, we plan to study the effect of LDL(-) in macrophages. Although CD14 is poorly expressed in this cell type, these cells possess TLRs (Krishnan, Selvarajoo et al. 2007) that could mediate the inflammatory response induced by LDL(-). We will also analyse the putative LDL(-)-induced foam cell formation in macrophages. Foam cell formation is a process in which TLRs have been also reported to participate (Chavez-Sanchez, Garza-Reyes et al. 2014). However, one putative receptor that could feasibly induce uptake of LDL(-) and foam cell formation is LRP-1. LRP-1 can uptake CER-enriched LDLs or LDLs treated with SMase, as well as aggregated LDL (Llorente-Cortes and Badimon 2005). We will then assess whether LDL(-) promotes foam cell through TLRs or through LRP-1.

FIfth, we hypothesize that the cytokine secretion induced by LDL(-) in monocytes from patients with an increased inflammatory state may differ from that in normolipemic subjects. In this regard, we are currently studying the effect of LDL(-) and monocytes isolated from type II diabetic patients and its association with the activation of CD14-TLR4 receptors. Although we do not yet have results, it has been reported that the amount of LDL(-) and the levels of CER and NEFA in LDL are increased in patients with diabetes mellitus type I and II (Amati, Dube et al. 2011, Dasu and Jialal 2011, Hussey, Lum et al. 2014). Evidence also shows that CD14-TLR4 signalling is increased in these patients (Cipolletta, Ryan et al. 2005, Reyna, Ghosh et al. 2008, Dasu, Devaraj et al. 2010). We will study the cytokine release induced by LDL(-) in these patients and the compensatory role of this lipoprotein in LPS-induced inflammation. These results will be compared in normolipemics.

And finally, we also plan to study the involvement of LDL(-) in other diseases where inflammation is a key phenomenon. In Alzheimer disease it is feasible that LDL(-) activates CD14 and TLR4 in the brain. This hypothesis is supported by the presence of modified lipoproteins (Danik, Champagne et al. 1999), and an increased content of CER (Panchal, Gaudin et al. 2014) and CD14-TLR4 receptors (Saresella, Marventano et al. 2014) in the brain tissue. In a similar manner, LDL(-) could also be involved in the inflammation underlying cancer (Joseph, Wright et al. 1994).

In conclusion, this thesis studies the role of CER in the inflammatory properties of LDL(-). It shows that the increased CER content in LDL(-) mediates the activation of CD14-TLR4 leading to cytokine release in human monocytes. These results open new paths of research to improve our knowledge about the inflammatory response activated by LDL(-) in plasma and its relation to atherosclerosis and other inflammatory diseases.

Chapter VI

CONCLUSIONS

From the results presented in this thesis, we present the following conclusions:

1. LDL(-) induces IL-6, IL-10 and MCP-1 release in monocytes, partly due to its increased CER content. The high levels of CER in LDL(-) are also involved in the increased susceptibility to aggregation of this lipoprotein. The origin of the greater CER amount is the presence of PLC-like activity intrinsic in LDL(-).

2. The incubation of LDL(-) with HDL promotes the transfer of PLC-like activity and its resulting products from LDL(-) to HDL. In LDL(-), this incubation triggers, a decrease in CER content, a lower cytokine release and a decreased susceptibility to aggregation of the lipoprotein.

3. The complex CD14-TLR4 mediates the release of IL-6, IL-10 and MCP-1 promoted by LDL(-) in monocytes. LDL(-) binds to the cell receptor CD14 and promotes the formation of a complex with TLR4, thereby inducing the intracellular signalling that leads to cytokine release. To a lesser degree, LDL(-) can directly interact with TLR4 to activate the intracellular signalling. However, TLR2 does not exert a significant effect in the LDL(-)-induced inflammatory action.

4. The increased CER content in LDL(-) plays a role in docking this lipoprotein to CD14, thereby activating TLR4 and leading to cytokine release in monocytes.

5. We observed a competition between LDL(-) and LPS for CD14 binding and for promoting cytokine release in monocytes. This might be explained because CD14 and TLR4 are also receptors used by LPS to exert its inflammatory response in these cells. This competition suggests that, under certain conditions and depending on their relative concentrations, LDL(-) could counteract the inflammatory effects of LPS.

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SUPPLEMENTARY DATA

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Review Article

Electronegative LDL: A Circulating Modified LDL with a Role in Inflammation

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Electronegative low density lipoprotein (LDL(−)) is a minor modified fraction of LDL found in blood. It comprises a heterogeneous population of LDL particles modified by various mechanisms sharing as a common feature increased electronegativity. Modification by oxidation is one of these mechanisms. LDL(−) has inflammatory properties similar to those of oxidized LDL (oxLDL), such as inflammatory cytokine release in leukocytes and endothelial cells. However, in contrast with oxLDL, LDL(−) also has some anti-inflammatory effects on cultured cells. The inflammatory and anti-inflammatory properties ascribed to LDL(−) suggest that it could have a dual biological effect.

1. Introduction

The inflammatory properties of modified LDLs are a main topic in atherosclerosis research. In addition to their inflammatory properties, modified LDLs are recognized by the scavenger receptor (SR), leading to the formation of lipid-loaded foam cells, typical of atherosclerotic lesions. LDL can be modified in arterial intima and in plasma circulation by several mechanisms, such as glycation, lipolysis, aggregation, and oxidation [1]. Oxidized LDL (oxLDL) and minimally modified LDL (mmLDL), a mild oxidized LDL, are the most widely studied modified LDLs in the literature. The involvement of oxLDL and mmLDL in atherogenesis and inflammation in the arterial wall is well established [2], but they have been detected in blood only at a very low concentration [3].

Electronegative LDL (LDL(−)) is a modified circulating form of LDL found in blood. It is an LDL subfraction with a high negative charge that constitutes about 3–5% of the total LDL in normolipidemic (NL) subjects. Its existence was first reported by Avogaro in 1988 [4]. Numerous studies focusing on LDL(−) have since been performed, and the most widely accepted idea is that LDL(−) is a pool of LDL particles modified by several mechanisms.

LDL(−) has several physicochemical characteristics that differ from native LDL (hereafter referred to as LDL(+)) [5, 6]. Regarding lipid and protein composition, LDL(−) has a higher content of triglycerides [7], nonesterified fatty acids (NEFA) and lysophosphatidylcholine (LPC) [8], and ceramide (CER) [9] than LDL(+). It also shows associated phospholipolytic activities that are absent in LDL(+) [10, 11]. LDL(−) has an abnormal apolipoprotein B (apoB) conformation, which seems to play a role in both its greater binding to proteoglycans (PG) and greater susceptibility to aggregation than LDL(+) [12]. These physicochemical properties are likely responsible for its biological effects in different cell types that participate in the atherosclerotic process.

