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NOVEL MECHANISMS FOR REGULATION OF CELL SURVIVAL AND MIGRATION BY CERAMIDE 1-PHOSPHATE

Doctoral Thesis

"Doctor Europeus"

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November, 2011

Index

Abbreviations

Abbreviations

AA	Arachidonic Acid
ABT	1-Aminobenzotriazole
APC	Antigen Presenting Cell
ATP	Adenosine Triphosphate
BCR	B cell receptor
BMDM	Bone Marrow-Derived Macrophages
BSA	Bovine Serum Albumin
C1P	Ceramide 1-phosphate
CCR2 (CRK2)	C-C chemokine receptor type 2
CD	Cluster of differentiation
Cer	Ceramide
CERK	Ceramide Kinase
CERS	Ceramide Synthase
CERT	Ceramide Transport Protein
$cPLA_2$	Cytosolic Phospholipase A_2
CTL	Cytotoxic T Lymphocyte
DAG	Diacylglycerol
DAGK	Diacylglycerol Kinase
DMEM	Dulbecco’s modified Eagle’s medium
DPI	Diphenyleneiodonium chloride
EDG	Endothelial Differentiation Gene
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular signal-regulated kinases
FBS	Fetal Bovine Serum
FI	Fluorescence Intensity
FITC	Fluorescein isothiocyanate
GCS	Glycosylceramide Synthase
GN8	N-Acetyl-d-glucosamine-coated polyamidoamine dendrimer
GPCR	G protein-coupled receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase

ICAM-1	Inter-Cellular Adhesion Molecule 1
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
L-NAME	L-NG-Nitroarginine methyl ester
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein (MAP) kinases
MCP-1 (CCL-2)	Monocyte Chemotactic Protein-1
M-CSF	Macrophage colony-stimulating factor
MMP	Matrix Metalloprotease
mTOR	Mammalian Target of Rapamycin
NAC	N-Acetyl Cystein
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural Killer cell
NOX	NADPH Oxidase
PA	Phosphatidic Acid
PBS	Phosphate-Buffered Saline
PC	Phosphatidyl Coline
PDGF	Platelet-Derived Growth Factor
PGE2	Prostaglandin E2
Phox	Phagocytic Oxidase
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PKB/Akt	Protein Kinase B
PKC	Protein Kinase C
PLA	Phospholipase A
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PMS	Phenazine Methosulphate
Ptx	Pertussis Toxin
RISC	RNA-induced silencing complex

ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute medium
S1P	Sphingosine 1-phosphate
S1PR	Sphingosine 1-phosphate Receptor
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
siRNA	Small interfering ribonucleic acid
SM	Sphingomyelin
SMase	Sphingomyelinase
SMC	Spleen Mononuclear Cells
SMS	Sphingomyelin Synthase
SOD	Superoxide Dismutase
Sph	Sphingosine
SphK	Sphingosine Kinase
SPT	Serine Palmitoyltransferase
STAT	Signal Transducers and Activators of Transcription
TAM	Tumour-associated Macrophage
TCR	T cell receptor
Th	Helper T Lymphocyte
TNF-α	Tumour necrosis factor-alpha
TTFA	2-Thenoyltrifluoroacetone
VCAM	Vascular cell adhesion protein
VEGF-A	Vascular endothelial growth factor
VEGFR	VEGF receptors

Introduction

1. INTRODUCTION

When all forces in a system are balanced to the point where no change is occurring, the system is said to be in a state of *static equilibrium*. This fully obeys the laws of thermodynamics, which establish that all systems in the universe tend imperatively to increase their equilibrium (absolute stability). In contrast to stable systems that are in thermodynamic equilibrium, systems that are far from equilibrium are inherently unstable.

Living organisms are open systems that are never at equilibrium until they die and the absolute stability is our understanding of death. Living organisms create internal organization thanks to a continuous flow of energy which is known as *dynamic equilibrium* (steady state). This dynamic equilibrium is actively regulated by living systems in order to avoid being affected by external changes. Claude Bernard reported in 1853 [1] that the difference between a living organism and a non-living one is the ability to regulate its internal environment, and Walter Cannon defined this active regulation as *homeostasis* [*2*]. Homeostasis is the condition of dynamic equilibrium between at least two system variables and it is known that the continuance of a living system depends on its ability to maintain the homeostasis. The capacity of maintaining the internal organization and characteristics, in spite of the always varying external environment, is what discerns life from death.

Normal development of an organism requires the intervention of complex biological processes that are strictly regulated to maintain cell and tissue homeostasis. Living organisms must be able to detect internal or external changes and activate the control mechanisms by which they can restore homeostasis. These include systems to control cell growth and survival, as well as mechanisms to prevent homeostatic imbalance. Strictly regulated and highly complicated cellular signaling processes make homeostasis possible. Alteration of any of these processes can lead to metabolic dysfunction or cause illnesses such as autoimmune diseases, chronic inflammation, neural degeneration, cardiovascular disorders, or cancer.

In multicellular organisms the immune system is a key factor managing tissue and cell homeostasis.

1. Components of the Immune System

All cells of the immune system originate from a hematopoietic stem cell in the bone marrow, which gives rise to two major lineages, a myeloid progenitor cell and a lymphoid progenitor cell. These two progenitors give rise to the myeloid cells (monocytes, macrophages, dendritic cells, megakaryocytes and granulocytes) and lymphoid cells (T cells, B cells and natural killer (NK) cells), respectively. These cells make up the cellular components of the innate (non-specific) and adaptive (specific) immune systems (Figure 1.1).

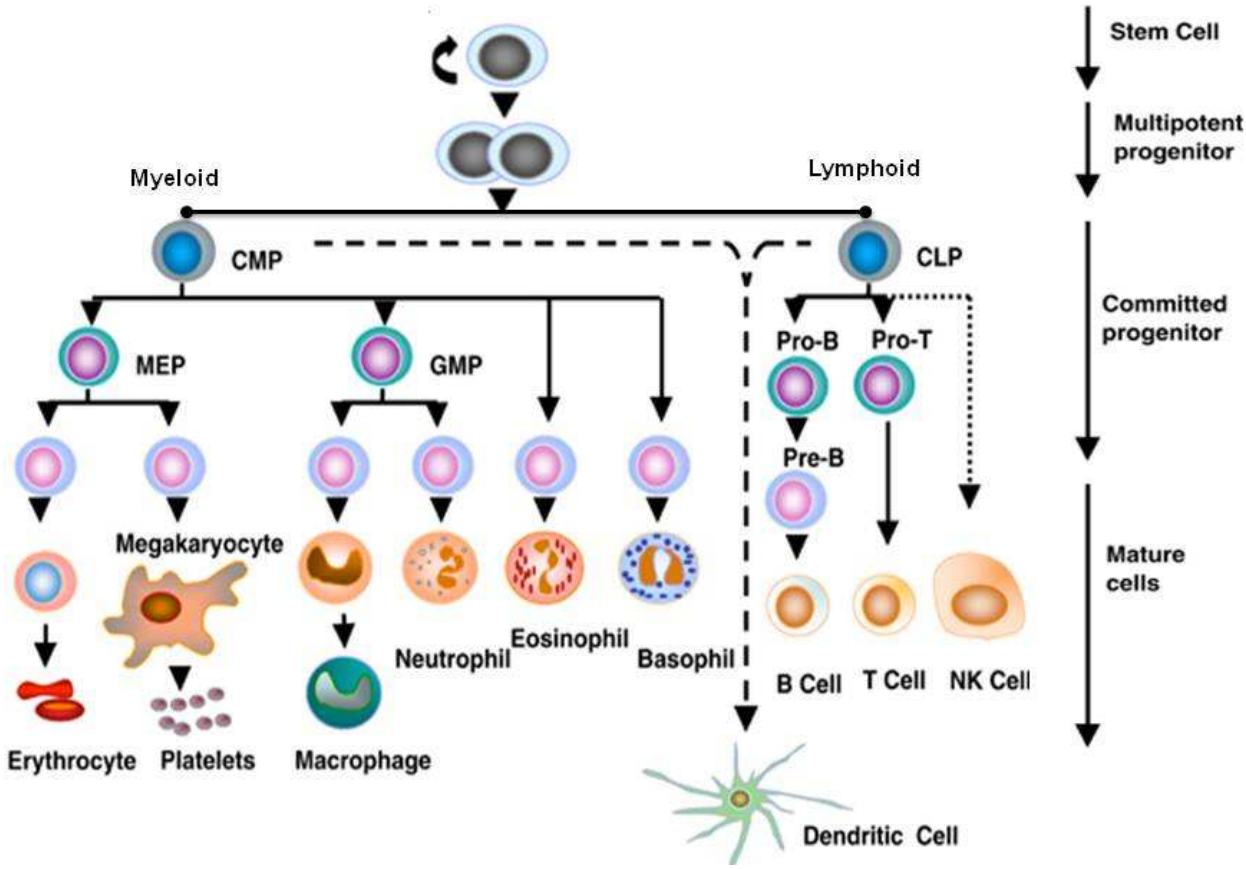


Figure 1.1. Scheme of hematopoiesis. Adapted from [3].

1.1. THE INNATE IMMUNE SYSTEM

The innate immune system responds to pathogens or damage stimuli in a non-specific way. The elements of the innate immune system include anatomical barriers, secretory molecules and cellular components.

Production of chemokines (which recruit leukocytes and other immune cells), activation of the complement cascade and activation of adaptive immune cells by antigen presentation are the most important responses of innate immune system.

The innate immune cells include: Natural Killer cells, mast cells, eosinophils, basophils; and the phagocytic cells including macrophages, neutrophils and dendritic cells. Nevertheless, concerning tissue homeostasis, the most important ones are macrophages because of their multiple functions in innate immunity and their capacity to activate the adaptive immune system.

1.2. ADAPTIVE IMMUNE SYSTEM

Adaptive immune system is composed of specialized cells and mechanisms that are able to recognize and eliminate pathogens efficiently. In addition, adaptive immunity develops memory cells for each eliminated pathogen in order to respond in a faster and stronger manner if the pathogen enters the organism again.

Lymphocytes are central to all adaptive immune responses. They originate from stem cells in the bone marrow and mature either in the bone marrow (B lymphocytes) or in the thymus (T lymphocytes), after exiting the bone marrow. T lymphocytes are further divided into functional subsets: cytotoxic T lymphocytes (CTL), which generate cell-mediated immune responses and helper T lymphocytes (Th cells), which regulate the immune system, governing the quality and strength of all immune responses.

1.3. MACROPHAGES

Macrophages (from *makros* "large" and *phagein* "eat") are the major leukocyte orchestrating the innate immune system. Their primary function is the clearance of invading pathogens, cell debris and apoptotic cells by a process called phagocytosis. Nevertheless, their function goes beyond this simple definition, as will be discussed throughout this thesis.

These cells were first described as macrophages by Elie Metchnikoff after he saw large mononuclear phagocytic cells on different tissues. Macrophages are the major phagocytic cells and they can acquire different morphology and localization in the body.

Macrophages are part of the innate and acquired immunity, therefore, they play very important functions in the immune system, as well as in the maintenance of tissue homeostasis and the regulation of other important processes, such as inflammation [4].

1.3.1 Macrophage generation and location

Macrophages are derived from bone marrow hematopoietic cells and constitute the terminally differentiated cell type from the mononuclear phagocytic cell lineage. Monocytes are the immediate precursors of macrophages and they also have relevant functions in inflammation. Monocytes are liberated from the bone marrow and they circulate in the bloodstream where they are recruited into the sites of injury or inflammation. During an inflammatory response monocytes differentiate into macrophages within the tissue where they remain until their function is completed. Tissue macrophages can be found constitutively in order to contribute to the maintenance of many biological processes.

1.3.2. Macrophage activation and function

Macrophage-activating stimuli are found in sites of inflammation and they are commonly produced by macrophages themselves, other inflammatory cell types or invading organisms. The surface of the macrophages is equipped with many receptors,

which interact with these stimuli and activate macrophage functions such as phagocytosis, adhesion, chemotaxis, secretion of different molecules and antigen processing and presentation. The most remarkable biological functions of macrophages are:

1.3.2.1. Phagocytosis

The process of phagocytosis is complex and requires the implication of recognition receptors and initiation of signaling pathways that lead to cytoskeleton rearrangements. The process begins with recognition of the particle to be engulfed. After recognition, macrophages induce cytoskeleton rearrangements so that they can extend the plasma membrane and surround the particle to be engulfed. The particle is internalized by vesicles called phagosomes and is digested in a specific cellular compartment. Phagocytosis is a necessary mechanism not only in pathogen elimination but also in antigen presenting processes, being an important link between acquired and innate immune systems.

1.3.2.2. Chemotaxis

Chemotaxis is described as the movement of cells in response to chemical stimuli usually released by foreign pathogens or inflammatory cells. These stimuli are detected by extracellular receptors and they initiate a signaling cascade that provides the rearrangement of cytoskeletal structures. These changes make possible the movement of the cell toward the origin of the stimulus and finally cell migration. Cell migration is a key component of tissue homeostasis. Wound repairing and inflammatory response are the examples of homeostatic processes in which cell migration is featured prominently.

1.3.2.3. Antigen processing and presentation

Antigen processing and presentation is necessary for acquired immunity because it is a mechanism to distinguish self antigens from foreign antigens. Macrophages, as well as dendritic cells and B lymphocytes, are professional Antigen Presenting Cells (APC), which means that they have the special machinery to process and present antigens. This is absolutely necessary for the specific detection of pathogens and the subsequent activation of acquired immunity. APCs can also promote recruitment and proliferation of T lymphocytes.

1.3.2.4. Secretion

Once they are activated macrophages secrete many signaling molecules that enhance or diminish inflammation as well as they contribute to antigen presentation, or assist in wound healing. Important molecules secreted by macrophages can be:

1.3.2.4.1. Enzymes

They are generally secreted in order to aid in the digestion of cell debris or pathogens. Some of them are released constitutively (i.e. lysozime) but others depend on previous activation of the macrophages; these include proteases, lipases, deoxyribonucleases, phosphatases, glycosidase, collagenases, elastases and sulfatases.

1.3.2.4.2. Cytokines

Cytokines (from *cyto* "cell" and *kinos* "movement") are small proteins generally secreted by immune system cells. Cytokines can influence cell growth, differentiation and they function in many different cell types. Macrophages can release different cytokines that regulate the immune system and they can exert different biological functions. Some examples of cytokines released by macrophages are TNF-a, IL-6, INF-γ or MCP-1.

1.3.2.4.3. Complement components

Macrophages can secrete components of both classical and alternative complement systems. The complement system participates in immunoregulatory functions including lysing of foreign cells and promoting the recognition and uptake of particles by phagocytosis. Complement factors can also promote or suppress inflammatory response by binding directly to complement receptors on the macrophage surface. These include C1, C2, C3, C4, C5, factor B, factor D, properdin, C3b inactivator and a-IH. In addition macrophage secreted proteases can generate active fragments of the complement system such as, C3a, C3b, C5a and Bb.

1.3.2.4.4. Coagulation factors

The coagulation cascade is an important pathway stimulated in response to injury. Coagulation factors recruit and activate platelets in order to promote blood clotting and deposition of fibrin. The coagulation cascade is essential for wound healing. Macrophages secrete coagulation factors VII, IX, X and V.

1.3.2.4.5. Reactive oxygen species (ROS)

Reactive oxygen species are highly reactive molecules that can oxidize and destroy many molecules. Macrophages can secrete large amounts of these cytotoxic molecules to the extracellular matrix contributing to the defense against a possible infection. Nevertheless, it has been demonstrated that low amounts of ROS can also act as second messengers in some signaling pathways, which usually lead to cell proliferation or angiogenesis. ROS are produced in activated macrophages by different enzyme activities but the main enzymes involved in the production of ROS in macrophages are NADPH oxidase and iNOS.

