ROLE OF ENZYMATICALLY MODIFIED LOW-DENSITY LIPOPROTEIN (E-LDL) ON MACROPHAGE GENE EXPRESSION

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Summary

Monocyte-derived macrophage gene regulation plays an important role not only in pathogenesis of atherosclerosis but also in many other inflammatory diseases, such as, cirrhosis, rheumatoid arthritis, glomerulosclerosis, pulmonary fibrosis and chronic pancreatitis. In addition, bioactive lipids as derivatives of lysophospholipids have been remarkably evidenced to contribute to many pathophysiological stages of these diseases. Therefore, the aim of this thesis work is to analyze global gene expression of monocyte-derived macrophages under the modulation of selected of bioactive lipids, including ceramide, S1P, SPC, LPA and LPA, and modified low-density lipoprotein as the source of the these bioactive lipids. Results archived from CD36 and Cla-1 analysis in phagocytic differentiation and foam cell formation was further confirmed the higher atherogenic properties of enzymatically modifification LDL, compared to other type of modifications (acetylation, oxidation). Furthermore, these results also supported the hypothesis of an autoregulatory loop for enhanced cholesterol uptake and provide a link between this modified LDL and the HDL metabolism. In addition, analyses of adipophilin and ABCA2 were also revealed that adipophilin could be considered as a new sensitive marker for lipid loading of human monocytes/macrophages as well as ABCA2 could play a role in intracellular LDL-derived free cholesterol trafficking and lipid homeostasis. Expression of the 13 up-to-date G-protein-coupled receptors for bioactive lipid derivatives; S1P, LPA, LPC, SPC in human monocytes/macrophages was also investigated. The serum-free phagocytic differentiation model was able to eliminate the discrepancy arrived from other studies. Since these bioactive lipid derivatives are products of platelets and involve in many cellular processes, these results suggested the importance of further study on cross-talk between platelets and monocytes/ macrophages. DNA chip analysis provided an overview of cellular effects of the above-mentioned bioactive lipids and cholesterol loading on macrophages global genes expression. The effects of cholesterol loading (E-LDL) on cholesterol metabolism pathways and homeostasis were investigated in-details. Interestingly, exogenous free cholesterol loading of human macrophages could lead to a complete blockage of cellular cholesterol metabolism pathway triggered by SREBP-2, while triacylglycerides and fatty acids metabolism pathways triggered by SREBP-1c were not influenced. The study also reported the expression of ABC transporter A2, A3, A8, B4, C9, D2, G1 and G4 in human macrophages and suggested the roles of ABCA2, ABCB4 and ABCG1 in macrophages cellular lipid rheostat.

Zusammenfassung

Genregulation in Makrophagen spielt eine grosse Rolle in der Pathogenese der Atherosklerose und weiteren inflamatorischen Erkrankungen wie Zirrhose, Rheumatoider Arthritis, Glomerulosklerose, Lungenfibrose und chronischer Pankreatitis. Lysophospholipid-Derivate und andere bioaktive Lipide scheinen verschiedene Schritte dieser Erkrankungen zu modulieren. Ein Ziel dieser Arbeit war deshalb die globale Analyse der Genexpression von Makrophagen unter den Einfluß bioaktiver Lipide einschließlich Ceramiden, S1P, SPC, LPA und LPA, und modifizierten LDL-Lipoproteinen.

Die durchgeführten DNA Chip Analysen zeigten einen dominanten Einfluss von bioaktive Lipiden auf die globale Genexpression der Cholesterinaufnahme. Expressionsanalysen von CD36 und Cla-1 bei der phagozytären Differenzierung und Schaumzell-Bildung bestätigen die hohe atherogene Eigenschaft von enzymatisch modifiziertem LDL gegenüber acetyliertem und oxydiertem LDL. Ausserdem unterstützen diese Ergebnisse die Hypothese einer autoregulatorischen Schleife der Cholesterin-Aufnahme mit dem HDL-Metabolismus.

Weitere Analyse zeigten, dass Adipopholin ein Marker der Aufnahme von Lipiden in humane Monozyten/Makrophagen ist und, dass ABCA2 eine Rolle im intrazellulären Cholesterintransport spielt.

Abschliessend wurde in dieser Arbeit die Expression von 13 G-Protein-gekoppelten Rezeptoren für bioaktive Lipid Derivate (S1P, LPA, LPC, SPC) in humanen Monozyten/Makrophagen untersucht.

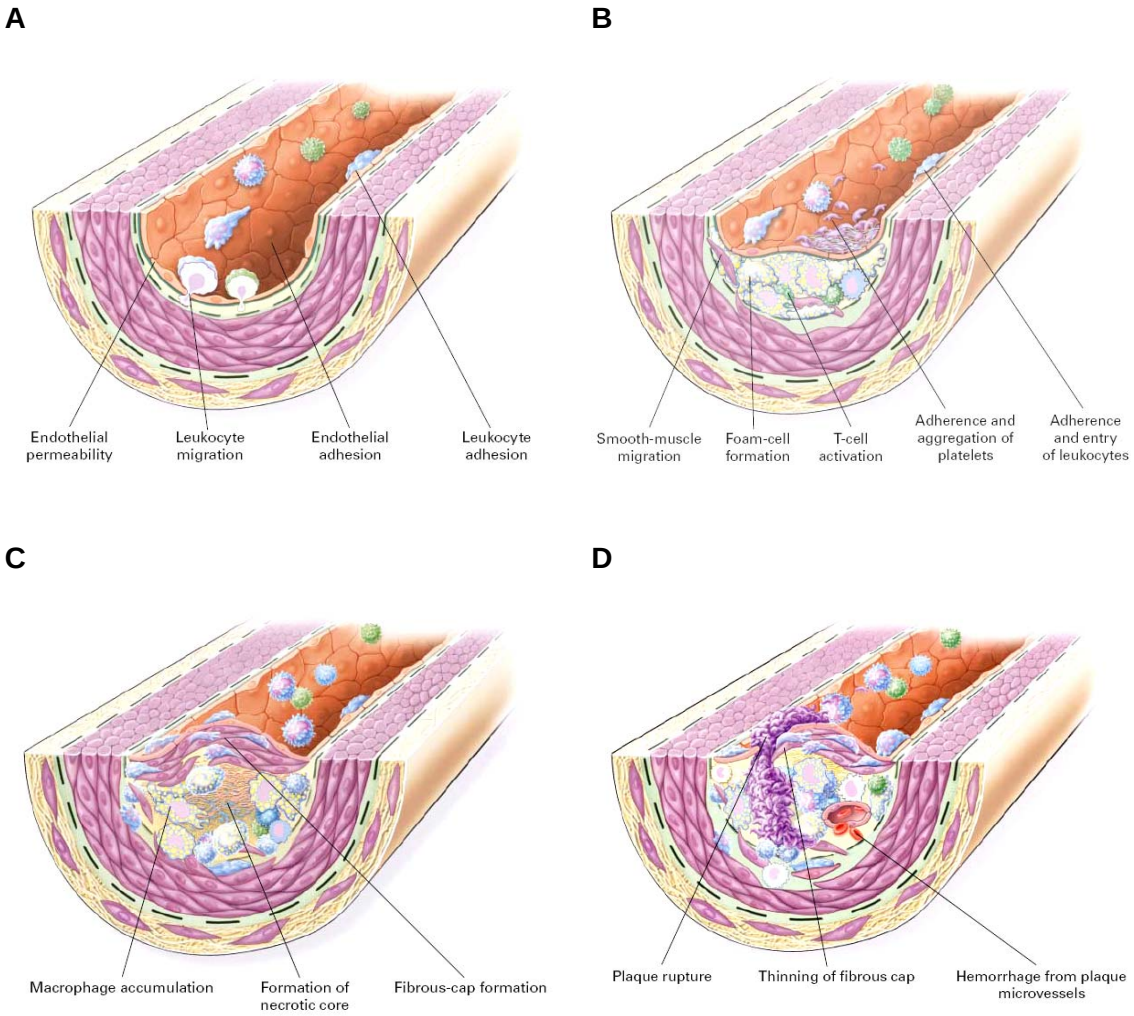
1. INTRODUCTION

1.1. ROLE OF MONOCYTES AND MACROPHAGES IN ATHEROSCLEROSIS

Cardiovascular disease is currently the leading cause of illness and death in industrialized countries and predicted to be the pre-eminent health problem worldwide [1]. Atherosclerosis, a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries, constitutes the single most important contributor to cardiovascular disease. This multiple-phase, decade-spanning of progressive pathological alteration of large and medium-sized elastic and muscular arteries may be developed since first decade of life [2].

Pathological studies in the last decades have revealed a defined series of changes in blood vessels during atherogenesis and demonstrated that blood-derived inflammatory cells, particularly monocytes/macrophages, play a key role in atherosclerosis. The development and progression of atherosclerosis is displayed in Figure 1.1 and can be shortly summarized as follows:

- Lipoproteins from circulating blood infiltrate the intima and accumulate in the arterial wall leading to diffuse intimal thickening.
- In response to lipid accumulation, blood monocytes migrate into thickened intimal area and differentiate to macrophages. Subsequently, macrophages undergo foam cell transformation by taking up lipids, leading to the development of fatty streak lesions. These events are accompanied by proliferation and migration of smooth muscle cells from the media into the intima.
- Local proliferation of specific macrophage populations.
- The centers of atherosclerotic plaques are surrounded by a dense population of foam cells leading to necrotic events.
- As consequence, plaque disruption occurs in the shoulder region, where macrophages have accumulated, leading to ulceration or arterial occlusion.
- Thrombosis occurs in the ulcer of the advanced complicated lesions, often accompanied by calcification in and around the atheromatous core.



Russell Ross, 1999 [2]

Figure 1.1: The development of atherosclerosis in the artery wall.

(A) Endothelial dysfunction leading to its high permeability for lipoproteins and other plasma constituents. **(B)** Fatty streak formation is caused by migration of monocytes, macrophages and foam cell transformation. **(C)** Formation of advanced complicated atherosclerotic lesion. Fatty streaks progress to intermediate and advanced lesions and tend to form fibrous caps that walls off the lesion from the lumen. **(D)** Disruption of the fibrous cap or ulceration of the fibrous plaque. These events can rapidly lead to thrombosis and usually occurs at the site of thinning of the fibrous cap that covers the advanced lesion.

Monocytes and macrophages are not only playing important roles in atherosclerosis but are also involved in other chronic inflammatory diseases, such as liver cirrhosis, rheumatoid arthritis, glomerulosclerosis, pulmonary fibrosis and chronic pancreatitis [2]. Therefore, gene expression analysis of these cells, under certain pathological circumstances and/or disease models, is currently under intensive research.

1.2. ENZYMATIC MODIFICATION OF LOW-DENSITY LIPOPROTEIN

Macrophages transform into atherogenic foam cells by taking up modified low-density lipoproteins (LDL) but not native LDL. To date, LDL has been reported to be altered either by oxidation (Ox-LDL), chemical modification (e.g. Ac-LDL) [3;4] or enzymatic modification (E-LDL) [5;6]. *In vitro* enzymatically modification of LDL was reported by Bhakdi *et al*., using the combination of trypsin, cholesteryl esterase and neuraminidase. Enzymatic modification converts native LDL into an atherogenic moiety that is remarkably similar to lipid deposits in atherosclerotic lesions [7-10]. Likewise, *in vitro* E-LDL has complement-activating capacity [7-10] and a higher potential to induce macrophage foam cell transformation compared to Ac-LDL and Ox-LDL [11]. Therefore, *in vitro* induction of macrophages using E-LDL might be considered as promising laboratory model for atherogenesis studies.

1.3. BIOCHEMISTRY, CELL ORGANELLE DISTRIBUTION AND REGULATION OF PHOSPHOLIPID PATHWAYS

Phospholipids are the major cell and organelle membrane lipids. Phospholipids and their derivatives form the building blocks of biological membranes and play important roles in proliferation, migration and programmed cell death. Phospholipids are constructed from four molecules; fatty acids, a platform where fatty acids are attached, a phosphate and an alcohol attached to the phosphate.

Glycerophospholipids (or phosphoglycerides) are phospholipids that contain glycerol. In glycerophospholipids, the hydroxyl groups at C-1 and C-2 of glycerol are esterified to the carboxyl groups of two fatty acid chains. The C-3 hydroxyl group of glycerol back-bone is esterified to phosphoric acid. Phosphatidate (diacylglycerol 3-phosphate), the simplest glycerophospholipid, is present in a small amount in membranes but acts as the key intermediate in biosynthesis of other glycerophospholipids. In that process, the phosphate group of phosphatidate becomes esterified to the hydroxyl group of several alcohols. The common alcohol moieties of glycerophospholipids are serine, ethanol-amine, choline, glycerol and inositol.

Glycerophospholipids are synthesized in aminoglycero-phospholipid synthesis, which consists of phosphatidylserine (PS) pathway and Kennedy pathway (figure 1.2). PS, phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidic acid are the most predominant phospholipids in endoplasmic reticulum.

Pool size of phosphatidic acid makes up only 1-2% of the endoplasmic reticulum membrane lipid, but the flux through this pool is extremely high due to its function as precursor for other phospholipids and triacylglycerol [12].

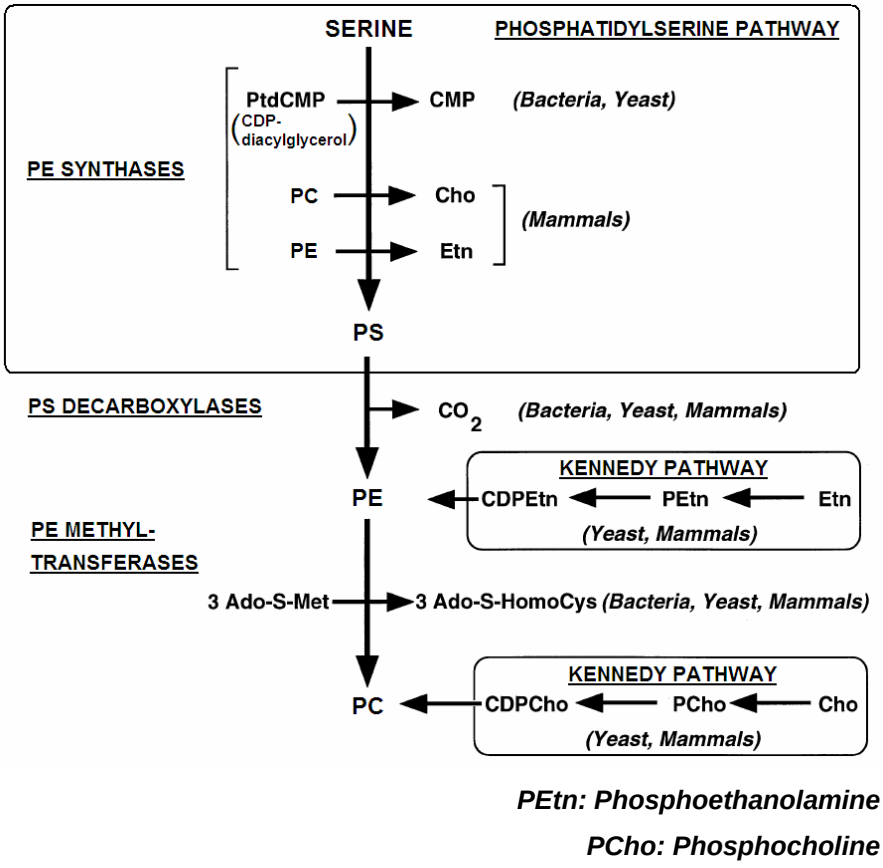


Figure 1.2: Biosynthesis pathways and interconversion of PS, PE and PC.
PS is synthesized in bacteria, yeast, mammals from serine in endoplasmic reticulum (ER) and disseminated throughout the cell for the assembly of new organelles [13]. PS can be converted to phosphatidylethanolamine (PE) by decarboxylation and PC is subsequently formed by methylation of PE. (Adapted from Voelker [14]).

Although endoplasmic reticulum synthesizes a dominant amount, Golgi is also contributing to the production of these phospholipids. Significant rates of PC and PI synthesis are found in the Golgi [15-17]. In addition, the mitochondria also synthesize their own pool of 3-phosphatidylglycerol that is believed to act as precursor for mitochondrial phosphatidylglycerol (PG) and cardiolipin [18] and these two phospholipids are retained within the mitochondria.

Following the synthesis, these phospholipids must be transported to membranes that lack the synthetic machinery to generate their own lipids. This transportation, for many

years, is believed to be carried out by phospholipid transfer-exchange proteins [19]. Recent findings, however, indicate that the transport of these phospholipids between organelles follows some specialized routes that are independent from those followed by membrane proteins [12]. The potential route that could account for above-mentioned observation is the movement via specialized zones between subcellular membranes. A mitochondria-associated membrane (MAM) of endoplasmic reticulum (ER) was evidenced as the way that PS is imported to mitochondria [20].

Interorganelle distribution of these phospholipids starts with PS synthesis in the endoplasmic reticulum and the mitochondria-associated membrane. Nascent PS is transported to mitochondria and Golgi-vacuoles via the pathways, designated PSTA and PSTB respectively. The transportation of PS to mitochondria is an ATP-dependent process in mammalian systems. This has been demonstrated for both intact and permeabilized cells [20-22]. Within mitochondria and Golgi, PS is metabolized to PE by PS decarboxylase (Psd1p or Psd2p). The PE can be retained within mitochondria and Golgi or exported to other organelles via pathways PEEA and/or PEEB [12]. Methylation of PE by methyltransferases (Pem1p and Pem2p) in endoplasmic reticulum leads to the formation of PC. In addition, the Golgi can synthesize PC and the ER can synthesize PE, PC using ethanolamine and/or choline precursors following the Kennedy pathway (figure 1.3).

To date, the mechanism of transporting phospholipids from their sites of synthesis to the plasma membrane has not been completely understood but probably involves both vesicular and cytosolic protein-mediated transfer mechanisms [23].

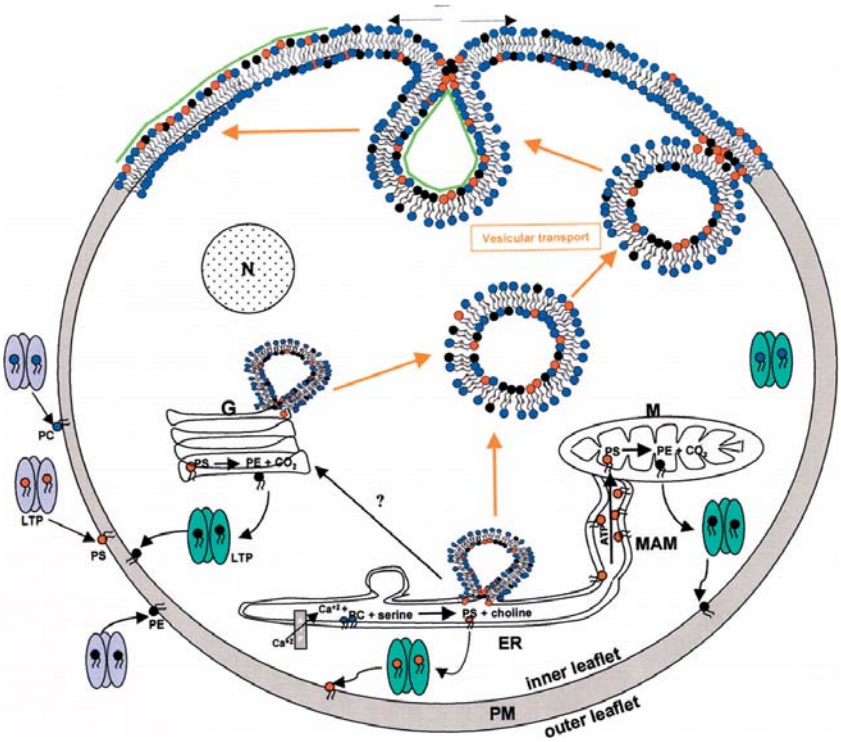


Figure 1.3: Biosynthesis and trafficking of aminoglycerophospholipids.
PS is synthesized in the endoplasmic reticulum (ER) and transported to the mitochondrial-associated membrane (MAM), which then delivers it to the outer membrane of the mitochondria (M) by an ATP-dependent process. The lipid is then transported to the mitochondrial inner membrane where it is converted to PE by PS decarboxylase I. PS is also transported from the ER to the Golgi (G) by an unknown mechanism, where it is converted to PE by PS decarboxylase II. The mechanism by which PE and PS is transported to the plasma membrane (PM) is not clear, but could involve vesicular or protein-mediated mechanisms [24].

1.4. THE ROLE OF BIOLOGICALLY ACTIVE DERIVATIVES OF THE GLYCERO-PHOSPHOLIPID AND AMINOPHOSPHOLIPID PATHWAYS

1.4.1. Biological role of lysophosphatidylcholine

Lysophosphatidylcholine (LPC), a naturally occurring lysophospholipid, serves as a highly active biological molecule involved in many cellular processes. LPC has been reported to play an important role in atherosclerotic lesion development [25;26] and in chronic inflammatory diseases [25;27;28]. LPC is a normal constituent of body fluids, including blood and ascites [29] but is greatly elevated in hyperlipidemic low-density lipoprotein (LDL) and in atherosclerotic lesions from humans and experimental animals compared to unaffected tissues [30-32].

Molecular species of LPC, distinguished by the length and saturation level of their acid chains, are the metabolites of cell membrane-derived PC. This conversion is the result of hydrolysis of PC by hormone-activated cytosolic phospholipase A_2 (PLA_2) [33]. Among the different forms of PLA_2, secretory forms of PLA_2 ($sPLA_2$) are released from macrophages and other cell types at the site on inflammation and tissue injury [34]. LPC is also derived from PC-containing lipoproteins as the result of oxidative processes via the action of endogenous PLA_2 [35].

LPC exists in various concentrations (5-180μM) [29] and physiological forms, including the free form, micellar, and bound to LDL or hydrophobic serum proteins (albumin) [36]. Under physiological conditions, plasma LPC is present mainly in albumin- and lipoprotein-bound forms. These forms may remain active in some nonreceptor-mediated functions, such as delivery of fatty acids and choline to tissues, but may be in the inactive forms for receptor-mediated activities [29;36]. Besides, different concentrations of LPC present in various cellular and tissue systems (i.e., different LPC compartmentations) may differently regulate cellular functions [37]. It has also been shown that serum proteins neutralize the toxicity of LPC [38]. Zhu *et al*. showed that in the presence of molar concentration of BSA, the ability to elicit an increase in intracellular calcium ($[Ca^{2++}]_i$) of LPC via its receptor, GPR4, was greatly diminished [39]. Biological effects and signaling properties of LPC have been most extensively investigated *in vivo* in cell types related to atherosclerosis, including endothelial cells (EC), smooth muscle cells (SMC), monocyte/macrophage and lymphocytes [25;40;41]. LPC has been shown to activate phospholipase C (PLC) [42] and protein kinase C (PKC) [40], induces $[Ca^{2++}]_i$ [42;43] and activates or inhibits mitogen-activated protein (MAP) kinase [44;45]. Although there are more lipid molecules reported as components of oxidized LDL (oxLDL) (i.e., 7β-hydroperoxylcholesterol and 4-hydroxynonenal) [46;47], recent findings indicated that LPC is the primary lipid responsible for oxidative LDL-mediated alterations in endothelial functions [48].

Recent evidence indicated that LPC plays a dual function as an atherogenic or an anti-atherogenic agent [41]. Due to its various physiological forms, it may be important to determine whether this dual function is related to receptor-mediated vs. nonreceptor-mediated effects, receptor compartmentalization and/or is mediated via different types of receptors. Mouse models have shown that LPC stimulates phospholipase D (PLD) activity that in turn elevates generation of other second messengers such as phosphatidic acid, lysophosphatidic acid and diacylglycerol, all of which are considered responsible for many cellular processes including atherogenesis and inflammation [49].

Other findings also reported that LPC might be responsible for Ox-LDL-induced apoptosis [50], showing a possible new role in endothelial dysfunctions in pathogenesis of atherosclerosis. LPC induces endothelial cell suicide by sensitizing endothelial cell to Fas-mediated apoptosis [51]. These findings add to the understanding of atherosclerosis but the mechanisms of LPC-induced apoptosis have not been fully elucidated.

Recently, three G protein-coupled receptors (GPCRs), OGR1/GPR68, GPR4 and G2A, are identified as receptors for LPC [39;52;53]. However, GPR4 and OGR1 have higher affinity to sphingosylphosphorylcholine (SPC) than LPC. Ligand-induced increase in $[Ca^{2++}]_i$, particularly released from intracellular stores, is characteristic of many GPCRs including OGR1, G2A and GPR4. Furthermore, these receptors function as immunosuppressor genes (G2A) [54], tumor suppressor regulated genes (OGR1) involved in apoptosis [55]. These findings are still preliminary and the receptor-mediated signal transduction of LPC needs further investigation.