Early studies regarding the biological effects of LDL(−) were performed in endothelial cells. It was found that LDL(−) promoted cytotoxicity [13, 14] and release of inflammatory cytokines [7]. The cytokine release effect has since been reported in monocytes and lymphocytes [15]. These observations support an atherogenic role for LDL(−). Nevertheless,

recent data suggest that LDL(−) may not only have such an inflammatory role as was first thought. Studies in mononuclear cells have shown that LDL(−) has the ability to induce anti-inflammatory cytokine IL10 [15] and counteract the inflammatory effect promoted by lipopolysaccharide (LPS) [16].

This review focuses on the biological effect of LDL(−) on cells, emphasizing its role in monocytes, which are pivotal to the inflammatory response in atherosclerotic lesions. We discuss the dual function of LDL(−), inflammatory and anti-inflammatory, and its physiological role.

2. A Heterogeneous LDL

Although LDL(−) was first considered an oxidized particle in the circulation, it is now widely accepted to be a pool of modified LDLs with different properties but sharing the common feature of increased electronegativity. Nowadays, LDL(−) heterogeneity is considered a consequence of its different origins.

The oxidative origin of LDL(−) is controversial. Avogaro et al. and Sevanian et al. reported that LDL(−) has a lower vitamin E content [17], a higher amount of lipoperoxides and oxidized cholesterol [14, 17], and a higher susceptibility to oxidation [18] than LDL(+). However, other studies do not replicate these findings [19, 20]. Chen and coworkers focused their research on the most electronegative LDL subfraction, the so-called L5, detected in dyslipidemic patients but not in NL subjects [21]. They described that L5 is a mild oxLDL subfraction contained in the whole pool of LDL(−). The observation that L5 is a minor LDL(−) subfraction is in agreement with the oxLDL proportion found in blood (0.1–0.5% of total LDL) [3] compared to the LDL(−) proportion (3–5%) [5].

It has been suggested that LDL modifications other than oxidation contribute to the generation of LDL(−). Such modifications include nonenzymatic glycosylation, NEFA enrichment, and modification by phospholipolytic enzymes: phophospholipase A2 (PLA2) and sphingomyelinase (SMase) [1]. These modifications are known to increase the negative charge of LDL and likely to occur not only in blood but also in the arterial intima. It is described that in the arterial intima of atherosclerotic lesions there is an overexpression of PLA2 and SMase [22, 23], which could generate LDL(−).

LDL(−) is heterogeneous in size and density. This heterogeneity seems to depend on the mechanism involved in the generation of the particle. LDL(−) are small-dense particles in NL subjects and large-buoyant particles in familial hypercholesterolemic (FH) subjects, whereas hypertriglyceridemic patients can present both dense and light particles [24].

LDL(−) is also heterogeneous in its lipid and protein content. Compared to native LDL, it has an increased content of several non-apoB apolipoproteins: apoE, apoCIII, apoAI, apoAII, apoD, apoF, and apoJ [25]. Besides apolipoproteins, LDL(−) has a higher content in platelet-activating factor acetylhydrolase (PAF-AH) than LDL(+), leading to an increase in its enzymatic activity. Another enzymatic activity found in LDL(−) is the phospholipase C (PLC)-like activity [11]; its origin in LDL(−) is unknown, and it is absent in LDL(+). Both enzymatic activities in LDL(−) could be responsible for the altered lipid content in LDL(−), including its higher content in NEFA, LPC, and CER than LDL(+). These three lipid components are related to the inflammatory effect of LDL(−) on cultured cells [8, 9, 26]. The increased NEFA and LPC content in LDL(−) seems to be generated by hydrolysis of choline-containing phospholipids by PAF-AH activity [5] and the increased CER content by hydrolysis of sphingomyelin by PLC-like activity [9].

Finally, the heterogeneity of LDL(−) is also suggested by the presence of a minor proportion of an aggregated subfraction (agLDL(−)). AgLDL(−) seems to be responsible for the PLC-like activity of LDL(−), since such activity is mainly present in agLDL(−) [27]. It has been described that the heterogeneity in the aggregation level is responsible for LDL(−) populations with a normal or high binding affinity to PG compared to native LDL [12]. A relationship between aggregation and the abnormal apoB conformation of LDL(−) also exists [12].

3. An Atherogenic LDL

Several inflammatory effects have been ascribed to LDL(−), and they are probably a consequence of the combination of the different LDL(−) physicochemical properties (Figure 1). These inflammatory effects and other evidence described in this section suggest that this modified LDL could play an atherogenic role and be a putative biomarker of cardiovascular risk, as suggested elsewhere [28, 29]. The usefulness of LDL(−) as a biomarker in the diagnosis of cardiovascular risk should be determined in large cohorts of patients, but methods to do this are still under development [28].

3.1. Increased LDL(−) Proportion in Inflammation. The first evidence of the relationship between LDL(−) and atherosclerosis is the increased proportion of LDL(−) in subjects with pathologies known to be associated with cardiovascular risk and inflammation. These pathologies include FH [30], hypertriglyceridemia [24], type 1 and type 2 diabetes mellitus (DM) [31, 32], chronic kidney disease requiring hemodialysis [33, 34], and rheumatoid arthritis [35]. LDL(−) is also increased in patients with acute myocardial infarction [36] and angiographically documented coronary artery disease [37]. In each pathology, the mechanisms involved in LDL(−) generation likely depend on the individual characteristics and the underlying disease of the patients. Some drugs administered to treat DM and FH, such as insulin and statins, decrease the proportion of LDL(−), besides decreasing the cardiovascular risk [30, 32].

Moreover, a high LDL(−) proportion has been associated with a worse lipid profile since there is a positive correlation of LDL(−) proportion with nonhigh density lipoprotein cholesterol (non-HDLc) and a negative correlation with HDLc [38].

3.2. Immunological Response Induced by LDL(−). It has been described that LDL(−) can trigger an adaptative immune response, leading to the production of anti-LDL(−)-autoantibodies and immunocomplexes, which can be quantified by ELISA [39]. The presence of these autoantibodies is increased in DM [40] and in acute coronary syndromes [41].

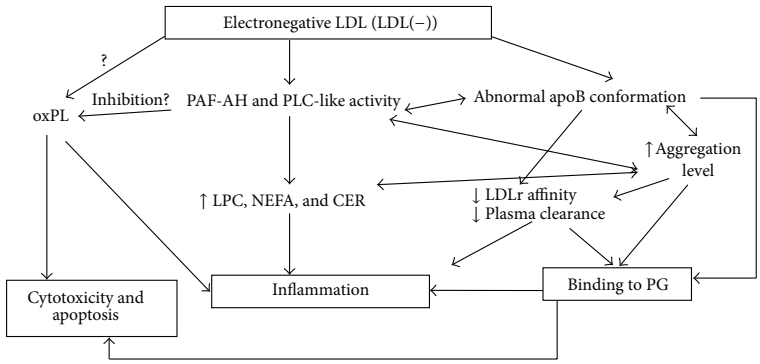

Figure 1: Putative relationships between the physicochemical properties of LDL(−) and its inflammatory actions. Phospholipolytic activities contained in LDL(−) increase its LPC, NEFA, and CER content. These compounds are involved in the inflammatory action of the particle. Phospholipolytic activities could also be related to the abnormal apoB conformation and high aggregation of LDL(−), which may contribute to its decreased plasma clearance and increased binding to PGs. The retention of LDL(−) to endothelium by PG would favor the inflammatory action of LDL(−) on the arterial wall cells. Some authors have suggested that the presence of oxPL in LDL(−) is responsible for the inflammatory, cytotoxic, and apoptotic effects of this particle. LDL(−): electronegative LDL, oxPL: oxidized phospholipids, PAF-AH: platelet-activating factor acetylhydrolase, PLC: phospholipase C, LPC: lysophosphatidylcholine, NEFA: nonesterified fatty acids, CER: ceramide, apoB: apolipoprotein B, LDLr: LDL receptor, PG: proteoglycans.