1.3.2.4.6. Arachidonic acid (AA) and its metabolites

Macrophages are known to be important sources of products of the AA pathway, such as, prostacyclin, thromboxane, prostaglandin E_2 (PGE_2) and leucotriene B4. Macrophages contain the necessary enzyme components for synthesis, metabolism and control of these products. For instance, it is well known that macrophages express PLA_2 activity, which generates AA by cleaving phospholipid precursors, as well as lipooxygenase and ciclooxygenase activities, which catalyze the conversion of AA into active prostacyclin, prostaglandins, leukotrienes or thromboxanes. The release of AA metabolites has multiple functions on inflammation and on the regulation of macrophage activation. For instance, macrophages are important sources of PGE_2 which regulate body temperature by its action on the hypothalamus, among other cell functions.

1.3.3. Macrophages in pathology

Macrophages are essential cells for tissue homeostasis and the regulation of many biological processes but their functions must be tightly regulated since they can be harmful for the organism. Dysfunction in maturity, migration or phagocytic response of macrophages can lead to a reduced clearance of an infection. In addition, increased and non-regulated inflammatory responses of macrophages can also be dangerous because of an increase in the release of toxic molecules.

Thus, there are many illnesses related to macrophage dysregulation, most of them due to the chronic inflammation produced by macrophages. In fact, chronic inflammation is a

major cause of many diseases including atherosclerosis, chronic obstructive pulmonary disease, rheumatoid arthritis, inflammatory bowel disease, or cancer, among others.

When inflammation fails to subside, this unresolved inflammation can promote tumour cell growth, survival and angiogenesis. Solid tumours are infiltrated with leukocytes and the cross-talk between neoplastic and blood cells has profound effects on tumour progression. Leukocytes account for up to 50% of the tumour mass, the most represented subsets being lymphocytes and macrophages [5].

It has been reported that the density of macrophages is correlated in most, though not all, tumours with increased angiogenesis, tumour invasion and poor prognosis [6, 7].

1.3.3.1. Tumour-associated macrophages (TAM)

Monocytes can be differentiated into two types of macrophages with different functions: tumour-suppressive and tumour-supportive macrophages. Tumour-supportive macrophages are active in matrix remodeling, tissue repair and angiogenesis, whereas tumour-suppressive macrophages have microbicidal activity, immunostimulatory functions and tumour cytotoxicity. During the accumulation of somatic mutations of oncogenes and oncosuppressor genes, various immunoreactive cell types, including tumour-suppressive macrophages, are thought to suppress the progression of cancer. However, when such macrophages fail to suppress the progression of tumours, other macrophages, possibly tumour-supportive or tumour-associated macrophage (TAM), may support tumour growth and suppress the immune response against cancer [8, 9]. Tumour-associated macrophages (TAM) are key regulators of the link between inflammation and cancer.

The interaction between many stromal cell types with cancer cells further enhances production of inflammatory cytokines, chemokines, matrix-proteases, prostanoids, growth factors, adaptive immunity suppressors and angiogenesis related factors. These substances transform the tumour microenvironment so that it favors the survival, growth and motility (Figure 1.3.3.1.1)

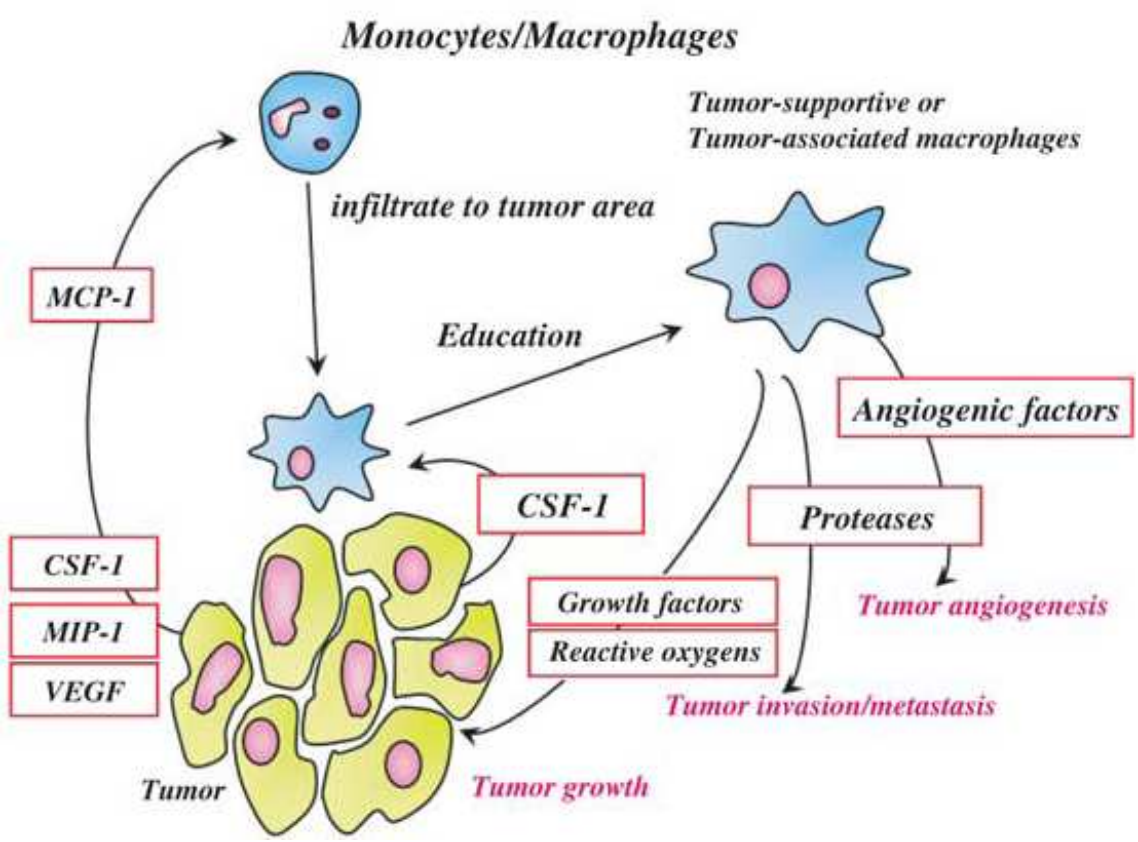


Figure 1.3.3.1.1. Monocytes/macrophages are educated to support malignant progression of cancer cells. Among the various inflammatory cell types and fibroblasts in tumor stroma, tumor-associated macrophages (TAM) are thought to play a critical role in supporting the progression of cancer. Monocytes/macrophages are recruited to the tumor, become TAM, and help creation of a microenvironment that favors angiogenesis, migration and growth of malignant cells. CSF-1, colony stimulating growth factor-1; MCP-1, monocytes chemoattractant protein-1; MIP-1, macrophage inhibitor protein-1; VEGF, vascular endothelial growth factor [10].

1.3.3.1.1. Secretion of growth factors, chemokines and matrix proteases

Many macrophage products released in the tumour stroma can directly stimulate the growth of tumour cells and/or promote tumour cell migration and metastasis. These include epidermal growth factor (EGF), members of the FGF family, TGFb, VEGF, chemokines and cytokines. It is well established that cytokines, such as IL-1b, augments metastasis.

Macrophages can produce enzymes and inhibitors that regulate the digestion of the extracellular matrix, thus favoring tumour invasion. TAMs produce several matrix-metalloproteases (e.g. MMP2, MMP9) and activators of MMPs, such as chemokines.

Strong evidence demonstrates that levels of MCP-1 are associated with TAM accumulation and that MCP-1 may play an important role in the regulation of angiogenesis [6].

1.3.3.1.2. Promotion of angiogenesis

TAMs contribute to tumour progression also by producing several factors which enhance angiogenesis and the dissolution and remodeling of the interstitial matrix. Tumour angiogenesis is often activated during the early, reneoplastic stages of tumour development and is controlled by a number of positive or negative regulators produced by cancer cells and tumour-associated leukocytes [11]. TAM accumulation is associated with increased angiogenesis and with the production of angiogenic factors such as VEGF and PDGF.

1.3.3.1.3. Stimulation of cell growth and inhibition of apoptosis. Implication of Reactive Oxygen Species (ROS)

Stimulation of cell growth and inhibition of apoptosis are necessary processes for tumour growth, either in macrophages or in tumour cells. Reactive oxygen species (ROS) such as superoxide ($O2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are found in a large number of tumours. ROS are conventionally thought as cytotoxic and mutagenic, and in high levels they induce cell death, apoptosis and senescence. In contrast, ROS at low levels function as signaling molecules to mediate cell growth, migration, differentiation and gene expression. It is known that in tumour cells ROS contribute to angiogenesis and mitogenesis (reviewed in [12]). Besides, most tumour cells seem to be more resistant to oxidative stress, and this resistance could be a selective advantage for tumour cell survival and growth. Several mechanisms may lead to oxidative stress in tumour-bearing patients, among others chronic inflammation. Inflammatory cells, especially macrophages and neutrophils, may produce ROS which participate in

carcinogenesis and tumour-associated immunosuppression. It has been reported that ROS production in neutrophils was higher in larynx carcinoma patients than in healthy controls and that the more advanced the cancer was, the more increased ROS production [13].

1.3.3.1.4. Suppression of adaptive immunity

An additional characteristic of tumour-associated macrophages is the ability to suppress the adaptive immune response through mechanisms including poor antigen-presenting activity and inhibition of T cell proliferation [14]. A major advance in the field of cancer immunology has been to understand how cancer cells achieve a global shutdown of immune-stimulating molecules, such as co-stimulatory molecules and cytokines, in the tumour microenvironment. TAMs produce and release several immunosuppressive cytokines, for instance IL-10. Moreover, they produce low levels of immunostimulatory cytokines such as TNFα. Suppression of adaptive immunity is a key process for cancer development.

1. Sphingolipids. Metabolism and regulation of cell homeostasis

Sphingolipids have been considered for many years as simple structural components of cells. Nevertheless, in the past few decades they have emerged as potent bioactive lipids able to regulate many essential cell functions. Sphingolipids are involved in cell and tissue homeostasis as well as in the establishment and progression of numerous diseases.

Sphingolipids are lipids derived from the aliphatic amino alcohol sphingosine (Figure 2.1). They were first discovered by Johann Ludwig Wilhelm Thudichum in 1870s in brain extracts and he named them for the mythological Sphinx because of their enigmatic nature.

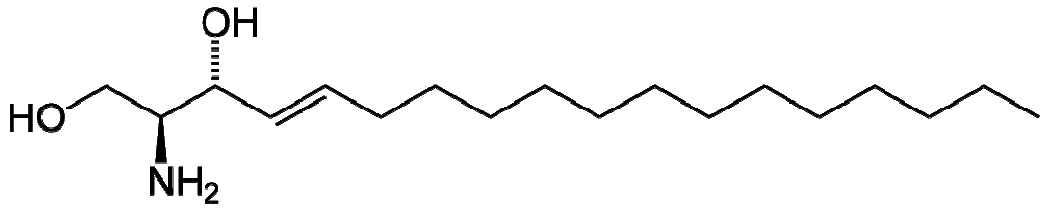


Figure 2.1. Sphingosine structure.

Sphingosine is the basic structure of more complex sphingolipids, such as, sphingomyelin, ceramides, glycosylceramides and complex glycosphingolipids. In addition to more complex sphingolipids, there are also some lyso-sphingolipids that lack N-acyl substituents, for instance, sphingosine 1-phosphate, sphingosine 1-phosphocoline or lyso-glycosphingolipids.

In particular, ceramides are the central core in sphingolipid metabolism and, apart from being an essential part of the cell membrane structure they are important signaling molecules capable of regulating cell proliferation, differentiation, adhesion, migration and apoptosis.

2.1. CERAMIDES

Ceramides consist of a sphingosine molecule linked to a fatty acid via an amide bond (Figure 2.1.1). Unlike the sphingoid precursors, they are not soluble in water and they are located in membrane compartments, including the plasma membrane where they participate in raft formation.

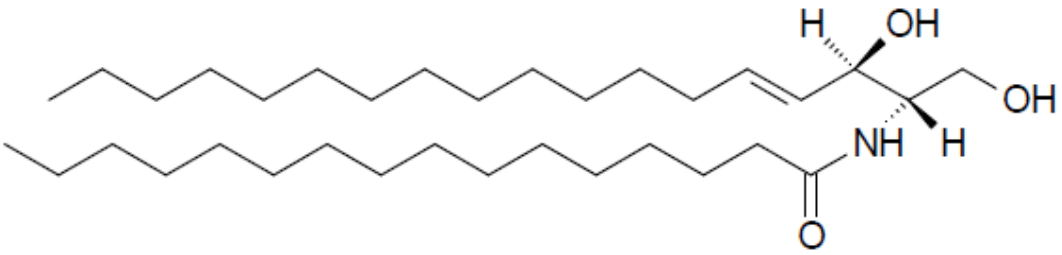


Figure 2.1.1. Ceramide structure.

There is a great variety of ceramides because their fatty acid can defer in length and in the number of unsaturations, and each organism or tissue can synthesize different ceramide species.

Ceramides can be generated by three major mechanisms (Figure 2.1.2):

1. The *de novo* synthesis pathway:

This is an anabolic route that begins with the condensation of the amino acid serine and palmitoyl-CoA to form 3-ketosphinganine in a reaction that is catalyzed by serine palmitoyltransferase (SPT). Reduction of 3-ketosphinganine to sphinganine follows immediately and afterwards acylation of sphinganine by dihydroceramide synthase (CERS, also known as Lass) generates dihydroceramide. The last step of this pathway is catalyzed by a desaturase through introduction of a trans-4, 5 double bond in the dihydroceramide molecule to yield ceramide.

2. Sphingomyelin hydrolysis:

The second major mechanism for ceramide generation is a catabolic pathway involving activation of sphingomyelinases (SMases). SMases hydrolyze sphingomyelin (SM) to generate phosphorylcholine and ceramide directly. The activation of SMases was suggested to be a major route for the production of ceramide in response to cellular stress. There are different types of SMases and they are classified by their optimal pH (acid, neutral and basic SMases). The opposite reaction is catalyzed by SM synthase (SMS). This important enzyme can control the levels of ceramides and sphingomyelin in cells. Specifically, SMS catalyzes the transfer of phosphorylcholine from phosphatidylcholine (PC) to ceramide, thereby releasing diacylglycerol (DAG) and lowering the levels of ceramide to produce SM.

3. The salvage pathway.

The third significant mechanism for generating ceramide is the sphingosine salvage pathway, in which sphingosine, produced from the metabolism of complex sphingolipids, is re-cycled to ceramide through the action of ceramide synthase (CERS).

Ceramide levels can be also reduced by synthesizing more complex sphingolipids such as glycosylceramides or complex glycosphingolipids.