1.4.2. Biological role of lysophosphatidic acid

Lysophosphatidic acid (LPA) is a bioactive lipid controlling a large number of cellular responses through the activation of specific GPCRs. LPA is present in several body fluids (serum, plasma, aqueous humor) and can also be secreted by several cell types, including platelets, fibroblasts, adipocytes and cancer cells [56]. LPA is the simplest phospholipid that contains long chain fatty acids (C16-C24). Production of LPA involves a number of enzyme activities, extracellularly ($sPLA_2$, PLD) and intracellularly (cytosolic PLA_2, glycerol-3-phosphate acyltransferase, monoacylglycerol kinase). Although platelets are described as the main source of LPA, several studies have revealed the existence of LPA in other cells (cancer cells, fibroblasts, adipocytes) and lipoproteins under physiopathological conditions [56].

In the vascular system, activation of platelets upon the injury of endothelial cells leads to release of LPA. In activated platelets, PLC and diacyl glycerol kinase are contributed to the conversion of phosphoinositides to phosphatidic acid (PA). PA is then degraded to LPA by PLA_2. Recent findings indicated that the production of LPA by platelets is sensitive to extracellular calcium [57-59]. Moreover, albumin binds LPA with high affinity and detection of LPA in medium is possible only in the presence of albumin. Other studies also revealed that biological activity of serum albumin on the stimulation of chloride efflux in *Xenopus* oocytes and actin stress fiber formation and cell

proliferation is due to its high content of LPA [60-62]. Another source of LPA may result from the conversion of LPC that accumulated in lipoproteins which contain unsaturated fatty acids. This conversion is carried out by PLD present in plasma. Whereas native LDL contains only a small amount of LPA, mild oxidation of LDL leads to the large contents of LPA. This explains why LPA is not only present in circulating blood but also accumulates in the intima of human atherosclerotic lesions in comparison to normal arterial tissues [63].

LPA may be one of the factors involved in the permeability of endothelium, one of the first steps in atherosclerosis Recent studies indicated that LPA and LPA present in mildly Ox-LDL are responsible for actin stress fiber formation, contraction of endothelial cells and intercellular gap formation, processes leading to endothelial permeability [63-70]. The decrease of the endothelial barrier function requires LPA concentrations above 1μM and exposure time for several hours [64]. This has been observed in human umbilical venous endothelial cells, human aortic endothelial cells, porcine brain capillary endothelial cells and the human endothelial cell lines [64;65;68-70]. Endothelial dysfunction may drive endothelial cells to undergo apoptosis and leads to plaque erosion and intravascular thrombosis without plaque rupture [71;72]. In other studies, LPA, however, has been found to stabilize the integrity of endothelial monolayer [73;74]. This contradictory observation may be explained by the different effects of LPA on different species, origin of endothelial cells and/or differences in cell culture conditions leading to expression of LPA receptors.

LPA is also reported to act directly on vascular smooth muscle cells (VSMCs) or indirectly by stimulating the release of vasoactive substances from endothelium. Platelet-derived LPA up-regulates the production of endothelin-1, a VSMC-contracting substance, in endothelial cells derived from rat aorta and piglet cerebral microvessels *in vitro* [75;76] and synergizes with endothelin-1 reinforcing vasoconstriction in animal models *in vivo* [77]. At high concentrations (10-100μM), LPA stimulates the proliferation of VSMCs and fibroblasts. The stimulatory or inhibitory effects of LPA on VSMCs migration depend on their phenotype and also the type of matrix on which the cells grow. Low concentrations of unsaturated LPA species (EC_{50}: 20-30nM) induce VSMCs differentiation, migration and proliferation, whereas saturated LPA species are inactive. Interestingly, the differentiated and de-differentiated VSMCs are expressing only LPA receptor 1 and 3 (LPA_1 and LPA_3) but not LPA receptor 2 (LPA_2) and the de-differentiation induced by LPA is mediated by activation of ERK- and p38 MAP kinases.

These unsaturated LPA species which induced phenotypic modulation of VSMCs are implicated as potent atherogenic factors [78].

In addition, LPA also shows its effects on adhesion molecules and monocyte adhesion. LPA (10μM) increased the expression of E-selectin and vascular cell adhesion molecule 1 (VCAM-1) on endothelial cell surface and stimulated monocyte binding. Expression of these molecules have been found to be stimulated by LPA via its receptors ($LPA_{1,\ 2\ and\ 3}$ or old nomenclature; EDG-2, EDG-4 and EDG-7, respectively) and inhibited by NPTyrPA and NPSerPA as LPA receptor antagonists [79-81]. These effects of LPA may be due to activation of the NF-kB transcription factor [82]. The increase of intracellular concentration of calcium, stimulation of chemotaxis and haptotactic migration of human monocytes were reported to be induced by LPA (at millimolar concentration) [83] via its receptors (Table 3).

Similar to sphingosine-1-phosphate, LPA is also having effects on endothelial cell migration and proliferation, which are important for vascular repair and re-endothelialization of the carotid, coronary or peripheral arteries after surgical endarterectomy, balloon angioplasty or stent implantation [84-86].

1.4.3. Biological role of ceramide

Ceramide belongs to the group of sphingosine-based lipid second messenger molecules that are involved in diverse cellular responses to exogenous stimuli. These cellular responses, including proliferation, differentiation and apoptosis, are believed as results of the coupling of ceramide to different signaling cascades in both stimulus and cell-type specific manners [87;88].

Ceramide can be synthesized de novo by condensation of serine and palmitoyl-CoA to form ketosphinganine, which is subsequently reduced to sphinganine (dihydrosphingosine) [89]. In the presence of ceramide synthase (sphinganine *N*-acyl transferase), sphinganine is catalyzed to form dihydroceramide and subsequently is catalyzed by dihydroceramide desaturase to form ceramide. Although it is known that dihydroceramide is generated in endoplasmic reticulum and in the mitochondria, the subcellular localization of the dihydroceramide desaturase has not been determined. This enzyme appears to be crucial because ceramide, but not dihydroceramide, is biologically active. Also, ceramide synthase is reported to be a stimulus-responsive enzyme. Several studies indicated that prolonged activation of ceramide can be

induced by daunorubicin treatment of some cell types, such as P388, U937 and HL-60 cells [90;91]. Ceramide can also be generated by breakdown of glycosphingolipid complexes through acid hydrolases although the regulation of this enzyme by exogenous stimuli is still unknown.

Activation of the sphingomyelin cycle subsequently increases the intracellular ceramide levels and leading to various cellular effects of ceramide, depending on the cellular complement of its effectors, activities of synthesizing and catabolizing enzymes as well as the subcellular localization in which ceramide is generated. There are several ceramide effector enzymes that have been identified. One of those is ceramide activated protein phosphatase (CAPP), which has been identified as a member of 2A class of protein phosphatases (PP2A). Fishbein *et al*. reported that PP2A, as the ceramide effector, mediates ceramide-induced growth arrest in yeast [92] and this has been confirmed by gene knockout studies [93]. Another ceramide effector enzyme is protein kinase known as ceramide acitvated protein (CAP) kinase [94]. It is known that CAP kinase participates in TNF inflammatory responses by activating Raf and subsequently the MAP kinase cascade [95]. Also, Raf itself may act as a direct target of ceramide in interleukin-1 signaling [96]. Therefore, it is suggested that CAP and Raf may be responsible for mediating ceramide activation of the MAP kinase pathway in response to inflammatory cytokines. Protein kinase Cζ is also reported to directly bind ceramide and modulate the cellular response to TNF [97]. Interestingly, kinase suppressor of Ras (KSR), Raf and protein kinase Cζ all have a cysteine-rich domain, which has been recently hypothesized to play a role in the binding of ceramide to these kinases [98]. While CAPPs, CAP kinase and Raf have been reported to play a role in growth arrest and inflammatory cytokine signaling, there is no decisive evidence provided to demonstrate the role of these ceramide effectors in apoptosis.

Signaling through the sphingomyelin pathway as a second messenger, via distinct receptors (CD28, CD95, TNF-α, IL-1β, progesterol, γ-interferon), ceramide shows diverse cellular functions, including fibroblasts proliferation, promyelocytes differentiation, inhibition of the respiratory burst in human neutrophils, survival of T9 glioma cells and regulation of apoptosis [87;88;99]. Ceramide analogue such as cell-permeable C_6-ceramide was first shown to have anti-proliferation effects in HL-60 cells, while cell-permeable C_2-ceramide induced HL-60 cells differentiation towards the monocytic lineage and mimics the action of agonists, such as 1α,25-dihyroxyvitamin D3, IFNγ and TNF-α [94;100]. In addition, mimicking of the effects of TNF-α is also

resulting from the induction of apoptosis by exogenously addition of ceramide to cells [101] (figure 1.4).

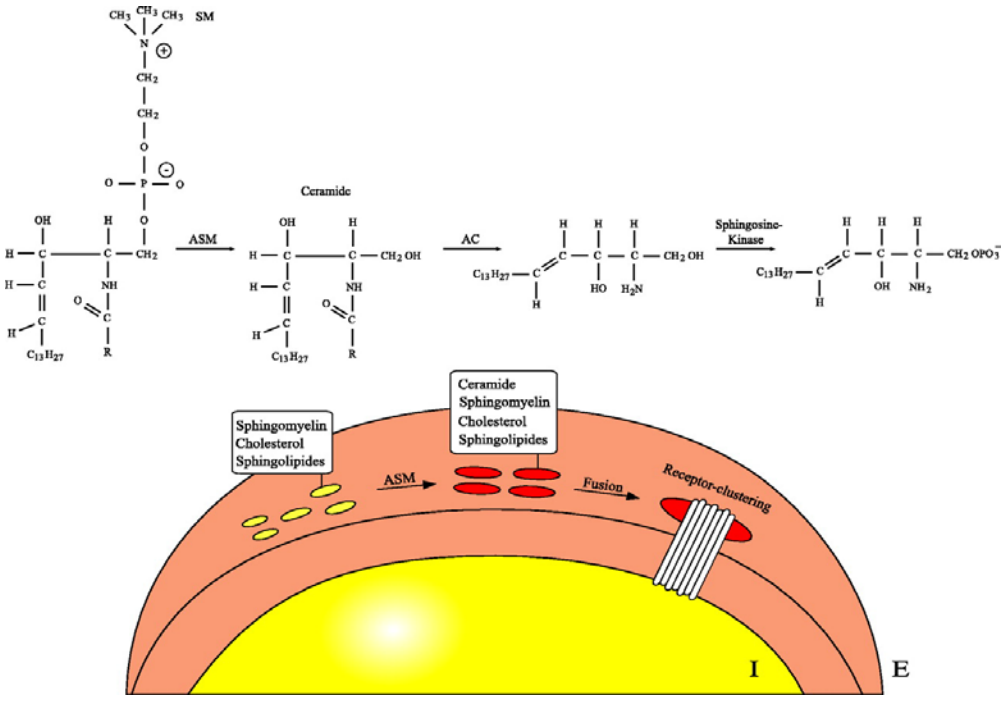


Figure 1.4: Signaling via ceramide

Activation of the acid sphingomyelinase results in hydrolysis of sphingomyelin, the release of ceramide, and the transformation of rafts to small ceramide-enriched microdomains that spontaneously fuse to a large ceramide-enriched membrane platform. SM, sphingomyelin; ASM, acid sphingomyelinase; AC, acid ceramidase; I, intracellular; E, extracellular. (Gulbins [102])

To date, the most highlighted importance of ceramide is its role in apoptosis, a physiological form of cell death in order to control cell populations. Apoptosis has been reported to be induced by receptor-mediated, stress stimuli and growth factor-deprivation-mediated processes. CD95, which may serve as paradigm for receptor-mediated apoptosis, has been shown to activate acid sphingomyelinase and trigger the release of ceramide. CD95 also leads to the formation of ceramide-enriched membrane platforms that mediate clustering of the receptor [103-105], which has been shown in several cell types [106]. Clustering of CD95 in ceramide-enriched membranes, as has been reported in several studies, is required for the production of apoptosis. In those studies, acid sphingomyelinase-deficient cells fail to release ceramide on CD95 stimulation and therefore resist apoptosis, while re-addition of natural C_{16}-ceramide to

these cells lead to apoptosis [104;105]. Likewise, acid sphingomyelinase deficient animals were protected from TNF-α-induced apoptosis of hepatocytes, hepatic failure and death [107].

In addition, the acid sphingomyelinase and ceramide-enriched membrane platforms are not only restricted to receptor-mediated apoptosis but also cause non-receptor-mediated apoptosis, or stress stimuli-trigger cell death. In particular, the acid sphingomyelinase has been shown to be central for the induction of cell death by gamma-irradiation and UV-A, UV-C light [108-111]. Other chemotherapeutic reagents, such as cisplatin and doxorubicin, were shown to stimulate acid sphingomyelinase that trigger the release of ceramide, formation of ceramide-enriched membrane platforms which served to cluster CD95 and, therefore, excluded death of tumor cells [112;113]. Ceramide is also found to play roles in growth factor-deprivation-mediated apoptosis. It was recently shown that several drugs, such as desiparmine and imiparmine [114], prevented apoptosis induced by inhibition of intergrins, while inhibition of $\alpha_v\beta_3$/ $\alpha_v\beta_5$ intergrins results in activation of acid sphingomyelinase and release of ceramide leading to protection of cell death.

Acid sphingomyelinase has been also reported by Tilly *et al*. to play role in developmental death in mice [113]. In mice as well as in human females, approximately 80% of all oocytes undergo cell death until birth and genetic deficiency of acid sphingomyelinase prevented and delayed developmental apoptosis of oocytes thus leading to hyperplasia at birth. This shows a fundamental function of acid sphingomyelinase in oocytes apoptosis.

1.4.4. Biological role of sphingosine

Sphingosine, as the intermediate metabolite of ceramide and sphingosine-1-phosphate (S1P), plays a key role in balancing the intracellular levels of ceramide and sphingosine-1-phosphate leading to either survival or death of cells. In the sphingomyelin pathway, sphingosine can be generated during metabolization of ceramide by ceramidase in the early stages of apoptosis. Sphingosine has been found to induce apoptosis when added exogenously to many cell types. Sphingosine can be further phosphorylated by sphingosine kinase to form S1P, which acts as a signaling molecule that antagonizes ceramide-mediate apoptosis processes (figure 1.5).

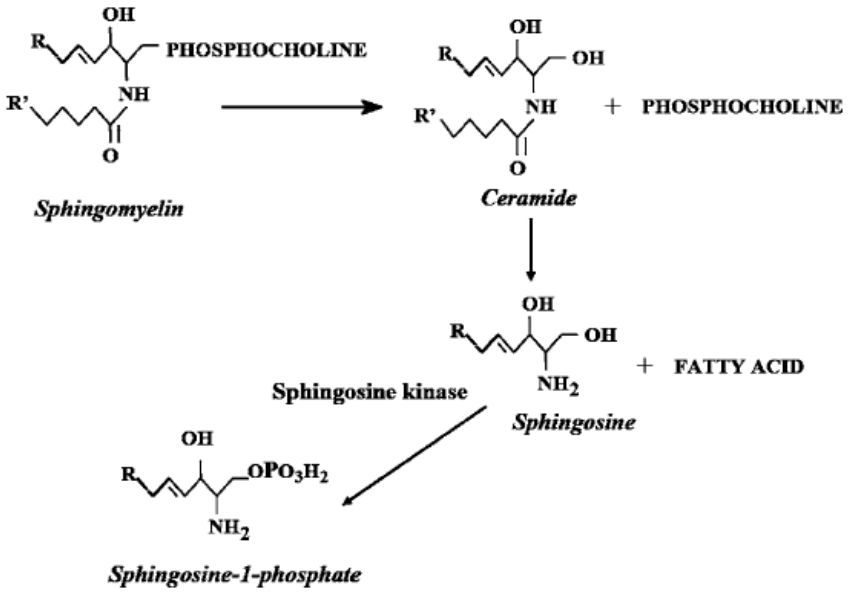


Figure 1.5: Sphingolipids metabolism

Sphingosine is generated from ceramide by ceramidase as well as phosphorylated to sphingosine-1-phosphate by sphingosine kinase.

The role of sphingosine in apoptosis was initially supported by the study of Igarashi *et al*. [115]. In this study, human neutrophils treated with TNF-α showed an increase in the level of both, ceramide and sphingosine during the first 60 min. prior to any apoptotic morphological changes. The increase of ceramide is more rapid than that of sphingosine suggesting that formed ceramide has been degenerated to sphingosine after cells were treated with TNF-α. The increased intracellular level of sphingosine reaches up to 5-10μM and a similar amount of exogenous added sphingosine both showed to induce apoptosis in neutrophils, mimicking TNF-α [115]. Even though ceramide and sphingosine are interconvertible metabolites, sphingosine induces apoptosis without being converted to ceramide inasmuch as the ceramide synthase inhibitor fumonisin B1 dose not affect sphingosine-induced apoptosis in several cell types, including HL-60, U937, Jurkat, TF1 erythroleukemic and Hep3B hepatoma cells [116-120].

Sphingosine is demonstrated to mediate apoptosis in leukemic cells or solid cancer cell lines via various mechanisms. Among those, a large body of evidence has been accumulated regarding the role of MAPKs. The p38 MAPK subgroups, known to present one of two independent parallel MAPK pathways, were found strongly activated by sphingosine in neutrophils. This finding suggested that sphingosine-mediated apoptosis could be p38 MAPK-dependent [121]. Other studies also demonstrated a

strong and virtually complete inhibition of p44-ERK1/p42-ERK2 activity in leukemic and solid cancer cells by sphingosine [117;122;123], implicating the direct contribution of sphingosine to ERK1/ERK2's regulation properties of cell proliferation. Furthermore, sphingosine is also reported to stimulate the cleavage of poly ADP-ribose polymerase (PARP), a well-known target for executioner caspases that plays a central role in executing mammalian cell apoptosis [120;124] (figure 1.6).

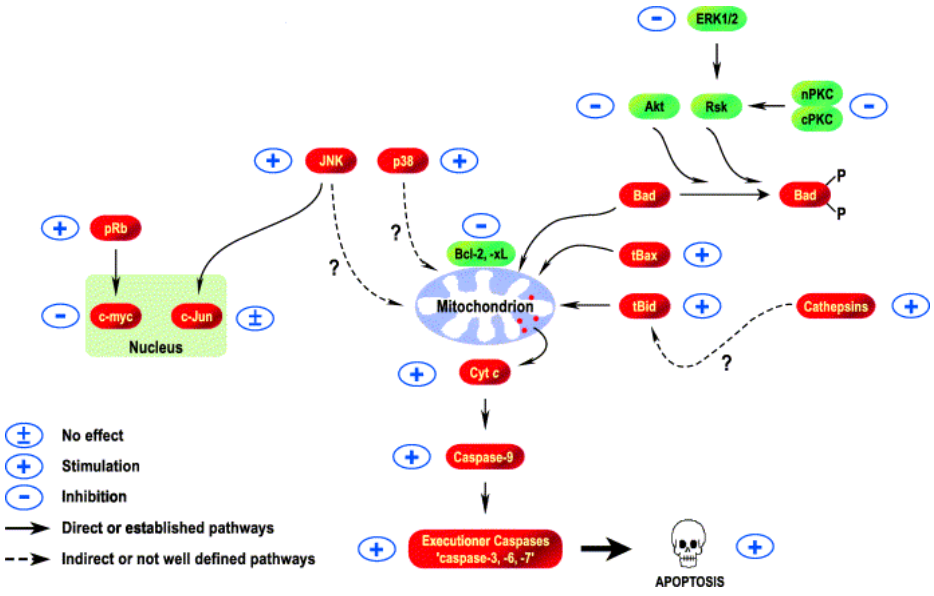


Figure 1.6: Mechanisms of apoptosis regulation by sphingosine [125]

Cyt c, cytochrome c; tBax, truncated Bax; tBid, truncated Bid; ERK, extracellular-regulated protein kinase; JNK, c-Jun N-terminal kinase; Rsk, ribosomal S6 kinase; cPKC, conventional protein kinase C isoforms; nPKC, novel protein kinase C isoforms; pRb, retinoblastoma gene product. Pro-apoptotic elements are represented in red background, and anti-apoptotic ones in green.

In addition to its dependence to MAPK cascades and the caspases, sphingosine is also believed to mediate apoptosis through the mitochondrial pathways. These include the decrease of Bcl-2, Bcl-xL enforced expression that abolished apoptosis [126-128], triggering of executioner caspases activation and cytochrome *c* exit by sphingosine [118;129]. Also, the involvement of other members of the pro-apoptotic Bcl-2 family, such as Bax, Bid and Bad, to sphingosine apoptosis signaling could implicate an alternative mechanism, while during apoptosis, sphingosine is shown to induce truncation of Bid and Bax [49;118;119]. Another direction of sphingosine-induced apoptosis may be its association to the Akt/Rsk/Bad pathway. Recently, several studies showed that sphingosine-induced apoptosis associates with inhibition of basal-/serum-

stimulated Akt kinase activity in hepatoma cells [124] and with inhibition of Bad phosphorylation, through inhibition of a PKC/Rsk pathway [130-133].

At last, while ceramide and sphingosine are considered as pro-apoptotic molecules, its metabolite, S1P, promotes cell survival in response to apoptotic stimuli such as TNF-α, Fas ligation, serum deprivation, cell-permeable ceramides [134-140]. In this 'ceramide/S1P rheostat', sphingosine kinase and S1P phosphatase play the important roles that determine whether cells die or survive.

1.4.5. Biological role of sphingosine-1-phosphate

Sphingosine-1-phosphate (S1P) appears as a normal lipid constituent of human plasma and serum [141]. It has been shown to elicit a great variety of responses, including stimulation of cell proliferation, survival, motility and cytoskeleton in a large number of cell types. S1P is also acting as an intracellular second messenger and extracellular mediator.

The formation of S1P is a dynamic balance between sphingosine kinase and S1P-lyase. Sphingosine kinase, which exists in cytosol and cell membranes, catalyzes sphingosine to S1P [142;143]. Whereas, S1P lyase cleaves S1P into fatty aldehyde and phosphoethanolamine and possibly is the key enzyme that keeps intracellular S1P levels low [144]. In addition to S1P lyase, S1P phosphatase may also play a role in the attenuation of S1P by converting S1P into sphingosine [145]. S1P may also be synthesized by hydrolysis of sphingosylphosphorylcholine (SPC) catalyzed by lysophospholipase D or by deacylation of ceramide-1-phosphate [146]. These, however, have never been reported in biological systems. Extracellularly, S1P has been reported to be synthesized and released by blood platelets [141]. The accumulation of S1P in platelets is due to the highly active level of sphingosine kinase and its activity is not depending on cell activation by physiological antagonists [147-149]. Extracellularly release of S1P from blood platelets is following the activation of protein kinase C directly or indirectly by stimulation with thrombin, 12-*O*-tetradecanoyl-phorbol 13-acetate or 1-oleoyl-2-acetyl-glycerol. Concentration of S1P in plasma and serum are very high; 190pmol/ml and 480pmol/ml, respectively [141]. As same as LPA, plasma S1P is also bound to albumin and this keeps S1P metabolically stable and might prevent it to elicit activities. S1P was also reported to be present in ascites from patients with ovarian cancer cells. In addition, high activity of sphingosine kinase was also found in other peripheral blood cells, including erythocytes, neutrophils and

mononuclear cells [150]. Considering the great variety of S1P-responsive cell types and wide distribution of S1P receptor expression, it may suggest that S1P is released from various cell types to influence target cells in paracrine or autocrine fashions [151].

Furthermore, many studies focusing on diverse biological effects of S1P released from blood platelets have demonstrated that S1P is involved in a variety of physiological and pathophysiological processes, including thrombosis, hemostasis, angiogenesis and atherosclerosis [83;151;152].

S1P has been reported to induce intracellular calcium mobilization, shape change reaction as well as primary aggregation in platelets. The effect of S1P on platelets requires a high concentration and much weaker than that of its metabolite, LPA [149]. The physiological role of S1P as an autocrine stimulator of platelets remains to be further investigated. In contrast to its week effects on platelets, S1P has been shown to have dramatic effects on endothelial cells. Its receptor transcripts, $S1P_1$ (EDG-1) and $S1P_3$ (EDG-3), at high and low level respectively, were reported to be expressed in human umbilical vein endothelial cells (HUVECs) [153;154]. S1P, at as low as nanomolar concentration, reportedly induces a variety of endothelial responses. S1P, as the intracellular second messenger, was reported to induce Ca^{2+} mobilization, MAPK activation and Rho family of small G protein activation [153-158], as confirmed by transfection studies [159-161]. Functionally, S1P stimulates endothelial cell survival or proliferation through its G_i-coupled receptor, $S1P_1$. In addition, S1P also induces adherent junction assembly, migration, capillary tube formation and promotion of angiogenesis [153-155;157]. As extracellular mediator, S1P is found to induce expression of adhesion molecules such as E-selectin and VCAM-1. S1P, but not its metabolites, ceramide or sphingosine, is able to mimic TNF-α to induce adhesion molecules that activate NF-*κ*B [135;162]. In addition, S1P receptors are also reported to respond to changes in fluid shear stress that modulates vascular structure, function and plays an important role in the pathogenesis of vascular diseases such as atherosclerosis, hypertension and restenosis. $S1P_1$ mRNA level in endothelial cells was confirmed to markedly increase in response to fluid flow [143;163].