Grosso et al. reported that anti-LDL(−)-autoantibodies administered intravenously in mice can play a protective role in atherosclerosis [42]. Taken together, it seems that anti-LDL(−)-autoantibodies could be useful biomarkers in patients with high risk for coronary events [39, 41].

3.3. Apoptotic and Cytotoxic Effects of LDL(−). Some authors have reported that LDL(−) has cytotoxic properties in cultured endothelial cells. This was considered due to its high content of oxidized cholesterol [14, 43]. In contrast, other authors have reported that LDL(−) has no cytotoxic effect [7, 15] or that its cytotoxic effect is due to mechanisms other than oxidation [13]. The divergence in results is probably a consequence of the LDL(−) heterogeneity.

There is an agreement that LDL(−) induces apoptosis. Chen and colleagues reported that the highly electronegative LDL subfraction L5 promoted apoptotic effects on endothelial cells through a decrease in fibroblast growth factor 2. This induction of apoptosis was found for L5 isolated from FH [44, 45], DM [46, 47], and smokers [48]. The apoptotic effect was suppressed in the presence of low concentration of aspirin [36]. These authors attributed the apoptotic ability of L5 to oxidation. However, the apoptotic effect could be due to the increased CER content in LDL(−) since CER is an inductor of apoptosis [49]. An apoptotic effect of LDL(−) was also shown in macrophages [50] and in cardiomyocytes [51]. In the latter study, it was found that apoptosis was induced by culture-conditioned medium of endothelial cells incubated with LDL(−). In addition, LDL(−) has been described to induce in lymphocytes and macrophages the gene expression and membrane-bound protein of Fas [50, 52], a factor that triggers extrinsic pathway of apoptosis [53].

At subapoptotic concentrations, however, L5 impairs differentiation of endothelial progenitor cells and inhibits endothelial cell regeneration and neovascularization [48]. In endothelial cells, L5 also inhibits reendothelization [46], growth, and survival signaling [54] and activates cell stress by promoting inflammation and mitochondrial dysfunction [55].

3.4. Inflammatory Properties of LDL(−). There is consensus that LDL(−) induces an inflammatory response on cells participating in the atherosclerotic process. The most important effect induced by LDL(−) is the release of cytokines, particularly in endothelial and mononuclear cells. Figure 2 summarizes the role of LDL(−) in atherogenesis in relation to the inflammatory effects promoted on cells.

3.4.1. Effects on Endothelial Cells. The endothelium is the physical barrier between blood and the vessel wall. Endothelial cells control important physiological processes, including cellular trafficking. They also control the recruitment of circulating monocytes and lymphocytes to the arterial endothelium. Infiltration of these circulating cells to sites of inflammation is one of the earliest events in atherosclerosis. It has been described that LDL(−) attracts monocytes and lymphocytes to endothelial cells [21, 56], suggesting its participation in the early phases of atherosclerosis. It has been reported that LDL(−) promotes this attraction by inducing adhesion molecules and chemokine release in endothelial cells. In relation to adhesion molecules, LDL(−) induces vascular cell adhesion molecule (VCAM) [56, 57]. The induction of chemokine release by LDL(−) was first reported by De Castellarnau et al. who observed that LDL(−) promotes monocyte chemotactic protein 1 (MCP1) and interleukin 8 (IL8) release in human umbilical vein endothelial cells (HUVEC) [7]. MCP1 and IL8, respectively, induce the recruitment of monocytes and T lymphocytes to the endothelium. The release of

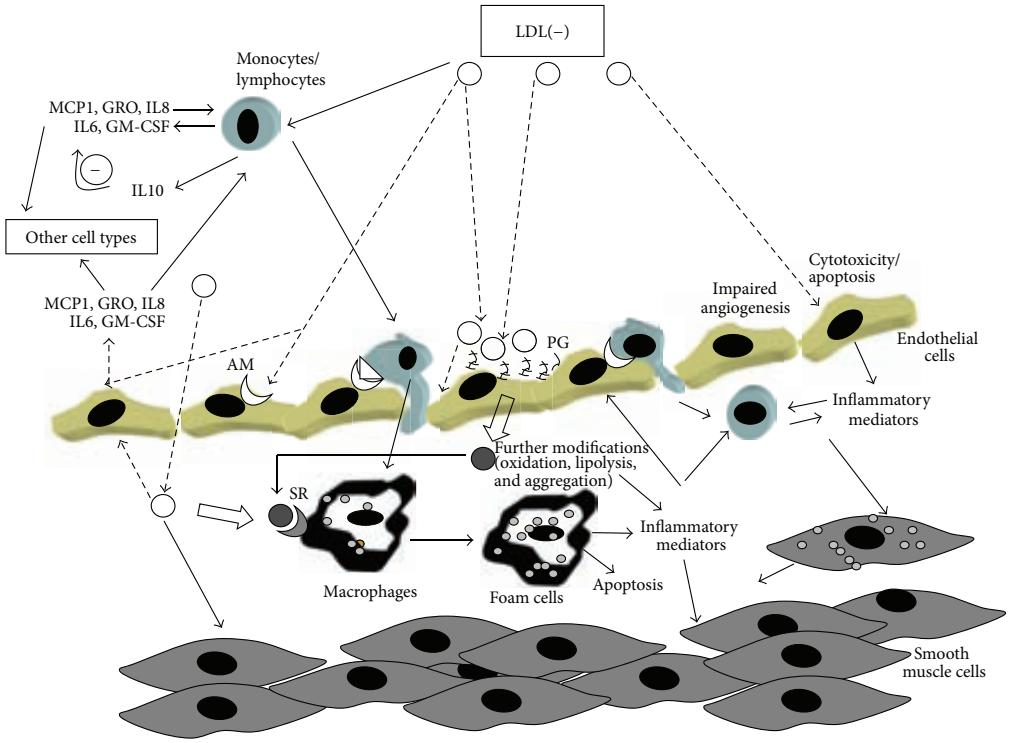

Figure 2: Biological actions of LDL(−) on circulating mononuclear cells (monocytes/lymphocytes) and arterial wall cells (endothelial cells, macrophages and smooth muscle cells) in relation to atherogenesis. LDL(−) can activate circulating leukocytes, mainly monocytes, and lymphocytes. LDL(−) also induces chemokine and adhesion molecules in endothelial cells, promoting the recruitment of more circulating leukocytes to endothelium. Cytokines released by endothelial cells can also act on other cell types of the arterial wall. LDL(−) retained in the subendothelial space by its increased binding to PG can also stimulate arterial wall cells. In this environment, LDL(−) could be further modified, leading to additional inflammatory actions on cells. It could also be uptaken by SR, promoting the formation of foam cells. LDL(−): electronegative LDL, MCP1: monocyte chemoattracting-protein 1, GRO: growth-related oncogen, IL6, IL8, and IL10: interleukin 6, 8, and 10, GM-CSF: granulocyte monocyte-colony stimulating factor, SR: scavenger receptor, AM: adhesion molecule, PG: proteoglycans.

these chemokines in HUVEC has been reported for LDL(−) isolated from NL [7], FH [20], and DM subjects [58]. As the LDL(−) proportion is higher in FH and DM than in NL, the inflammatory effect promoted by LDL(−) should be greater in these patients than in NL subjects.