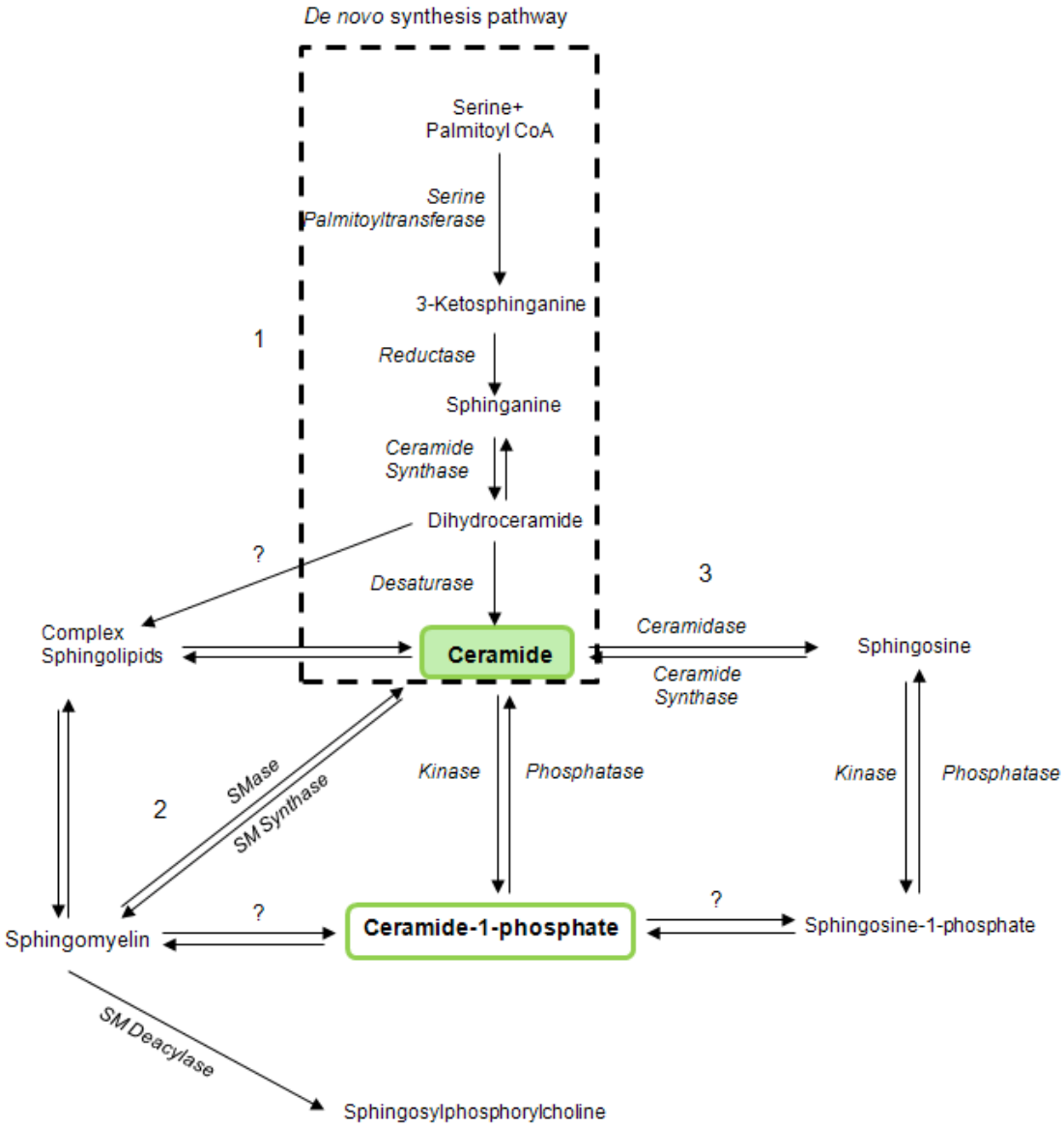


Figure 2.1.2. Sphingolipid metabolism. Ceramide can be generated by three major mechanisms 1) *De novo* pathway 2) Sphingomyelin hydrolysis 3) salvage pathway.

Apart from being the central lipid in the metabolism of sphingolipids, ceramide is also involved in the regulation of many signaling pathways.

It has been reported that ceramides are able to induce cell cycle arrest and promote apoptosis, a form of programmed cell death [15, 16]. Also, ceramides play important roles in the regulation of autophagy, cell differentiation, survival, and inflammatory responses [17-25], and have been associated with insulin resistance through activation of protein phosphatase 2A and the subsequent dephosphorylation and inactivation of

Akt (also known as protein kinase B (PKB)) [26-28]. In addition, ceramides are key mediators of radiation and chemotherapy effects on tumours, bacterial and viral infections, heat or UVA injury and ischemia-reperfusion injury (Reviewed in [29]).

Formation of ceramide is also relevant considering its role as precursor of important bioactive sphingolipids that can also regulate cellular functions. Ceramide and its downstream metabolites have been suggested to be involved in a number of pathological states including cancer, neurodegeneration, diabetes, microbial pathogenesis, obesity, and inflammation.

Once synthesized, ceramide can be used for synthesis of complex sphingolipids, through intervention of different biosynthetic enzymes, including glycosyl or galactosyl ceramide synthases to form cerebrosides or gangliosides, or it can incorporate a phosphocholine head group from phosphatidylcholine (PC) to form SM through the action of SM synthases. Formation of glycosylceramide is particularly important because of its role in conferring drug resistance to tumour cells [30].

2.2. SPHINGOSINE

Sphingosine (2-amino-4-octadecene-1, 3-diol) is an 18-carbon amino alcohol with an unsaturated hydrocarbon chain (Figure 2.1).

Sphingosine can be metabolized from ceramide by the action of specific ceramidases (Figure 2.1.2) and as well as ceramide, sphingosine is also bioactive. There are many reports showing that protein kinase C (PKC) is inhibited by exogenous sphingosine, and it has been also demonstrated that endogenously generated sphingosine can inhibit PKC very potently [31]. In turn, sphingosine can control the activity of other key enzymes involved in the regulation of metabolic or cell signaling pathways such as the Mg^{2+} dependent form of phosphatidate phosphohydrolase (PAP) [32, 33], phospholipase D (PLD) [34], or diacylglycerol kinase (DAGK) [35, 36] in a variety of cell types.

2.3. SPHINGOSINE 1-PHOSPHATE (S1P)

Phosphorylation of sphingosine by sphingosine kinases (SphKs) produces sphingosine 1-phosphate (S1P) (Figure 2.3.1), which can regulate a variety of cellular functions including cell growth and survival, differentiation, and angiogenesis [37-40].

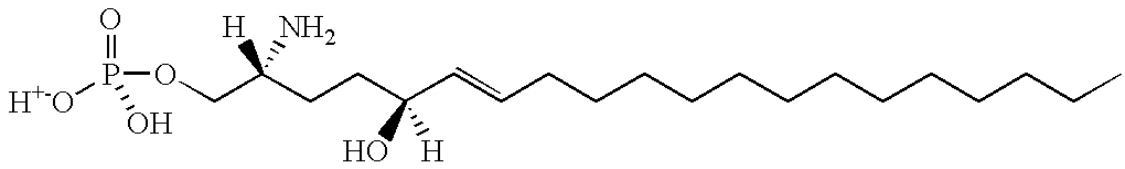


Figure 2.3.1. Sphingosine 1-phosphate.

S1P was originally thought to be an intracellular second messenger, but it was later discovered that is also a ligand for G protein-coupled receptors S1PR [41]. It is now known that S1P receptors are members of the lysophospholipid receptor family. These were originally named endothelial differentiation gene (EDG) receptor family. Up to date, five S1P receptors have been described, which are named S1P 1-5. Most of the biological effects of S1P are triggered by signaling pathways through these cell surface receptors.

2.4. CERAMIDE 1-PHOSPHATE (C1P)

A major metabolite of ceramide is ceramide 1-phosphate (C1P). C1P was thought to be not biologically active until 1995, when it was first described as a stimulator of DNA synthesis in rat fibroblasts [42, 43]. C1P is mainly produced by ceramide kinase (CERK) which phosphorylates ceramide at the C1 position, and can be dephosphorylated by phosphatase activity (Figure 2.4.1).

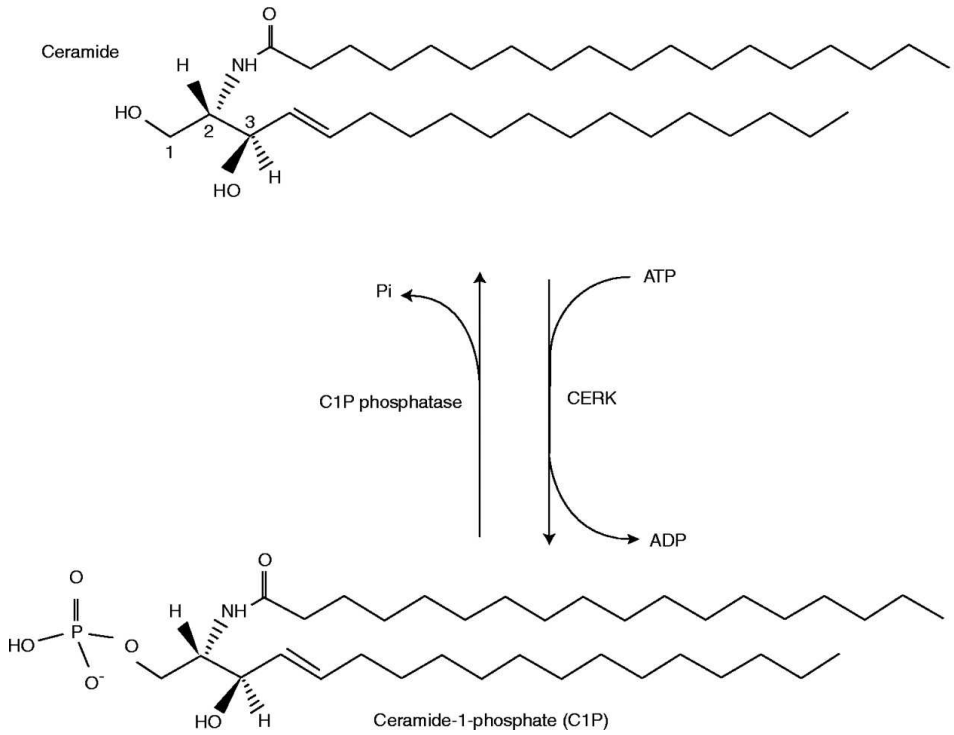


Figure 2.4.1. C1P can be synthesized by CERK. Figure taken from [44]**.**

2.4.1. Ceramide Kinase (CERK)

CERK (EC 2.7.1.138) was first identified in brain synaptic vesicles and it was reported that this kinase phosphorylate ceramide specifically [45].

Human CERK has been cloned [46]. The protein sequence has 537 amino acids with two protein sequence motifs, an N-terminus that encompasses a sequence motif known as a pleckstrin homology (PH) domain (amino acids 32–121), and a C-terminal region containing a $Ca^{2+/}$calmodulin binding domain (amino acids 124–433). Using site-directed mutagenesis, it was found that leucine 10 in the PH domain is essential for its catalytic activity [46] and the integrity of the PH domain is essential for the activity and the location of the enzyme.

It has been established that CERK activity is Ca^{2+} dependent and that it can be regulated by phosphorylation/dephosphorylation. Apart from these processes, the PH domain of CERK also has a myristoylation site and this could be another regulatory center [47]. The interaction between the PH domain of CERK and phosphatidylinositol 4,5-bisphosphate regulates the plasma membrane targeting and C1P levels [48], and the subcellular localization of CERK requires the interplay of their PH domain-containing

N-terminal regions together with the C-terminal domains [49]. More recently, it has been published that a conserved cystein motif in CERK is also essential for its functionality [50].

It has been postulated that a transport protein (ceramide transport protein or CERT) is required for CERK to phosphorylate ceramide. This protein utilizes the ceramide transported to the trans-Golgi apparatus. This fact was discovered by silencing CERT with specific siRNA which resulted in a strong inhibition of newly synthesized C1P [51]. However, this postulation remains controversial as it was also reported that this ceramide transport was not dependent on CERT [52]. Hence, there are still some questions concerning this concept that require further investigation.

At the present time, CERK is considered to be the only C1P source in mammalian cells. However, it has been reported that bone marrow-derived macrophages from CERK null mice (CERK-/- mice) still have significant levels of C1P [52]. This suggests that there could be other metabolic pathways for generating C1P. We have previously speculated two alternative pathways for generation of C1P in cells might be the transfer of a long acyl-CoA chain to S1P by a putative acyl transferase, or cleavage of SM by a PLD-like activity, similar to the one discovered in arthropod or bacterial SMase D. However, work from our own lab [53] and that of others [52] have shown that acylation of S1P to form C1P does not occur in mammalian cells. In addition, we found no evidence for the implication of SMase D activity in rat fibroblasts. Nonetheless, these possibilities should be further explored in other cell types.

There are many papers reporting that C1P can be generated intracellularly. For instance, it has been published that C1P can be generated in neutrophils after addition of exogenous C6-ceramide [54]. Also, upon the recycling pathway of SM-derived ceramide or ganglioside catabolism-derived sphingosine, C1P generation can occur in cerebral granule cells [55]. In addition, in A549 lung carcinoma cells C1P levels increase when cells are treated with interleukine-1β [56] and in bone marrow-derived macrophages C1P is generated upon addition of M-CSF to the culture medium [57].

2.4.2. Putative receptor of C1P

Most of the biological functions of C1P can be reproduced by increasing intracellular C1P levels. However, it has recently been reported that not all of them can be achieved with this strategy. For instance, C1P can induce cell migration in Raw 264.7 cells but it is not possible to stimulate chemotaxis by increasing intracellular C1P levels (i.e. with IL-1β or with the calcium ionophore A23187 [58]). This result suggests that there may be some kind of interaction between C1P and the plasma membrane that cannot be achieved intracellularly. For this reason binding experiments between C1P and cell membranes were performed and so that a putative receptor for C1P could be detected. This receptor turned out to be a Gi protein-coupled receptor (GPCR) with low affinity for its substrate (Kd=7.8 μM) [58].

C1P is a key regulator of many proliferative pathways. It has been implicated in the induction of many proliferating signaling pathways and also in anti-apoptotic signaling cascades. Moreover, C1P has emerged as a proinflammatory molecule in different cell types.

3. Sphingolipids and the immune system

In immune cells, sphingolipid metabolism results in the formation of lipid second messengers, including ceramide (Cer), sphingosine (Sph), ceramide 1-phosphate (C1P), and sphingosine-1-phosphate (S1P); all of them can be generated in a common signaling pathway that might control major cell functions including, cell proliferation, survival, differentiation, or immune cell development. Although, little is known about the role of sphingolipids in immune cell functions, there are some interesting data that underscore the importance of sphingolipids in cell immunity (reviewed in [59]).

3.1. SPHINGOLIPIDS AND MACROPHAGES

As mentioned before, macrophages are key cells, involved in orchestrating inflammatory processes. Hence, their activation must be tightly regulated in order to avoid tissue damage or chronic inflammation. For this reason, understanding the macrophage homeostasis and the regulation of inter- and intracellular signaling pathways is necessary to enlighten the importance of sphingolipids in controlling cell activation. This knowledge provides useful information to develop proper therapeutical tools for the treatment or prevention of this kind of illnesses.

3.1.1. CERAMIDE 1-PHOSPHATE IS A KEY REGULATOR OF MACROPHAGE PROLIFERATION AND SURVIVAL

Our group has pioneered the studies concerning the implication of C1P in cell biology [60-62]. As mentioned above, it was first demonstrated that C1P stimulates cell proliferation in fibroblasts and macrophages [42, 43, 57], and it was also found to be a potent inhibitor of apoptosis [53, 63-65]. Some of the signaling pathways involved in these actions have already been described, and they are listed below:

3.1.1.1. Mitogen-activated Protein Kinase (MAPK)

Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens, osmotic stress, heat shock and proinflammatory cytokines) and can regulate many cellular activities. These include gene expression, mitosis, differentiation, proliferation, and cell survival [66]. MAP kinases are activated within the protein kinase cascades called “MAPK cascade”. Each one consists of three enzymes: MAP kinase, MAP kinase kinase and MAP kinase kinase kinase which are activated sequentially. There are many MAPK subfamilies, such as, extracellular signal-regulated protein kinases (ERK1-2), c-Jun N-terminal kinases (JNK), or p38. It has been demonstrated that C1P can activate ERK1-2 and JNK in BMDM and that this activation leads to cell proliferation [57].