Transfection studies were reported that S1P, via its $S1P_2$ receptor, induces Ca^{2+} mobilization, MAPK activation and adenylyl cyclase activation in human aortic smooth muscle cells [164-166]. While endothelial cell migration is induced by S1P, vascular smooth muscle cell migration (induced by PDGF) is demonstrated to be inhibited by this bioactive lipid [166]. This cell type-dependent discrepancy in S1P effects on cell

migration is intriguing and maybe explained by different expression patterns of S1P receptor subtypes. Kon J. *et al*. described that $S1P_1$ and $S1P_3$ receptors, expressed in endothelial cells, are able to induce migration of this cell type in response to S1P, whereas $S1P_2$ failed to promote migration activity. Among S1P receptor subtypes, $S1P_2$ exerted the most potent effect on adenylyl cyclase stimulation leading to the inhibition of smooth muscle cell migration [165]. The discrepancy in S1P effects on cell migration, however, requires further investigation.

1.4.6. Biological role of sphingosylphosphorylcholine

In contrast to S1P and LPA, only little information is available regarding the formation, degradation and biological action of sphingosylphosphorylcholine (SPC). However, it has been shown to be involved in many cellular processes and is produced under physiological and pathological conditions. Recently, SPC was detected in normal rabbit plasma and serum [167]. Its higher concentration in serum, compared to that in plasma, suggests that SPC is also formed during blood clotting. Similar to other bioactive lipids, SPC is also found to be associated with high-density lipoprotein (HDL) [168;169]. Despite its presence in plasma, SPC seems to undergo a clearance mechanism during circulation [170]. The pathways of SPC production under physiological conditions have still to be analyzed. SPC may also be produced by sphingomyelin deacylase in certain pathological conditions. It has been found accumulated in Niemann-Pick disease, which is characterized by a deficiency of sphingomyelinase [171;172]. Other data suggest that SPC also accumulated in atopic dermatitis [173], in ascites caused by ovarian cancer and in certain meningeomas [174].

As with S1P, SPC has been reported to promote both intracellular and extracellular actions. Many of SPC actions are shared by its structurally related S1P [175-178] suggesting that these two lipids have common cites of action. S1P-GPCRs are believed to be activated by SPC, although a much higher concentration is needed, compared to that of S1P. The best-studied intracellular action of SPC is release of Ca^{2+} from intracellular stores such as microsomes [179], permeabilized smooth muscle cells [180], and permeabilized EA.hy926 human endothelial cells [181]. The release of Ca^{2+} that is observed in permeabilized cells and microsomes is reported to require as high as micromolar concentrations of SPC (EC_{50} of 3-6μM). In some studies, it is reported to be up to 50μM of SPC [179;180]. At these concentrations, SPC promotes Ca^{2+} mobilization by activating S1P-GPCRs. These two actions of SPC have been studied separately as reported for HEK 293 cells; Extracellular SPC induced a stereospecific

Ca^{2+} mobilization in intact cells most likely via activation of S1P-GPCRs, whereas in permeabilized cells it caused Ca^{2+} release at same potency and efficacy [182]. Other cells such as CFPAC-1 (a pancreatic duct adenocarcinoma cell line) and CFNP9o (an immortalized airway epithelial cell line) had no responses to SPC at concentrations of 10μM and 20μM, respectively [183;184]. This may suggest that the threshold of action of SPC in these cells is much higher.

Nevertheless, there are still open questions raised about intracellular SPC-induced Ca^{2+} release as the exact target of SPC's action is not known and, more importantly, the physiological significance of in-vitro observed actions is unclear. Compared to its structurally related S1P, SPC's intracellular formation is not reported or takes as long as several hours, which is too slow to induce rapid process of Ca^{2+} mobilization [183]. Furthermore, SPC's contractile effect in permeabilized smooth muscle cells is not inhibited by pretreatment with thapsigagin or an antibody against myosin light chain, both of which block inositol 1,4,5-trisphosphate (IP_3)-induced contraction, but sensitive to protein kinase C and to an antibody against MAPK [185]. Besides, the effects of GPCRs are preserved during smooth muscle cells permeabilization and therefore it can not be stated whether SPC acted as intracellular or extracellular mediator [186].

SPC have been shown to share some actions with S1P [175-178], however it also exerts biological actions that are not shared by S1P such as inhibition of Ca^{2+} influx in GH_4C_1 cells [187], induction of superoxide anions formation in HL-60 granulocytes [188] and stimulation of inositol phosphate production in airway epithelial cells [189]. This suggested that, besides the signal transduction via S1P-GPCRs, SPC may act on its own receptors. Initially, OGR1, GPR4, GPR12 and G2A are reported as receptors for SPC [39;53;190]. However, Ludwig *et al.* recently have demonstrated that GPR4 and OGR1 are not responsive to SPC [191]. This raised a question about the identity of these receptors and needed to be further investigated. Therefore, one can assume that SPC acts only on OGR1 (high-affinity) and G2A (lower affinity) compared to that of LPC [52]. In addition to its direct action to release Ca^{2+} from intracellular stores, extracellularly applied SPC also induces rapid increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a number of cell types, including vascular endothelial cells and VSMCs [192;193]. The SPC-induced $[Ca^{2+}]_i$ is pertussis toxin sensitive and can be observed with G2A receptor [52], interestingly, in the absence of inositol phosphate production [188]. SPC is also demonstrated to act on cell growth as it is reported in quiescent 3T3 fibroblasts [194] and other cells such as endothelial cells [192],

keratinocytes [195] and VSMCs [193], as well as play roles in cell migration [196;197], adhesion, stress fiber formation and cytoskeleton rearrangements [198].

Beside the extra and intracellular mediator functions, SPC also shows its effects in tissues and organs. The induction of $[Ca^{2+}]_i$ in vascular endothelial cells [199] and VSMCs [193;200] and induction of endothelial cells migration and differentiations [196] by SPC clearly showed that it has an influence on vascular tone. Cells of immune system are recently reported to be activated and stimulated by SPC. HL-60 leukemia cells and human neutrophils are activated by SPC [188;201]. In HEY cells, SPC is also reported to increase mRNA levels of IL-8 at the concentration 1-10μM.

1.5. THE LYSOPHOSPHOLIPID RECEPTORS

1.5.1. G protein-coupled receptors for lysophospholipids (GPCRs)

Lysophospholipid receptors form a super family of GPCRs, a large family of plasma membrane receptors, activated by a wide variety of ligands, including proton, H^+ ion, small molecules, odorants, peptides, proteins and lipids. GPCRs are used ubiquitously and widely for signal transduction across the plasma membranes. Lysophospholipid receptors as well as other GPCRs, share a common structure that is encoded by seven membrane spanning helix containing proteins. The basic structure motif of these receptors form an extracellular and/or transmembrane ligand binding pocket and an intracellular domain that interacts with signal transducer molecules. The most prominent family being the heterotrimeric G proteins (G_s, $G_{i/o}$, $G_{q/11}$, $G_{12/13}$ and G_{16}). Ligand binding to these receptors results in their conformation changes and therefore allows productive interaction with the G proteins and other effector molecules. It is believed that the basic structural motif of seven transmembrane helices is well suited for cell-cell communication events that utilize diffusible mediators. Lysophospholipid receptors as well as most of lipid receptors belong to the Rhodopsin super family of GPCRs. Herein, only a group of bioactive lysophospholipid receptors is being discussed, including receptors for S1P, LPA, LPC, SPC.

As discussed in previous chapters, lysophospholipids are bioactive lipids emerged as important mediators and involved in various physiological actions as well as cellular effects. Many of these functions associate to the appropriate GPCRs. To date, there are fifteen mammalian GPCRs identified as high affinity cellular surface receptors for these lysophospholipids: LPA (LPA_{1-5}), S1P ($S1P_{1-5}$, GPR3 and GPR12), LPC (G2A,

GPR4) and SPC (OGR1, GPR4, GPR12 and G2A). Receptors for S1P and LPA are also known as endothelial differentiation gene (EDG) receptors.

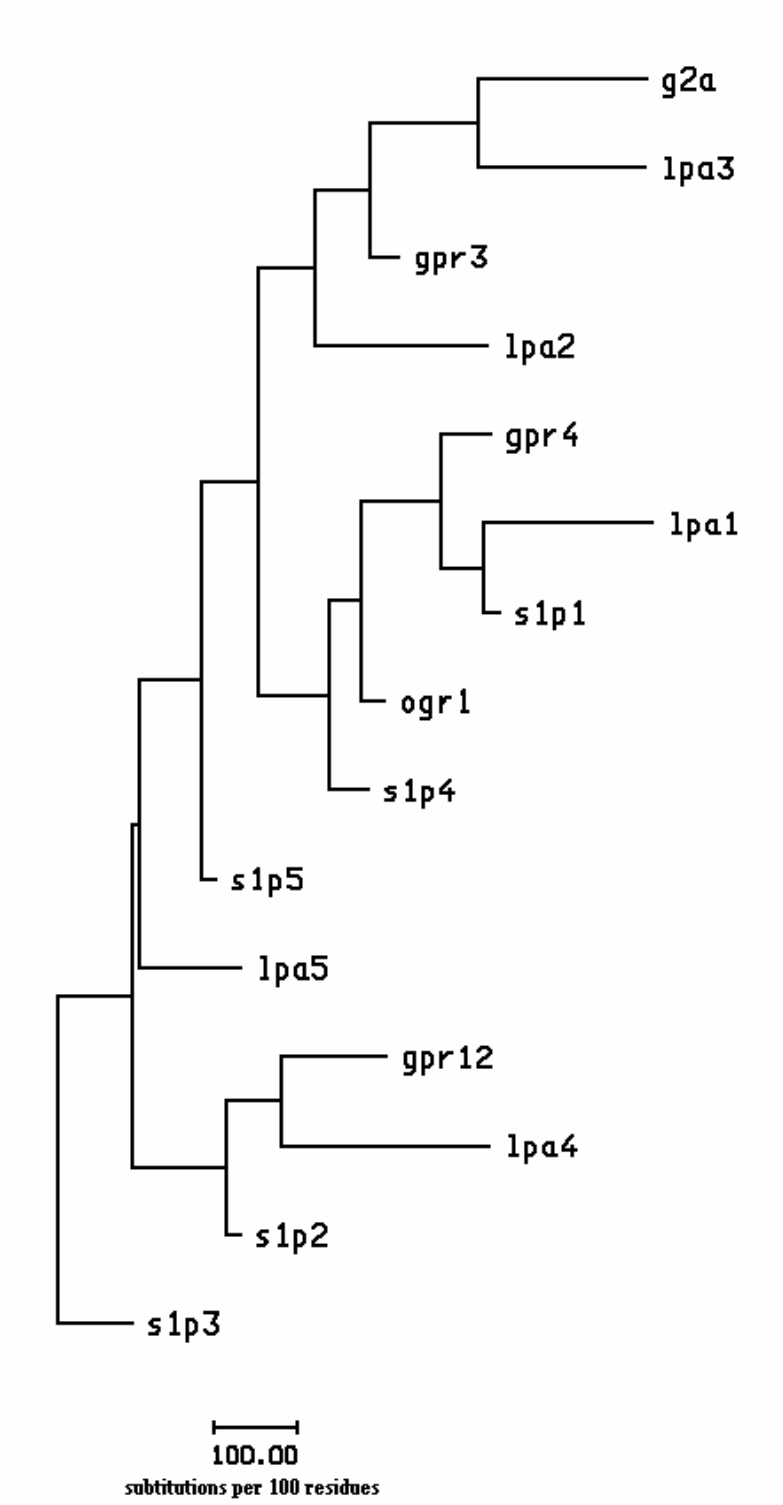


Figure 1.7: Evolutionary dendrogram of lysophospholipid receptors

Evolutionary tree made by comparison of lysophospholipid receptor amino acid sequences, archived from NCBI database, using HUSAR bioinformatics lab software.

LPA receptors are reported in many studies to meditate LPA downstream signaling, including MAPK activation, adenylyl cyclase (AC) inhibition/activation, phospholipase C (PLC) activation/ Ca^{2+} mobilization, arachidonic acid release, Akt/PKB activation and the activation of small GTPase, Rho, Rac, and Ras (figure 1.8). LPA receptors are found to be expressed in various tissues such as; brain, heart, placenta, colon, small intestine, prostate, testis, ovary, pancreas, spleen, kidney, skeletal muscle, and thymus [202]. So far, five receptors (LPA_1- LPA_5) have been identified for LPA and three of those (LPA_1-LPA_3, previously named as EDG-2, 4, 7) share a high amino acid sequence similarity.

LPA_1 was the first to be identified as high affinity receptor for LPA [203]. Functional analysis using mammalian heterologous receptor expression system revealed the multi-functionality of this receptor [204;205]. LPA_1 coupled with three types of G proteins; $G_{i/o}$, G_q and $G_{12/13}$ [206;207] and activation of these proteins leads to LPA-mediated induction of many cellular responses including proliferation, serum-response element (SRE) activation, MAPK activation, AC inhibition, PLC activation, Ca^{2+} mobilization, Akt activation and Rho activation [204;205].

LPA_2 was identified by homology search of orphan GPCR from GenBank. LPA_2 was also found to couple with $G_{i/o}$, G_q and $G_{12/13}$ in order to mediate LPA-induced cellular signaling. Targeted deletion of LPA_2 in mice does not result in any obvious phenotypic abnormality but significant loss of normal LPA signaling (e.g., PLC activation, Ca^{2+} mobilization and tress fiber formation) is observed in primary cultures of mouse embryonic fibroblasts (MEFs) [208]. Creation of $LPA_1^{(-/-)}$/$LPA_2^{(-/-)}$ double-null mice showed the absence or severe reduction of many LPA-induced responses which include proliferation, AC inhibition, PLC activation, Ca^{2+} mobilization, JNK and Akt activation and stress fiber formation while $LPA_2^{(-/-)}$ itself does not show any similar effects [208].

LPA_3 was isolated as an orphan GPCR gene by degenerated polymerase chain reaction (PCR)-based cloning and homology search [209;210]. LPA_3 distinct from LPA_1 and LPA_2 due to its capability of coupling with only $G_{i/o}$ and G_q but not $G_{12/13}$ [207]. LPA_3 is much less responsive to LPA species, which contain saturate acyl chain, in comparison to LPA_1 and LPA_2 [209;210]. LPA_3 also shares common affinity to LPA to induce PLC activation, Ca^{2+} mobilization, AC inhibition/ activation and MAPK activation [207;209;210] although its effects on AC likely depends on cell types and expression levels.

LPA_4 (or GPR23) was identified from orphan GPCR gene libraries within another evolutionary branch of the LP receptor superfamily [211]. Unlike the other three LPA receptors, LPA_4 is found to be encoded by only a single exon and shared greater similarity to platelet-activating factor GPCR. Like other LPA receptors, LPA_4 mediates LPA-induced Ca^{2+} mobilization and cAMP accumulation and possibly couples to Gs protein to activate AC, although its capability to couple to other G proteins is not clear.

A new LPA receptor named as LPA_5 (or GPR92) was identified by Lee *et al*. [212]. In this study, LPA_5 was shown to share 35% of similarity to LPA_4 but lower identities compared to LPA_{1-3}. LPA_5 is capable to couple to G_q and to increase cAMP levels, while LPA_{1-3} and LPA_4 signaling by coupling to G_i and G_s, respectively. Although LPA_5-mediated signaling is relevant to normal function is likely in concert with other previously identified LPA receptors, however, its expression at single cell types is unclear.

As for LPA receptors, S1P receptors have been extensively investigated because of their importance in mediating S1P signaling. There are eight receptors identified for S1P including $S1P_1$-$S1P_5$ (previously named as EDG-1, 5, 3, 6, 8) and GPR3, 6, 12. $S1P_1$ was the first identified S1P receptor, isolated initially as an orphan GPCR in human endothelial cells. The $S1P_1$ gene contains two exons but the entire coding region is located in exon 2 [204]. $S1P_1$ is primarily coupled to $G_{i/o}$ protein and mediates S1P-induced MAPK activation, AC inhibition, PLC activation, Ca^{2+} mobilization, cell aggregation, Rac and Rho activation and cell migration (figure 1.8) [204;213-215]. There is evidence suggesting the involvement of PDGF-induced cellular responses to $S1P_1$ signaling. PDGF-induced Src activation, focal adhesion kinase activation and cell migration are defective in $S1P_1$ null mice, whereas neither PDGF receptor autophosphorylation nor DNA synthesis are altered in this setting [216;217]. Also, PDGF activates sphingosine kinase, inducing its membrane translocation which may lead to an increase of S1P levels in local areas where the $S1P_1$ is presented [216-219]. In addition, immunoprecipitation experiments suggested a possible protein interaction between DPGF and $S1P_1$ [218;219]. Other studies also revealed the role of $S1P_1$ in embryonic development [220;221].

The second S1P receptor, $S1P_2$ was isolated as an orphan GPCR gene from rat cardiovascular and nervous system and is encoded by a single exon. Similar to $S1P_3$, $S1P_4$ and $S1P_5$, $S1P_2$ appears as a high-affinity receptor for S1P and low-affinity

receptor for SPC. $S1P_2$ couples with $G_{i/o}$, G_q and possibly G_s proteins and meditates S1P-induced cell proliferation, survival, cell rounding, activation of SRE, MAPK, AC, PLC and Rho as well as Ca^{2+} mobilization [213-215]. In contrast to $S1P_1$, $S1P_2$ inhibits Rac activity and therefore prevents cell migration [222]. The S1P-induced Rho activation is significantly impaired, whereas PLC activation and Ca^{2+} mobilization remained intact in $S1P_2$ knock-out MEFs, suggesting that $S1P_2$ is critical for Rho activation but not for PLC activation and Ca^{2+} mobilization [223].

$S1P_3$ is also an orphan GPCR gene isolated by screening of a human genomic library [224]. Similar to $S1P_2$, $S1P_3$ is also encoded by a single exon and couples to $G_{i/o}$, G_q, $G_{12/13}$ and maybe also to Gs to mediate S1P-induced cell proliferation, cell survival, cell rounding, MAPK activation, SRE activation, AC activation/inhibition, PLC activation, Ca^{2+} mobilization, Rho activation, Rac activation, and cell migration [213-215]. Targeted disruption of $S1P_3$ in mice showed no obvious phenotypic abnormality but significant loss of S1P signaling especially PLC activation and Ca^{2+} mobilization are observed in $S1P_3^{(-/-)}$ MEFs [223;225].

$S1P_4$ was isolated from *in vitro* differentiated human and murine dendritic cells [226] as an orphan GPCR gene. It couples with $G_{i/o}$, $G_{12/13}$, and possibly G_s, and mediates S1P-induced MAPK activation, PLC activation, Ca_{2+} mobilization, AC activation, Rho activation, cytoskeletal rearrangement (including stress fiber formation and cell rounding), and cell motility [225;227-229]. To date, the *in vivo* roles and functions of $S1P_4$ are still unclear.

$S1P_5$ was isolated as an orphan GPCR gene from rat pheochromocytoma 12 cells [230] and is encoded by a single exon. Like the other S1P receptors $S1P_5$ couples with $G_{i/o}$ and $G_{12/13}$ to mediate S1P-induced AC inhibition and Ca^{2+} mobilization but unlike others, $S1P_5$ mediates the inhibition of MAPK activation/cell proliferation [225;231-233]. As for $S1P_4$, physiological roles of $S1P_5$ are still under investigation.

The newly identified receptors GPR3 and GPR12 have been reported as receptors for S1P [234] while other experiments showed that GPR12 has a higher affinity to SPC and therefore it is considered as a receptor for SPC rather than S1P [235]. GPR3 has been demonstrated to couple not only with G_s but also $G_{i/o}$ proteins and therefore exhibits constitutive signaling towards the G_s and $G_{i/o}$ pathways [236].

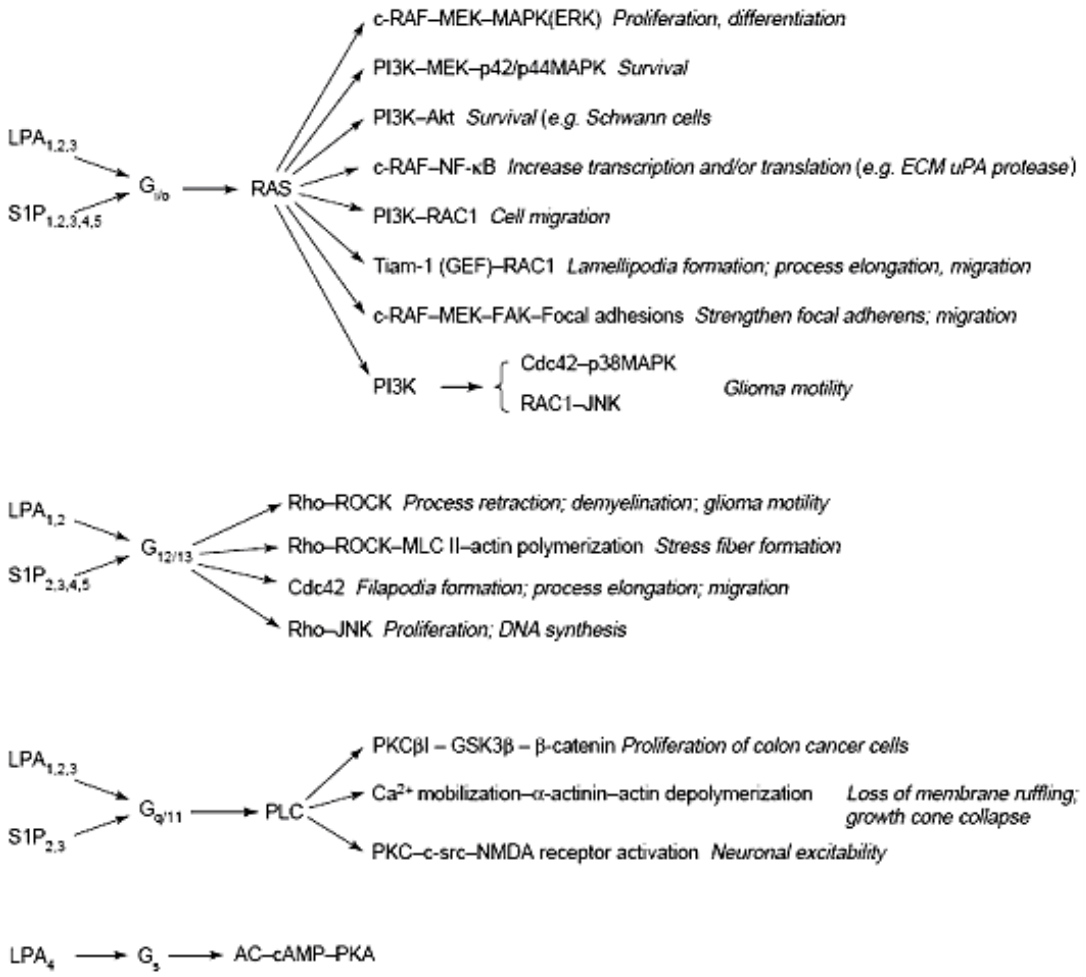


Figure 1.8: Biological roles of receptors for LPA and S1P

LPA and S1P receptors coupled to G-proteins to activate multiple pathways

In addition to its low-affinity receptors ($S1P_1$-$S1P_5$), there are three receptors including OGR1, GPR4 and GPR12 that have been investigated as high-affinity receptors for SPC. All of these receptors were isolated as the orphan GPCR genes and reported as receptors for SPC [39;53;190]. Nevertheless, recent studies from Ludwig and Uhlenbrock pointed out that OGR1 and GPR12 showing no response to SPC, and therefore, raised questions about the identity of OGR1 and GPR12 [191;237]. These receptors all couple with $G_{i/o}$ protein and mediate SPC-induced Ca^{2+} mobilization, while OGR1 additionally couples with G_q to activate MAPK and GPR12 couples with G_s to activate AC [237]. GPR4 is reported to mediate cell proliferation, SRE activation and cell mobility [39]. These SPC receptors are demonstrated to be widely expressed in human tissues; ovary, liver, lung, kidney, heart, placenta, brain, spleen, testis, small intestine, and peripheral blood lymphocytes [39;238-240]. However, the identity of

these receptors must be clarified beyond provisional classification as receptors for SPC.

The last receptor, G2A, has been confirmed as low-affinity for SPC but high-affinity for LPC. G2A was also an orphan GPCR gene delivered from mouse bone marrow cells [55]. G2A appears to couple with $G_{12/13}$ to mediate LPC-induced activation of small GTPase (Rho, Rac and Ras), stress fiber formation and SRE activation [241;242]. It also mediates Ca_{2+} mobilization and MAPK activation in a PTX-sensitive manner, suggesting a coupling with $G_{i/o}$. G2A, additionally, it can mediate cell migration and apoptosis [52;243]. Recently, G2A has been reported to mediate LPC-induced activation of PLC and AC in the PTX-sensitive fashion, implicating a possibility to couple with G_q and G_s [243]. G2A expression is restricted to lymphoid tissue, such as thymus, spleen and bone marrow in mice [241]. G2A has been reported to play roles in immunological function as well as lymphocyte homeostasis [54].