Further studies in HUVEC have shown that LDL(−) induces other inflammatory cytokines, such as interleukin 6 (IL6), growth-related oncogen (GRO), granulocyte-monocyte-colony stimulating factor (GM-CSF) [59], and epithelial cell-derived neutrophil-activating peptide 78 [56]. The cytokine release promoted by LDL(−) has been reproduced in cultured human endothelial cells of arterial origin [60]. In bovine arterial endothelial cells, the most electronegative subfraction L5 also induces secretion of matrix metalloproteinases and vascular endothelial growth factor expression [45].

3.4.2. Effects on Monocytes and Lymphocytes. Besides endothelial cells, lymphocytes and particularly monocytes play a pivotal role in atherogenesis and inflammation by secreting cytokines and growth factors. As they are present in blood, it is highly feasible that they interact with LDL(−). For this reason, the interaction between mononuclear cells and LDL(−) has been a focus for study in recent years. It has been observed that LDL(−) induces the release of the same cytokines in mononuclear cells, monocytes, and lymphocytes, as in endothelial cells [15]. However, LDL(−) induces anti-inflammatory IL10 in mononuclear cells [15], but not in endothelial cells [59]. The putative physiological role of the IL10 production and other theoretically anti-inflammatory actions promoted by LDL(−) will be discussed further on.

Cytokine induction by LDL(−) in monocytes and lymphocytes occurs both at RNA and protein levels [15]. In a genomic study it was shown that LDL(−) modifies the transcription of other genes related to inflammation and atherosclerosis in mononuclear cells. Among these modifications, LDL(−) promotes Fas upregulation, colony stimulating factor 1 receptor (CSF1R), and CD36 downregulation [52]. Fas

has been reported to be involved in apoptosis and in cytokine induction [53, 61]. Therefore, Fas induction could be related to these biological effects of LDL(−).

3.4.3. Increased Affinity to Proteoglycans. LDL(−) presents higher affinity to PG than LDL(+). Aggregation of LDL(−), mediated by its PLC-like activity, seems to be important in its binding to PG since agLDL(−) is the LDL(−) subfraction that has the highest affinity to PG [12]. It has been hypothesized that alterations in the N-terminal extreme of apoB could be responsible for this increased binding [12]. LDL(−) could also act as a seeding factor since its aggregation stimulates aggregation of native lipoproteins. This process could promote the subendothelial retention of lipoproteins in vivo. The higher LDL(−) binding to PG and subendothelial retention could favor LDL(−) exerting its inflammatory action locally in the microenvironment of the arterial wall, besides acting on circulatory cells. Moreover, LDL(−) retention in the arterial intima would allow induction of cytokine release for a longer period of time.

3.4.4. Global Inflammatory Effect of LDL(−). It is noteworthy that LDL(−) promotes an inflammatory action on several cell types that participate in the atherosclerotic process. The biological effects found in "in vitro" experiments with a cell type cannot be considered individually because in a physiological context all the cell types interact. These interactions enhance the effect promoted by LDL(−) since some cytokines can induce the release of other cytokines, and, moreover, cytokines induced in a cell type can act on other cell types, as shown in Figure 2.

LDL(−) in the circulation induces cytokine release in monocytes and lymphocytes. LDL(−) also promotes chemokine and adhesion molecule expression in endothelial cells, and these molecules promote the recruitment of circulatory leukocytes to endothelium. In addition, cytokine released by endothelial cells can act on cells that are already in the subendothelial space, such as recruited monocytes, macrophages, and smooth muscle cells. These cell types are also exposed to LDL(−) retained in the subendothelial space by PG. In addition, LDL(−) retained in the arterial wall could be further modified by oxidation since it is not protected by the plasma antioxidants and by enzymatic hydrolysis. These modifications of LDL(−) could lead to additional inflammatory actions on cells or to further aggregation of LDL(−). This latter effect could favor LDL(−) recognition by SR, promoting the formation of foam cells.

The biological effects described for LDL(−) are, in part, similar to that for mmLDL/oxLDL, whose involvement in the atherosclerotic process has been extensively reported. Nevertheless, there are several differences between the biological properties of these modified LDLs, shown in Table 1.

4. An Antiatherogenic LDL?

Early observations regarding the cytotoxic effect of LDL(−) on endothelial cells typecasted this modified LDL as a "bad guy" in the atherosclerotic process. Further findings describing an apoptotic and inflammatory effect for LDL(−) also supported this idea. However, in recent years, other studies ascribed some putative anti-inflammatory and regulatory properties to LDL(−), questioning whether LDL(−) is really so "bad".

The main modulatory property promoted by LDL(−) is the induction of the anti-inflammatory cytokine IL10 in monocytes and lymphocytes. The relationship between IL10 and protection against atherosclerosis has been widely established in human clinical studies and in mice [62, 63]. The protective role of IL10 has also been demonstrated in studies with cultured cells, in which IL10 regulates the production of proinflammatory cytokines [64]. All data support a physiological function of IL10 as a controller of inflammatory response, as it seems to be the role of IL10 induced by LDL(−). IL10 diminishes the release of the inflammatory cytokines promoted by LDL(−) in monocytes and lymphocytes [15]. The addition of exogenous IL10 and blocking of IL10 action with antibodies, respectively, inhibit and increase the cytokine release promoted by LDL(−). Therefore, if LDL(−) does not induce IL10 in mononuclear cells, its inflammatory response will be higher. IL10 also promotes its inhibition by negative feedback to avoid the absence of an inflammatory response [15]. Taken together, these data show that LDL(−) counteracts its inflammatory cytokine induction in leukocytes through IL10 to avoid an excessive inflammatory response. Otherwise, this counteracting mechanism does not occur in endothelial cells because they do not produce IL10 in response to LDL(−) [59].