3.1.1.2. Phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (PKB) pathway

Phosphatidylinositol 3-kinases (PI3Ks) are a family of enzymes involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking, which in turn are involved in the development of cancer. PI3Ks are signal transducer enzymes capable of phosphorylating the 3rd position hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns). The pleckstrin homology domain of PKB binds directly to PtdIns(3,4,5)P3 and PtdIns(3,4)P2, which are produced in the plasma membrane by the active form of PI3K. Thus PKB translocates to the plasma membrane where it becomes phosphorylated. Phosphorylated PKB is further activated in the cytosol where it exerts its functions by activating signaling cascades that lead to cell proliferation and survival. Our group found that C1P also activates the PI3K/PKB pathway [57].

3.1.1.3. Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB)

NF-κB is a transcriptional factor involved in many cellular functions. However, with regards to C1P-stimulted cell proliferation, our group has demonstrated that NF-κB is downstream of PI3K/PKB, MAPK/ERK1-2 or JNK, and that activation of this transcriptional factor is necessary for the proliferative effect of C1P in macrophages [57].

3.1.1.4. Acid Sphingomyelinase (A-SMase)

It has been reported that apoptotic bone marrow-derived macrophages (BMDM) posses high A-SMase activity and high levels of ceramides compared to healthy cells [63, 67] and that C1P blocked apoptosis in BMDM [64]. Investigation into the mechanism whereby C1P exerts its anti-apoptotic effects demonstrated a complete inhibition of both A-SMase and ceramide accumulation by C1P in intact macrophages [53]. C1P also blocked the activity of A-SMase in cell homogenates suggesting that inhibition of this enzyme occurs by a direct physical interaction with C1P. Furthermore, it was also reported that PIP3 (a major product of PI3K activity) is a direct inhibitor of A-SMase [68] and as mentioned before, PI3K is a target of C1P in BMDM.

3.1.1.5. Serine palmitoyl transferase (SPT)

Our group also reported that ceramide levels were increased in apoptotic alveolar macrophages (NR8383) and that C1P decreased intracellular ceramide accumulation when these cells became apoptotic [65]. However, in these macrophages A-SMase was only slightly activated, suggesting that this pathway was not the major mechanism involved in the production of ceramides. Of interest, activation of serine palmitoyl transferase (SPT) appeared to be the major mechanism by which ceramide levels were increased in these cells. As mentioned before, SPT is the key regulatory enzyme for synthesis of ceramides by *de novo* pathway. We reported that C1P inhibits SPT activity, causing a significant reduction in the levels of intracellular ceramides, and this prevented the macrophages from entering apoptosis [65].

3.1.1.6. Mammalian Target of Rapamycin (mTOR)

mTOR is an important factor for the regulation of cell proliferation. Our group has demonstrated that C1P stimulates macrophage proliferation through activation of the mammalian target of rapamycin complex 1 (mTORC1) [69]. In this work we also reported that activation of RhoA/ROCK is essential for the induction of cell proliferation and that this event is upstream of mTORC1 activation.

3.1.1.7. Inducible Nitric Oxide Synthase (iNOS)

An important metabolite with cell signaling properties is nitric oxide (NO). NO can be generated by three distinct nitric oxide synthases: neuronal NOS (nNOS or NOS I), endothelial NOS (eNOS or NOS II), and inducible NOS (iNOS or NOS III). While nNOS and eNOS are constitutively expressed, iNOS is an inducible enzyme that can be stimulated by different cytokines or endotoxins. Our group has reported that upregulation of iNOS and the subsequent production of NO is another mechanism by which C1P inhibits apoptosis and promotes cell survival [70].

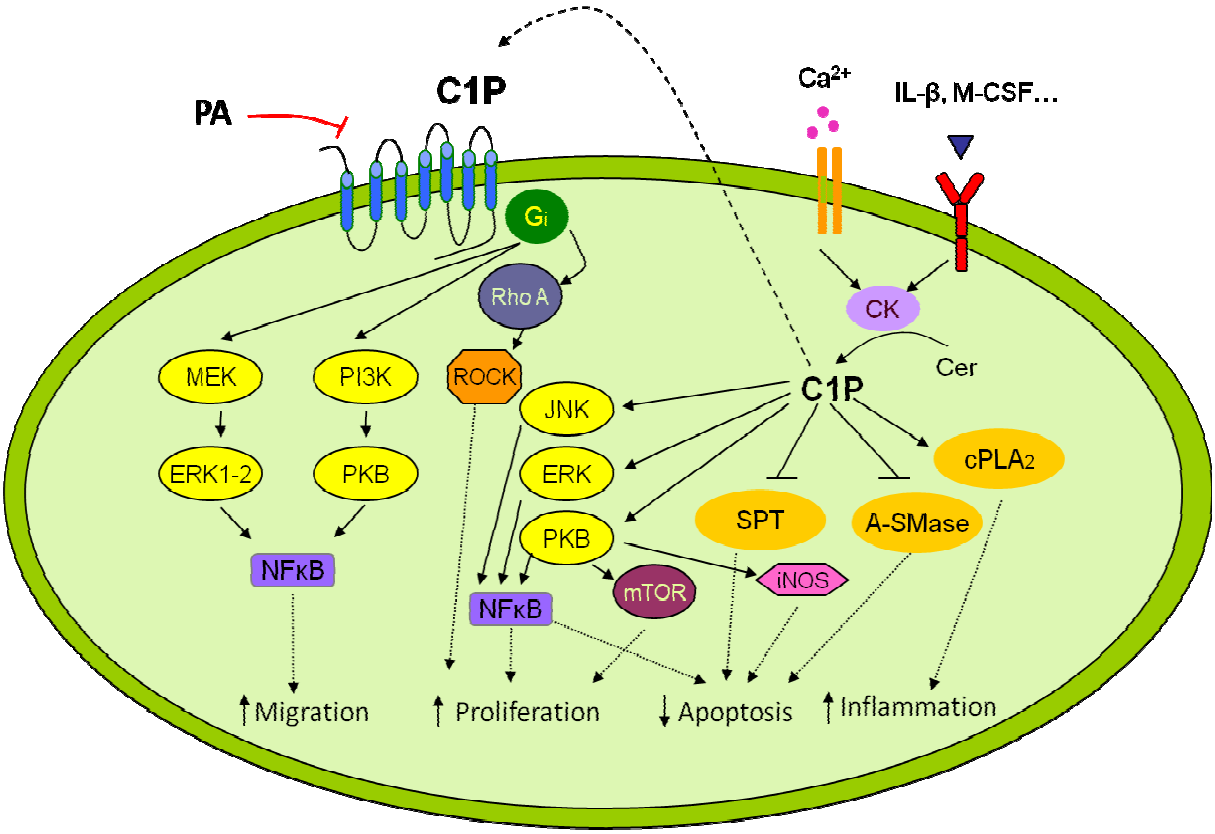
3.1.2. CERAMIDE 1-PHOSPHATE AND THE CONTROL OF INFLAMMATION

Many inflammatory mediators are secreted by macrophages; these include chemokines, cytokines, vasoactive amines, phospholipases and eicosanoids.

Of interest, it was reported that C1P can stimulate arachidonic acid (AA) release and prostaglandin formation [56]. AA is a polyunsaturated fatty acid that is present in phospholipids and can act as a second messenger in inflammatory pathways. AA can be also secreted to the extracellular medium and activate other cells in a paracrine manner. AA can be generated by the action of phospholipase A_2 (PLA_2) activity, which cleaves the fatty acid in the second position within the phospholipid molecule. Also, AA can be generated from diacylglycerol (DAG) through cleavage by diacylglycerol lipase. AA is also a precursor of other pro-inflammatory molecules, such as, porstaglandins, leukotrienes or epoxyeicosatrienoic acid. It has been reported that C1P can activate group IV $cPLA_2$ [71] by increasing the enzyme affinity for its substrate, mainly phosphatidylcholine [72].

3.1.3 C1P AND THE REGULATION OF CELL MIGRATION

Recently, our group found that C1P can induce cell migration in Raw 264.7 leukemic macrophages [58]. Macrophage chemotaxis was stimulated by extracellular C1P and not by intracellularly formed C1P. This fact suggested that C1P could interact with cell membrane sites or receptors to achieve this action. It was observed that C1P interacted with a putative membrane receptor [58]. Cell migration was potently induced by exogenous C1P but not by any other related sphingolipid, including S1P and ceramides, or structurally related phospholipids, such as phosphatidic acid. In addition to this, the signaling pathways by which C1P exerted its chemotactic function were described. These pathways include the MAPK/ERK, and PI3K/PKB pathwa which converge in the activation of the transcription factor NF-κB.



3.1.1. Schematic representation of the multiple regulatory roles of C1P in macrophage responses.

3.2. SPHINGOLIPIDS AND NATURAL KILLER CELLS

Natural killer cells (or NK cells) are a type of cytotoxic lymphocytes that constitute a major component of the innate immune system. NK cells play a significant role in tumour rejection and suppression of viral infections. They kill cells by releasing small cytoplasmic granules containing proteins called perforin and granzyme that cause the target cell to undergo apoptosis.

NK cells express EDG receptors (EDG-1, 3,6 and 8), which are receptors for S1P, as mentioned before [9]. Besides, it has been reported that S1P, along with its closely related phospholipid, dihydrosphingosine-1-phosphate (DHS1P), can activate NK cell chemotaxis through activation of heterotrimeric G proteins and PI3K [9].

There are also evidences suggesting that A-SMase, SMS and GCS are implicated in interleukin-2 (IL-2) induced ceramide reduction in NK cells, which rescues cells from apoptosis [73]. The authors of the latter reference also reported that IL-2 withdrawal

activates A-SMase and inhibits GCS and SMS, and that these effects can be suppressed by adding IL-2 into the culture medium. Interestingly, these authors observed that these cells could not be rescued when the cells were previously treated with the PI3K inhibitor Ly 294002.

3.3. SPHINGOLIPIDS AND CYTOTOXIC T LYMPHOCYTES (CTL)

The regulation of the surface expression levels of T cell receptor (TCR) is probably an important mechanism by which T cell responsiveness is controlled. The ability of ceramide to induce a rapid TCR up-regulation in both CD4+ and CD8+ cells has been reported previously [74]. However, in Jurkat T cells, ceramides can also exert the opposite effect on TCR expression, depending on incubation time and ceramide concentration [75]. Thus, there are still many questions to be answered concerning the role of sphingolipids in the regulation of T lymphocyte responses.

3.4. SPHINGOLIPIDS AND CELL-MEDIATED CYTOTOXICITY

Cytotoxic T lymphocytes (CTL) and NK cells induce apoptosis in the target cell by two major mechanisms:

1) Ca^{2+}-dependent secretion of preformed cytoplasmic granules containing cytotoxic molecules such as perforin and several serine proteases. Perforin, a pore-forming protein, allows the entry of granzymes (most importantly serin proteases) into the cytoplasm of target cells, where they induce apoptotic cell death. Perforin/granzyme-based cytolysis has been reported to occur without increasing the cellular ceramide content, therefore ruling out the possible contribution of the sphingomyelinase pathway to this mechanism of cell death [76].

2) CD95 (Fas), a TNF receptor-like molecule, with its ligand. CD95 system-mediated cytotoxicity involves cross-linking of the Fas antigen with an agonist monoclonal antibody resulting in a coordinated increase in sphingomyelinase activity, ceramide levels, and cytotoxicity in susceptible tumour cells [77].

Cytolysis induced by both granule-mediated and CD95-based mechanisms exhibited the typical features of apoptosis.

3.5. SPHINGOLIPIDS AND B LYMPHOCYTES

B lymphocytes require the expression of a surface BCR (B cell receptor) for development and survival and the signals conveyed through the B cell antigen receptor are crucial for B cell maturation and activity. It is well established that in mature B cells, BCR signaling results in activation while in immature B cells signaling through the BCR results in apoptosis, receptor editing or anergy. Some reports have led to the identification of glycosphingolipid and cholesterol-rich plasma membrane microdomains, or lipid rafts, which have been proposed to function as platforms for both signal transduction and membrane trafficking for immune cells. The presence of phosphorylated proteins in the rafts following BCR cross-linking suggests that the signal cascades are initiated and propagated from the rafts. In addition, several reports indicated the translocation of important proteins, such as phospholipase C2, into lipid rafts upon BCR stimulation [78]. However, the importance of ceramides and glycosphingolipids on raft-associated signaling cascades remains to be determined.

The stimulation via antigen receptor in immature B cells and B cell lines leads to cell death through apoptosis. The detailed signaling cascade of this apoptotic pathway is still unknown, but sphingolipid metabolites seem to be involved in this signaling.

Another route by which ceramide could regulate the immune response is by activating IL-6 production. IL-6 is a potent immunomodulator which regulates B cell and macrophage differentiation as well as T cell stimulation. Ceramide was discovered to be a potent inducer of IL-6 release in human fibroblasts and exogenous addition of SMase seemed to have similar effects [79].

Objectives

2. OBJECTIVES

From the above comments, it is clear that C1P is a bioactive sphingolipid metabolite that is capable of regulating vital cellular functions. However, the mechansims by which C1P exerts its biological effects have only begun to be understood.

The present thesis was undertaken to elucidate additional mechanisms by which C1P controls cell proliferation and cell migration, and to assess whether C1P plays a role in the activation of the immune system. Accordingly, the specific objectives of this work are as follows:

1. To study the mechanism by which C1P stimulates macrophage migration. Although some of the pathways involved in the stimulation of cell chemotaxis by C1P have already been put forward by our group, it is not clear whether C1P can induce the release of chemoattractant molecules. Therefore, the possible implication of monocyte chemoattractant protein-1 (MCP-1) in this process will be exhaustively studied.

2. Our group previously established that C1P is mitogenic for macrophages. However, mitogenesis is a complex process that implicates numerous pathways and mechanisms. In the present work, the possible stimulation of endothelial growth factor (VEGF) secretion by C1P, as well as the mechanisms involved in this process, will be examined.

3. Another feature of macrophages is the production of reactive oxygen species (ROS), which have been involved in the regulation of both stimulation of cell proliferation and apoptosis. Therefore, the possible stimulation of ROS production by C1P, and its possible participation in the mitogenic or antiapoptotic effects of C1P will be investigated.