1.5.2. Scavenger receptors

Scavenger receptors are involved in many processes including innate immune response, removal of apoptotic cells, transportation of long-chain fatty acids, mediating collagen and thrombospondin action as well as the development of atherosclerosis. CD36 (an 88kDa class B scavenger receptor or platelet glycoprotein IV) was identified as a facilitator of fatty acid uptake and inhibitor of fatty acid transport. CD36 is expressed in endothelial cells, adipocytes, smooth and skeletal muscle cells, cardiomyocytes, platelets, monocytes, and macrophages. CD36 recognizes and binds many ligands, such as oxLDLs, long-chain fatty acids, collagen, thrombospondin 1, apoptotic cells, anionic phospholipids, and Plasmodium falciparum-infected erythrocytes. The class B scavenger receptor family also includes the HDLs receptor type B class I (SR-BI or Cal-1) that is expressed in a wide range of tissues such as adrenal gland, liver, testis as well as monocytes/ macrophages and is involved in the selective uptake of cholesterol ester, recognized modified lipoproteins and plays a role in HDL metabolism [244;245]. CD36 and Cla-1 share a hairpin membrane topology with two transmembrane domains and with both termini in the cytoplasm [246;247].

1.6. AFFYMETRIX DNA MICROARRAYS

To manage the large quantity of gene expression analysis of monocytes-differentiated macrophages and stimulated macrophages in this thesis work, a set of Affymetrix GeneChip™ microarrays, Human Genome U133A, was employed. Using this GeneChip™ microarray set, the expression of approximately 14,800 human gene transcripts were screened in parallel, under different *in vitro* stimulations.

GeneChip™ microarrays contain of millions of oligonucleotides (referred to probes), chemically synthesized and at specific locations on a small coated quartz surface. GeneChip™ microarrays are produced using photolithographic manufacturing process derived from the integration of semiconductor fabrication techniques, solid phase chemistry, combinatorial chemistry, molecular biology and sophisticated robotics.

The photolithographic manufacturing process begins with 5-inch square quartz wafer. At first, the wafer is covered by a light-sensitive chemical compound that prevents the binding of the first nucleotide of DNA probe being created. Lithographic masks are then used to either block or transmit light onto specific locations to allow the coupling. When the surface is flooded with the solution containing either adenine, thymine, cytosine, or guanine, and coupling occurs only in those regions on the glass that have been unblock through illumination. The bound nucleotide also bears a light-sensitive protecting group that is able to be blocked or unblocked in the next synthesizing cycle. The process is cycled until the probes reach their full length, usually 25 nucleotides (figure 1.9). Commercially available arrays are typically manufactured at a density of over 1.3 million unique features (the locations where specific probe is synthesized) per array.

The 25-mer probes on Affymetrix microarray are designed with highest specificity and are able to distinguish one sequence from billions of similar sequences. For each probe on the array that perfectly matches to its complement sequence in the sample, Affymetrix also built a paired mismatch probe which contains a mismatch nucleotide directly in the middle of the probe sequence. While the perfect match probe gives measurable fluorescence mean, the mismatch probe allows detecting and eliminating any false or contaminating fluorescence within that measurement. Therefore, the mismatch probe is served as internal control of the perfect match probe for the quantification and subtraction of any spurious signals, i.e. cross hybridization, from a gene expression analysis.

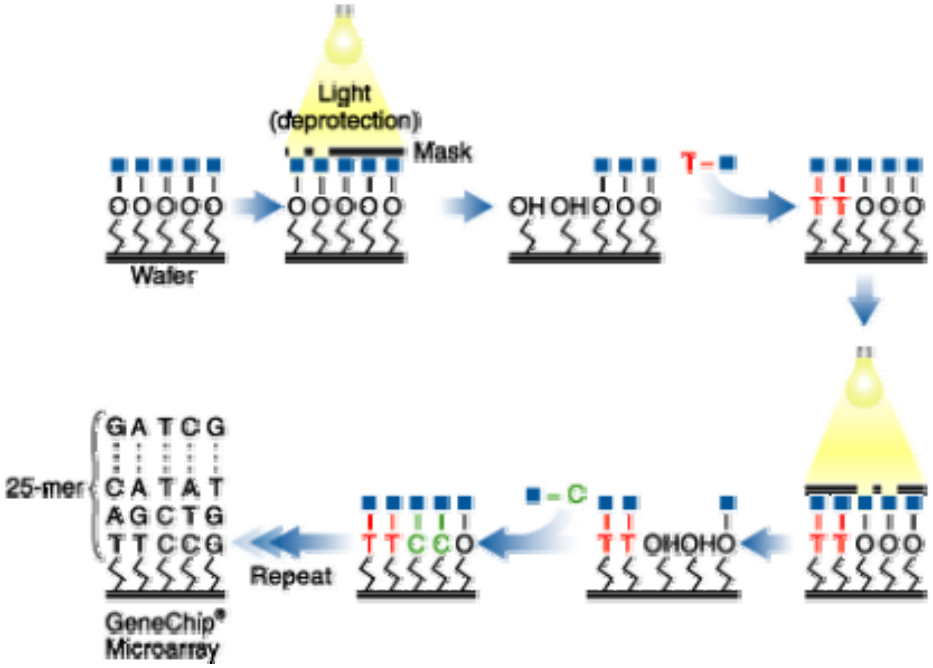


Figure 1.9: Principle of GeneChip™ array production using photolitho-graphic and combinatorial chemical processes.

After hybridization, the microarray is scanned and the fluorescence signal of each hybridized probe is imported to the database of Affymetrix microarray analysis software suite. The imported signal is then normalized to background and adjusted to noise in order to be given the proper *detection call*; present, marginal present or absent. The system is also able to create the expression regulation analysis by comparing the signals and provides one of five possibilities of *different call* for the comparison as; increase, marginal increase, decrease, marginal decrease and no change. In addition, a *p* value is added for each of *detection call* and *different call* to confirm the reliability of the calculated data. Further details for the GeneChip™ production and data calculation can be archived from Affymetrix website: www.affymetrix.com.

1.7. AIMS OF THE STUDY

- To analyze the expression of marker genes in macrophages using our own *in vitro* phagocytic differentiation and macrophage foam cell formation models.
- To analyze the expression of lysophospholipid receptors on human monocytes, macrophages and cholesterol loaded/de-loaded macrophages.
- To analyze global gene expression of macrophages during *in vitro* foam cell formation and bioactive lipids stimulation using Affymetrix DNA chip.
- To analyze more specific monocyte/macrophage gene expression using known literature pathways.

2. MATERIALS AND METHODS

2.1. MATERIALS

Table 2.1: List of used reagents and kits

Reagent and kit	Provider
Agarose	Biozym, Hameln, Germany
Ampicillin	Roche, Mannheim, Germany
Bacto-Agar	Difco-Laboratories, Detroit, USA
Bacto-Trypton	Difco-Laboratories, Detroit, USA
Bacto-Yeast extracts	Difco-Laboratories, Detroit, USA
Blue-dyed Phagobeads (0,8 ?m)	Sigma, Deisenhofen, Germany
BSA (Lipid-free)	Sigma, Deisenhofen, Germany
DABCO (Triethylendiamin)	Sigma, Deisenhofen, Germany
EDTA (Di-sodium)	Pharmacia Biotech, Freiburg, Germany
Fluoromount-G Southern	Biotech, Birmingham, USA
Fluoesbrite microparticles	Polysciences, Eppelheim, Germany
H2O Nuclease-free	Promega, Madison, AL, USA
Kanamycin	Roche, Ingelheim, Germany
L-glutamine	Gibco BRL, Berlin, Germany
Lubrol WX	Serva, Heidelberg, Germany
MEM (Non-essential Amino acid)	Gibco BRL, Berlin, Germany
Minimal SD Agar Base	Clontech, Palo Alto, USA
Nickel-Chelating Resin	GenoTech Biosciences, Lohmar, Germany
Penicillin/Streptomycin	Gibco BRL, Berlin, Germany
Polyvinyl alcohol	Sigma, Deisenhofen, Germany
Protease inhibitors	Calbiochem, Bad Soden, Germany
Protein A-Dynabeads	Dynal, Hamburg, Germany
Second strand buffer	Invitrogen, Glasgow, UK
Triton X-100	Boehringer, Mannheim, Germany
Urea	Pharmacia Biotech, Freiburg, Germany
1 kb Ladder, DNA	Gibco BRL, Berlin, Germany
100 bp Ladder, DNA	Pharmacia, Freiburg, Germany
AMV-Reverse Transcriptase	Promega, Madison USA
BCA Protein Assay Kit	Pierce, Rockford, IL, USA
Cell Line Nucleofector Kit	Amaxa GmbH, Cologne, Germany

List of used reagents and kits (*continued*)

ECL™ Western Blotting Analysis System	Amersham, Braunschweig, Germany
GeneChip Sample Cleanup Module	Affymetrix, Santa Clara, CA, USA
HighYield RNA Transcript Labeling Kit	Enzo Life, Farmingdale, NY, USA
Limulus endotoxin assay	Sigma, Deisenhofen, Germany
Oligotex mRNA Kit	Qiagen, Hilden, Germany
Oligolabeling Kit	Pharmacia, Freiburg, Germany
Rainbow Coloured Molecular Marker	Amersham, Braunschweig, Germany
Reverse Transcription System	Promega, Madison, AL, USA
RNA 6000 Nano Chip	Agilent, Palo Alto, CA, USA
RNeasy Mini Kit	Qiagen, Hilden, Germany
SuperScript Choice System	Invitrogen, Glasgow, UK
T7-Oligo(dT) Promoter Kit	Affymetrix, Santa Clara, CA, USA
QIAEX II Gel Extraction Kit	Qiagen, Hilden, Germany
Qiashredder	Qiagen, Hilden, Germany
Qiaprep (Miniprep) Kit	Qiagen, Hilden, Germany
[3H]Choline phospholipid	Amersham, Braunschweig, Germany
[14C]Cholesterol	Amersham, Braunschweig, Germany
Cholesterol Esterase	Roche, Mannheim, Germany
Klenow-Enzyme	Pharmacia, Freiburg, Germany
Long Template PCR System	Roche, Mannheim, Germany
Rnase A	Fluka, Deisenhofen, Germany
T4-DNA-Ligase	Gibco BRL, Berlin, Germany
T4-DNA-Polymerase	Roche, Mannheim, Germany
T4-Polynukleotidkinase	Roche, Mannheim, Germany
Taq-DNA-Polymerase	Roche, Mannheim, Germany
TaqMan PCR Mastermix	ABI, Darmstadt, Germany
Trypsin / EDTA	Sigma, Deisenhofen, Germany
Bacto-Agar	Difco-Laboratories, Detroit USA
Bacto-Trypton	Difco-Laboratories, Detroit USA
Bacto-Yeast extract	Difco-Laboratories, Detroit USA
DMEM with L-Glutamin	Bio WHITTAKER, Walkersville USA
DMEM with L-Glutamin, w/o Phosphate	Bio WHITTAKER, Walkersville USA
Fetal Calf Serum (FCS)	Gibco BRL, Berlin, Germany
Luria Broth Base	Gibco BRL, Berlin, Germany
Macrophage-SFM-Medium	Gibco BRL, Berlin, Germany

List of used reagents and kits (*continued*)

PBS w/o Ca+2/Mg+2	Gibco BRL, Berlin, Germany
RPMI 1640	Gibco BRL, Berlin, Germany
YPD Medium	Clontech, Palo Alto USA
YPD Agar Medium	Clontech, Palo Alto USA
Human Genome U95Av2 Array	Affymetrix, Santa Clara, CA, USA
Human Genome U133A	Affymetrix, Santa Clara, CA, USA
Human Genome U133 Plus 2.0 Array	Affymetrix, Santa Clara, CA, USA

Table 2.2: List of equipments and providers

Equipment	Provider
2100 Bioanalyzer, Caliper	Agilent, Palo Alto, CA, USA
Autoclave Steam Sterilizer Type 24	Melag, Berlin, Germany
Automatic Gamma Counter 1470 WIZARD	Berthold, München, Germany
Biofuge 15R	Heraeus, Hanau, Germany
Cell culture Incubator 6000	Heraeus, Hanau, Germany
ELISA-reader	Tecan, Stuttgart, Germany
FACScan	BD, Heidelberg, Germany
GeneQuant pro RNA/DNA Calculator	Amersham, Braunschweig, Germany
GeneChip® Fluidics Station 450	Affymetrix, Santa Clara, CA, USA
GeneChip® Scanner 3000	Affymetrix, Santa Clara, CA, USA
Horizontal Shaker GFL-3016	GFL, Großburgwedel, Germany
Hybridization Oven 640	Affymetrix, Santa Clara, CA, USA
Incubator B 6120	Heraeus, Hanau, Germany
Instant Camera MP4	Polaroid, Offenbach, Germany
Kodak X-Omat 2000 processor	Kodak, Rochester, NY, USA
LaminAir Hood	Heraeus, Hanau, Germany
Liquid Scintillation Counter Wallac 1410	Berthold, München, Germany
Lumi Imager F1	Boehringer, Mannheim, Germany
Megafuge 1.0 R	Heraeus, Hanau, Germany
Microscope (Visible) Leitz Laborlux S	Leitz GmbH, Wetzlar, Germany
Milli-Q UF Plus System	Millipore, Bradford, VT, USA
MiniSpin Plus Centrifuge	Eppendorf, Hamburg, Germany
Mini Transblot Cell	BioRad, München, Germany
Mini Protean-3 Electrophoresis Cell	BioRad, München, Germany
Mini-Sub Cell GT Electrophoresis	BioRad, München, Germany

List of equipments and providers (*continued*)

pH-Meter pH537	WTW, Weilheim, Germany
Precision Balance Sartorius MD BA 200	Sartorius, Göttingen, Germany
Power Supply PAC 300	BioRad, München, Germany
Princeton MicroMax CCD-1317-K/1	Roper Scientific, Trenton, NJ, USA
Shaking Incubator GFL-3032	GFL, Großburgwedel, Germany
Shaking Water Bath Julabo SW-20C	Julabo, Seelbach, Germany
Spectrophotometer UV/VIS Lambda 2	Perkin Elmer, Überlingen, Germany
Stirrer with Heating Surface	IKAMAG Labor Center, Nürnberg, Germany
SpeedVaq Alpha RVC	Christ, Osterode, Germany
Sysmex Micro-Cell Counter F-300	Digitana AG, Hamburg, Germany
Thermocycler Gene Amp PCR System 9600	Perkin Elmer, Uberlingen, Germany
Thermomixer Comfort	Eppendorf, Hamburg, Germany
Ultrasonic Disintegrator Soniprep 150	MSE, Watford Herts, United Kingdom
Ultracentrifuge (fixed angle) J2-21 M/E	Beckman, München, Germany
Ultracentrifuge L-70	Beckman, München, Germany
Ultracentrifuge Optima TLX	Beckman, München, Germany
Vortex-Mixer REAX 2000	Heidolph, Kelheim, Germany
Zeiss Axiovert S-100 Spectral Microscope	Carl Zeiss, Goettingen, Germany
ECL Hyperfilm	Amersham, Braunschweig, Germany
Fluorotrans Membrane (PVDF)	Pall Filtron GmbH, Dreieich, Germany
Instant Picture Film Typ 667	Polaroid, Offenbach, Germany
X-Ray Films Biomax	Kodak, Rochester, NY, USA

2.2. DONORS FOR MONOCYTES ISOLATION

Leukocyte-enriched apheresates or heparinized blood samples were obtained from healthy, normolipidemic, from 25 to 45 years of age volunteers, bearing the apolipoprotein E3/E3 genotype after informed consent.

2.2.1. Density gradient centrifugation of heparinized blood samples

Heparinized blood samples were subjected to the following procedures within 4hrs after donation. Mononuclear cells, comprising monocytes and lymphocytes, were isolated from 3 to 15ml of whole blood diluted with an equal volume of PBS (w/o Ca^{2+}, Mg^{2+}) through 35 min. of brakeless centrifugation at 600g upon a layer of Histopaque-1077.

The upper plasma layer was removed and a white band of mononuclear cells was quantitatively isolated from the gradients mid. After 2 washing steps with PBS (w/o Ca^{2+}, Mg^{2+}) each followed by centrifugation at 425g the cells were resuspended in a small volume (1-2ml) of PBS (w/o Ca^{2+}, Mg^{2+}) and the concentration of the suspension was determined with a sysmex counter.

2.2.2. Elutriation of monocytes

Suspensions enriched in human peripheral blood leukocytes were isolated by leukapheresis in a Spectra cell-separator (Gambro BCT), supplemented with the anticoagulant ADCA and diluted with an equal volume of PBS (w/o Ca^{2+}, Mg^{2+}). The diluted apheresate was then subjected to counterflow centrifugation (J2-MC centrifuge with JE-6B Rotor, Beckmann) [248] using buffers and centrifuge adjustment as follows:

Running buffer: PBS (w/o Ca^{2+}, Mg^{2+})
1.0 % (v/v) penicillin / streptomycin-solution
0.5 % (w/v) BSA
0.1 % (w/v) Glucose

Centrifugation protocol:

Rotation: 2040 rpm. Rotor temperature: 15°C

	Flow rate (ml/min)	Volume of fraction (ml)*
Loading	7	-
Pre-run	9	150
Fractions 1 - 4	12	50
Fractions 5 - 8	15	50
Fractions 9 - 12	18	50
Fractions 13 - 16	20	50
Fractions 17 - 19	22	50
Fractions 20 - 22	24	50

*In case of a marked erythrocyte contamination of the apheresate it may be necessary to enlarge the volume of initial fractions till the content of erythrocytes substantially decreases.

The monocyte content of each fraction was determined using flow cytometry based on the different scatter properties of lymphocytes, granulocytes and monocytes Fractions that were devoid of granulocytes with a monocyte content of at least 90% of leukocytes were pooled, washed with PBS (w/o Ca^{2+}, Mg^{2+}) and resuspended in culture medium. The concentration of the suspension was determined with a sysmex counter.

2.3. ISOLATION AND MODIFICATION OF LIPOPROTEINS

2.3.1. Isolation of lipoproteins

Lipoproteins were isolated from human plasma or serum by sequential preparative ultracentrifugation in KBr gradients according to [249] followed by extensive dialysis and filter sterilization. All lipoprotein concentrations mentioned are protein concentrations determined by Lowry´s method [250]. Lipoprotein fractions were stored at 4°C and used within two weeks from end of dialysis.

2.3.2. Enzymatic modification of low-density lipoprotein (E-LDL)

To gain the same atherogenic properties of LDL found in atherosclerotic plaque, isolated LDL was enzymatically modified before loading to cells.

- As described in [9]. Briefly, LDL was diluted to 2 mg/ml protein in PBS (w/o Ca^{2+}, Mg^{2+}). Enzyme treatment was conducted with trypsin (6.6µg/ml) and cholesterol esterase (40µg/ml) for 2 h at 37°C. Subsequently, the pH of the solution was adjusted to 5.5 by addition of morpholinoethane sulfonic acid (MES) buffer, pH 5.0. Finally, neuraminidase (79mU/ml) and Mg-ascorbate solution (30 mg/ml) were added for 14 h at 37°C.
- As described in [251]. Briefly, LDL was diluted to 2 mg/ml protein in PBS (w/o Ca^{2+}, Mg^{2+}). Enzyme treatment was conducted with trypsin (6.6µg/ml) and cholesterol esterase (40µg/ml) for 24-36 h at 37°C.

These two preparation procedures led to similar results concerning lipid loading potency and competed with each other for binding and uptake with identical potencies (data not shown). The absence of oxidation products in E-LDL was verified by the determination of thiobarbituric acid-reactive substances (TBARS) to quantify lipid peroxidation products [5]. Modified lipoproteins were stored at 4°C and used within a

week. During LDL preparation and subsequent modification, general precautions were taken to avoid lipopolysaccharide (LPS) contamination.

2.4. MONOCYTE CELL CULTURE

2.4.1. Cell culture

After isolation, monocytes were seeded at 10^6 cell/ml in macrophage serum-free medium supplemented with monocyte-colony stimulating factor (M-CSF, 50ng/ml) on plastic Petri dishes (10cm diameter). Monocytes were incubated for up to 4 days at 37°C/ 5% CO_2 (Heraeus 6000 Incubator, Heraeus Instruments) to induce phagocytic differentiation, as described by Stohr *et al*. [252]. Atherogenic lipoprotein (E-LDL) and/or other stimuli were added according to the two following independent experiments:

2.4.1.1. Foam cells induction and removal of cholesterol by HDL_3 loading

Macrophages at day 4 were induced to foam cells by adding of 40µg/ml atherogenic lipoprotein (E-LDL) and incubated for 24h. Subsequently, the culture medium was refreshed and supplemented with 100µg HDL_3 for 24h in 5% CO_2 at 37°C.

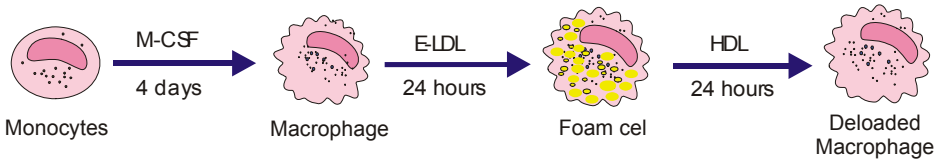


Figure 2.1: *In vitro* differentiation of monocyte to macrophage, foam cell induction and cholesterol deloading of foam cells.

2.4.1.2. Lysophospholipids stimulation of monocyte-derived macrophages

After 4 days of differentiation, macrophages were stimulated for 24h in 5% CO_2 at 37°C with the following lysophospholipids:

Table 2.3: Substances used for macrophage stimulation

Substance	Dilution	Concentration
Ceramide C8 (CerC8)	Ethanol	10µM
Sphingosine-1-phosphate (S1P)	H_2O	10µM
Lysophosphatidylcholine (LPC)	H_2O	10µM
Lysophosphatidic acid (LPA)	H_2O	10µM
Sphingosylphosphorylcholine (SPC)	Ethanol	100µM

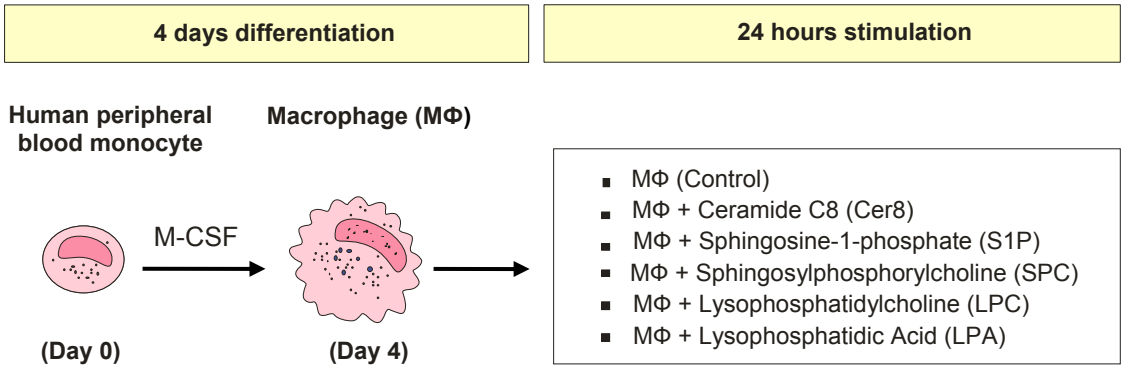


Figure 2.2: Illustration of monocytes/macrophage differentiation and stimulation

2.4.2. Cells harvesting

To all cell stimulation, monocytes at day 0, macrophages day 4 and stimulated cells (day 5 and day 6) were collected for downstream experiments. After cultivation, medium was removed and dishes were rinsed with pre-chilled 1xPBS (w/o Ca^{2+}, Mg^{2+}) to avoid removal of cells. Depending on downstream experiments, cells were put in Trizol™ for total RNA isolation or in 1x PBS with protease inhibitors cocktail for total cell lysates.

2.5. TOTAL RNA ISOLATION FROM MONOCYTES AND MACROPHAGES

Reagents: 1xPBS
Trizol™
Ethanol
Chloroform
Isopropanol
RNase-free water

Protocol:

- After culture, discard medium and rinse dishes with pre-chilled 1xPBS
- (The cultured medium could be spin, discard cell pellet and store at –80°C for ELISA analysis)
- Add 1ml Trizol to dish (approx. $1x10^7$ cells) and harvest cells by cell-scraper. Cell-suspension then could be stored at –80°C for at least 30 min.
- Fast thaw cell-in-Trizol then resuspend cells by syringe (3-5 times) and leave at RT for 2 min.
- Apply about 700µl of cell suspension to the QIAshreder column (QIAGEN 79654) and spin at max. speed for 2min. Collect solution to a new, fresh tube and leave at RT for 5 min. (the QIAshreder column could be used 2-3 times).
- Divide the cells suspension to the centrifuge tube (maximum 2/3 of the tube's total vol.)
- Add chloroform (1/5 vol. of cell suspension), mix by inverting the tube for few times. Incubate tube at RT for 2 min.
- Spin at max. speed (15000 rpm) for 15 min. Collect aqueous phase to the new, fresh tube and put on ice.
- Add 1/5 vol. of Chloroform and mix by inverting few times. Incubate tube at RT for 2 min.
- Spin at max. speed (15000 rpm) for 15 min. Collect aqueous phase to the new, fresh tube and put on ice.
- Precipitate RNA by isopropanol (0.7 vol. of cell suspension), invert gently and leave on ice for at least 10 min.
- Spin at max. speed (15000 rpm) for 30 min. Discard solution and carefully wash pellet with 70% pre-chilled ethanol (do not vortex). And spin again at max. speed for 15 min. Repeat this step if necessary.
- Carefully discard ethanol and dry pellet under hood. Resuspend pellet with RNAse-free water (approx. 50µl per $1x10^7$ cells).
- Control RNA quality and quantity by spectrophotometer and/or running on 1.2% formamide gel.