Another modulatory action promoted by LDL(−) is the induction of nuclear translocation of the transcription factor Nrf2 in macrophages [50]. Nrf2 decreases apoptotic activity and modulates the metabolic response to oxidative stress. Accordingly, LDL(−) promotes cell survival and adaptation to oxidative stress in macrophages and endothelial cells [65]. Nrf2 production by LDL(−) in macrophages attenuates their LDL(−)-induced apoptosis [50]. IL10 production by LDL(−) could also be involved in the regulation of apoptosis since IL10 promotes antiapoptotic effects in macrophages [66]. However, Nrf2 activation does not overcome the proapoptotic effect of LDL(−), and IL10 induction does not avoid inflammatory cytokine release either. These compensatory mechanisms could limit the atherogenic effects of LDL(−) but could not inhibit them altogether.

A study by Bancells et al. showed that LDL(−) could avoid monocyte differentiation to macrophages [52], in contrast to oxLDL [67, 68]. LDL(−) downregulates the expression of molecules involved in monocyte differentiation: CSF1R, CD36, and peroxisome proliferator-activated receptor γ (PPARγ) [52]. The inhibition of PPARγ by LDL(−) could promote the CD36 downregulation since PPARγ is a transcription factor that induces CD36 expression [69]. In contrast to these results, Pedrosa et al. observed that LDL(−) induces CD36 in macrophages [50]. On the other hand, it has been described that LPS downregulates the expression of CD36 and CSF1R in inflammatory situations, hindering excessive cell activation [70].

It has been proposed that the combination of PAF-AH and phospholipase C-like enzymatic activities associated with LDL(−) could play a role in the inactivation of oxidized

Table 1: Differences in the properties of oxLDL/mmLDL and LDL(−).

oxLDL/mmLDL	LDL(−)
(i) Oxidized particle	(i) Resistance to oxidation. Oxidized LDL?
(ii) 0.1–0.5% of total plasma LDL	(ii) 3–5% of total plasma LDL (increased in some pathologies)
(iii) No increased PG affinity	(iii) Increased PG affinity
(iv) No phospholipolytic activity	(iv) Associated phospholipolytic activities
(v) Recognition by SRA, EC accumulation	(v) No recognition by SRA, no EC accumulation
(vi) TNF induction, no IL10 induction	(vi) No TNF induction, IL10 induction.
(vii) CD36 upregulation and PPARγ upregulation	(vii) CD36 downregulation (and PPARγ) in monocytes, CD36 upregulation in macrophages
(viii) Cytotoxicity	(viii) Discrepances in cytotoxic effect
(ix) No induction of LDL fusion	(ix) Induction of LDL fusion
(x) Altered immunoreactivity to antibodies anti-apoB	(x) Altered immunoreactivity to antibodies anti-apoB, but different than oxLDL
(xi) No competition with LDL(−) for binding to monocytes	(xi) No competition with oxLDL for binding to monocytes, competition with LPS

(oxLDL/mmLDL) and LDL(−). oxLDL: oxidized LDL, mmLDL: minimally modified LDL, PG: proteoglycans, SRA: type A scavenger receptor, TNFα: tumor necrosis factor α, IL10: interleukin 10, EC: esterified cholesterol, PPARγ: peroxisome proliferator-activated receptors, LPS: lipopolysaccharide.

phospholipids (oxPL), inflammatory components of oxLDL, and mmLDL [6]. PAF-AH activity hydrolyzes PAF-like phospholipids, which could prevent LDL oxidation, but it yields LPC that is an inflammatory molecule. Therefore, LPC could be hydrolyzed by the PLC-like activity of LDL(−) since it is the main substrate. According to this theory, LDL(−) develops a protective function since it avoids the presence of oxLDL or mmLDL, which have greater atherogenic effects than those of LDL(−) [6].

Finally, the most recent observation showing an anti-inflammatory action for LDL(−) is the counteraction of LPS-induced inflammation in monocytes [16]. This counteracting action of LDL(−) seems to be a consequence of the competition between LPS and LDL(−) for the same pathway in monocytes. Both LPS and LDL(−) promote cytokine release in monocytes through the activation of two receptors, CD14 and toll-like receptor 4 (TLR4) [16]. This observation suggests a putative protective action of LDL(−) by decreasing systemic LPS toxicity in cases of overwhelming inflammation, such as a sepsis syndrome arising from bacterial infection.

There is controversy regarding a putative competition between modified LDLs and LPS. Some authors describe an inhibitory action of oxLDL on the LPS effect in monocytes [71, 72]. In contrast, others have reported that native LDL [73] and oxLDL [74] present a synergic proinflammatory effect on monocytes when incubated with LPS. These discrepancies are probably related to the concentrations of LPS and LDL and to the type and degree of LDL modification. OxPL have been described to compete with LPS in the inflammatory effect [75]. In spite of TLR4 binding to small amounts of oxPL [76], oxPL are considered weak agonists for TLR4. The most accepted idea is that oxPL could inhibit TLR signaling by preventing LPS interaction with accessory proteins involved in TLR4 binding [75, 77, 78]. In the atherosclerotic lesion there could be oxPL and mmLDL. However, their presence in plasma is not so feasible, whereas circulating LDL(−) is a likely physiological TLR-ligand.

5. Molecular Mechanisms Involved in LDL(−) Effect on Cells

As reviewed above, several LDL(−) actions on cells have been described. Nevertheless, the components or the physicochemical characteristics of LDL(−) responsible for its effect on cells are not totally understood. The receptors that bind and mediate the biological effects of LDL(−) are reasonably well established, but the intracellular pathways activated by LDL(−), which would lead to its inflammatory and anti-inflammatory effects on cells, are not well known.

5.1. Inflammatory Components of LDL(−). Some authors suggest that oxidation is the mechanism responsible for the inflammatory and cytotoxic effects of LDL(−) [13, 14]. Other authors do not attribute an oxidative origin to LDL(−) [20] and do not find a cytotoxic effect either [7, 15]. They suggest other explanations for the atherogenic properties of LDL(−), such as the increased content in LPC, NEFA, and CER.

The increased PAF-AH activity associated with LDL(−) [10] might be the origin of the increased amount of LPC and NEFA in LDL(−). Both components are involved in the cytokine release promoted by LDL(−) in endothelial cells [8]. The increased NEFA content of LDL(−) is also involved in the induction of cytokine release promoted by LDL(−) in monocytes [26]. In these cells, the presence of HDL caused a diminution in both the NEFA content in LDL(−) and the cytokine release induced by LDL(−) [26], thereby supporting a relationship between NEFA and inflammation promoted by LDL(−).

PLC-like activity of LDL(−) seems to be involved in the cytokine release promoted in monocytes through the generation of CER. PLC-like activity, CER content, and cytokine release are reduced by preincubation of LDL(−) with HDL, suggesting a relationship between these LDL(−) properties [26]. PLC-like activity hydrolyzes the polar head of choline-containing phospholipids and preferentially

degrades LPC, with intermediate medium efficiency for sphingomyelin (SM) and with lower efficiency for phosphatidylcholine (PC). The products of this hydrolysis are CER, monoacylglycerol (MAG), diacylglycerol (DAG), and phosphorylcholine (Pchol). Pchol is water soluble and presumably leaves the LDL particle, but the other products are hydrophobic and remain retained in the LDL particle. Even though LPC is rapidly degraded by the PLC-like activity, MAG would be scarce in LDL since the amount of LPC is much lower (2-3% of total phospholipids in LDL) than PC (70%) and SM (20%). For this reason, CER and DAG are more abundant products of PLC-like activity than MAG in LDL(−). CER and DAG are considered as bioactive and inflammatory molecules that promote cell signal transduction. A relationship between PLC-like activity and increased CER and DAG content in LDL(−) has been shown. The involvement of CER content in LDL, but not of DAG, in cytokine release in monocytes has been demonstrated [9].