4. To study the possible implication of C1P in lymphocyte activation

Materials and Methods

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Chemical products

Supplier	**Reagents and products**
Sigma-Aldrich	1-Aminobenzotriazole (ABT) Acrylamide/bisacrylamide Allopurinol Ammonium persulfate Apocynin Bovine Serum Albumin (BSA) diphenyleneiodonium chloride (DPI) Eosin Fibronectin Gentamicin Hematoxylin IL-2 JE (MCP-1) from mouse L-glutamine LY 294002 N-acetylcysteine (NAC) L-NG-Nitroarginine methyl ester (Hydrochloride) (L-NAME) PD 98059 Pertussis Toxin Protease inhibitor cocktail

	rotenone rottlerin RPMI 1640 RS 102895 Scott′s Tap Water Substitute Concentrate (Blueing reagent) SP 600125 Tween-20 ZM 39923 hydrochloride
Matreya, LLC	N-hexadecanoyl-D-erythro-sphingosine-1-phosphate (N-Palmitoyl-Ceramide 1-phosphate) (C1P)
Gibco (Invitrogen)	Fetal Bovine Serum (FBS) Opti-MEM
Cell Signalling Technology **(Beverly MA, USA)**	Ab Akt Ab MCP-1 Ab p42/p44 Ab p47phox Ab p65 Ab p85 subunit of PI3K Ab p-Akt (Ser 473) Ab p-cPLA2a (Ser505) Ab p-p40phox (Thr154) Ab p-p42/p44 (Thr202/Tyr204) Ab p-p65 (Ser536) Ab p-STAT3 (Ser 727)

	Ab p-VEGFR2 (Tyr 1175) (D5B11) Ab rabbit IgG HRP-linked Ab STAT3 Ab VEGFR2
Santa Cruz Biotechnology, Inc.	Ab cPLA2 Ab p-38 (THr 180/Tyr 182)-R Ab p40phox Ab total p40-phox (D-8) Ab β-actin (H-196) Akt2 siRNA CKR-2 (M-50) CKR-2 siRNA Control siRNA MCP-1 siRNA
Calbiochem- Novabiochem corporation (USA)	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) "*Annexin-V/PI detection kit*" "*NucBuster Protein Extration Kit*" N-{(2S,4R)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide (pyrrolidine-2) phenazine methosulfate (PMS) Phorbol-12-myristate-13-acetate (PMA)
BIO RAD	BCA assay reagents Nitrocellulose membranes

	Protein markers
Cayman Chemical	*"cPLA$_2$ assay kit"*
Becton–Dickinson	Propidium Iodide (PI)
ebioscience	CD11b-FITC antibody CD3 CD4 CD54 (ICAM-1)-FITC antibody CD69-FITC CD8a Dx5 MCP-1 neutralizing antibody (cell tested) Mouse CCL2 (MCP-1) ELISA Ready-SET-Go! NK1.1 NKG2D
R&D Systems	"Mouse VEGF Quantikine" ELISA kit
Molecular Probes (Invitrogen)	5-(and 6-)-chloromethyl-20, 70-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA) Oligofectamine^{TM} Reagent
Perkin Elmer	[^{3}H-Methyl]-thymidine (2.0 Ci/mmol) [5,6,8,9,11,12,14,15-^{3}H(N)]-Arachidonic acid
Tocris Bioscience	10-DEBC Go6976
GE HealthCare	Ficoll-Paque PREMIUN (d=1.084 g/mL)

Lonza	DMEM
Promega	CellTiter96® AQ_{ueous} One Solution (MTS)
Applied Biosystem (Ambion)	Akt1 siRNA Mapk1 (Erk2) siRNA Pik3r1 (PI3K) siRNA
Merk Chemicals	TLC silicagel 60
Other chemicals and reagents	Analytic grade reagents

BHNB-C1P was synthesized as previously described [80] in the Department of Chemistry and Biochemistry, Queens College of The City University of New York, Flushing, New York (USA).

M-CSF was obtained from a fibroblast cell line, L-929 cells, which were generously donated by Dr. U. P. Steinbrecher (University British Columbia, Vancouver, Canada). L-929 fibroblasts were incubated in T-175 flasks with 30 mL of DMEM supplemented with 10% fetal bovine serum (FBS) for 2 weeks after reaching confluence. Then, medium was centrifuged and filtered through 0.22 μm pore filters. Harvested medium was stored at -20 ºC until use. This medium is called L-conditioned medium.

N-Acetyl-d-glucosamine-coated polyamidoamine dendrimer (GN8) was kindly provided by Prof. T.K. Lindhorst (Christiana Albertina University in Kiel, Germany) and Prof. V. Kren (Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic).

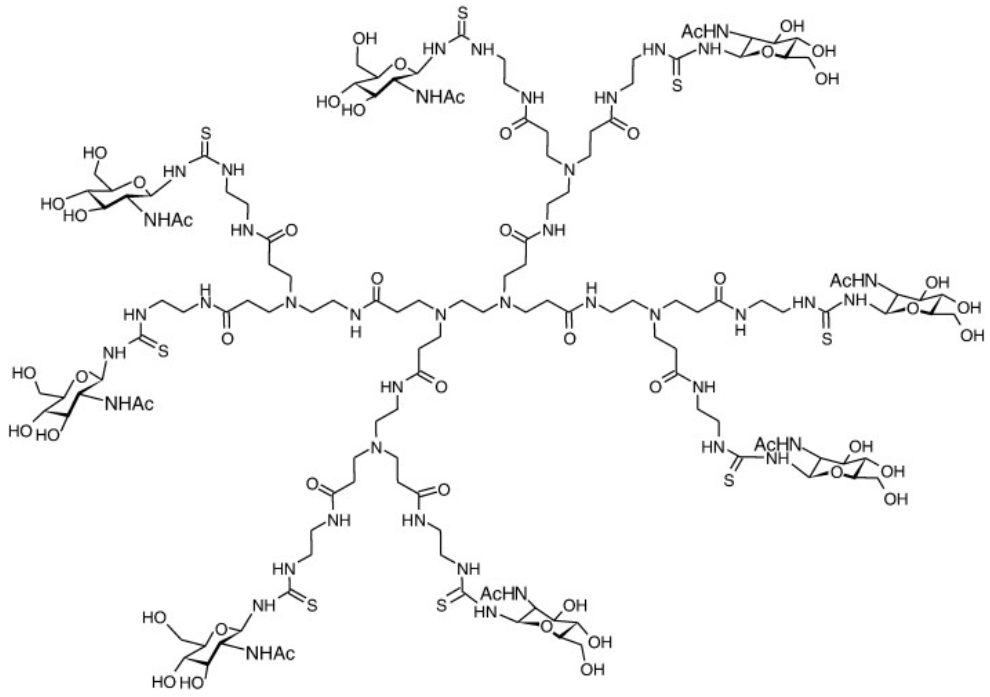


Figure 3.1.1.1. Scheme of *N*-Acetyl-d-glucosamine-coated polyamidoamine dendrimer (GN8) [81].

3.2. CELL LINES AND PRIMARY CELLS

3.2.1. J774A.1 cell line

J774A.1 cell line was purchased from ATCC (Manassas, VA, USA) and cultured following the manufacturer's indications. J774A.1 cell line is a monocyte/macrophage cell line obtained from a BALB/c mice with reticulum-cell sarcoma. Cells were grown in 175 cm^2 flasks in DMEM supplemented with 10% FBS, 20 µM L-glutamine and 50 mg/L gentamicin. Cells were incubated at constant temperature (37 ºC) and regulated atmosphere (humidified atmosphere containing 5% CO_2) and subcultured every 2-3 days maintaining cell density between $0.5\text{-}2\cdot10^6$ cells/10 mL.

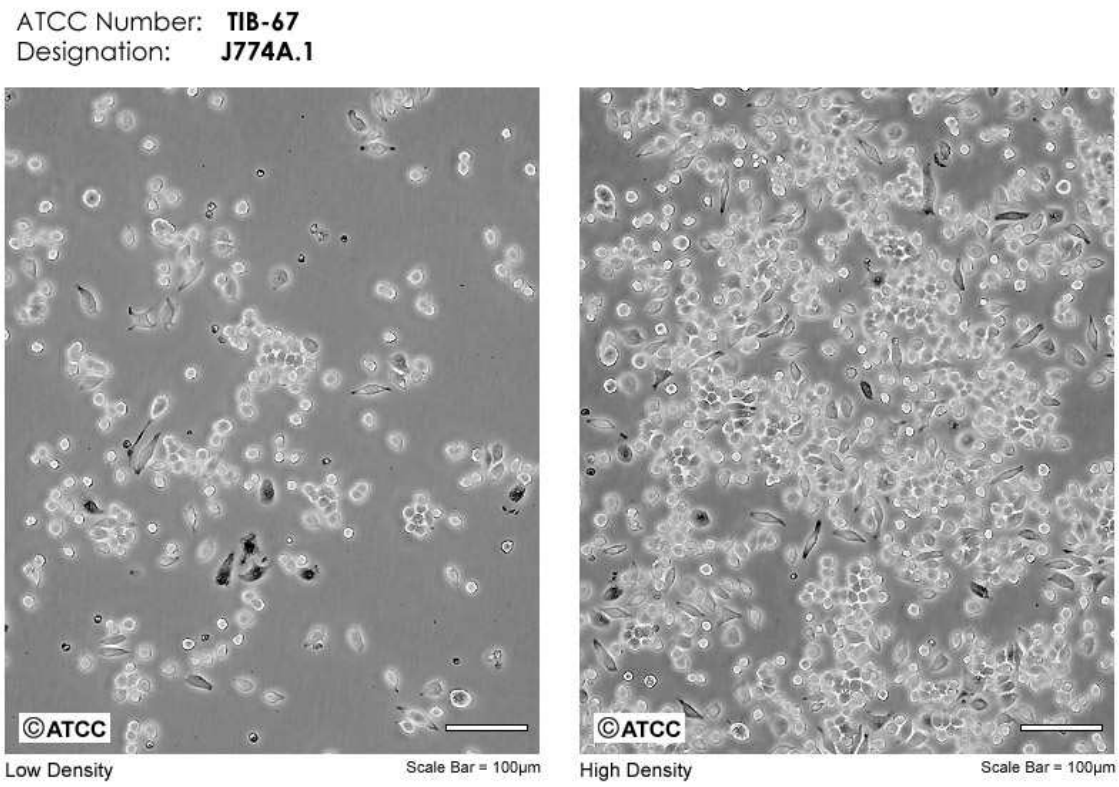


Figure 3.2.1.1. Micrograph of J774A.1 cells (taken from the ATCC website).

3.2.2. Bone Marrow-derived Macrophages (BMDM)

Bone marrow-derived macrophages (BMDM) were isolated from femurs of 6-8-week old female CD-1 mice as described [82]. Ice-cold phosphate-buffered saline (PBS) was used to perform perfusion of femurs and collected cells were plated in RPMI 1640 containing 20 µM L-glutamine, 100 mg/L gentamicin 10% fetal bovine serum (FBS) and 20% L-cell conditioned medium, as the source of macrophage-colony stimulating factor (M-CSF) for 24 h [83]. The next day, non-adherent cells were collected and counted to finally be cultured for 4-6 days in the same medium. After this incubation, about 80% confluence was reached and 85% of the harvested monocytes had been differentiated into macrophages.

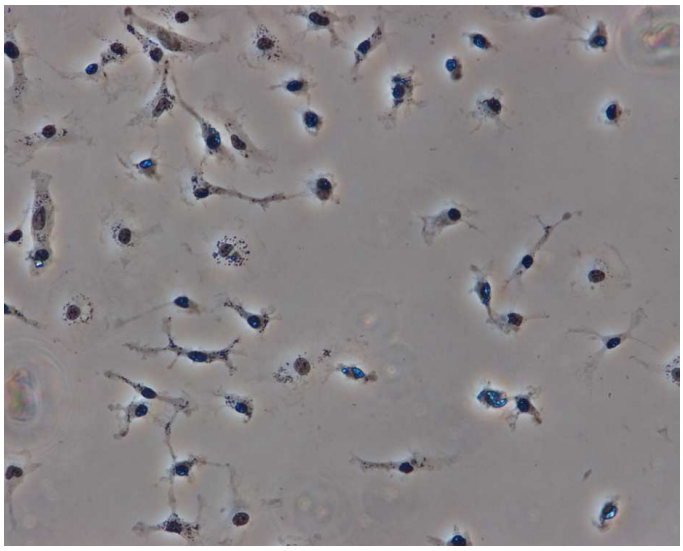


Figure 3.2.2.1. Phase-contrast image of bone marrow-derived monocytes cultured for 4-6 days in RPMI 1640 containing 10% FBS and 20% L-cell conditioned medium.

Cells were then incubated for 24 h in RPMI 1640 containing 10% FBS and 1.5% L-cell conditioned medium, before being used in experiments. It was established that this concentration of L-cell conditioned medium is enough to ensure cell viability, without stimulating cell proliferation (Figure 3.2.2.2).

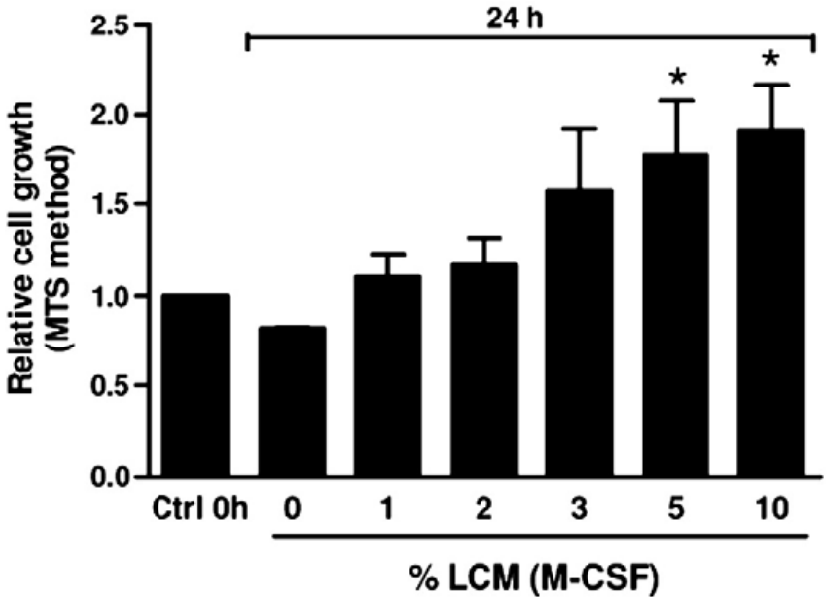


Figure 3.2.2.2. The M-CSF concentration determines cell viability in BMDM. Cells were incubated for 24 h in RPMI supplemeted with 10% FBS and the indicated concentrations of L-conditioned medium. Cell viability was measured by the MTS-formazan method. Results are the mean ± SEM of three independent experiments performed in triplicates (*$p<0.05$).

3.2.3. Isolation and culturing of Spleen Mononuclear Cells (SMC)

The spleen is an important organ in regards to senescent red blood cells and immune system. Due to its functions, spleen is full of red blood cells, lymphocytes (T and B), Natural Killer cells (NK cells) and monocytes. Although lymph nodes and peripheral blood can be used as sources of immune system cells, spleen is the most often used lymphocyte source because it constitutes the most accessible way to obtain them.

Spleens were isolated from 6-8-week old female C57BL/6 mice from Charles River Laboratories International Inc. After a sterile extraction, the spleen was dissociated through a nylon mesh collecting all SMCs but connective and fatty tissue in RPMI 1460 containing plate. The cell suspension was gently pipetted inside a centrifuge tube containing Ficoll-Paque (without mixing both liquids) and centrifuged at 400 × g for 30 min. Ficoll-Paque is a sterile Ficoll PM400/sodium diatrizoate solution of the proper density (d=1.084 g/L), viscosity and osmotic pressure to be used in mononuclear cell isolation. After gradient centrifugation, cell suspension was divided in three phases (Figure 3.2.3.1).