2.6. RNA QUALITY ASSESSMENT AND QUANTITATION

- To qualify, 5-10 µg of total RNA was apply for 1% agarose gel and run at 12mA and visualized under UV light after staining with ethiliumbromide. Good quality total RNA on the gel showed clear bands of 18s and 23s ribosomal RNAs.

- Also, RNA concentration and purity can be measured by GeneQuant *pro*™ (Amersham). The wave length 260 nm is used to calculate quantification and the purity is determined via 260/230 and 260/280 ratios.

- Alternatively, monitoring of RNA can be performed with RNA 6000 nano assay (Agilent technology). This assay provides excellent output feature of RNA parameters using Bioanalyzer 2100 and RNA 6000 Nano LabChip. The chip can detect RNA concentration between 25 - 500 ng/µl for 12 samples at the same time and shows concentration, purity level together with a gel-like graph and a fluorescence scheme of individual sample.

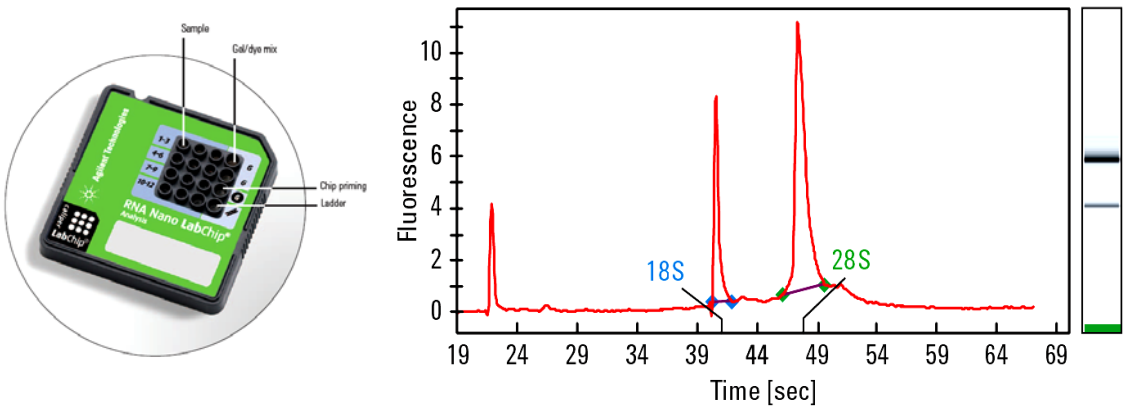


Figure 2.3: RNA quantification and quality assessment using Agilent 2100 Bioanalyzer.

2.7. AFFYMETRIX DNA MICROARRAY GENE EXPRESSION ANALYSIS

The procedure of cRNA samples for GeneChip array hybridization is as following:

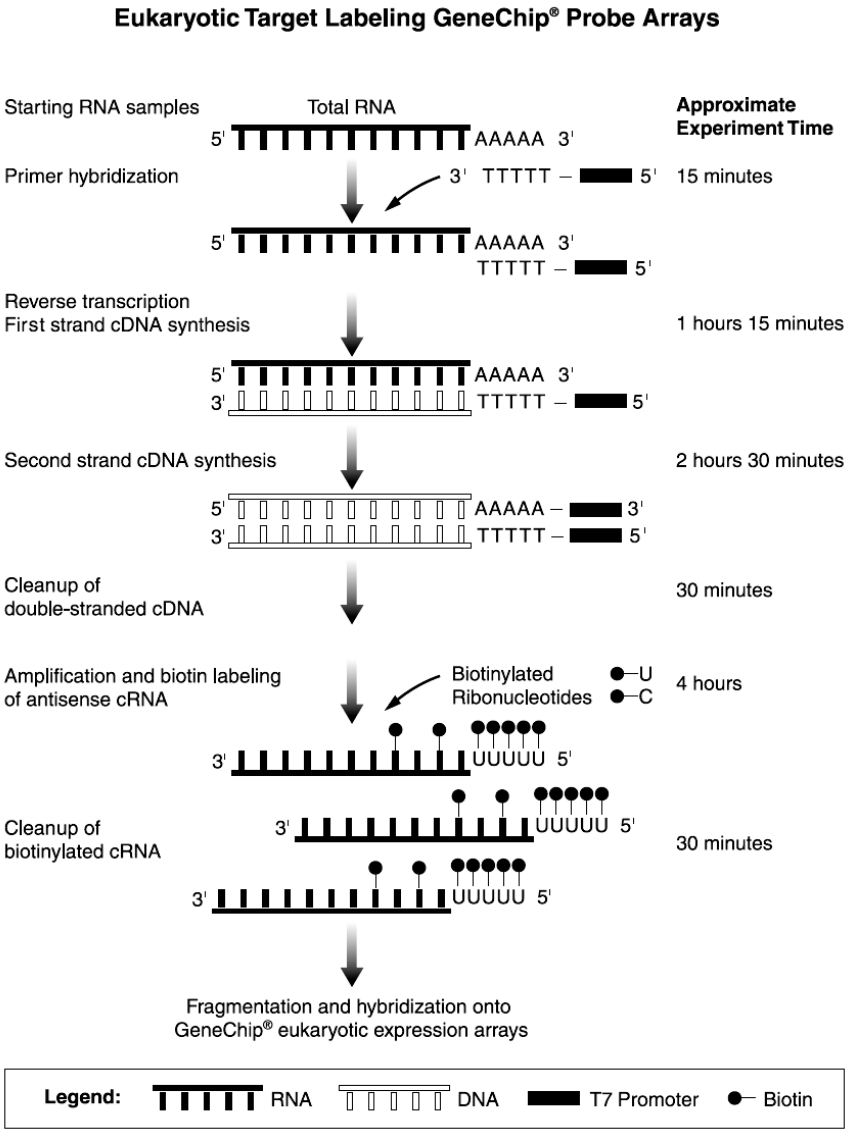


Figure 2.4: principle of GeneChip™ cRNA probe labeling

- First-strand cDNA was synthesized from 10 µg of total RNA of each simulation, using using 50 µM of T7-oligo-dT primer and 400U of SuperScript II RT (Invitrogen Life Technologies SuperScript Choice system).
- Second-strand cDNA was synthesized 10U of T4 DNA polymerase using using T7-oligo(dT) primer (Life Technologies).
- cDNA is cleaned by the Gene Chip Sample Cleanup Module (Affymetrix).

- Antisense cRNA strand was amplified and biotin-labeled by Enzo BioArray HighYield RNA Transcript Labeling Kit based on the manufacturer's protocol.
- Biotinlabeled cRNA was purified, fragmented and quantified by measuring the spectrophotometric absorbance at 260 nm; the purity was determined by the use of the 2100 Bioanalyzer (Agilent technologies).
- After addition of control oligonucleotide B2 (3 nM) and 20x Eukaryotic Hybridization Controls (bioB, bioC, bioD, cre) the cRNA was hybridized to Affymetrix H-U133A GeneChips (Santa Clara, CA) for 16 h at 45°C.
- The chips were stained with Streptavidin Phycoerythrin (SAPE), and washed in the Fluidics Station 450 with protocol EukGE-WS2 which includes an antibody amplification step.
- The arrays were scanned on the Affymetrix array scanner and data was imported to the gene expression data analysis sofware (Affymetrix Microarray Suite, version 5.0).
- Calculated hybridization data was exported for further analyses using Microsoft® Excel™ and Access™ suite.

2.8. GENE EXPRESSION ANALYSIS BY QUANTITATIVE RT-PCR

2.8.1. Reverse transcription reaction (First strand cDNA synthesis)

The reverse transcription reaction was based on Promega reverse transcription system kit using AMV reverse transcriptase.

Table 2.4: Reverse transcription reaction components

Reagent	Volume
Reverse transcription 10x buffer	2µl
$MgCl_2$ (25mM)	4µl
dNTP mix (10MmM)	2µl
Recombinant RNase inhibitor	0.5µl
AMV reverse transcriptase (High Conc.)	0.6µl (15u)
Oligo(dT)	0.5µl
1µg RNA	-
Nuclease-free water	-
Total	**20**

Protocol:

- Place 1µg of total RNA in a microcentrifuge tube and add nuclease-free water up to the volume of 10.4µl and incubate at 70°C for 10 min. Spin briefly and place the tube on ice.
- Prepare the mixture of reagents (table 2.4) and add to the RNA microcentrifuge tube above. Mix well and incubate the reaction at 42°C for 15 min.
- Heat the reaction tube at 99°C for 5 min. and then incubate at 0-5°C for 5 min.

2.8.2. Relative quantitation of gene expression with TaqMan® PCR

- The relative quantification of gene expression analysis was carried out on ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems). The amplification and detection of the target gene sequence based on the gene-specific primers, TaqMan florescent probe using TaqMan Universal PCR Master Mix (UMM). UMM 2x stock solution contains heat-activated AmpliTaq Gold DNA Polymerase, AmpErase UNG for carryover prevention, dNTPs (containing dUTP), Passive Reference Dye (ROX) and optimized buffer components.
- Messenger RNA sequences of target genes were archived from GenBank and gene-specific primers and probes were designed using Primer Express™ software version 2.0 (Applied Biosystems) which optimized for TaqMan quantitative PCR assays. The primers and probes were then ordered from MWG-Biotech. TaqMan probe for target gene is labeled with two different fluorescence dyes; 6-carboxylflourescein (FAM) at 5' end and 6-carboxyltetramethylrhodamine at 3' end (TAMRA). For GAPDH endogenous control, the 3' end of its probe was labeled with VIC™. Using combination of the two dyes (FAM/TAMRA or VIC/TAMRA) enables the system to detect and count every single amplicon formed after every cycle.

Reagent:

- TaqMan GAPDH control (human)
- TaqMan universal PCR master mix

Protocol:

- Prepare the serial dilution of cDNA as following: 50, 25, 12.5, 6.125 and 3.0625 ng for calibration curve generation. These can be a pool of samples cDNA or another cDNA from monocytes/macrophages. H_2O used as negative control.

- For target gene quantification, 25 or 50ng of sample cDNA was used as template.
- Each run was performed in triplicates for both calibration curve generation and target gene quantification (table 2.5 and 2.6).

Table 2.5: Reaction mixture for target gene

Reagent	Volume
cDNA (2-50ng)	5µl
Master mix	10µl
Forward primer (18µM)	1µl
Reverse primer (18µM)	1µl
Labeled TaqMan probe (5µM)	1µl
Nuclease-free water	2µl
Total	**20**µl

Table 2.6: Reaction mixture for GPADH control

Reagent	Volume
cDNA (2-50ng)	5µl
Master mix	10µl
GAPDH control	1µl
Nuclease-free water	4µl
Total	**20**µl

- The running protocol as follow:

Table2.7: PCR program

PCR step	Temp. (°C)	Time	No. of cycle
1. Carryover decontamination via UNG	50	2 min.	1
2. AmpliTaq Gold pre-activation	90	10 min.	1
3. Melting step	90	15 sec.	40
4. Anneal/extend step (combined)	60	1 min.	

- Data analysis was performed with Sequence Detector Software (SDS) 2.0 (Applied Biosystems). Calibration curve generation was generated for every sample which showed ΔRn on the *y* axis (where Rn is the fluorescence emission intensity of the reporter dye normalized to a passive reference, and ΔRn is the Rn of no reacted samples minus that of reacted sample) against cycle number on the *x* axis. From each amplification plot, a threshold cycle (C_T)

value was calculated, which is defined as cycle at which a statistically significant increase in ΔRn is first detected and is display in the graph as the intercept point of the amplification curve with the threshold.

- The obtained C_T values were used further analysis by Microsoft® Excel™. The C_T value of each target gene was used to calculate the initial input amount of cDNA template. Thereafter the results were normalized to endogenous control, GAPDH. At last, the fold changes were calculated by comparison of the expression of target gene upon different stimulations.

2.9. TOTAL CELL LYSATES PREPARATION AND WESTERN BLOT ANALYSIS OF PROTEINS

2.9.1. Preparation of total cell lysates

Total cell lysates were prepared from monocytes and macrophages as following:

Reagents

Protease inhibitors:

Leupeptin 25μM
Aprotinin 25μM
AEBSF 25μM

Protein lysate buffer (100ml):

1M Tris	1ml
0.5M EDTA	0.4ml
5M NaCl	3ml
10% Brij 96	8.75ml
10% NP40	1.25ml
Protease inhibitors	150μl
ddH_2O up to 100ml	

2x loading buffer (10ml):

Brommophenol blue	pinch
0.5M Tris pH 6.8	2.5 ml
Glycerol	2.0 ml

10% SDS	2.0 ml
ddH_2O	2.5 ml
Beta-mecaptoethanol	1.0 ml

Protocol:

- After cell culture, cell culture medium was removed and dishes were rinse with pre-chilled 1x PBS and collect cells to 1.5 ml centrifuge tube by cell scraper. Centrifuge the tube at 10000 rpm, 4°C for 7 min., discard PBS and add 500µl (approx. 10^7 cells) of lysate buffer.
- Sonicate the cell pellet for 45 seconds and keep the pellet on ice for 2 min. Repeat sonication for 2 more times.
- Centrifuge at 14000 rpm, 4°C and transfer protein lysates to a new fresh tube.
- Keep protein lysates at -80°C for long storage.

2.9.2. Western blot analysis of protein

Material and equipment:

- PVDF membrane
- Whatman 3MM paper
- Mini trans-blot cell (Bio-Rad)
- Tris-HCl Ready gell system (Bio-Rad)
- Full-Range Rainbow™ protein molecular marker (Amersham: RPN800)
- ECL plus western blotting detection reagents (Amersham: RPN21323)

Solution

- 5x AA running buffer: (1000 ml)
  - 15g Tris
  - 72g Glycin
  - 5g SDS (or 250 ml 10% SDS)
  - ddH_2O up to 1000 ml

- Transfer buffer
  - 100 ml MeOH
  - 3.03g Tris
  - 14.4g Glycin
  - ddH_2O up to 1000 ml

- 1x PBS: 1 mM sodium phosphate, 15 mM NaCl pH 7.4
- PBS/Tween: PBS + 0.05% Tween-20 (500µl/1l)

Procedure:

Protein separation:

- Boil protein samples and marker for 5 min. and put immediately on ice. Spin down briefly before use.
- Apply the amount of 10 – 100 ng of protein samples and 10 ng of marker to Tris-HCl ready gel and run for approx. 1 hour, at 25mA/gel.
- After running, rinse the gel briefly with water and put in transfer buffer

Western blotting and probing of antibodies:

- Blotting of protein was carried out according to manufacture's guidance (Bio-Rad)
- After blotting, remove membrane and rinse blot several times with PBS/Tween.
- Block the blot in 5% milk/PBS/Tween for 2 hours, at room temperature on shaker.
- Primary antibody probing: dilute antibody (according to product guidance) in 5% milk/PBS incubate with blot for 2 hours at room temperature on shaker.
- Wash the blot 3 times for 10 min. in PBS/Tween
- Secondary antibody probing: dilute antibody (according to product guidance) in 5% milk/PBS, incubate for 2 hours at room temperature on shaker.
- Wash the blot 3 times for 10 min. in PBS/Tween

Detection:

- Incubate blot in diluted detection reagent complex for 5 min. at room temperature. Drain off excess detection reagent with a dry tissue, wrap up the blot and gently smooth out air bubbles.
- Expose the autoradiography film for different time period to detect the protein bands.

Stripping and re-probing of blots:

- Add approx. 25 ml stripping buffer to the blot, incubate in 50°C water bath for 30 min. with occasional agitation.
- Wash blot 2 times for 10 min. in PBS/Tween

- Incubate (optional) the blot with detection reagent and expose to film to ensure that the blot has been adequately stripped
- Block blot in 5% milk/PBS/Tween for 2 hours at room temperature on shaker.

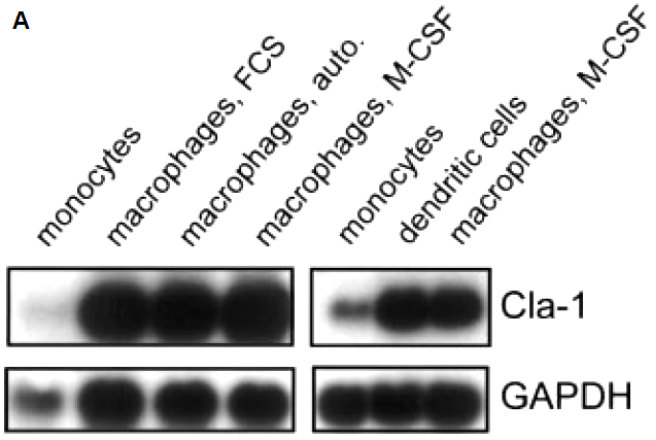
3. RESULTS

3.1. ANALYSIS OF MARKER GENES IN MONOCYTES, MACROPHAGES, CHOLESTEROL LOADED/DELOADED MACROPHAGES

3.1.1. Expression of scavenger receptors, Cla-1 and CD36, in monocytes, monocyte-derived macrophages and E-LDL treated macrophages

Scavenger receptor, CD36, is known to be expressed in monocytes and its expression is elevated during *in vitro* and *in vivo* macrophage differentiation and Ox-LDL-induced foam cell formation [253;254]. In addition, scavenger receptor type B, class I (SR-BI, or Cla-1) that sharing a high homology to CD36 is also hypothesized to increase its expression during macrophage differentiation. Therefore, the expression of these receptors was subjected to analyze with *in vitro* models of M-CSF-induced macrophage differentiation and E-LDL-induced macrophage foam cell formation.

For the analysis of Cla-1, freshly isolated human monocytes were differentiated to macrophages in serum-free medium, supplemented with M-CSF. After 4 days of differentiation, total RNA and total cell lysate from differentiated macrophages were prepared. Northern blot and western blot analyses of above-mentioned samples showed that Cla-1 was weakly expressed in monocytes but induced its expression in monocyte-derived macrophages (figure 3.1a,b). GAPDH probe and β-actin protein were used as control of northern and western analyses, respectively.



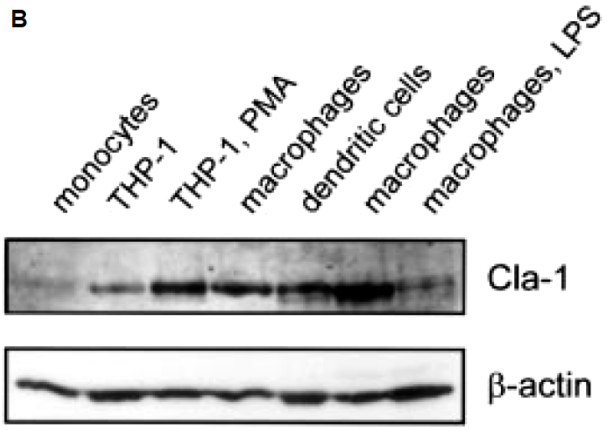


Figure 3.1: Induce mRNA expression of Cla-1 in differentiated macrophages. (A) mRNA expression of Cla-1 in monocytes and monocyte-derived macrophage. (B) Protein expression of Cla-1 in monocytes, monocyte-derived macrophages and other cells

Also, the expression alteration of CD36 scavenger receptor by E-LDL was investigated. In this experiment, monocyte-derived macrophages were incubated with E-LDL according to three different time-points (2, 4, 24 hours of incubation). The quantitative RT-PCR results showed that the expression of CD36 was highly elevated in macrophages incubated with E-LDL compared to macrophages incubated with enzyme cocktail (EC, combination of enzymes that used to modified native LDL) as control. In contrast, the expression of CD36 in macrophages was partially elevated by E-LDL in comparison to that of native LDL-stimulated in macrophages. These results also revealed that the E-LDL-induced expression of CD36 is time-dependent. Control run of RNA from enzyme cocktails incubation showed no changes in macrophages expression of CD36.

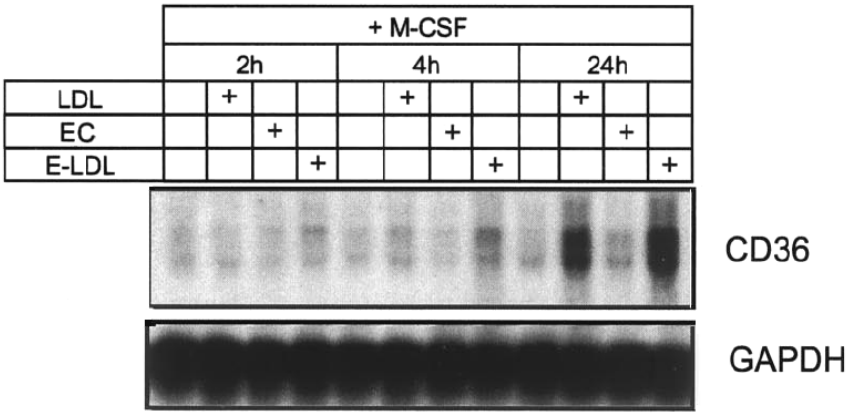


Figure 3.2: Elevation of macrophage CD36 expression by E-LDL.

3.1.2. Adipophilin is a sensitive marker for lipid loading in human blood monocytes

Expression of adipophilin, or adipose differentiation related protein, is shown to be elevated during Ox-LDL-induced foam cell formation [255]. Therefore, it could be considered as a marker gene for *in vitro* induction of foam cell formation.

At first, the expression of adipophilin on human monocytes was analyzed. Monocytes were freshly isolated from whole blood of 11 healthy individuals. Total cell proteins of these cells were subjected to test the expression of adipophilin using western blot analysis. As the results, adipophilin was found to be expressed in monocytes from four donors (3, 6, 9, 11). However, the results showed an unequal amount of total proteins from isolated monocytes among individuals and explained the differences in expression of adipophilin in monocytes among different donors. To control, the blot was stripped and hybridized with β-actin (figure 3.3a)

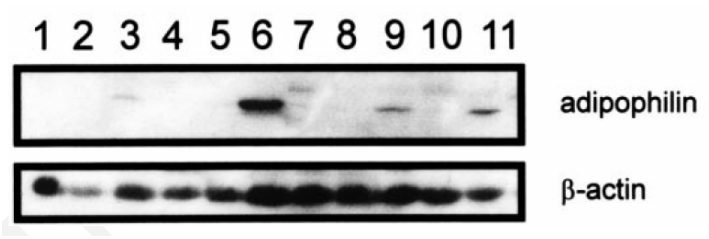


Figure 3.3a: Expression of adipophilin in human monocytes

Also, several publications as well as results of our group experiment indicated that E-LDL is more similar to atherogenic LDL isolated from atherogenic lesions than other types of modified lipoprotein (ox-LDL, ac-LDL) [9;256] and rapidly induced foam cell formation in monocytes and macrophages. Therefore, the regulation of adipophilin in macrophages treated with E-LDL has been tested. In this experiment, freshly isolated monocytes were differentiated to macrophages using M-CSF, incubated with E-LDL (40µg/ml) and cell sample harvesting according to time periods (2, 4 and 24 hours of incubation). The quantitative RT-PCR analysis showed that adipophilin expression was induced by E-LDL when compared to macrophages differentiated by M-CSF. The expression was up-regulated and time dependent, while the incubation with enzyme cocktails (EC) did not change its expression. In this experiment, GAPDH was used as control gene (figure 3.3b).

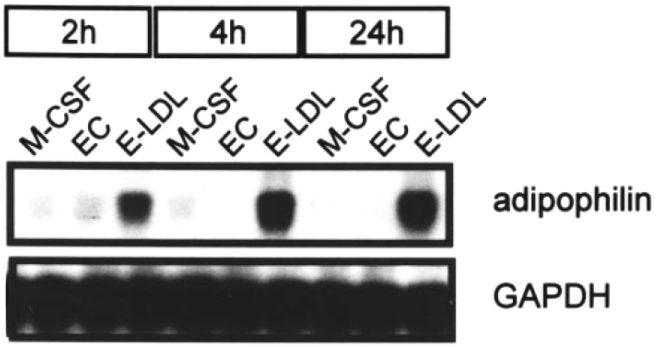


Figure 3.3b: Upregulation of adipophilin mRNA expression in foam cells

Western blot analysis of these cells also revealed a similar result. Adipophilin protein was induced in a time-dependent manner in macrophages stimulated with E-LDL and a slight induction was observed as early as 2 hours of incubation. In this analysis, β-actin was used as the control protein (figure 3.3c).