The role of CER and NEFA in the cytokine release promoted by LDL(−) in monocytes could be explained by the fact that both compounds can bind to CD14 [79]. It is well known that CD14 binds to inflammatory ligands and afterwards interacts with TLR4 to mediate cytokine release. However, apart from CER and NEFA, other factors seem to contribute to the inflammatory effects of LDL(−). LDL modified "in vitro" to increase its content of CER or NEFA to a similar or higher degree than LDL(−) promotes a lower inflammatory action than LDL(−). This suggests that a combination of several LDL(−) properties contributes to its inflammatory effect.

LDL(−) presents a higher aggregation level than LDL(+), probably as a consequence of its increased CER and NEFA content. However, the high aggregation of LDL(−) as a cause of its inflammatory properties has been ruled out. In vitro aggregation of LDL does not promote cytokine release in monocytes compared to native LDL [9]. But as discussed previously, aggregation is responsible for the increased binding to PG of LDL(−), where it would remain retained favoring its inflammatory action.

5.2. LDL(−) Cell Receptors. The first step in the knowledge of the mechanisms involved in the biological effects for LDL(−) is to determine the receptor or receptors that recognize LDL(−) and mediate the starting signals in the activation of intracellular pathways. Several physicochemical properties ascribed to LDL(−), such as electronegative charge, higher aggregation level, conformational changes in apoB, and increased content in inflammatory lipids, suggest that LDL(−) interacts with different cell receptors than LDL(+). This would influence the clearance of LDL(−) from the circulation and the activation of certain intracellular pathways involved in the induction of cytokine release promoted by LDL(−).

Early studies regarding cell binding focused on LDL receptor (LDLr). LDL binds to LDLr through its apoB lysine residues. As LDL(−) has a higher negative charge than LDL(+), it was expected that LDL(−) would bind to LDLr with lesser affinity. The first study performed in this regard observed that LDL(−) presented loss of affinity for LDLr [4]. These results concur with those of Benitez et al. who found that LDLr affinity was 3-fold lower for LDL(−) than for LDL(+) [80]. The lower affinity for LDLr could be partly explained by the higher NEFA content in LDL(−) [80], its increased degree of aggregation [27], and the abnormal conformation of its apoB [12]. The global consequence of the loss of affinity would be a diminished clearance of LDL(−) from plasma circulation, making this particle susceptible to further modifications. In contrast, other studies reported that LDL(−) binds to LDLr with a similar or increased affinity compared to LDL(+) [13, 19, 81]. The increased binding was attributed to the increased content in apoE of LDL(−).

As LDL(−) possesses an electronegative charge, some SR could uptake this subfraction, as occurs in the case of other modified LDL, such as oxLDL or acetylated LDL [82]. Once again, there is no concensus on this point as some authors describe no differences in the uptake through type A SR [4, 80, 83] while others suggest that LDL(−) could be recognized by SRs [84, 85]. In any case, LDLr and SR should not be related to cytokine release but to plasma cholesterol uptake and accumulation of intracellular cholesterol, respectively. So which cell receptor or receptors are involved in the inflammatory effects of LDL(−)?

Chen et al. suggested that the PAF receptor plays a role in mediating apoptotic effects of L5 in endothelial cells [44]. However, as LDL(−) presents high PAF-AH activity [10], its PAF content can be expected to be low. More recently, Chen and coworkers also reported that lectin-like oxidized LDL receptor (LOX-1) plays a role in L5 recognition. As a consequence of binding to LOX-1, L5 induces several biological effects in endothelial cells, including apoptosis and LOX-1 upregulation [46, 48, 54]. LOX-1 is the main SR in endothelial cells, whereas low LOX-1 expression can be found in monocytes [86]. Moreover, oxLDL, the typical ligand for LOX-1, does not compete with LDL(−) for its binding to monocytes [16]. For these reasons, it is unlikely that LOX-1 is the mediator of the cytokine release promoted by LDL(−) in monocytes. Other SRs, such as SRA, are expressed in low amounts in monocytes, increasing its expression during the differentiation of this cell type to macrophages.

The involvement of TLRs in the biological effects of LDL(−) had been suggested [87] and recently demonstrated [16]. TLRs are immune response receptors against pathogens, which are related to atherosclerosis [88]. TLR ligands, such as LPS, bind to CD14, a differential marker of monocytes, which associates with TLR2 or TLR4 to induce intracellular signal transduction [89]. TLR2 and TLR4 can bind directly to LPS and also modified lipoproteins. The activation of the system CD14-TLR4 by mmLDL has been studied in depth by Miller and coworkers, particularly in macrophages. They found that CD14 binds to mmLDL, the binding site being different from that for LPS [90]. This binding promotes CD14 and TLR4 association and leads to stimulation of phagocytosis [90], macropinocytosis, and cholesterol accumulation [91]. mmLDL also induces inflammatory cytokines in macrophages, such as MCP1, IL6, and tumor necrosis factor α (TNFα), in a TLR4-dependent or -independent manner [92]. Studies by Chávez-Sánchez et al. show that, in monocytes and macrophages, mmLDL induces IL1, IL6,

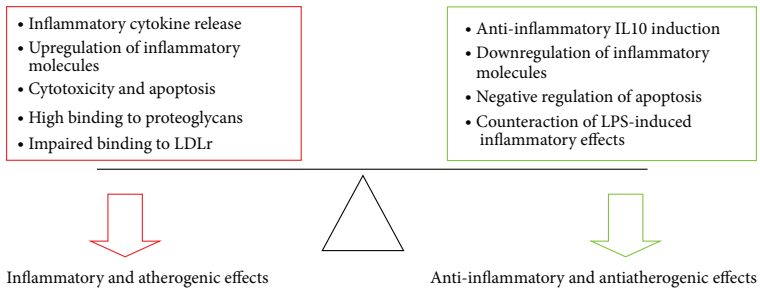

Figure 3: Balance of inflammatory and anti-inflammatory effects of LDL(−) on cells. LDLr: LDL receptor, IL10: interleukin 10, LPS: lipopolysaccharide.

IL10, and TNFα secretion through CD14, TLR4, and TLR2 [93, 94]. Other authors have reported that oxLDL promotes MCP1 and IL8 release and upregulates TLR4 in monocytes [95], and mmLDL also induces TLR4 in macrophages [96]. Because of the role of CD14-TLR4 in the inflammatory action of mmLDL, the involvement of TLRs in the LDL(−) effects on cells seems to be feasible. According to this, recent findings from our group have demonstrated that CD14 is the main receptor of LDL(−) in monocytes. CD14 association with TLR4 triggers the subsequent intracellular machinery leading to cytokine release [16]. The fact that LDL(−) shares the CD14-TLR4 pathway with LPS explains the previously mentioned cross-competition between LDL(−) and LPS in binding to monocytes and in cytokine release.