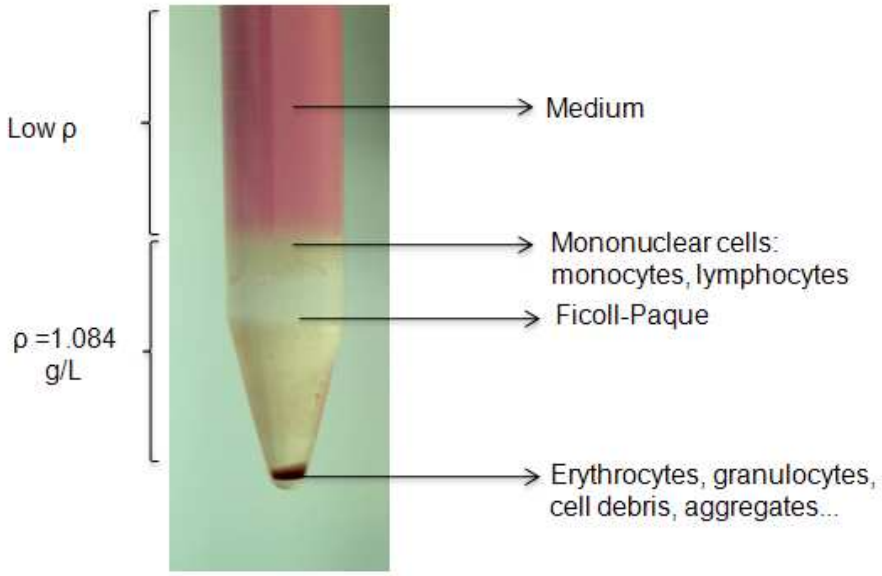


Figure 3.2.3.1. Photograph of a SMC suspension after gradient centrifugation using Ficoll-Paque.

The mononuclear cell ring was collected and transferred to a new sterile centrifuge tube where cells were washed three times with fresh medium by centrifugation at 500 × g for 5 min. After washing, the cell suspension was counted and immediately used for different experiments.

3.2.4. Raw 264.7 cell line

Raw 264.7 cell line was generously donated by Dr. Urs Steinbrecher (University British Columbia, Vancouver, Canada). Raw 264.7 cells are a monocyte/macrophage cell line obtained from belson murine leukemia virus-induced tumour of BALB/c mice. Cells were grown in 75 cm^2 flasks in DMEM supplemented with 10% FBS, 20 µM L-glutamine and 50 mg/L gentamicin. Cells were incubated at constant temperature (37 ºC) in a regulated atmosphere (humidified atmosphere containing 5% CO_2) and subcultured every 2-3 days.

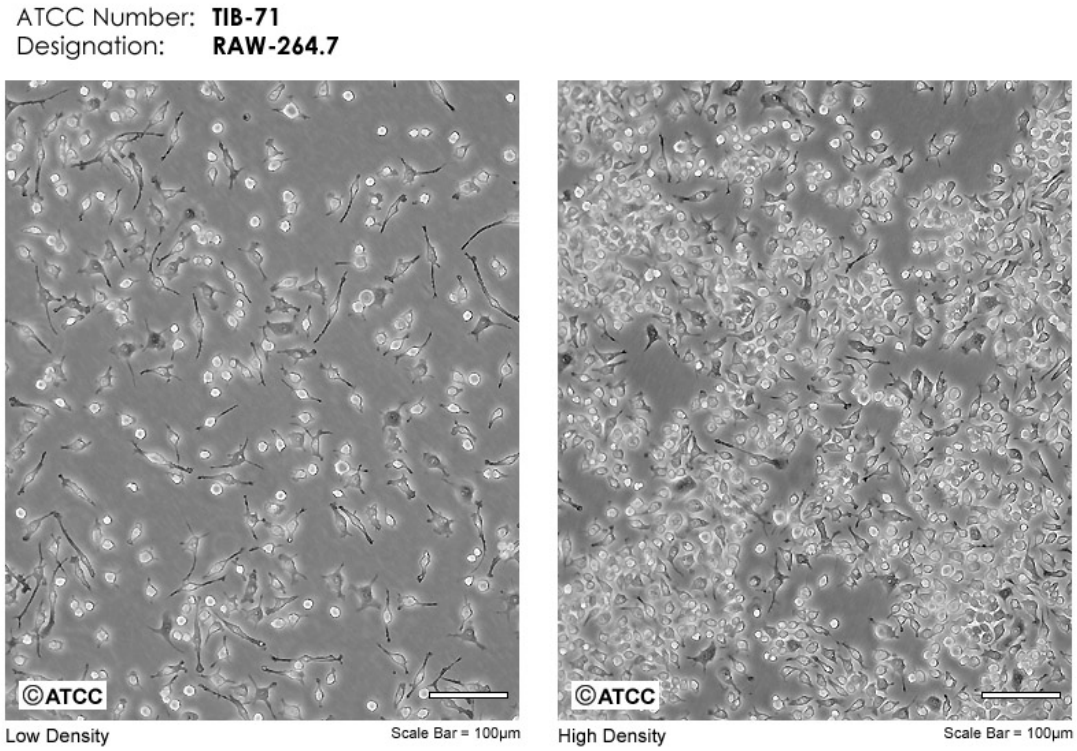


Figure 3.2.1.1. Micrograph of Raw264.7 cells (taken from the ATCC website).

3.3. METHODS

3.3.1. Delivery of C1P to cells in culture

An aqueous dispersion (in liposome form) of C1P was added to cultured macrophages as previously described [53, 57, 64]. Specifically, stock solutions were prepared by sonicating C1P (5 mg) in sterile nanopure water (3 mL) on ice using a probe sonicator until a clear dispersion was obtained. The final concentration of C1P in the stock solution was approximately 2.62 mM. This procedure is considered preferable to dispersions prepared by adding C1P in organic solvents because droplet formation is minimized and there are no organic solvent effects on cells.

C1P was added to the culture medium in the micromolar range, 20-50 µM. It is important to keep in mind that this C1P is delivered in aqueous liposome and cells only internalize little amount of these liposomes. It has been demonstrated that after 2 h of incubation with 25 µM C1P delivered in aqueous liposomes only few nmoles of C1P are incorporated per million cells [43]. Similar increase in intracellular C1P levels (4 nmoles/million cell) were achieved after treatment of A549, HeLa or macrophages with physiological concentrations of ATP (0.1 mM) or Ca^{2+} ionophore A23187 (1 µM) [84].

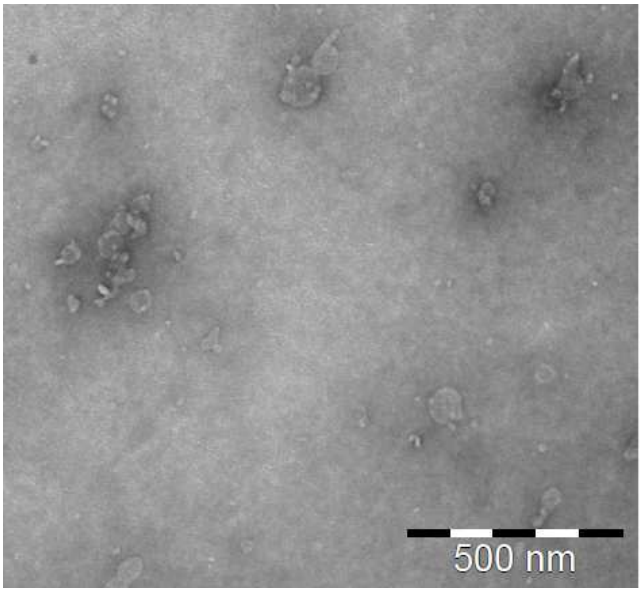


Figure 3.3.1.1. Electron microscopy of C1P liposomes.

We also delivered C1P to cells by using the photolabile caged C1P analog, BHNB-C1P [80], which was dissolved in ethanol at 1.62 mM. The cells were exposed to 400-500 nm light in a transilluminator equipped with a 9 W lamp for 60 min at a distance of 1.5 cm at 37 °C, in order to allow the release of C1P into the cytosol. In this case lower concentrations of caged-C1P were used because caged-C1P is soluble in ethanol and it diffuses across cell membranes.

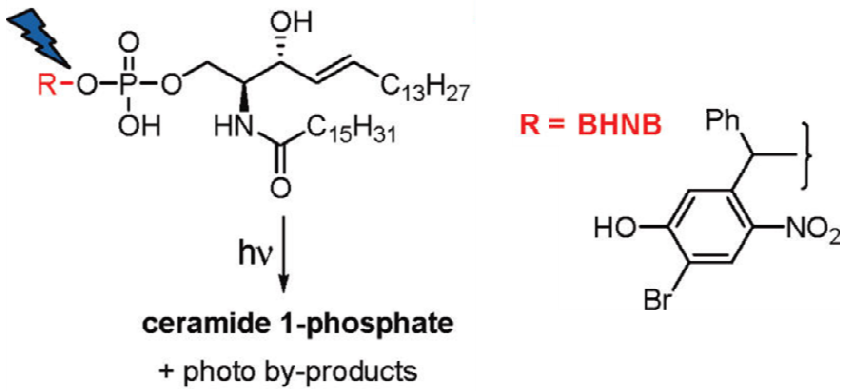


Figure 3.3.1.2. Structure of caged-C1P (adapted from [80]).

3.3.2. Quantitative Enzyme-Linked Immunosorbent Assays (ELISA)

3.3.2.1. Determination of MCP-1 concentration in the culture medium

J77A.1 cells were seeded in 24-well plates (10^4 cells/well) and incubated in DMEM containing 10% FBS, overnight. The next day, cells were washed twice with PBS, the medium was replaced by DMEM containing 1% FBS and cells were incubated for 2 h before adding any agonist. This step is important because it has been published that refreshing the medium can generate rapid sphingolipid and DAG formation [31]. Thus, by incubating cells for 2 h before starting the assay we can discard any interference in our results. After 2 h, inhibitors or agonists were added and cells were incubated further for the indicated periods of time. After incubation, the cell medium was collected into microcentrifuge tubes and cells were scrapped for cell counting. The harvested medium was centrifuged at 10,000 × g for 5 min at 4 ºC and supernatant was diluted for performing ELISA assay.

The MCP-1 concentration in the medium was determined using a kit named "Mouse CCL2 (MCP-1) ELISA Ready-SET-Go!" according to the manufacturer's instructions.

The coating of 96-well plates was performed adding the specific antibody to MCP-1 into the wells and incubating the plate overnight at 4 ºC. Once capture antibody was adhered to the plate, the wells were washed and blocked with assay diluent for 1h. After blocking, the wells were washed and the samples were added in duplicates to the wells. Along with the samples, serial dilutions of a standard solution of MCP-1 were added. Samples were incubated overnight at 4 ºC. After incubation, the wells were washed and the biotinylated-detection antibody was added. This antibody binds to the MCP-1-capture antibody complex. After 1 h of incubation at room temperature and subsequent washes, an avidin-HRP solution was added to the wells and it was incubated for 30 min. This enzyme binds to the biotinylated-detection antibody during this time period. Finally, after the last wash, a 3,3′,5,5′-Tetramethylbenzidine (TMB) solution was added as a substrate into the wells and this reaction generated a chromophoric product. The reaction was then stopped with 1 N H_2SO_4 and the absorbance was read at 450 and 570 nm using the PowerWave™ XS Microplate Reader by BioTek and Gen5 software. Before analyzing data, A570 values were subtracted to A450 values, in order to avoid possible interferences, and the standard solutions were used to perform a calibration curve. Sample concentration values (pg/mL) obtained by this calibration curve were normalized by the number of cells counted in each well (ng/million cell).

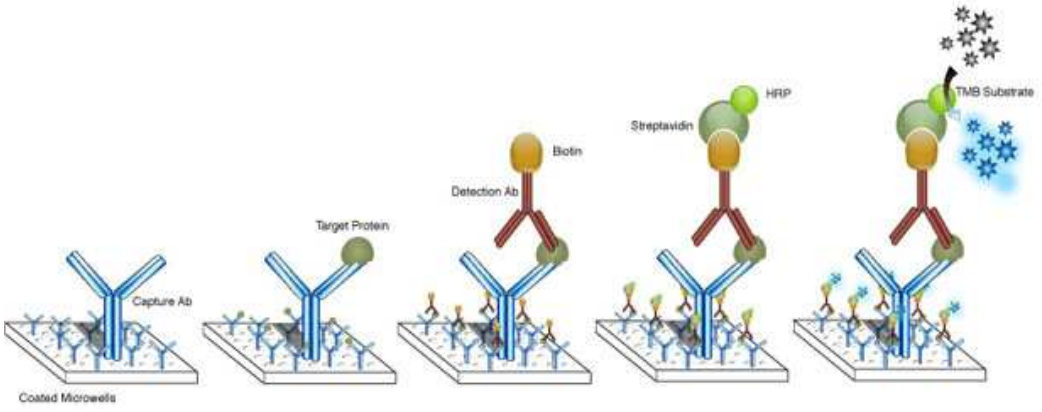


Figure 3.3.2.1.1. Schematic representation of an ELISA experiment.

3.3.2.2. VEGF determination in the culture medium

Raw 264.7 cells were seeded in 6-well plates ($0.75 \cdot 10^6$ cells/mL) in DMEM supplemented with 10% FBS and incubated overnight to allow cells to attach to the plates. The next day, cells were washed and incubated in DMEM supplemented with 1% FBS. Agonists were added and 24 h later the cell medium was collected in microcentrifuge tubes. Samples were centrifuged at 10,000 × g for 5 min and the supernatants were used for VEGF measurement. VEGF concentration in the supernatants was measured using a "Mouse VEGF Quantikine" kit following manufacturer's instructions. This kit provides precoated ELISA plates so that samples and standard solutions were directly added to 96-well plates and incubated for 2 h at room temperature. In order to remove unbound materials, the wells were washed and a HRP-conjugated detection antibody was added. After 2 h of incubation, the wells were washed again and TMB solution was added as a colorigenic substrate. Colour development was stopped using the "Stop Solution" and the absorbance was measured as indicated above.

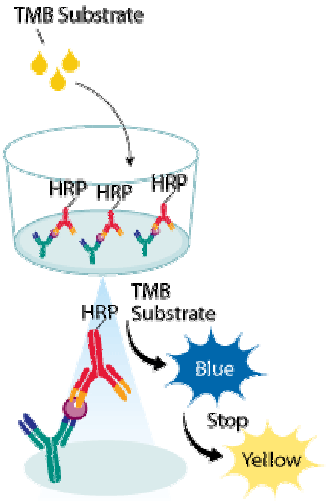


Figure 3.3.2.2.1. Schematic representation of an ELISA experiment.

3.3.3. Determination of cell migration. Boyden chamber assay

Macrophage migration was measured using a Boyden chamber-based cell migration assay, also called trans-well migration assay. 24-well chemotaxis chambers (Transwell, Corning Costar) were used for the experiments and before starting experiments, they

were coated with 30 µg of fibronectin to allow cell attachment. Cell suspensions (100 µl, $5 \cdot 10^4$ cells) were then added to the upper wells of 24-well chemotaxis chambers. Agonists were then added to the lower wells diluted in 300 µl medium supplemented with 0.2% fatty acid-free bovine serum albumin (BSA) and activated carbon. When used, inhibitors were added to the upper and lower wells and pre-incubated for 1 h prior to agonist addition. The cells were incubated in the upper chamber for 1 h inside the incubator in order to ensure cell adhesion. Next, the upper chambers were located inside the lower wells (Figure 3.3.3.1).