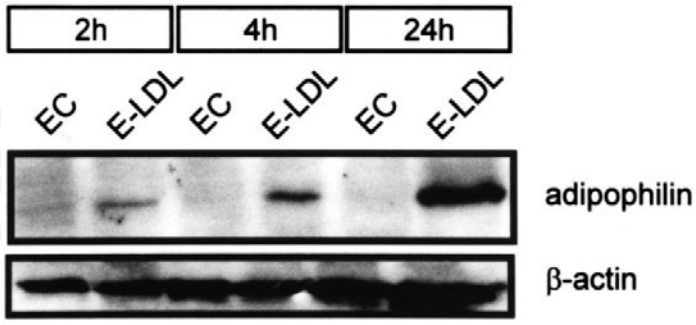


Figure 3.3c: Upregulation of adipophilin protein expression in foam cells

3.1.3. Expression analysis of ATP-binding cassette transporter 2 gene (ABCA2) in monocytes, macrophages and foam cells

In addition to the above-analyzed marker genes, the expression of the ABC-transporter gene 2 (ABCA2) was also investigated. The basis of this investigation is that two other ABC transporter genes (ABCA1 and ABCA7, sharing highest homology to ABCA2) are expressed in macrophages in a sterol-independent manner. Therefore, it is assumable that expression of ABCA2 in macrophages is also influenced by cholesterol. To test this hypothesis, monocytes were allowed to differentiate to macrophages and subsequently stimulated with cholesterol. Regulation expression of ABCA2 by cholesterol has been analyzed using semi-quantitative RT-PCR. The semi-quantitative RT-PCR results

showed that ABCA2 is slightly induced in macrophages compared to monocytes but show a very high expression in macrophages treated with cholesterol (E-LDL). The house-keeping gene (GAPDH) was used as a control gene in this experiment. The result confirmed the hypothesis that ABCA2 expression in human macrophages is regulated by cholesterol loading of macrophages.

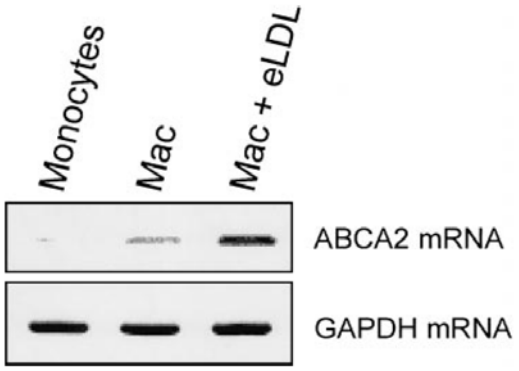


Figure 3.4: Upregulation of ABCA2 expression in monocyte-derived macrophages and cholesterol-loaded macrophages

Notes:

- *The Cla-1 and CD36 results were collaborated to the publication of Buechler and Ritter, BBRC, 1999 [257] and Kapinsky, ATVB, 2001 [11], respectively.*
- *The adipophilin results were contributed to the publication of Buechler, BBA, 2001 [258].*
- *The ABCA2 results were contributed to the publication of Kaminski, BBRC, 2001 [259].*

3.2. ANALYSIS OF LYSOPHOSPHOLIPID RECEPTORS EXPRESSION IN MONOCYTES, MACROPHAGES, AND CHOLESTEROL-LOADED/ -DELOADED MACROPHAGES

3.2.1. mRNA expression of lysophospholipid receptors in human monocytes

To investigate the mRNA expression levels of lysophospholipid receptors in human monocytes, an equal amount of total RNA from 4 different donors were employed for TaqMan® quantitative RT-PCR. The standard curve for each of receptors was generated from a pool of RNAs from all donors. The final results were calculated to standard curve and normalized to the GAPDH gene expression as the endogenous

control (Fig. 3.5). Experimental results showed that the mRNAs of all tested lysophospholipid receptors were detected in human blood monocytes. Of those, G2A showed the highest mRNA expression level and the strength of the expression followed by $S1P_3$, $S1P_4$, GPR3, LPA_2, $S1P_1$, LPA_4, LPA_1, GPR6, GPR12, $S1P_2$, $S1P_5$ and LPA_3. mRNA transcript from LPA_3 was also detected but expressed about fivefold lower than that of G2A. Also, the standard deviation given in figure 3.5 resembles the variance of the lysophospholipid receptor mRNAs abundance from the four different investigated donors. These data show that monocytes of different donors express similar levels of the lysophospholipid receptors mRNAs with less than 2 folds difference.

Table 3.1: Primers and probes for TaqMan® RT-PCR analysis of lysophospholipid receptors

Receptor	* Forward primer 5'-3'	Hybridization probe 5'-3'	Reverse primer 5'-3'
$S1P_1$	TGCGGGAAGGGAGTATGTTT	TGGCCCTGTCAGCCTCCG	CGATGGCGAGGAGACTGAAC
$S1P_2$	GCCTCTCTACGCCAAGCATTA	CTGTGCGTGGTGACCATCTTCTCCAT	TTGAGCGGACCACGCAGTA
$S1P_3$	TGATTGTGGTGAGCGTGTTCA	CGCCTGCTGGTCCCCACTCTTC	GGCCACATCAATGAGGAAGAG
$S1P_4$	GGAGCCCCTGTCCAGCAT	TCCAGCGTGCGGAGCATCTGAA	TCCACACGCAAGACTGCAA
$S1P_5$	GCTTGCTCCACTGTCTTGCC	TCGCCTTCGTGGGCATCC	TAGAGTGCACAGATAGCGGCC
LPA_1	CAATACTCGGAGACTGACTCTTAGC	ATGGCTCCTGCGTCAGGGCCT	CCGTCAGGCTGGTGTCAAT
LPA_2	GGCTGTGAGTCCTGCAATGTC	AGGCCAACTCACTGGTCAATGCTGC	TCTCAGCATCTCGGCAAGAGT
LPA_3	CTTGACTGCTTCCCTCACCAA	TTGCTGGTTATCGCCGTGGAGAGG	CGCATCCTCATGATTGACATG
G2A	GCCATCCCTCTCTCCATCATC	CCTTCACCAACCACCGGATTTTCAGG	TGCTTCACCTTGGCCTTCTG
GPR3	GCTCCCTGCCACCTACAACTC	CTACGCCTTCCGCAACCAGGAT	GACAGCCCACAGCACTTTCTG
GPR6	ACTCCATGATCAATCCCATCATCTA	CGCAACCAGGAGATCCAGGCG	GGCACTTTGGACTGGAAACAG
GPR12	TCTCGGTGTCCTTCCTCTTCAT	TGCGCTCATGCATCAGCTCTAC	GGCGTGCCTCATCACAATCT

* LPA_4 probe was ordered as an Assay-On-Demand probe from Applied Biosystems, Darmstadt, Germany.

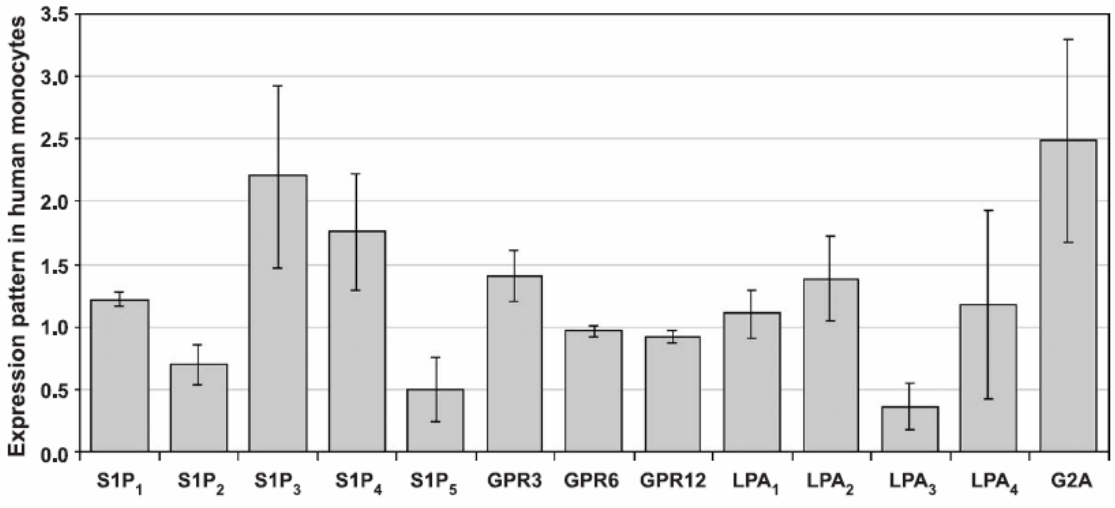


Figure 3.5: mRNA expression of lysophospholipid receptors in human monocytes

3.2.2. mRNA expression of the lysophospholipid receptors during in-vitro differentiation of monocytes to macrophage

In order to analyze the mRNA expression of these lysophospholipid receptors during the phagocytic differentiation of macrophages, monocytes from above-mentioned donors were allowed to differentiate to macrophages for four days in a serum-free medium using M-CSF. Total RNA from those cells was isolated and the mRNA expression was determined by TaqMan® RT-PCR using standard curves for each receptor. After normalization to the housekeeping gene GAPDH, fold changes of mRNA expression of these receptors in macrophages compared to the corresponding monocytes were calculated by division of the corresponding values. As it is shown in figure 3.6, the lysophospholipid receptors expressed differently during macrophage differentiation and the value 1 represented for a no-change line. The mRNA of GPR3 and G2A were 3 and 2.5 folds down-regulated in macrophages, respectively, when compared to non-differentiated monocytes. Other receptors, including $S1P_1$, $S1P_2$, $S1P_3$ $S1P_4$, $S1P_5$, GPR6, GPR12, LPA_1, LPA_2, LPA_3 and LPA_4 showed a less than 2.5-fold changes of the mRNA expression levels (figure 3.6).

3.2.3. mRNA expression of the lysophospholipid receptors during foam cell formation and lipid deloading

To further investigate the effects of modified lipoprotein on lysophospholipid receptors, differentiated macrophages were incubated with E-LDL (40 µg/ml, 24h) and subsequently incubated with purified HDL_3 (100 µg/ml, 24h) to allow lipid efflux. Experimental results showed that LPA_3 and LPA_4 mRNAs were found induced in E-LDL treated cells, mRNA levels were, respectively, 2.7-fold and 4.2-fold increased in E-LDL laden cells compared to 4 days differentiated macrophages. However, LPA_3 and LPA_4 were not found reduced in HDL_3 treated foam cells (figure 3.6). The mRNAs of the remaining receptors showed no regulation upon E-LDL loading and HDL_3 de-loading. This indicates that the mRNAs of the lysophospholipid receptors are not significantly regulated by sterols or fatty acids.

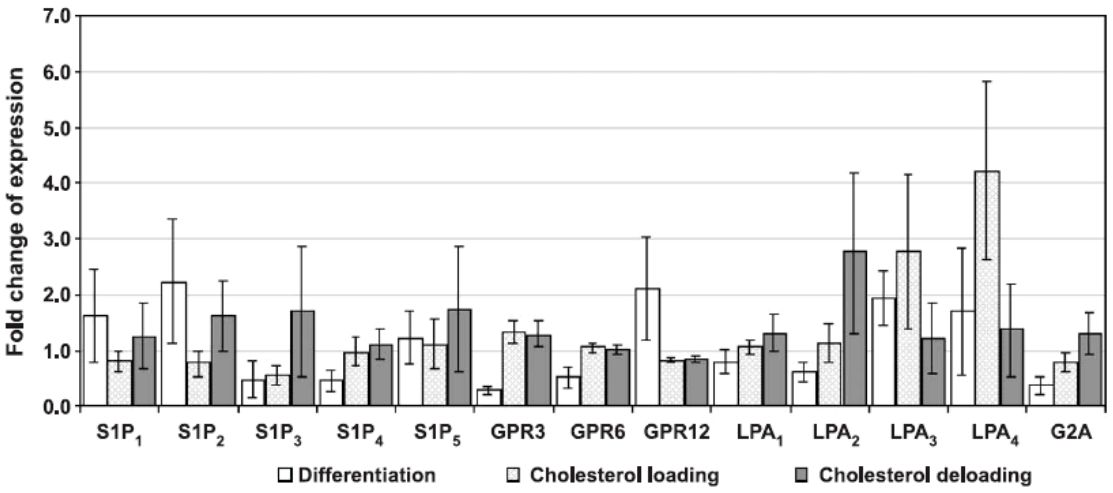


Figure 3.6: mRNA expression of lysophospholipid receptors in human differentiated macrophages, foam cells and HDL_3-treated macrophages.

3.2.4. Protein expression of the lysophospholipid receptors

To investigate the expression of the lysophospholipid receptors on the protein level in this *in vitro* model, immunoblots with currently available antibodies were performed. Protein isolated from the prostate (BD Biosciences, CA, USA) was used as positive control and β-actin abundance as a loading control. Cell lysates were prepared from monocytes, 4-day differentiated macrophages, foam cells and foam cells treated with HDL_3. In addition, 4-day differentiated macrophages were treated with HDL_3 without prior E-LDL treatment to investigate the effects of HDL_3 on differentiated macrophages. The experiments were performed with cells lysates from at least 2 different donors.

Immunoblots results showed that $S1P_1$, $S1P_2$, $S1P_4$, LPA_1 and LPA_2 expressed in monocytes and the expression was similar in monocytes, macrophages, foam cells and HDL_3 treated foam cells. $S1P_5$ and LPA_3 proteins could not be detected in any of the stimulated cells. $S1P_3$ is not expressed in monocytes but is strongly induced during differentiation, whereas incubation with E-LDL or HDL_3 showed a reduced abundance of $S1P_3$ when compared to macrophages. LPA_1 is already expressed in monocytes but down-regulated in macrophages, foam cells and HDL_3 treated macrophages. LPA_2 protein expression was equal in all stimulations.

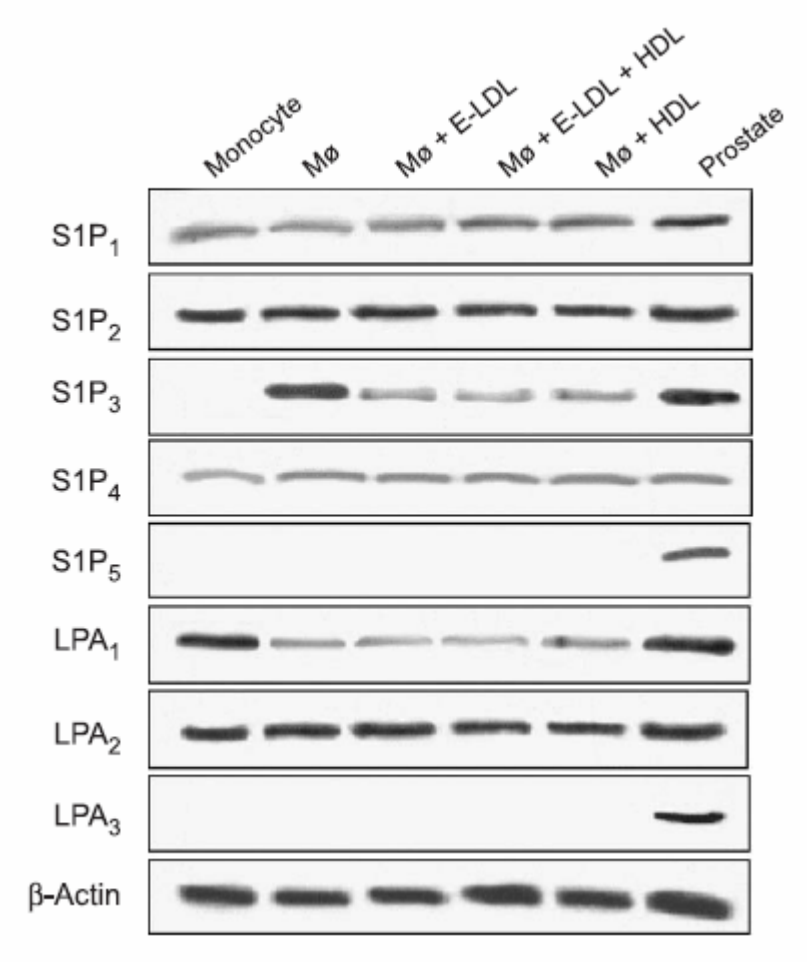


Figure 3.7: Immunoblots of lysophospholipid receptors in monocytes, macrophages, foam cells and HDL_3-treated macrophages.

3.3. DNA MICROARRAY EXPERIMENTS AND BIOSTATICTICS ANALYSIS OF GENES REGULATED BY E-LDL AND LYSOPHOSPHOLIPIDS

3.3.1. Principles of large-scale gene expression analysis by DNA microarray

To identify the regulation of genes and pathways in foam cell induced by E-LDL and in macrophages stimulated by lysophospholipids, the Affymetrix GeneChip™ U133A was employed. The principle microarray analysis is shown below.

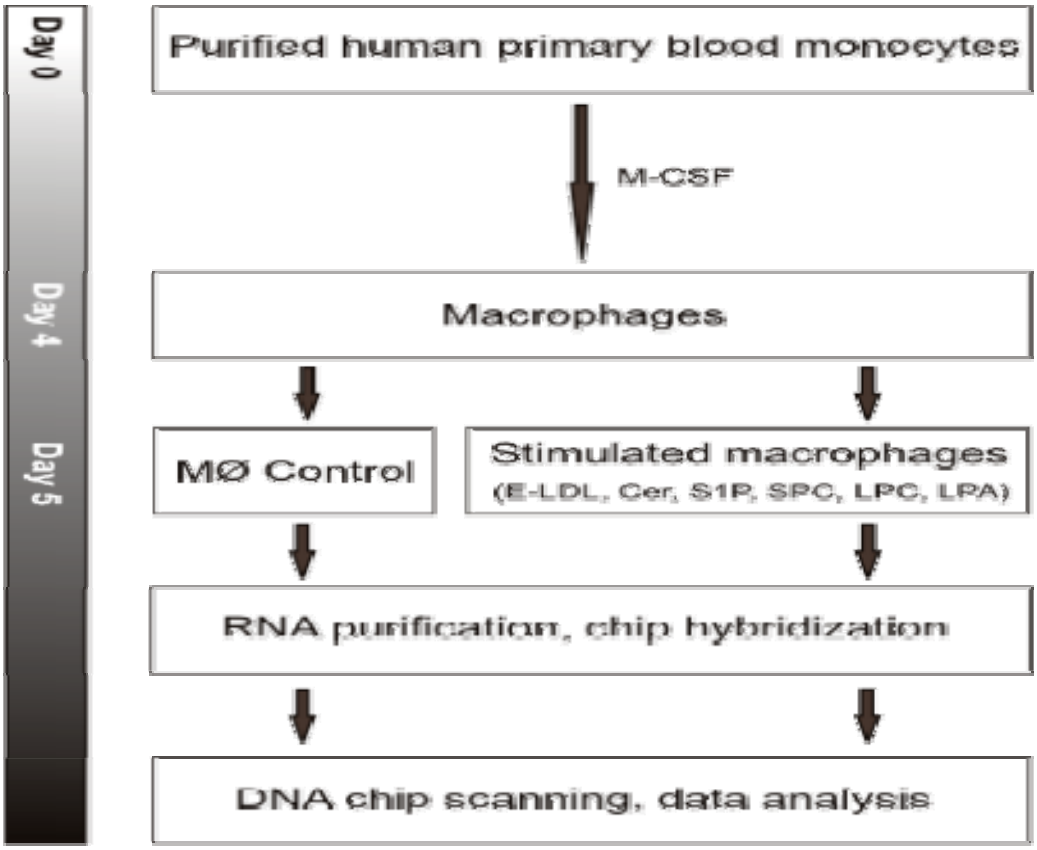
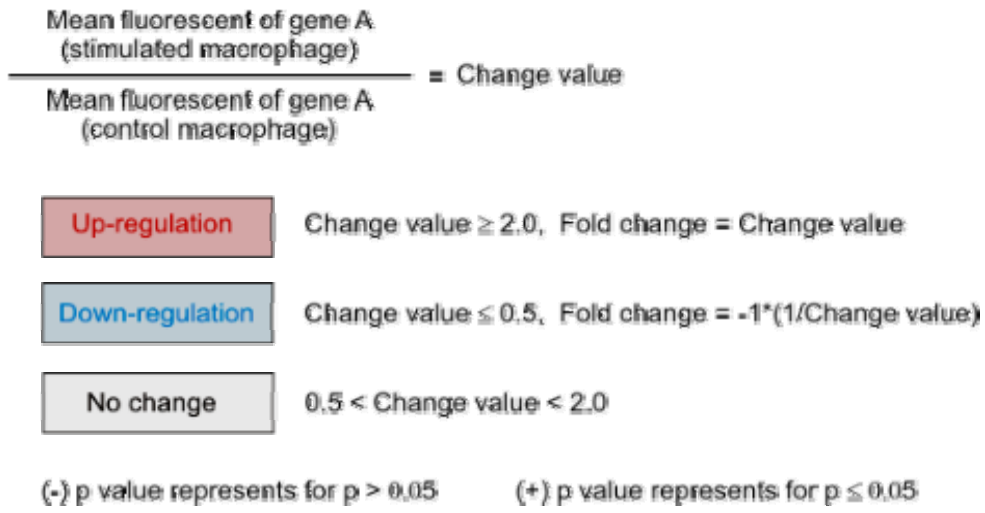


Figure 3.8: Experimental model of microarray analysis of macrophages

Total RNAs from healthy donors were used for lysophospholipid stimulation and DNA microarray analysis. The cRNAs were labeled with streptavidin phycoethrin fluorescent and hybridized to the chip. After scanning, the result of hybridizations were archived as high-resolution images and analyzed with the Affymetrix Microarray Suite 5.0^{TM}. Gene information (including name, UniGene, chromosome localization, and cellular function) was then merged to every probeset on the chip to generate the complete gene database for further analysis.

In this experimental model, the expression level of the gene of interest during stimulation was represented by its mRNA accumulation level conserved during cRNA synthesis and fluorescent labeling. Subsequently, the accumulation level of the probes those hybridized to the DNA microarray was detected in terms of mean fluorescent of the probeset. Finally, the change of expression patterns of genes was calculated according to the comparison of mean fluorescent among different stimulations. The regulation of gene expression was calculated as following:



3.3.2. Expression analysis of control genes with DNA microarray

In addition to internal expression control of house-keeping genes spotted on Affymetrix GeneChip, expression patterns of CD36, adipophilin, ABCA2, low density lipoprotein receptor (LDLR) and Cla-1 were analyzed. Consistent with the previous works (see 3.1) the RNA expression of CD36, adipophilin and ABCA2 were 2.1, 2.4 and 3.9 folds up-regulated, respectively, in macrophages stimulated with E-LDL. Scavenger receptor class B, member 1, Cla-1, was slightly elevated (1.5-fold) whereas low density lipoprotein receptor expressed a 10-fold down-regulation in RNA expression in comparison to the non-stimulated control macrophages. All of these regulations showed a (+) p-value (indicates a p-value ≤ 0.05) which demonstrated the reliability of the changes. Together with the house-keeping genes on the GeneChip, these results further prove the reliability of the experimental and computational archived data.

Table 3.2: DNA Microarray analysis of marker genes expression in E-LDL stimulated macrophages.

Name	Unigene	Short	Chr.Loc.	Macrophage Control	E-LDL	p value	Fold change
Collagen type I receptor, thrombo-spondin receptor	Hs.75613	CD36	7q11.2	1500	3179.4	+	2.1
Adipose differentiation-related protein (Adipophilin)	Hs.3416	ADFP	9p21.2	1541.1	3688.3	+	2.4
ATP-binding cassette, sub-family A (ABC1), member 2	Hs.121561	ABCA2	9q34	3.9	15.2	+	3.9
Low density lipoprotein receptor (familial hypercholesterolemia)	Hs.213289	LDLR	19p13.3	223.8	28.8	+	-10
Scavenger receptor class B, member 1 (Cla-1)	Hs.298813	SCARB1	12q24.32	194.4	288.4	+	1.5

(-) fold change represents a down regulation

3.3.3. Biostatistic ranking of regulated genes in microarray analysis

The Affymetrix GeneChip™ U133A contains 22,283 gene transcripts (duplicates excluded). As the results archived from DNA microarray analyses, there were n=2962 (13%) genes found to be regulated by E-LDL stimulation. Similarly, n=1701 (7.6%) genes found to be regulated by ceramide, n=1762 (7.9%) genes for S1P, n=2019 (9.1%) genes for SPC, n=1876 (8.4%) genes for LPC and n=1721 (7.7%) genes for LPA. Among these, E-LDL and SPC were having higher influence on macrophage genes than that of ceramide, S1P, LPC and LPA. Furthermore, the calculation also revealed that these stimuli suppressed more genes than elevated them. The percentage of suppressed genes ranges from 54% (LPA) to 73% (E-LDL) of total regulated genes. In total, there were n=5534 gene transcripts in macrophages found to be significantly regulated upon at least one of the stimulations (table 3.3).