5.3. Intracellular Mechanisms Activated by LDL(−). Knowledge about intracellular signaling pathways activated by LDL(−) that lead to cell response is scarce. In contrast, the activation of several signaling pathways by mmLDL is better known, particularly in macrophages. Some of these pathways could also be activated by LDL(−).

In macrophages, mmLDL activates phosphoinositide-3-kinase (PI3k) by TLR4-dependent or -independent pathways, [90, 92] initiating Akt signaling [92]. It has also been suggested that LDL(−) activates PI3k and nuclear factor κB (NFκB) in cardiomyocytes leading to induction of apoptosis [51]. However, these findings contrast with those reported for the electronegative L5 subfraction in endothelial cells and endothelial progenitor cells, where the PI3k-Akt pathway is inhibited via LOX-1 [46, 48, 54]. As endothelial progenitor cells derive from circulating monocytes, LDL(−) could also have an inhibitory effect on the PI3k-Akt pathway in monocytes.

It has been described that mmLDL induces the recruitment of spleen tyrosine kinase to TLR4 in macrophages [91, 97, 98]. This leads to phosphorylation of endothelial cell signal-regulated kinase (ERK1/2) and of c-Jun N-terminal kinase, which finally induces activating-protein 1 (AP1) [98]. In endothelial cells, the stimulation of TLR4 by oxLDL is described to induce the activation of ERK and p38 mitogen-activated protein kinase [99]. The involvement of these kinases on the biological effects of LDL(−) has not yet been studied.

Several observations show that AP1 and NFκB seem to be involved in the inflammatory effects of LDL(−). In HUVEC, an increased nuclear translocation of some components of these transcription factors was observed (p65 and p50 for NFκB and c-jun, cfos, and ATF2 for AP1) [100]. AP1 and NFκB have also been reported to be involved in VCAM induction by LDL(−) [57]. A gene expression study in leukocytes suggests the activation of NFκB and downregulation of PPARγ [52]. The involvement of NFκB and AP1 activation in the inflammatory effect of LDL(−) in monocytes has also been recently reported [16].

6. Physiological Effects of LDL(−)

It is difficult to ascertain the physiological effects that LDL(−) could exert in vivo, where other factors can contribute to modify its action on cells. The role displayed by LDL(−) will probably depend on the cell environment in each particular situation. The presence of other lipoproteins or cell activators, such as HDL and LPS, could modulate the biological action of LDL(−). Moreover, LDL(−) can promote different biological effects depending on the cell type. For example, LDL(−) downregulates CD36 expression in monocytes, probably to inhibit activation of these cells and differentiation to macrophages [52]. In contrast, LDL(−) upregulates CD36 in macrophages [50] to eliminate toxic compounds, including oxidized lipids, leading to foam cell formation.

The fact that LDL(−) is recognized by innate immune receptors on monocytes suggests, a priori, that it could be a "self-pathogen" particle that the immune system has to eliminate. This is supported by the detection of antiLDL(−)-autoantibodies and immunocomplexes [39]. Although some anti-inflammatory actions on cells have been ascribed to LDL(−), the abundant atherogenic properties would lead to a global inflammatory effect rather than to an atheroprotective effect, as shown in Figure 3. Probably, it would be more appropriate to consider the anti-inflammatory actions described for LDL(−) as regulatory/modulatory mechanisms to minimize the inflammatory effect of this modified LDL.

Thus, the classification of the biological effect of LDL(−) as positive or negative is not so categorical since it would depend on the situation. Cytokine release promoted by LDL(−) could be considered as an atherogenic action, but,

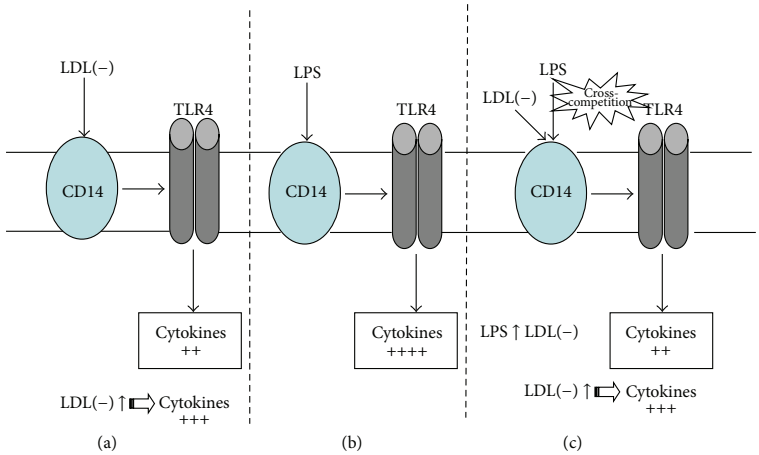

Figure 4: Cytokine induction through CD14-TLR4 by LPS and LDL(−). In the absence of bacterial infection, CD14-TLR4 activation mediated through LDL(−) triggers an inflammatory response that would be deleterious in case of high LDL(−) concentration (a). This effect would be lower than that induced by LPS at high concentrations (infection) in the absence of LDL(−) (b). When LPS and LDL(−) coexist, there is a competition between the two stimuli. The global effect will depend on the relative concentration of both molecules (c). TLR4: toll-like receptor 4, LPS: lipopolysaccharide, LDL(−): electronegative LDL.

in turn, this inflammatory response would be beneficial in counteracting an external aggression. Regarding the physiological role of LDL(−)-induced apoptosis, it is not so clear whether this is an atherogenic effect. Apoptosis could be considered detrimental in late atherosclerotic lesions, but, in early atherosclerotic lesions, the clearance of apoptotic cells is associated with decreased lesion progression [101]. Therefore, these two "atherogenic" properties may not be so bad, and, only when these processes are uncontrolled or excessive, they became detrimental. On the other hand, a putative protective action may not be so good. The counteraction by LDL(−) of the LPS-induced inflammatory effect could be protective. Nevertheless, LDL(−) exerts an inflammatory action that could also be harmful when LDL(−) concentrations increase, even though it is less deleterious than LPS, as shown in Figure 4.

LDL(−) could play a role as a modulator of the inflammatory response to avoid detrimental and inappropriate immune responses. The proportion of LDL(−) is increased in inflammatory situations, such as rheumatoid arthritis or DM. In such events, it could modulate the immune response to some degree. It can be hypothesized that LDL(−) would emerge as a negative feedback to counteract an excessive/overwhelming inflammatory response and play a protective role. It thus seems likely that LDL(−) is more of a consequence of inflammatory situations than a cause.