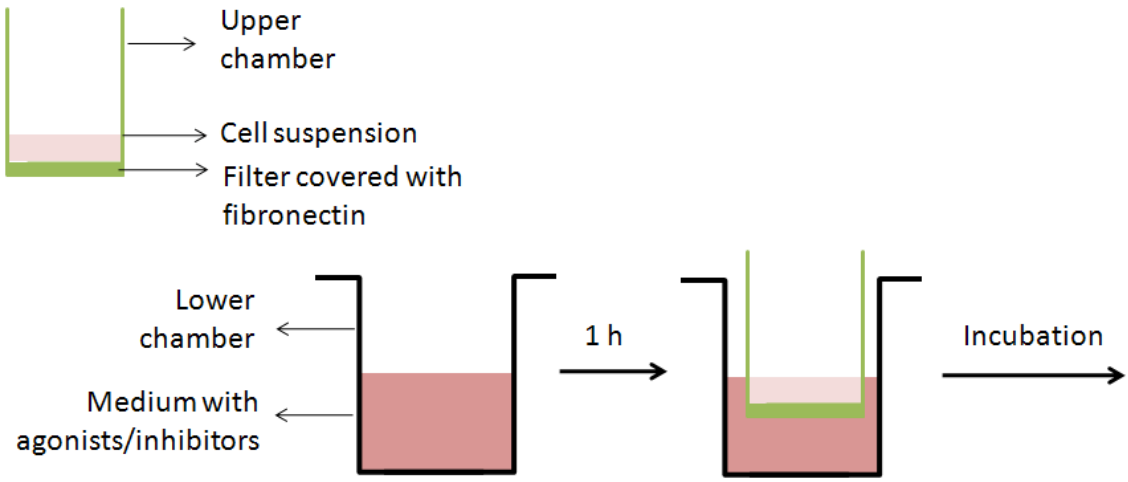


Figure 3.3.3.1. Schematic representation of a Boyden-chamber based cell migration assay.

After the indicated incubation time, non-migrated cells were removed with a cotton swab, and the filters were fixed with formaldehyde (5% in PBS) for 30 min. Then, formaldehyde was removed and the filters were stained with hematoxylin for 2 h. After removing hematoxilyn with water, the filters were immersed in an acid alcohol solution (70% ethanol/hydrochloric acid, 50:1 v/v) for few seconds and they were then submerged in blueing agent for 2 min. Next, the filters were dehydrated with ethanol and stained with eosin for 2 min. After hematoxilyn-eosin staining, the filters were placed on microscopy slides using mineral oil (avoiding bubbles between slides and coverslips). Cell migration was measured counting the number of migrated cells in a Nikon Eclipse 90i microscope equipped with NIS-Elements 3.0 software. Cells were counted in six randomly selected microscopy fields per well at 20× magnification. The

number of migrated cells was normalized by the number of migrated cells on the control filter.

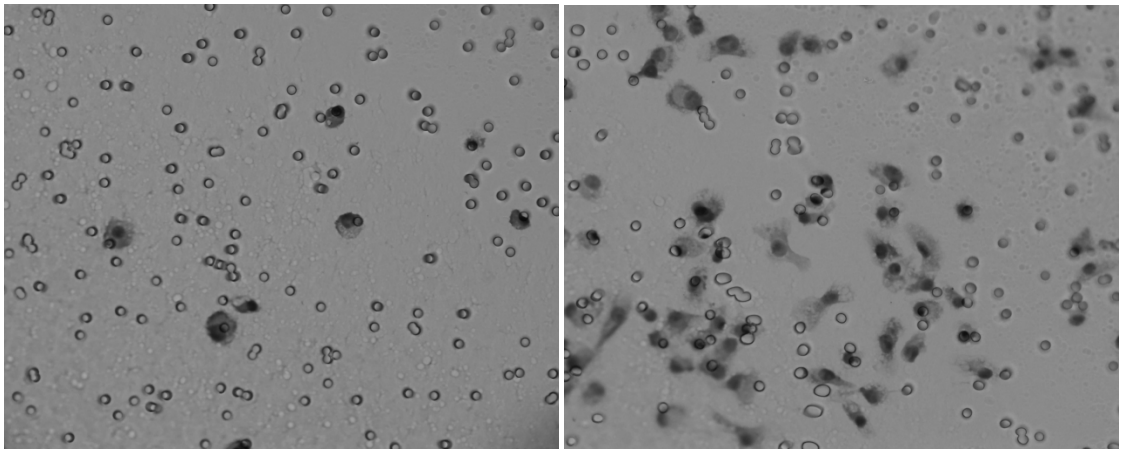


Figure 3.3.3.1. Pictures of analyzed filters. Cells were incubated with vehicle (left panel) or 20 μM C1P (right panel). Micrographs obtained with Nikon Eclipse 90i microscope equipped with NIS-Elements 3.0 software using a 20 x magnification.

3.3.4. Small interfering RNA transfection protocol

Small interfering RNAs (siRNAs) assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). RISC is a multiprotein complex that incorporates one strand of a small interfering RNA (siRNA). to be used it as a template for recognizing complementary mRNA. When it finds a complementary strand, RISC activates a ribonuclease and cleaves the RNA. Cleavage of cognate RNA takes place near the middle of the bounded region by the siRNA strand.

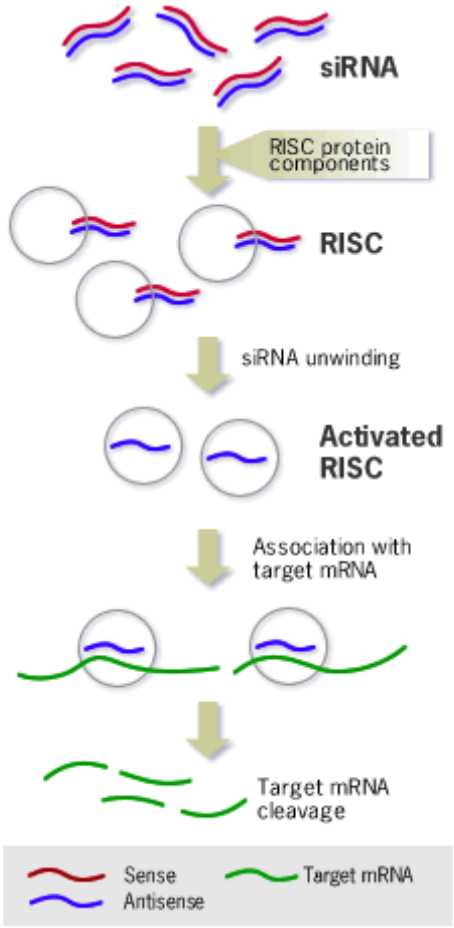


Figure 3.3.4. The mechanism of RNA interference (RNAi)

After the cleavage of the target mRNA, translation of the protein is inhibited, so that it is said that the protein has been silenced.

siRNA transfection protocols were performed following manufacturer's instructions.

- **siRNA transfection protocol for MCP-1 release experiments in J774A.1**

J774A.1 cells were seeded in 24-well plates (10^4 cells/well) in DMEM containing 10% FBS. 4 h later, the medium was removed and the cells were washed twice with sterile PBS. Cells were incubated for 24 h in 200 µL of opti-MEM (without antibiotics) and siRNA was added.

The following solutions were prepared (quantities per each well):

- Solution A: 1 µL of oligofectamine + 7.5 µL of opti-MEM (mixed and incubated for 5-10 min)

- Solution B: 2.5 of µL siRNA (from 20 µM siRNA stock) + 40 µL of opti-MEM

Solution A was added into solution B and mixed gently by pipetting. The mixture was incubated at room temperature for 15 min and 50 µL of the siRNA mixture was added into each well. Cells were incubated for 4-5 h and after that time 250 µL of opti-MEM supplemented with 20% FBS was added, without removing transfection mixture. This culture was further incubated for 24 h and the medium was replaced by DMEM containing 10% FBS. Cells were assayed for MCP-1 release experiments 24-48 h after the addition of fresh growth medium.

- **siRNA transfection protocol for migration experiments in J774A.1**

Macrophages were seeded in 60 mm diameter plates ($1.5 \cdot 10^5$ cells/well) in DMEM containing 10% FBS. The medium was replaced by 1.6 mL opti-MEM and cells were then incubated for 24 h. The siRNA was added following the next protocol:

- Solution A: 6 µL of oligofectamine + 30 µL of opti-MEM (mixed and incubated for 5-10 min).

- Solution B: 20 µL of siRNA (20 µM siRNA stock) + 350 µL of opti-MEM.

Solution A was added into solution B and mixed gently by pipetting. The mixture was incubated at room temperature for 15 min and 400 µL of the siRNA mixture were added into each well. Cells were then incubated for 4-5 h and after that time 2 mL of opti-MEM containing 20% FBS was added into the wells, without removing the transfection mixture. This culture was further incubated for 24 h and the medium was replaced by fresh DMEM containing 10% FBS.

24 h after medium replacement, the cells were scrapped and counted in order to be seeded ($5 \cdot 10^4$ cells/well) in the upper wells of 24-well chambers coated with fibronectin. Migration protocol was performed as mentioned before. Remaining cells were lysed and analyzed by Western blotting in order to determine the efficiency of the siRNA treatment.

- **siRNA transfection protocol for VEGF release experiments in Raw 264.7 cells**

Raw 264.7 cells were seeded in 6-well plates (10^5 cells/well) in DMEM containing 10% FBS. Then, the medium was removed and cells were washed twice with sterile PBS. Cells were then incubated for 24 h in 800 µL of opti-MEM (without antibiotics) and then, siRNA was added.

The following solutions were prepared (quantities per each well):

- Solution A: 4 µL of oligofectamine + 30 µL of opti-MEM (mixed and incubated for 5-10 min)

- Solution B: 10 µL of siRNA (from 20 µM siRNA stock) + 175 µL of opti-MEM.

Solution A was added into solution B and mixed gently by pipetting. The mixture was incubated at room temperature for 15 min and 200 µL of the siRNA mixture were added into each well. The cells were then incubated for 4-5 h and after then 1000 µL of opti-MEM supplemented with 20% FBS was added to the wells, without removing the transfection mixture. Cells were further incubated for 24 h and the medium was replaced by DMEM containing 10% FBS. The cells were assayed for VEGF release experiments 24-48 h after the addition of fresh growth medium.

3.3.5. Cell surface receptor staining

- **Determination of ICAM-1 or CD11b expression in J774A.1 cells**

J774A.1. cells were seeded in 60 mm plates ($2.5 \cdot 10^5$ cells/well) and incubated in DMEM containing 10% FBS, overnight. The medium was replaced with DMEM containing 1% FBS and further incubated for 2 h. Agonists were added to the cell medium and, after the indicated incubation times, cells were washed and scrapped in 0.5 mL PBS containing 1% BSA. Cells were then collected in microcentrifuge tubes and, after counting, the cell suspension was diluted to obtain $0.5 \cdot 10^6$ cells/0.5 mL. The tubes were centrifuged at 500 × g for 5 min at 4 ºC and resuspended in 200 µL of the blocking solution (PBS containing 1% BSA and 10% FBS). Cells were then blocked for 15 min on ice, in order to avoid unspecific antibody bindings. After blocking, the cell

suspension was washed and resuspended in 50 µL of PBS containing 1% BSA and the fluorescent antibody (1:1000 dilution) and incubated for 1 h at 4 ºC in the dark. Then, the cells were washed and resuspended in 200 µL of a paraformaldehyde solution (2% in PBS) for 15 min at 4 ºC in the dark. After fixing, cells were washed with PBS containing 1% BSA and resuspended again in 0.5 mL PBS with 1% BSA. Cell suspensions were transferred into cytometry tubes. FITC-fluorescence from specific antibodies was measured by flow cytometry using an air-cooled 488 nm argon–ion laser (FACSCalibur, BD Biosciences) and CellQuest software (Becton Dickinson), according to the manufacturer's instructions.

- **Measurement of CD69 surface expression in spleen mononuclear cells (SMCs).**

SMCs were obtained as mentioned above and they were seeded in microcentrifuge tubes ($1.5 \cdot 10^6$ cells/tube) in 1.5 mL of medium. After addition of the indicated agonists, cell suspensions were placed into a culturing incubator. After incubation, the cells were centrifuged (500 × g, 5 min) and resuspended with fresh medium. Cells were then seeded in 96-well round-bottom plates in triplicate.

Cells were then centrifuged (500 × g, 5 min) and resuspended in a blocking solution (10% mice serum diluted in PBS supplemented with 0.01% sodium azide). After 30 min of incubation on ice, the serum was removed by centrifugation and the cells were washed with PBS supplemented with 0.01% sodium azide. The cell phenotyping was performed using an appropriate dilution of the following fluorescent antibodies: CD3-FITC (1:40), CD4-PerCP (1:200), CD8a-Alexa700 (1:200), Dx5-PE (which binds CD49b marker) (1:100), NK1.1-APC (1:100) and CD69-PC7 (1:150). All antibodies were diluted in PBS containing 0.01% of sodium azide and 10 µL of this solution were added into each well. Cells were then incubated with appropriate antibody solution for 1 h on ice in the dark. After incubation, the cells were washed and resuspended in the culture medium and a propidium iodide (PI) solution was added in the last step for discarding non-viable cells. Single-stain controls were included for further offline compensation. The samples were analyzed by the FACS LSRII (Becton–Dickinson, Franklin Lakes, NJ, USA). Evaluation of data was performed using a FlowJo version 6.1.1 software (Tree Star, Ashland, OR, USA). All data analyse were performed using PI negative cell population (live cells).

Cell phenotype was determined using the following combination:

Cell Type	CD markers
Monocyte	CD3-,CD49b-, NK1.1-
Helper T cells (Th)	CD3+, CD4+, CD8a-
Cytotoxic T Lymphocyte (CTL)	CD3+, CD4-, CD8a+
Natural Killer cell (NK cell)	CD3-, CD49b +, NK1.1+
Natural Killer T cell (NKT cell)	CD3+, CD49b +, NK1.1+

3.3.6. Measurement of intracellular reactive oxygen species

ROS production was determined using 5-(and 6-)-chloromethyl-20,70-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA). This is a cell-permeant indicator that after entering the cell it remains inside the cells. Intracellular ROS de-esterify this compound and it is converted to fluorescent DCF.

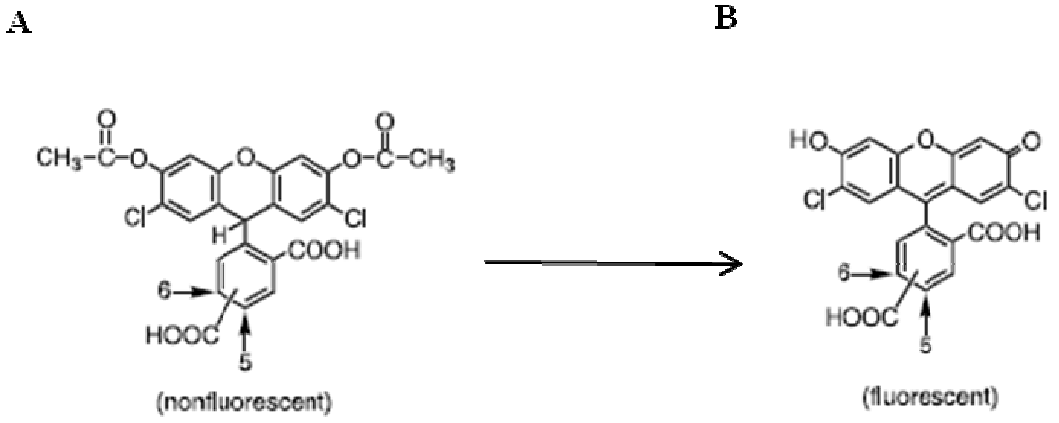


Figure 3.3.6.1. The structures of A. carboxy-H2DCFDA and B. the deacetylated and oxidized product, dichlorofluorescein (DCF).

BMDMs were incubated in 35 mm diameter dishes at $5 \cdot 10^5$ cells/dish and they were grown in RPMI 1640 containing 10% FBS and 20% L-cell conditioned medium (as the source of M-CSF) for 4-6 days until they were about 80% confluent. Cells were then

incubated for 24 h in RPMI 1640 containing 10% FBS and 1.5% L-cell conditioned medium as reported previously [57]. Macrophages were incubated in the presence or absence of agonist, without refreshing the medium, and CM-H2DCFDA was added at 5 μM during the last 30 min of incubation, at 37 ºC in the dark. After incubation cells were washed twice with PBS and lysed with 10 mM Tris-HCl buffer containing 0.5% Tween-20 (pH 7.4). The homogenates were centrifuged at 10,000 × g for 10 min to remove cell debris. DCF fluorescence in the supernatant was measured using a spectrofluorimeter with excitation and emission wavelengths of 490 nm and 522 nm, respectively. Data were processed and normalized to values obtained from untreated controls.