Table 3.3: Genes regulated by individual stimulation in macrophages

Stimuli	Up	Down	Total	Percentage
E-LDL	797	2165	**2962**	**13%**
Ceramide	617	1084	1701	7.6%
S1P	569	1193	1762	7.9%
SPC	878	1141	**2019**	**9.1%**
LPC	534	1252	1786	8.4%
LPA	788	933	1721	7.7%

In addition, statistic analysis of regulated genes revealed that there is remarkable number of genes being influenced by two or more stimuli. Therefore, it is necessary to look at the role of structure-related and/or function-related stimuli in genes regulation. For this purpose, four groups of genes were assembled to ceramide/S1P, S1P/SPC, LPC/LPA and S1P/LPA biostatistics ranking lists. Subsequently, five sub-groups were generated for each of comparison, including common up, common down, specific up, specific down and inversely regulated genes (table 3.5).

Table 3.4: Global genes co-regulated by different stimulations in macrophages

	Common up	Common down	Specific up	Specific down	Inversely regulated	Sum
Cer/S1P	253	442			62	2706
Cer			327	617		
S1P			291	714		
S1P/LPA	275	365			75	2768
S1P			271	776		
LPA			461	545		
LPC/LPA	265	382			84	2779
LPC			250	808		
LPA			461	529		
S1P/SPC	287	385			75	3034
S1P			263	752		
SPC			535	737		

Table 3.5: Pathway genes co-regulated by different stimulations in macrophages

	Common up	Common down	Specific up	Specific down	Inversely regulated	Total
Cer/S1P	137	203			34	1294
Cer			142	324		
S1P			129	325		
S1P/LPA	128	175			33	1357
S1P			146	346		
LPA			216	313		
LPC/LPA	110	195			39	1424
LPC			144	415		
LPA			232	289		

3.4. DNA MICROARRAY ANALYSIS OF MACROPHAGE GENE EXPRESSION REGULATION IN LITERATURE-BASED PATHWAYS

3.4.1. Assemble of genes into literature-based pathways and categories

In order to have more insights of the gene regulation in macrophages, the GeneChip-presented genes were group into different pathway-related and/or structure-related categories. These pathway-defined and category-arrangement were performed according to literature surveys, database searches and unpublished data from our laboratory groups. The current assemble table having 65 pathways/categories (n=5673 genes), exclude unidentified-pathway genes and unknown transcripts. There are duplicates of gene entries in the pathways list due to the multiple contributions of numerous genes to different pathways.

Table 3.6: Functional pathways collected from literatures and databases.

Nr.	Pathway	Nr.	Pathway
1	Wnt/Frizzled	34	Integrins
2	Notch	35	TGFβ-family/BMP/TGFβ/Activins/Inhibins and signalling
3	Hedgehog	36	Collagens
4	Stimulatory and Inhibitory receptors in differentiation and apoptosis	37	Laminins
5	ABC Transporter	38	Proteoglycans
6	Ion Channels/Porins	39	Hyaluronan
7	Phagosome	40	Elastin/Fibrillin/Keratin/Fibulin
8	Rab/Ras pathway	41	Nuclear matrix
9	Rac/Cdc42 regulatory pathways	42	EGF family
10	Lamellar body	43	Junctional control
11	Adapter proteins	44	other ECM proteins
12	PDZ family	45	G protein receptors and G proteins
13	Other vesicular traffic proteins	46	Kinases + Phosphatases
14	Migration/polarization	47	NFkB signalling
15	TNF receptor signalling	48	Other second messengers
16	TNF/PI3 kinase coupling	49	General transcription POLII/TAFs/CDK/Cyclins/CDCs/CDT-kinases in transcriptional control
17	Apoptosis/Proteasome	50	Nucleosome
18	Mitochondrion	51	Nuclear receptors
19	Glucose/Insulin/Glutathione/Glucuronides	52	PPAR/LxR/PxR/RxR/FxR nuclear receptor target genes
20	Heat shock, Thioredoxin & target proteins	53	ZNF TCFs
21	Detoxification Phase I	54	Homeobox
22	Detoxification Phase II	55	NFkB target genes
23	Heat shock	56	Other specific transcription factors
24	NO/Thioredoxin	57	Splicing
25	Cholesterol metabolism/ SREBP1/2	58	Translation machinery
26	Fatty acid metabolism	59	IL-1β
27	Glycerophospholipids	60	IFNα regulated genes
28	Phosphatidylinositol and Ca^{++}	61	Homocysteine
29	Sphingolipids	62	Lipid sensitive genes in C. elegans
30	Gangliosides	63	Complement and defence
31	Sulfatides	64	IDB candidate loci
32	Glycosylation/Sialation	65	Colon express genes
33	Proteinases/Serpins/MMPs/ADAMS	66	Known genes not in pathways and unknown gene transcripts

Table 3.7: Pathways that influenced by E-LDL and lysophospholipids stimulation.
Percentage was calculated based on division of regulated genes to total number in corresponding pathway (red = up-regulation, blue = down-regulation).

Path. Nr.	ELDL		Cer		S1P		SPC		LPC		LPA		SUM
	up	down	up	down	up	down	up	down	up	down	up	down	
1	4.8%	12.7%	4.2%	5.5%	1.8%	9.1%	4.8%	5.5%	3.0%	10.3%	3.6%	6.1%	165
2	5.1%	7.7%	2.6%	5.1%	2.6%	2.6%		2.6%		5.1%	2.6%	2.6%	39
3		26.9%	3.8%	11.5%		13.5%	1.9%	5.8%		19.2%	1.9%	11.5%	52
4	5.4%	16.1%	5.7%	5.7%	4.7%	7.3%	7.5%	4.4%	3.4%	9.1%	4.4%	6.2%	386
5	7.3%	13.4%	1.2%			4.9%	1.2%	4.9%	1.2%	4.9%	2.4%	6.1%	82
6	3.1%	24.5%	3.8%	14.5%	8.2%	12.6%	11.3%	12.6%	5.7%	8.8%	8.2%	8.8%	159
7	6.4%	5.5%	1.8%	0.9%	0.9%	1.8%	2.8%	2.8%	0.9%	2.8%	0.9%	1.8%	109
8	4.8%	7.2%		2.4%	1.2%	6.0%	2.4%	6.0%		7.2%		7.2%	83
9		11.4%		2.9%		2.9%				2.9%			35
10	7.5%	12.6%	3.1%	3.5%	2.4%	6.7%	5.1%	4.3%	2.8%	8.7%	3.9%	4.3%	254
11	6.1%	7.3%		6.1%	1.2%	3.7%	4.9%	4.9%	1.2%	3.7%	2.4%	6.1%	82
12	8.5%	18.3%	5.6%	7.0%	2.8%	11.3%	4.2%	9.9%	4.2%	9.9%	2.8%	8.5%	71
14	5.3%	12.3%	3.5%	3.5%	3.5%	3.5%	3.5%	1.8%	1.8%	8.8%	1.8%	3.5%	57
15	2.0%	8.8%	0.7%	3.4%	2.0%	2.0%	1.4%	4.1%	1.4%	3.4%	2.0%	4.7%	148
16		10.3%			2.6%			2.6%				2.6%	39
17	1.9%	7.8%	1.3%	3.8%	1.9%	3.2%	2.4%	4.0%	1.6%	4.3%	2.2%	2.4%	371
18	1.4%	4.7%	1.7%	3.4%	2.0%	2.0%	2.7%	1.7%	1.0%	2.0%	0.7%	2.7%	295
19	7.3%	10.1%	2.8%	8.9%	4.5%	7.3%	5.6%	6.7%	2.8%	8.4%	5.0%	7.8%	179
20	5.7%	17.1%	2.9%	8.6%	17.1%	5.7%	14.3%	8.6%	8.6%	20.0%	14.3%	11.4%	35
21	8.9%	17.8%	5.0%	10.9%	5.0%	9.9%	9.9%	13.9%	5.0%	8.9%	11.9%	7.9%	101
22	9.7%	5.6%	5.6%	4.2%	8.3%	11.1%	8.3%	6.9%	4.2%	4.2%	5.6%	6.9%	72
23	2.9%				2.9%			8.6%		2.9%	2.9%	5.7%	35
24	5.6%	12.2%	3.3%	7.2%	4.4%	3.3%	6.7%	2.2%	3.3%	6.1%	5.0%	3.9%	180
25	1.3%	26.7%	4.0%	6.7%	1.3%	4.0%	2.7%	6.7%	1.3%	4.0%	1.3%	4.0%	75
26	5.7%	12.8%	6.4%	7.1%	6.4%	5.7%	5.0%	3.5%	5.0%	6.4%	3.5%	7.8%	141
27		8.3%		4.2%		4.2%	4.2%			4.2%		4.2%	24
28	4.3%	8.7%	2.9%	7.2%	2.9%	4.3%	5.8%	5.8%	4.3%	4.3%	5.8%	2.9%	69
29	5.3%	5.3%			5.3%		5.3%	5.3%				5.3%	19
30	2.3%	11.6%	2.3%	7.0%	2.3%	2.3%	2.3%	7.0%		11.6%	7.0%	2.3%	43
31		9.1%				9.1%							11
32		10.5%		10.5%		5.3%	5.3%	10.5%		10.5%	5.3%	5.3%	19
33	5.7%	8.9%	4.1%	8.1%	4.1%	10.6%	8.9%	8.9%	4.1%	8.1%	6.5%	8.9%	123
34		14.3%		14.3%	3.6%	7.1%		10.7%	3.6%	17.9%		7.1%	28
35	7.5%	17.2%	6.3%	13.8%	4.0%	8.0%	5.7%	8.0%	4.6%	10.9%	6.3%	5.2%	174
36	8.3%	20.8%	4.2%	12.5%	2.1%	2.1%	6.3%	10.4%	2.1%	12.5%	2.1%	6.3%	48
37	9.7%	25.8%	3.2%	12.9%	3.2%	16.1%	6.5%	9.7%	6.5%	16.1%	12.9%	3.2%	31
38	5.4%	16.2%	2.7%	10.8%		8.1%	8.1%	10.8%	5.4%	16.2%	2.7%	10.8%	37
39	5.1%	13.3%	4.1%	7.1%	3.1%	6.1%	5.1%	6.1%	5.1%	8.2%	3.1%	4.1%	98
40	6.7%	20.0%	2.2%	6.7%	4.4%	8.9%	7.8%	6.7%	4.4%	7.8%	3.3%	6.7%	90
41	3.1%	12.5%	1.6%	6.3%	1.0%	3.6%	3.1%	5.7%	2.1%	7.8%	1.0%	4.2%	192
42	7.1%	15.9%	7.1%	7.1%	5.3%	6.2%	12.4%	8.0%	3.5%	9.7%	12.4%	8.0%	113
43	10.6%	16.7%	6.1%	9.1%	6.1%	10.6%	9.1%	7.6%	6.1%	6.1%	7.6%	7.6%	66
44	8.0%	15.9%	8.0%	8.0%	6.8%	4.5%	13.6%	6.8%	5.7%	12.5%	12.5%	8.0%	88
45	5.7%	22.7%	3.7%	9.7%	5.0%	13.7%	9.7%	10.3%	4.7%	20.3%	8.3%	11.7%	300
46	4.6%	10.2%	3.4%	6.3%	2.8%	5.1%	3.6%	5.3%	2.5%	5.3%	3.2%	5.5%	527
47	2.3%	10.5%	2.3%	2.3%	1.2%	2.3%	2.3%	4.7%	1.2%	2.3%	2.3%	2.3%	86
49	2.6%	6.7%	2.1%	4.1%	2.1%	3.6%	2.1%	3.1%	2.1%	2.6%	2.6%	4.1%	194
50	9.8%	18.3%	9.8%	9.8%	7.3%	11.0%	14.6%	9.8%	8.5%	12.2%	6.1%	12.2%	82
51	6.5%	23.9%	4.3%	15.2%		19.6%		10.9%	4.3%	17.4%	4.3%	15.2%	46
52	5.4%	21.1%	5.4%	3.4%	3.4%	2.0%	9.5%	7.5%	4.1%	4.1%	7.5%	2.7%	147
53	4.8%	16.2%	5.7%	3.8%	3.8%	4.8%	8.6%	5.7%	2.9%	1.9%	3.8%	8.6%	105
54		26.7%	6.7%	6.7%	3.3%	13.3%	6.7%	10.0%	6.7%	13.3%	3.3%	16.7%	30
56	2.7%	11.5%	0.9%	2.7%	2.7%	3.5%	1.8%	3.5%	2.7%	7.1%	1.8%	2.7%	113
57			7.1%									7.1%	14
58	1.1%	1.1%		1.1%		1.1%	2.1%	1.1%		1.1%		1.1%	95
59	3.1%	12.5%		3.1%		3.1%	6.3%	15.6%		6.3%		12.5%	32
60	5.3%	8.2%	3.4%	4.8%	2.4%	2.9%	3.9%	5.3%	4.3%	3.4%	2.9%	1.9%	207
61	10.3%	6.9%	6.9%	10.3%	3.4%		10.3%	6.9%	6.9%		3.4%	6.9%	29
62	3.1%	10.8%	2.7%	6.3%	1.8%	8.1%	3.1%	8.5%	2.7%	9.0%	3.6%	7.6%	223
63	4.9%	11.8%	5.9%	3.9%	8.8%	2.9%	8.8%	2.9%	8.8%	3.9%	7.8%	2.9%	102
64	4.3%	14.5%	2.8%	8.5%	3.0%	9.2%	4.3%	7.6%	2.8%	8.9%	4.1%	7.4%	740
65	3.7%	9.2%	2.6%	3.7%	2.1%	4.4%	3.6%	4.7%	2.1%	4.4%	3.5%	3.2%	1601

Collected pathway genes were matched to microarray data using UniGene numbers. By this approach, the results from stimulated macrophages experiments were added to genes in pathways as presented in table 3.6 (categories 13, 48 and 55 are not

presented in this table due to no current update is available). The result table 3.6 summarized the contribution of pathways regulation of the stimuli in macrophages. Differ from other stimulation, E-LDL remarkably showing its influences on 45 pathways and most of them were down-regulated.

3.4.2. Candidate genes and pathways regulation in stimulated macrophages

3.4.2.1. Analysis of cholesterol metabolism pathway in stimulated macrophages

Lipid homeostasis in vertebrate cells is regulated by a family of membrane-bound transcription factor named as sterol regulatory element-binding proteins (SREBPs). The mammalian genome encodes three SREBP isoforms, designed SREBP-1a, -1c and SREBP-2. These three isoforms directly activate the expression of more than 30 genes dedicated to the synthesis and uptake of cholesterol, fatty acids, triglycerides, phospholipids as well as the NADPH cofactors required to synthesis of these molecules. Using DNA microarray, the synthesis pathways of these molecules in lipid-stimulated macrophages have been analyzed. Total of n=75 related genes were screened and n=30 genes have been found to be regulated in these stimulations (table 3.8).

There are two pathways that activated by SREBP-1c and SREBP-2. The microarray analysis revealed that enzymatically-modified LDL was having highest influences in comparison to other stimuli and E-LDL showing its most effects on the cholesterol metabolism pathway that activated by SREBP-2. E-LDL suppressed the expression of SREBP-2 activator and almost all related enzymes with only an exception of HMG CoA synthase. HMG CoA synthase isoform 1 was down-regulated by E-LDL, ceramide and SPC while isoform 2 was up-regulated by E-LDL and ceramide. Contradictory with SREBP-2 activated pathway, expression of SREBP-1c and most of SREBP-1c activated enzymes were not influenced by any of tested stimuli. Ceramide, S1P, SPC and LPC were regulated several genes but not seemed to alter the synthesis pathway. In addition to the suppression of SREBP-2, SERBP cleavage-activating protein (SCAP) as escort protein for SERBP and sensor of sterols was not regulated in any stimulations.

Table 3.8: Cholesterol metabolism pathway genes regulated by lipid stimulation of macrophages. (red = up-regulation, blue = down-regulation, (-) for down-regulation, value = fold change).

Name	Unigene	Short name	ChromLoc	ELDL vs. Ctrl	Cer8 vs. Ctrl	S1P vs. Ctrl	SPCvs. Ctrl	LPC vs. Ctrl	LPA vs. Ctrl
SREBP cleavage-activating protein	Hs.78442	SCAP	3p21.31	1.2	-1.3	1.1	1.2	1.0	-1.3
sterol regulatory element binding transcription factor 2	Hs.108689	SREBF2	22q13	-2.0	-1.1	-1.1	1.0	-1.1	-1.3
acetyl-Coenzyme A acetyltransferase 2 (acetoacetyl Coenzyme A thiolase)	Hs.278544	ACAT2	6q25.3-q26	-2.5	-1.1	-1.1	1.0	1.0	1.0
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	Hs.77910	HMGCS1	5p14-p13	-3.3	-2.0	-1.7	-2.0	-1.4	-1.7
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)	Hs.59889	HMGCS2	1p13-p12	2.0	3.5	1.1	1.5	1.9	-2.0
3-hydroxy-3-methylglutaryl-Coenzyme A reductase	Hs.11899	HMGCR	5q13.3-q14	-2.0	-1.1	1.1	-1.1	1.1	-1.1
mevalonate kinase (mevalonic aciduria)	Hs.130607	MVK	12q24	-3.3	-1.7	-1.1	-1.4	3.9	-1.7
mevalonate (diphospho) decarboxylase	Hs.3828	MVD	16q24.3	1.2	2.3	2.4	2.1	1.7	1.8
isopentenyl-diphosphate delta isomerase	Hs.76038	IDI1	10p15.3	-5.0	-1.1	1.0	-1.3	1.0	1.0
farnesyl-diphosphate farnesyltransferase 1	Hs.48876	FDFT1	8p23.1-p22	-5.0	1.0	1.0	-1.1	1.0	1.0
squalene epoxidase	Hs.71465	SQLE	8q24.1	-5.0	-1.4	-1.1	-1.3	1.0	1.0
Human lanosterol 14-alpha demethylase (CYP51P2) processed pseudogene	Hs.226213			-3.3	-1.4	-1.1	-1.3	-1.3	-1.3
7-dehydrocholesterol reductase	Hs.11806	DHCR7	11q13.2-q13.5	-3.3	-1.3	1.1	1.2	-1.1	-1.1
low density lipoprotein receptor (familial hypercholesterolemia)	Hs.213289	LDLR	19p13.3	-10.0	-1.3	1.0	-1.4	1.0	-1.1
sterol O-acyltransferase (acyl-Coenzyme A: cholesterol acyltransferase) 1	Hs.14553	SOAT1	1q25	1.1	-1.4	1.2	-2.5	1.0	1.0
malic enzyme 1, NADP(+)-dependent, cytosolic	Hs.14732	ME1	6q12	-2.5	1.2	1.1	1.4	1.0	1.1
hexokinase 2	Hs.198427	HK2	2p13	-2.0	-1.3	1.1	-1.1	-1.1	-1.1
insulin induced gene 1	Hs.56205	INSIG1	7q36	-5.0	-1.3	1.1	-1.1	1.0	-1.1
peroxisomal trans 2-enoyl CoA reductase; putative short chain alcohol dehydrogenase	Hs.281680	PECR	2q35	1.4	1.2	-2.0	1.3	1.2	-1.7
proprotein convertase subtilisin/kexin type 1	Hs.78977	PCSK1	5q15-q21	-1.1	-1.1	-1.3	2.6	1.3	2.7
proprotein convertase subtilisin/kexin type 1 inhibitor	Hs.256311	PCSK1N	Xp11.23	-3.3	-3.3	-3.3	1.5	-1.7	-2.5
proprotein convertase subtilisin/kexin type 2	Hs.93164	PCSK2	20p11.2	-2.5	-1.4	-1.4	-1.1	-2.0	-1.7
proprotein convertase subtilisin/kexin type 7	Hs.32978	PCSK7	11q23-q24	1.7	-2.5	1.1	-1.4	-1.7	1.2
elongation of very-long-chain-fatty-acids like 2	Hs.246107	ELOVL2	6p22.3	-1.3	-3.3	-5.0	-3.3	-5.0	-2.0
Sterol regulatory element binding transcription factor 1	Hs.592123	SREBPF1	17p11.2	-1.7	-1.3	1.0	1.1	-1.4	1.1
acetyl-Coenzyme A carboxylase alpha	Hs.7232	ACACA	17q21	-1.7	-3.3	1.1	-2.5	1.1	1.2
acetyl-Coenzyme A carboxylase beta	Hs.183857	ACACB	12q24.1	-1.1	2.5	-1.1	1.3	1.0	-1.3
fatty acid desaturase 1	Hs.132898	FADS1	11q12.2-q13.1	-2.5	-1.1	1.0	1.0	-1.1	1.0
fatty acid desaturase 2	Hs.184641	FADS2	11q12-q13.1	-10.0	-1.1	1.0	1.2	-1.3	1.3
fatty-acid-Coenzyme A ligase, long-chain 4	Hs.81452	FACL4	Xq22.3-q23	-2.0	-1.3	-1.1	-2.0	-1.3	1.2
fatty-acid-Coenzyme A ligase, long-chain 6	Hs.14945	FACL6	5q31	-2.5	-2.0	-1.4	1.1	-2.0	-2.0

3.4.2.2. Analysis of ABC transporter genes expression in lipid-stimulated macrophages

In addition to the previously described ABCA2 gene, a list of ABC transporter genes family regulated in stimulation of macrophages has been created. Table 3.9 showing ABC genes that regulated by E-LDL, ceramide, S1P, SPC, LPC and LPA. 35 genes from the total of 51 ABC gene members of 7 subfamilies have been analyzed with microarrays and n=15 (43%) genes from 5 subfamilies found to be regulated during

macrophages stimulations. Interestingly, several ABC genes have been found highly up or down regulated in lipid stimulated macrophage. ABCA2, known to transport estramustine and steroids, was 3.9-fold up-regulated. ABCA8, known for drug transportation, was 7-fold up-regulated. Whereas, ABCD2, as a very-long-chain fatty acid transporter, was 10 and 3.3 folds down-regulated by E-LDL and SPC, respectively. Other genes; ABCA3 (surfactant phospholipids transporter), ABCA12 and ABCB9 were 3.3 folds down-regulated by E-LDL and LPA.

Table 3.9: Regulation of ABC transporter genes upon stimulation

(red = up-regulation, blue = down-regulation, (-) for down-regulation, value = fold change).

Name	Unigene	Short name	ChromLoc	ELDL vs. Ctrl	Cer8 vs. Ctrl	S1P vs. Ctrl	SPC vs. Ctrl	LPC vs. Ctrl	LPA vs. Ctrl
ATP-binding cassette, sub-family A (ABC1), member 2	Hs.121561	ABCA2	9q34	3.9	-1.1	1.9	1.1	1.1	1.8
ATP-binding cassette, sub-family A (ABC1), member 3	Hs.26630	ABCA3	16p13.3	-3.3	-2.0	1.1	-1.3	-1.1	-1.1
ATP-binding cassette, sub-family A (ABC1), member 6	Hs.15780	ABCA6	17q24.3	-1.7	-1.4	-1.1	-2.0	-2.0	-1.1
ATP-binding cassette, sub-family A (ABC1), member 8	Hs.38095	ABCA8	17q24	7.0	1.3	-2.0	-1.4	-1.3	2.2
ATP-binding cassette, sub-family A (ABC1), member 12	Hs.134585	ABCA12	2q35	-1.4	-1.0	-1.4	1.2	-1.7	-3.3
ATP-binding cassette, sub-family B (MDR/TAP), member 1	Hs.21330	ABCB1	7q21.1	-1.7	-1.7	-1.1	-2.5	1.2	1.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4	Hs.73812	ABCB4	7q21.1	3.1	-1.0	-1.7	1.1	-1.7	-2.0
ATP-binding cassette, sub-family B (MDR/TAP), member 8	Hs.118634	ABCB8	7q36	1.3	-1.4	1.6	1.2	2.2	-2.0
ATP-binding cassette, sub-family B (MDR/TAP), member 9	Hs.6129	ABCB9	12q24	-1.1	-1.0	1.6	1.3	-1.3	-3.3
ATP-binding cassette, sub-family C (CFTR/MRP), member 9	Hs.248960	ABCC9	12p12.1	-2.0	-1.1	-1.1	1.1	-1.3	1.1
ATP-binding cassette, sub-family D (ALD), member 2	Hs.117852	ABCD2	12q11-q12	-10.0	1.4	-1.0	-3.3	1.3	1.2
ATP-binding cassette, sub-family G (WHITE), member 1	Hs.10237	ABCG1	21q22.3	2.2	1.2	1.6	1.5	-1.3	1.4
ATP-binding cassette, sub-family G (WHITE), member 2	Hs.194720	ABCG2	4q22	1.6	-1.7	-2.0	-1.4	-1.0	-1.1
ATP-binding cassette, sub-family G (WHITE), member 4	Hs.126378	ABCG4	11q23.3	-2.0	-1.1	-1.0	-1.4	-1.3	-1.1
ATP-binding cassette, sub-family G (WHITE), member 5 (sterolin 1)	Hs.132992	ABCG5	2p21	1.2	2.2	1.7	2.1	1.3	1.4

4. DISCUSSION

Monocyte-derived macrophage gene regulation plays an important role not only in pathogenesis of atherosclerosis but also in many other inflammatory diseases, such as, cirrhosis, rheumatoid arthritis, glomerulosclerosis, pulmonary fibrosis and chronic pancreatitis. In addition, bioactive lipids as derivatives of lysophospholipids have been remarkably evidenced to contribute to many pathophysiological stages of these diseases. For example, lysophospholipids are significantly involved in pathogenesis of atherosclerosis by modulating a broad spectrum of processes including platelet aggregation, cell growth and differentiation as well as recruitment of vascular cells. Therefore, the aim of this thesis work is to analyze global gene expression of monocyte-derived macrophages under the modulation of selected of bioactive lipids, including ceramide, S1P, SPC, LPA and LPA, and modified low-density lipoprotein as the source of the these bioactive lipids. All experiments were performed with human peripheral blood monocytes from ApoE 3 homozygous phenotype from healthy donors. Phagocytic differentiation was carried out in serum-free culture media to minimize potential artefacts.