7. Conclusions

In summary, LDL(−) is a heterogeneous modified LDL which promotes several inflammatory actions on cells. LDL(−) also promotes some anti-inflammatory actions to control an excessive inflammatory response. The global effect of LDL(−) will be the result of the combination of its inflammatory/anti-inflammatory properties. The importance of each individual property in the global action of LDL(−) depends on the physicochemical characteristics of LDL(−) and the cell milieu. Taken together, all data concur that, depending on the context, LDL(−) promotes or inhibits inflammation, playing a dual role in atherogenesis.

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PAPEL DE LOS PRODUCTOS DE DEGRADACIÓN DE CERAMIDA EN LA INDUCCIÓN DE CITOQUINAS PROMOVIDA POR LA LDL ELECTRONEGATIVA EN MONOCITOS

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Introducción y Objetivos

La LDL electronegativa (LDL(-)) es una fracción minoritaria de LDL en circulación y con propiedades aterogénicas. La LDL(-) induce la liberación de citoquinas inflamatorias en monocitos, debido en parte a su contenido aumentado en ceramida (CER) y en ácidos grasos no esterificados (NEFA). El contenido en CER y NEFA se incrementa a 37 °C (temperatura de incubación de la LDL(-) con las células). Este incremento sería debido, en el caso de la CER, al aumento de la actividad tipo fosfolipasa C de la LDL(-), pero la causa del aumento en NEFA se desconoce.

El objetivo del estudio fue evaluar si el contenido aumentado en NEFA a 37 °C se podría generar a partir de la degradación de ceramida en la LDL(-). Esta degradación también formaría esfingosina (SPH), la cual podría dar lugar a esfingosina-1-fosfato (S1P). Se evaluó si estos productos podrían ser responsables de la liberación de citoquinas inducida por la LDL(-) en monocitos.

Resultados

La incubación a 37 °C de las muestras de LDL(+) y LDL(-) con ceramida marcada a nivel del NEFA indujo la degradación de dicha ceramida y se observó la liberación de NEFAs fluorescentes por cromatografia en capa fina (Figura 1).

Figura 1. Cromatografía en capa fina representativa de extractos lipídicos de LDL(+) y LDL(-) incubadas a 37 °C con ceramida marcada.

El aumento en la degradación de la CER por parte de la LDL(-) generaría a su vez SPH y, si se fosforila, S1P, posibles responsables de la liberación de citoquinas promovida por esta lipoproteína en monocitos.

Se estudió la liberación de citoquinas inducida por estos compuestos por sí solos y no se observaron diferencias en la liberación de IL-6, IL-10 ni MCP-1, respecto las células control (BL) (Tabla 1).

Producto	MCP1	IL-6	IL10
BL	0,084 ± 0,023	0,0164 ± 0,002	0,014 ± 0,001
SPH	0,104 ± 0,005	0,011 ± 0,001	0,009 ± 0,009
S1P	0,108 ± 0,006	0,015 ± 0,006	0,007 ± 0,004

Tabla 1. IL-6, IL-10 y MCP1 liberadas en sobrenadante del cultivo de monocitos tras la estimulación con SPH y S1P, o sin estimular (BL) n=5.

Aunque estos compuestos por sí solos no promovieron la inducción de citoquinas, su aumento en la LDL(-) podría contribuir a su efecto inflamatorio en monocitos. Se evaluó, pues, el efecto sobre la LDL(-) de inhibidores de las actividades enzimáticas que generan estos productos derivados de la CER.

Material y Métodos

La LDL se aisló por ultracentrifugación a partir de plasma de donantes normolipémicos y se fraccionó en LDL(+) y LDL(-) por cromatografía de intercambio aniónico. La degradación de ceramida se analizó a partir de extractos lipídicos y cromatografía en capa fina de muestras de LDL(+) y LDL(-) incubadas a 37 °C durante 20 h con ceramida marcada fluorescentemente con *bodipy* en la posición del ácido graso

Se aislaron monocitos a partir de plasma de donantes humanos por gradiente de densidad. Se cultivaron y se incubaron con SPH y S1P comerciales, así como con LDL(+) o LDL(-) en presencia o ausencia de inhibidores de la degradación de CER: MAPP (0, 10 y 20 µM), inhibidor de la actividad ceramidasa y, por tanto, de la generación de SPH; y DMS (0, 1, 2 y 5 µM), inhibidor de la SPH quinasa. Estos inhibidores se adicionaron al mismo tiempo que las LDLs (coincubación) a los monocitos, o bien se preincubaron las LDLs con los inhibidores (preincubación) y posteriormente se añadieron a las células. Se evaluó la liberación de IL-6, IL-10 y MCP-1 en el sobrenadante celular mediante ELISA.

Se valoró la liberación de IL-6, IL-10 y MCP-1 en presencia o ausencia de estos inhibidores. Los resultados mostraron como la inhibición de la formación de SPH, mediante la coincubación de los monocitos con MAPP y LDL(-), disminuía significativamente la liberación de IL-10 y MCP-1 inducidas por la LDL(-) (Figura 2). La preincubación de MAPP con la LDL(-) antes de la adición en cultivo no provocó diferencias en la liberación de citoquinas respecto a la coincubación.

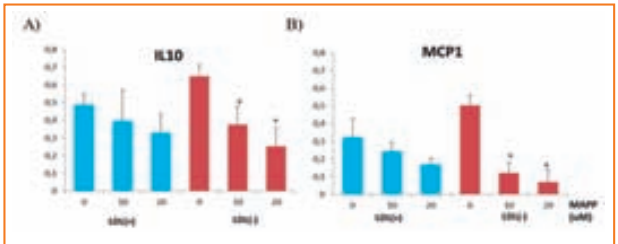

Figura 2. Liberación de IL-6 (A), IL-10 (B) y MCP1 (C) inducidas por la LDL(+) y LDL(-) en monocitos previamente tratados con MAPP (0, 10 y 20 µM) n=5.

La inhibición de la formación de S1P mediante la adición de DMS, produjo un leve descenso de los niveles de IL-10 y MCP1, aunque solo a la máxima concentración estudiada (Figura 3). La liberación de IL-6 no se vio modificada por los inhibidores de la degradación de ceramida en la LDL(-) (no se muestran los datos).

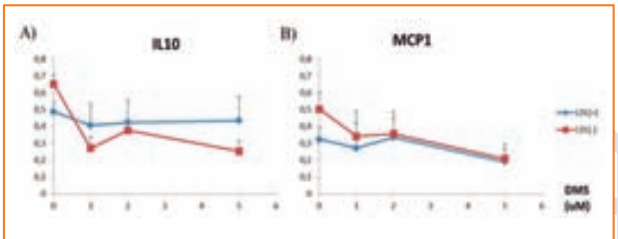

Figura 3. Liberación de IL-6 (A), IL-10 (B) y MCP1 (C) inducidas por la LDL(+) y LDL(-) en monocitos previamente tratados con DMS (0, 1, 2 y 5 µM) n=5

Conclusiones

En la incubación de la LDL(-) a 37 °C se degrada ceramida, produciendo NEFAs y SPH. Los presentes resultados muestran que la SPH contribuye a la liberación de IL-10 y MCP-1, aunque no a la de IL-6, promovida por la LDL(-).

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Montse