Expression analysis of marker genes in in vitro phagocytic differentiation and foam cell formation models.

Scavenger receptors (CD36, Cla-1), adipophilin and ABCA2 were analyzed as marker genes to test the *in vitro* model of phagocytic differentiation and foam cell formation induced by enzymatic modified lipoprotein.

Phagocytic differentiation is accompanied by enhanced cholesterol uptake activity and reported with the accumulation of number of cell surface receptors including scavenger receptors, as receptors for lipoproteins. CD36 and its homologue, Cla-1 (SR-BI), are both detected in monocytes and macrophages. CD36 recognizes and binds many ligands, including modified LDL. Expression of CD36 is reported to be elevated during *in vivo* macrophage differentiation and Ox-LDL-induced foam cell formation [253;254]. From my experiments, CD36 was abundantly expressed in monocytes and strongly up-regulated during phagocytic differentiation. Similar to current literatures addressed on atherogenesis, E-LDL incubation of monocyte-derived macrophages enhanced the expression of CD36 on both mRNA and protein levels, while other modified LDL (Ac-LDL, Ox-LDL) had the reverse effects [11]. Our collaborated study published by Kapinsky *et al.* also revealed that E-LDL but not Ac-LDL or Ox-LDL induced foam cell

formation in freshly isolated peripheral blood monocytes. Similarly, Cla-1 was also detected to be highly up-regulated during macrophage differentiation and in E-LDL loaded macrophages, while it was hardly detected in monocytes [11]. Recent findings also revealed that Cla-1 involved in the selective uptake of cholesterol ester, recognized modified lipoproteins and played a role in HDL metabolism [244;245]. Therefore, these results support the hypothesis of an autoregulatory loop for enhanced cholesterol uptake and provide a link between this modified LDL and the HDL metabolism.

The excess lipid deposition in macrophages has been reported to modulate expression of several genes, including adipophilin. Adipophilin or adipose differentiation related protein (ADRP) is currently reported to be abundance in macrophage-rich areas of atherosclerotic lesions. Its expression in atherosclerotic plagues was calculated as 3.5-fold higher compared to healthy areas of the same arteries. In their study, Larigauderie *et al*. showed that the stimulation of adipophilin expression by Ac-LDL promotes triglycerides and cholesterol storage and reduces cholesterol efflux [260]. In my study, the expression of adipophilin was analyzed with E-LDL-induced macrophage foam cell formation. Levels of adipophilin mRNA and protein were rapidly elevated within 2 hours and approached its peak at 24 hours of E-LDL incubations. Other study also revealed that the expression of adipophilin is increased in Ox-LDL-induced foam cell formation of human macrophage. However, the induction of adipophilin expression by Ox-LDL was not detected within 6 hours of incubation [255]. Therefore, these data suggested a high atherogenic potent of E-LDL and adipophilin could be considered as a sensitive marker for lipid loading in human blood monocytes.

Together with ABCA1, ABCA4 and ABCA7, ABCA2 is found to be expressed in human monocytes/macrophages and enhanced expression in macrophages treated with cholesterol. In line with these findings, my experiments also revealed that ABCA2 is already expressed in human peripheral blood monocytes and elevated its expression during phagocytic differentiation. In addition, the high level of accumulation of ABCA2 was observed in foam cells induced by E-LDL compared to macrophages. The repeated experiment using DNA chip (chapter 3.4.2.2) also confirmed an upregulation of ABCA2 during foam cell formation induced by enzymatically modified LDL. These data also assume that ABAC2 plays a role in intracellular trafficking of LDL-derived free cholesterol and cholesterol homeostasis.

All together, the above-mentioned data strongly indicate that the *in vitro* model of phagocytic differentiation and E-DLD-induced foam cell formation reflects the *in vivo* processes occurred during the development of atherosclerosis.

Expression analysis of lysophospholipid receptors.

To date, there are 13 G-protein-coupled receptors have been identified to recognize and bind to S1P, SPC, LPA and LPC. However, only a few studies have been published investigating the expression of these receptors in human blood monocytes and macrophages, as major cells involved in the pathogenesis of atherosclerosis. In one study, mRNA expression of $LPA_{1,}$ LPA_2 and $S1P_1$, $S1P_2$, $S1P_4$ were reported in human monocytes and macrophages whereas $S1P_3$ and $S1P_5$ mRNAs could not be detected using RT-PCR [261]. Another study reported the expression of $S1P_1$, LPA_1, LPA_2 and LPA_3 mRNAs in human alveolar macrophages but no mRNA for $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$ were detected in these cells [262]. These results showed a contradictory in the expression of $S1P_2$, $S1P_4$ and LPA_3 and it become unclear weather these receptors are expressed in macrophages or not. However, these macrophages have been cultured in the present of serum, which already contains high concentration of S1P and LPA. This may, at least in part, explain the contradictory of the above-mentioned results. The *in vitro* model of phagocytic differentiation in this thesis work was developed with no additional serum to investigate the expression of these receptors in both mRNA and protein levels. The advantage of mRNA expression analysis with TaqMan RT-PCR is to allow relative quantify the gene expression using calibration curves thus eliminate the varying efficiencies of single PCR reaction. The mRNAs of all 13 lysophospholipid receptors could be amplified in freshly isolated human monocytes but no significant regulation detected in further stimulation of monocytes and/or macrophages. With the exception of $LPA_{4, 5}$, GPR3, 4, 12 and G2A (commercial antibody unavailable), the protein expression analysis revealed the present of $S1P_{1, 2, 4}$ and $LPA_{1, 2}$ in monocytes and macrophages. In addition, Rikitake *et al*. reported an expression of G2A protein in human and rabbit macrophages as well as in murine atherosclerotic lesion [263]. Expression analysis of these receptors using microarray was not giving any significant data as the detected mean florescence were generally low.

Expression analysis of global macrophages genes with DNA microarray.

Large scale analysis has revealed an overview of macrophages global gene expression under the stimulations of the above-mentioned lysophospholipids. Herein, however, the discussion is only restricted to the effects of modified lipoprotein (E-LDL) on genes related to cholesterol metabolism pathways and ABC transporters during macrophage foam cell formation.

The lipid homeostasis in vertebrate cells is regulated by a family of membrane-bound transcription factors designated as sterol regulatory element-binding transcription factors, or proteins (SREBPs). Although the mammalian genome encodes three SREBP isoforms but only SREBP-1c and SREBP-2 are having predominant roles in regulation of cholesterol metabolism. Despite the fact that at high expression level, all three SREBP isoforms could activate all genes listed in fig. 3.9, the SREBP-1c is preferentially activate the fatty acid and lipoproteins biosynthesis and SREBP-2 preferentially activate cholesterol biosynthesis pathways, at normal physiological expression level.

The regulation activity of SREBPs depends on an escort protein SCAP (SREBP cleavage-activating protein) and two enzymes designate Site-1 and Site-2 protease. At normal stage, SREBPs are synthesized as inactive precursor, bound to endoplasmic reticulum. When cells become depleted in cholesterol, SCAP escorts SREBPs to Golgi apparatus, where it is cleaved by the two proteases and translocated to nucleus to activate the promoter and/or enhancer on multiple target genes of the metabolism pathways. The lipid homeostasis activity of SREBPs is manifested when cellular cholesterol content rises up. In which, the SCAP/SERBPs complex is no longer incorporated into ER transport vesicles and therefore SREBPs remained inactivated. In knockout and transgenic mice studies the role of SREBPs in cholesterol regulation is referred to SCAP as the sensor [264]. Mice with disrupted SCAP gene exhibited a fall of 70-80% of cholesterol and fatty acid synthesis in liver. In cultured cells, the processing of SERBP is reported to be inhibited by sterols by suppression of SCAP activity. Transgenic mice with a single amino acid substitution in the sterol-sensing domain (D443N) overproduced cholesterol. In addition, transgenic mice carried this mutation in liver also exhibited a similar phonotype. The levels of nucleus SREBP-1 and 2 were elevated leading to an increase of expression of all SREBP target genes even when these animals are fed with cholesterol-rich diet. Therefore, it is assumable that SCAP activity *in vivo* is normally under partial inhibition by endogenous

cholesterol. Thus the synthesis of cholesterol and fatty acids is partially repressed in the liver.

In my study, the role SREBP in lipid metabolism of human macrophages was investigated. Although both of SREBP isoforms 1c and 2 were detected in human macrophages, their expression levels in E-LDL-induced foam cells were not similar. Interestingly, mRNA expression SERBP-2 isoform is suppressed by exogenous cholesterol leading to a complete downregulation of almost all target genes of the cholesterol synthesis pathway (fig. 3.9). With the only exception of SMGCS2, which was 2-fold upregulated, other SREBP target genes were 2 to 5-fold downregulated in E-LDL loading macrophages. Thus, loading of E-LDL is completely closing down this pathway and it further confirmed by additional downregulation of malic enzyme 1 (ME1) that support energy for the cholesterol metabolism pathway.

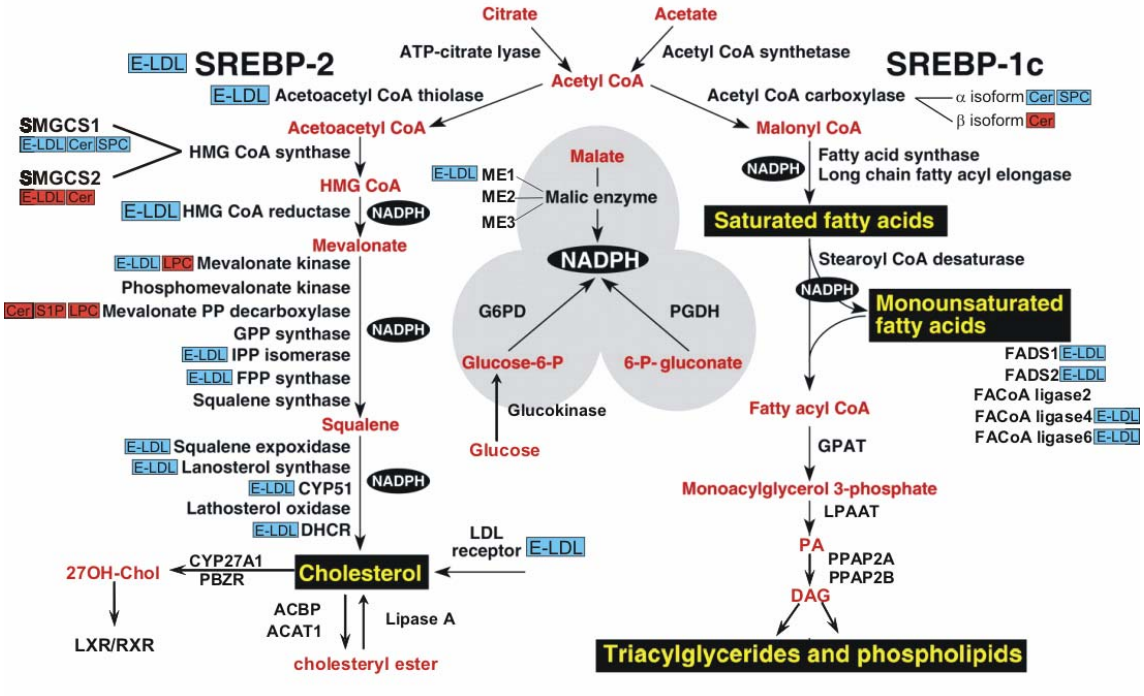


Figure 3.9: Regulation of cholesterol metabolism pathway genes in simulated macrophages. (red = up-regulation, blue = down-regulation, NC = no change, text in the boxes represent the stimulation). Figure adapted from [265].

It is known that SREBPs enhance lipid synthesis but it also stimulate LDL receptor expression [266], so their net effect on plasma lipoprotein levels depend on a balance between opposing effects. Mice that have an overexpressed or underexpressed of SREBPs showing a reduction of plasma levels of lipoproteins. Similarly, transgenic mice that overexpressed SREBPs also reduce its level of cholesterol and triglycerides in comparison to control, although they massively overproduced fatty acids and/or cholesterol. These observations could be explained that these lipids have been rapidly degraded in a process that mediated by LDL receptor. Mice that have a LDL-receptor deficiency exhibit a ten-fold of cholesterol and triglyceride induction levels. In conformity with the above conclusions, mRNA expression analyses in this thesis work revealed a 10-fold suppression of LDL-receptor during foam cell formation of macrophages. Thus, exogenous cholesterol turns down cholesterol metabolism pathways and also partially prevents cholesterol influx by LDL-receptor suppression in macrophages.

However, these mRNA expression analyses also revealed no change of fatty acid and triglycerides metabolism pathways in human macrophages upon extracellular lipid loading, except the suppression of conversion of monounsaturated fatty acids to fatty acyl CoA, triggered by FADS1, FADS2 and FACoA ligase 4 and 6.

Cellular cholesterols homeostasis is also regulated by several mechanisms involved high-density lipoproteins, apolipoproteins (apoA-I, apoE) and lipid-regulated ABC transporters. ABC transporters translocate a broad spectrum of molecules across cell membranes, including amino acids, carbohydrates, peptides, vitamins, ions, xenobiotics and lipids [267] and therefore, exert various cellular functions. Many of inherited diseases have been found to be caused or associated with mutations that occurred within ABC transporter genes family. Among these are familial HDL deficiency (or Tangier disease) cause by a mutation of ABCA1 [268;269] and accumulation of dietary cholesterol in sitosterolemia that account for ABCG5/ABCG8 [270;271] Herein, the regulation of these ABC transporters in human macrophage foam cells induced by exogenous E-LDL loading were investigated. From 35 ABC genes represented on the DNA chip, there were 8 genes regulated during foam cell formation, including ABCA2, ABCA3, ABCA8, ABCB4, ABCC9, ABCD2, ABCG1, and ABCG4.

Among those, ABCG1 is sharing highest functional similarity to the ABCA1 gene, the first ABC transporter found to be expressed in human macrophage [272]. While ABCA1 exhibited no response to lipid loading of macrophages, ABCG1 showed a 2.2 fold

upregulation in these cells. Initially, ABCG1 was reported to be expressed in human monocytes and its expression elevated during phagocytic differentiation as well as upon sterol-loading of macrophages using acetylated LDL. The sterol response of ABCG1 was further confirmed by an experiment in which cholesterol deloading of macrophages with HDL_3 lead to a suppression of ABCG1 mRNA and protein [273]. Furthermore, the induction of ABCG1 expression is not only observed with Ac-LDL but also other types of modified LDL such as ox-LDL and E-LDL, while native LDL and free cholesterol exhibited no effects on ABCG1 expression [274;275]. The role of ABCG1 in cholesterol efflux was initially reported by our Institute, when ABCG1-specific antisense oligonucleotides caused a significantly impaired cholesterol and phospholipid efflux to HLD_3 [273]. Recently, other investigations reported that ABCG1 can function either alone or in combination with other ABCG-family members, specifically ABCG4, to regulate cellular lipid efflux. DNA chip experiments from this thesis work exhibited an inversed regulation of ABCG1 and ABCG4 upon cholesterol loading. However, this may be explained by the very low expression of ABGC4 in macrophages in comparison to that of ABCG1.

Table 3.9: Regulation of ABC transporter genes upon stimulation

(red = up-regulation, blue = down-regulation, (-) for down-regulation, value = fold change).

Name	Unigene	Short name	ChromLoc	ELDL vs. Ctrl	Cer8 vs. Ctrl	S1P vs. Ctrl	SPC vs. Ctrl	LPC vs. Ctrl	LPA vs. Ctrl
ATP-binding cassette, sub-family A (ABC1), member 2	Hs.121561	ABCA2	9q34	3.9	-1.1	1.9	1.1	1.1	1.8
ATP-binding cassette, sub-family A (ABC1), member 3	Hs.26630	ABCA3	16p13.3	-3.3	-2.0	1.1	-1.3	-1.1	-1.1
ATP-binding cassette, sub-family A (ABC1), member 8	Hs.38095	ABCA8	17q24	7.0	1.3	-2.0	-1.4	-1.3	2.2
ATP-binding cassette, sub-family B (MDR/TAP), member 4	Hs.73812	ABCB4	7q21.1	3.1	-1.0	-1.7	1.1	-1.7	-2.0
ATP-binding cassette, sub-family C (CFTR/MRP), member 9	Hs.248960	ABCC9	12p12.1	-2.0	-1.1	-1.1	1.1	-1.3	1.1
ATP-binding cassette, sub-family D (ALD), member 2	Hs.117852	ABCD2	12q11-q12	-10.0	1.4	-1.0	-3.3	1.3	1.2
ATP-binding cassette, sub-family G (WHITE), member 1	Hs.10237	ABCG1	21q22.3	2.2	1.2	1.6	1.5	-1.3	1.4
ATP-binding cassette, sub-family G (WHITE), member 4	Hs.126378	ABCG4	11q23.3	-2.0	-1.1	-1.0	-1.4	-1.3	-1.1

Table 3.10: ABC genes transported molecules and functions.

Gene	Domain structure	Tissue expression, cellular location	Known or putative transported molecule
ABCA2	(TMD-ABC)2	Brain	Estramustine, steroids
ABCA3	(TMD-ABC)2	Lung	Surfactant phospholipids
ABCA8	(TMD-ABC)2	Ovary	xenobiotic
ABCB4	(TMD-ABC)2	Liver, apical membrane	Phosphatidylcholine
ABCC9	TMD0-(TMD-ABC)2	Heart, muscle	Sulfonylureas
ABCD2	TM-ABC	Peroxisomes	Very-long-chain fatty acids
ABCG1	ABC-TMD	Ubiquitous	Phospholipids, cholesterol
ABCG4	ABC-TMD	Liver	

Our DNA chip analysis also exhibited the regulation of other ABC transporter genes that are predominantly expressed in different tissues. Among those, ABCA3 and ABCB4 are reported to play role in cellular lipid rheostat. ABCA3 is predominantly expressed in lung and localizes to the limiting membrane of lamellar bodies in alveolar type II cells. Mutations in ABCA3 gene cause a neonatal respiratory failure of humans. ABCA3 is reported to transport pulmonary surfactant and is involved in the accumulation of phospholipids and cholesterol in lipid vesicles. Nevertheless, my data showed a 3.3-fold downregulation of ABCA3 in macrophages upon lipid loading, which could be potentially explained by its low expression in macrophages. Inversely, ABCB4 was 3.1-fold elevated upon lipid loading of those macrophages. It is however reported to be expressed predominantly in liver rather than in macrophages (table 3.10). Similarly, the results from ABCA8, ABCC9 and ABCD2 might be technical artefacts since no expression has been reported in monocytes/macrophages using other techniques.

Conclusions

Results archived from CD36 and Cla-1 analysis in phagocytic differentiation and foam cell formation was further confirmed the higher atherogenic properties of enzymatically modifification LDL, compared to other type of modifications (acetylation, oxidation). Furthermore, these results also support the hypothesis of an autoregulatory loop for enhanced cholesterol uptake and provide a link between this modified LDL and the HDL metabolism. In addition, analyses of adipophilin and ABCA2 were also revealed that adipophilin could be considered as a new sensitive marker for lipid loading of human monocytes/macrophages as well as the role of ABCA2 in intracellular LDL-derived free cholesterol trafficking and lipid homeostasis.

The expression of up-to-date G-protein-coupled receptor superfamily for the bioactive lipid derivatives; S1P, LPA, LPC, SPC in human monocytes/macrophages was also investigated. The serum-free phagosytic differentiation model was able to eliminate the discrepancy arrived from previous studies. Since these bioactive lipid derivatives are products of platelets and involve in many cellular processes, results archived from this thesis work would sugguest the importance of further study on cross-talk between platelets and monocytes/ macrophages.

DNA chip analysis provided an overview of cellular effects of the above-mentioned bioactive lipids and cholesterol loading on macrophages global genes expression. Due

to the limit of the thesis work, only the effects of cholesterol loading (E-LDL) on cholesterol metabolism pathways and homeostasis were investigated in-details. Interestingly, exogenous free cholesterol loading of human macrophages could lead to a complete blockage of cellular cholesterol metabolism pathway triggered by SREBP-2, while triacylglycerides and fatty acids metabolism pathways triggered by SREBP-1c were not influenced. The study also reported the expression of ABC transporter A2, A3, A8, B4, C9, D2, G1 and G4 in human macrophages and suggested the roles of ABCA2, ABCB4 and ABCG1 in macrophages cellular lipid rheostat.

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Appendix A: Abbreviations

Ac-LDL	Acetylated low-density lipoproteins
ADRP	Adipose differentiation related protein
CAP	Ceramide acitvated protein
EC	Endothelial cells
E-LDL	Enzymatically modified low-density lipoproteins
ER	Endoplasmic reticulum
ERK	Extracellular-regulated protein kinase
GPCR	G protein-coupled receptor
HDL	High-density lipoprotein
KSR	Kinase suppressor of Ras
LDL	Low-density lipoproteins
LDLR	Low density lipoprotein receptor
LPA	Lysophosphatidic acid
LPA_2	Phospholipase A_2
LPC	Lysophosphatidylcholine
MAPK	Mitogen-activated protein kinase
Ox-LDL	Oxidized low-density lipoproteins
PA	Phosphatitdic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PP2A	Protein phosphatases class 2A
PS	Phosphatidylserin
S1P	Sphingosine-1-phosphate
SMC	Smooth muscle cells
SPC	Sphingosylphosphorylcholine
SRE	Serum-response element
VCAM-1	Vascular cell adhesion molecule 1
VSMC	Vascular smooth muscle cell

Appendix B: List of figures

Appendix C: List of tables

Publications, workshops and presentations

Publications:

1. **Chinh Quoc Duong**, Salim Maa Bared, Ahmed Abu-Khader, Christa Buechler, Anna Schmitz and Gerd Schmitz. Expression of the lysophospholipid receptor family and investigation of lysophospholipid-mediated responses in human macrophages. Biochim Biophys Acta. 2004 Jun 1;1682(1-3):112-9

2. Buechler C, Ritter M, **Quoc CD**, Agildere A, Schmitz G. Lipopolysaccharide inhibits the expression of the scavenger receptor Cla-1 in human monocytes and macrophages. Biochem Biophys Res Commun. 1999 Aug 19; 262(1):251-4.

3. Buechler C, Ritter M, **Duong CQ**, Orso E, Kapinsky M, Schmitz G. Adipophilin is a sensitive marker for lipid loading in human blood monocytes. Biochim Biophys Acta. 2001 May 31; 1532(1-2):97-104.

4. Kapinsky M, Torzewski M, Buchler C, **Duong CQ**, Rothe G, Schmitz G. Enzymatically degraded LDL preferentially binds to CD14(high) CD16(+) monocytes and induces foam cell formation mediated only in part by the class B scavenger-receptor CD36. Arterioscler Thromb Vasc Biol. 2001 Jun;21(6):1004-10.

5. Kaminski WE, Piehler A, Pullmann K, Porsch-Ozcurumez M, **Duong C**, Bared GM, Buchler C, Schmitz G. Complete coding sequence, promoter region, and genomic structure of the human ABCA2 gene and evidence for sterol-dependent regulation in macrophages. Biochem Biophys Res Commun. 2001 Feb 16; 281(1):249-58.

6. Buechler C, Bodzioch M, Bared SM, Sigruener A, Boettcher A, Lapicka-Bodzioch K, Aslanidis C, **Duong CQ**, Grandl M, Langmann T, Dembinska-Kiec A, Schmitz G. Expression pattern and raft association of NIPSNAP3 and NIPSNAP4, highly homologous proteins encoded by genes in close proximity to the ATP-binding cassette transporter A1. Genomics. 2004 Jun; 83(6):1116-24.

Workshop and presentation:

6/2000 GeneSpring software training workshop for DNA chip data analysis. Heidelberg, Germany.

10/2002 Poster at 16th annual meeting of European Macrophages and Dendritic Cells Society (EMDS), University Basel, Switzerland.

Eidesstattliche Erklärung

Family name: Duong First name: Quoc Chinh
Date of birth: 22/08/1975

Ich erkläre hiermit an Eides statt, daß ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet (Siehe Kapitel „Citations“).

Bei der Auswahl und Auswertung folgenden Materials haben mir die nachstehend aufgeführten Personen in der jeweils beschriebenen Weise entgeltlich/unentgeltlich geholfen:

1-.....
2-.....
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Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhamg mit dem Inhalt der vorliegenden Dissertation stehen.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Ich versichere an Eides statt, daß ich nach bestem Wissen die reine Wahrheit gesagt und nichts verschwiegen habe.

Vor Aufnahme der obigen Versicherung an Eides statt wurde ich über die Bedeutung der eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung belehrt.

Regensburg, 21.11.2007

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Duong, Quoc Chinh