3.3.7. Superoxide anion assay kit

BMDMs were incubated in 60-mm diameter dishes at 3.5 x 10^6 cells/dish and they were grown in RPMI 1640 containing 10% FBS and 10% L-cell conditioned medium until they reached about 80% confluence. The cells were then incubated for 24 h in RPMI 1640 containing 10% FBS and 1.5% L-cell conditioned medium as reported previously. Macrophages were incubated in the presence or absence of 40 μM C1P, without refreshing the medium. After 8 h cells were scrapped and collected in the assay medium that was provided with the kit. Cells were then lysed with a Dounce homogenizer. Part of the homogenate was centrifuged at 10,000 × g for 30 min in order to remove mitochondria and nuclei. Superoxide anion production was measured using a "Superoxide anion assay kit" according to the manufacturer's instructions. Superoxide dismutase (SOD)-containing wells were used as negative controls.

3.3.8. Determination of the phospholipase A_2 (PLA_2) activity in vivo

The PLA_2 activity in vivo can be measured quantifying the generation of its product, arachidonic acid (AA).

Macrophages were seeded in 35 mm diameter plates and incubated overnight in the presence of 0.25 μCi [^{3}H]-AA. The next day, cells were washed in RPMI medium containing 0.2% BSA in order to remove non-incorporated [^{3}H]-AA and then they were

further incubated in RPMI supplemented with 0.1% BSA for 2 h. The assays were started treating cells with the indicated agonists for different periods of time. The cell supernatants and the adherent cells were collected and [^{3}H]AA was measured using a liquid scintillation counter *Packard*, TRI CARB 2700 model.

Stimation of released AA was determined as indicated below:

$$\text{Released AA} = \frac{[3H]\text{AA in the medium}}{[3H]\text{AA in the medium} + [3H]\text{AA in the adherent cells}}$$

3.3.9. Determination of cPLA$_2$ activity in vitro

BMDMs were incubated in 60 mm diameter dishes at 3.5 x 10^6 cells/dish and they were grown in RPMI 1640 supplemented with 10% FBS and 20% L-cell conditioned medium until they reached about 80% confluence. Cells were then incubated as described above. Macrophages were treated with the indicated agonists or inhibitors for 4 h. Cells were washed and harvested in ice-cold buffer (1 mM EDTA, 50 mM HEPES, pH 7.4). Samples were sonicated and centrifuged at 10,000 × g for 15 min at 4ºC. The cPLA$_2$ activity in the supernatant was determined using a commercial kit (see the Materials section) according to the manufacturer's instructions.

3.3.10. Arachidonic Acid (AA) delivery to the cell culture

Arachidonic acid was treated with KOH (in equimolar concentrations) prior to addition to cells.

Arachidonic Acid + KOH ↔ Potassium Arachidonate + H_2O.

The mixture was warmed to 37 ºC and gently mixed until the solution was transparent. Arachidonate was added to the cell culture at the indicated concentrations.

3.3.11. Determination of PKC activity

BMDMs were incubated in 60 mm diameter dishes at $3.5 \cdot 10^6$ cells/dish and they were grown in RPMI 1640 containing 10% FBS and 20% L-cell conditioned medium until

they reached about 80% confluence. Macrophages were then incubated in RPMI 1640 containing 10% FBS and 1.5% L-cell conditioned medium, and incubated as described before. The cells were washed and harvested in ice-cold homogenization buffer (50 mM HEPES, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% (v/v) NP-40, glycerol 10% (v/v), 2.5 mM EDTA, $Na_4P_2O_7$ 10 mM, 1 μg/mL protease inhibitor cocktail) as described [67]. The samples were lysed and the nuclei were removed by centrifugation at 500 × g for 5 min at 4ºC. Membrane and soluble fractions were separated by ultracentrifugation at 100,000 × g at 4 ºC for 30 min. Then, the concentration of each sample was determined by a BioRad commercial kit (see the *Materials and Methods* section) and the activity of PKC was determined using the Protein Kinase Non-Radioactive kit from Calbiochem, according to the manufacturer's instructions (see *Materials* section).

3.3.12. Determination of DNA synthesis

BMDMs were seeded at $5 \cdot 10^5$ cells/well in 12-well plates and they were grown in RPMI 1640 as previously described. The day before performing the experiment, the medium was removed and replaced with fresh RPMI 1640 supplemented with 10% FBS and 1.5% L-cell conditioned medium. After 24 h, the cells were incubated in the presence or absence of agonists for 24 h. [^{3}H]thymidine (0.5 μCi/mL) was added for the last 2 h of incubation. The medium was then removed and the cells were washed twice with PBS. Next, the cells were treated with trichloroacetic acid 10 % for 5 min and the precipitated material was dissolved in 0.3 M NaOH 1% SDS for 2 h at 37 ºC. Aliquots of these solutions were mixed with scintillation liquid and [^{3}H]Thymidine incorporation into DNA was measured in the liquid scintillation counter *Packard*, TRI CARB 2700, TR.

3.3.13. Cell viability and proliferation assay

Cell viability and proliferation can be determined using the MTS-formazan colorimetric assay. This assay is based on the rate of reduction of the tetrazolium dye, MTS. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), in the presence of phenazine methosulfate (PMS) (5% v/v diluted in PBS containing Mg^{2+} and Ca^{2+}), reacts with mitochondrial dehydrogenases producing a

formazan product that has a maximum absorbance at 490-500 nm in phosphate-buffered saline. Generated formazan is proportional to the number of viable cells in culture (Figure 3.3.13.1).

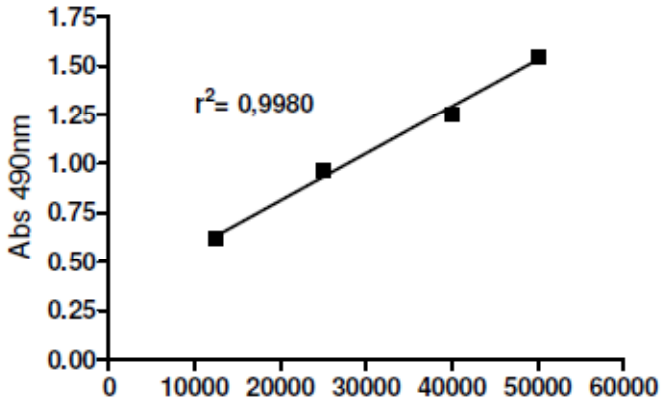


Figure 3.3.13.1. Generated formazan is proportional to the number of viable cells in culture. Different number of cells were seeded in 96-wells plates in RPMI 1640 supplemented with 10% FBS and 20% L-cell conditioned medium. 20 µL of MTS/PMS was added into each well and after 2 h absorbance was measured (490 nm). Absorbance of the medium (without cells) was subtracted from each absorbace value and results are the mean of three independent experiments. R^2= 0.998 indicates the linearity between cell number and absorbance values.

- SMCs were seeded at 10^5 cells/well in 96-well plates and the indicated agonists were added in triplicates (100 µL). Cells were then incubated for the indicated periods of time.
- BMDMs were seeded at $5 \cdot 10^4$ cells/well in 96-well plates and incubated overnight in RPMI 1640 supplemented with 10% FBS and 20% L-cell conditioned medium as the source of M-CSF. The medium was then replaced with fresh RPMI 1640 supplemented with 10% FBS and 1.5% L-cell conditioned medium in the presence or absence of agonists and/or inhibitors, as appropriate, and cells were incubated for 24 h.
- Raw 264.7 cells were seeded at 10^4 cells/well in 96-well plates and incubated overnight in DMEM supplemented with 10% FBS. The next day, the medium was replaced by DMEM containing 1% FBS with or without agonists and cells were incubated for 48 h.

After the indicated incubation time periods, 20 µL of MTS/PMS were added and cells were further incubated for 2 h. The absorbance was measured at 490 nm and absorbance

of the medium (without cells) was subtracted from all absorbance values. The control values were indicated as 100% of cell viability.

3.3.14. Measurement of [^{3}H] choline-labeled sphingomyelin levels

BMDMs were incubated in 60 mm diameter dishes at $3.5 \cdot 10^5$ cells/dish and they were grown in RPMI 1640 containing 10% FBS and 20% L-cell conditioned medium (as the source of M-CSF) for 4-6 days until they were about 80% confluent. Macrophages were labeled with [methyl-^{3}H] choline chloride (0.5 µCi/mL) for O8 h in RPMI 1640 supplemented with 10% FBS and 1.5% L-cell conditioned medium. After this time, radioactivity was removed washing cells with PBS containing 0.2% BSA. Cells were then further incubated for 2 h in fresh RPMI 1640 containing 1.5% L-conditioned medium and 10% FBS in order to avoid rapid sphingolipid formation. Cells were then incubated in the presence or absence of agonists for the indicated times, and harvested in 600 µL of distilled water. 2.5 mL of chloroform/methanol (2:1, v/v) were added and after mixing the samples they were centrifuged at 1000 × g for 15 min. The organic phase was dried down under nitrogen atmosphere and the total lipid extracts were subject to a mild alkaline hydrolysis. This hydrolysis was initiated by adding 0.25 mL of chloroform and 0.25 mL of a 0.5 M NaOH solution (diluted in methanol) to the samples and they were incubated at 37 ºC overnight. The reaction was stopped by adding 0.85 mL of chlorophorm, 0.25 mL of a 0.5 M HCl solution (diluted in methanol), 0.43 mL of distilled water and 0.5 mL of chloroform/methanol (2:1, v/v). Tha samples were mixed and centrifuged in order to separate aqueous and organic phases, and the organic phase was collected and dried down. Lipid extracts were loaded in a silicagel plate and lipid separation was performed by a thin layer chromatography in chloroform/methanol/acetic acid (50:30:8:5, v/v). [^{3}H]sphingomyelin radioactivity was determined by liquid scintillation counting.

3.3.15. Western blotting

Each cell type was seeded in different conditions in order to obtain optimum protein concentrations.

- BMDMs were incubated in 60 mm diameter dishes at $3.5 \cdot 10^5$ cells/dish and were grown in RPMI 1640 containing 10% FBS and 20% L-cell conditioned

medium (as the source of M-CSF) for 4-6 days until they were about 80% confluent. The cells were then incubated for 24 h in RPMI 1640 containing 10% FBS and 1.5% L-cell conditioned medium as reported previously.

- SMCs were obtained as mentioned before. Then, the cells were counted and seeded in 1.5 mL centrifuge tubes at $5 \cdot 10^6$ cells/tube in RPMI 1640.
- J774A.1 cells were seeded in 60 mm diameter dishes ($0.25 \cdot 10^6$ cells/plate) and incubated overnight in DMEM containing 10% FBS.
- Raw 264.7 cells were seeded in 60 mm diameter dishes (10^6 cells/plate) and incubated overnight in DMEM containing 10% FBS.

Then, all cell types were incubated with or without agonists for the indicated incubation times. Cells were washed with PBS and harvested with ice-cold homogenization buffer (50 mM HEPES, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% (v/v) NP-40, 10% (v/v) glycerol, 2.5 mM EDTA, 10 mM $Na_4P_2O_7$, 1 µl/mL protease inhibitor cocktail), as described [67]. Samples were lysed by sonication and protein concentration was determined by a BioRad commercial kit (see the *Materials and Methods* section).

Samples (20-40 µg protein/sample) were mixed with loading buffer 4x (125 mM Tris pH 6.8, 50% (v/v) glycerol, 4% SDS, 0.08% (p/v) bromophenol and 50 µL/mL β-mercaptoethanol). Samples were then heated at 90 ºC for 10 min and loaded onto polyacrilamide gels (12% or 7.5% acrylamide) to perform protein separation by SDS-PAGE. Electrophoresis was run (120 V for 2 h aprox.) in electrophoresis buffer (1.92 M glycin, 0.25 M Tris-HCl and 1% SDS).

Proteins were then transferred onto nitrocellulose paper. Transference was run using 400 mA for 1 h and 15 min in ice-cold transfer buffer (14.4 g/L glycin, 3 g/L Tris, Methanol 20%). In order to avoid unspecific antibody bindings, nitrocellulose paper was blocked for 1 h with 5% skim milk in Tris-buffered saline (TBS) containing 0.01% NaN_3 and 0.1% Tween 20, pH 7.6. The skim milk was then removed and nitrocellulose paper was incubated overnight with the primary antibody diluted in TBS/0.1% Tween (1:1000) at 4 ºC. After three washes with TBS/0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 1:4000 dilution in TBS/0.1% Tween for 1 h. Bands were visualized by enhanced chemiluminescence and exposed films were analyzed by ImageJ software in order to measure arbitrary intensity.

3.3.16. Determination of p47phox or PKCα translocation

BMDMs were incubated in 60 mm diameter dishes at $3.5 \cdot 10^5$ cells/dish and were grown in RPMI 1640 containing 10% FBS and 20% L-cell conditioned medium (as the source of M-CSF) for 4-6 days until they were about 80% confluent. Cells were then incubated for 24 h in RPMI 1640 containing 10% FBS and 1.5% L-cell conditioned medium, as described before. Cells were incubated in the presence or abscence of indicated agonists for the necessary incubation time. Macrophages were washed and harvested in ice-cold homogenization buffer. Samples were lysed with a Dounce homogenizer and nuclei were removed by centrifugation at 500 × g for 5 min at 4ºC. Membrane and soluble fractions were then separated by ultracentrifugation at 100,000 × g at 4 ºC for 30 min. Then, the concentration of each sample was determined by BioRad commercial kit and 20-30 µg of protein was loaded and separated by SDS–PAGE, using 12% separating gels. The translocation of p47phox and PKCα was analyzed measuring the presence of these proteins in the membrane fraction by Western blotting, using specific antibodies to p47phox or p-PKCα.

3.3.17. Determination of STAT3 and NF-κB translocation into the nucleus

J774A.1 cells were seeded ($0.25 \cdot 10^6$ cells/plate) in 60 mm diameter plates in DMEM containing 10% FBS and incubated overnight. The next day, the cells were washed and medium was replaced by DMEM containing 1% FBS. After 2 h agonists were added and cells were further incubated for the indicated periods of time. Then, the cells were washed and the nuclei were isolated using "NucBuster™ Protein Extraction Kit" from Calbiochem according to the manufacturer's instructions. The concentration of membrane and soluble fractions was determined by a BioRad commercial kit and 20-30 µg of protein was loaded and separated by SDS–PAGE, using separating gels containing 9% of acrylamide. The presence of p-STAT3 and p-NF-κB in nucleus or cytoplasm was determined by Western blotting.

3.3.18. Analysis of apoptosis by Annexin-V/PI assay

Apoptosis is a fundamental mode of programmed cell death. Apoptotic cells suffer characteristic cell changes. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation.

In the normal viable cells, phosphatidyl serine (PS) is located on the cytoplasmic side of the cell membrane. Upon induction of apoptosis, rapid alterations in the organization of phospholipids in most of the cell types occurs, leading to the exposure of PS on the cell surface. Recognition of PS by phagocytes *in vivo* results in the removal of cells that are programmed to die. *In vitro* detection of externalized PS can be achieved through interaction with the anticoagulant Annexin V. In the presence of calcium, rapid and high affinity binding of Annexin V to PS occurs. Besides, PS translocation to the cell surface precedes nuclear breakdown, DNA fragmentation, and the appearance of most apoptosis-associated molecules, making Annexin V binding a marker of early-stage apoptosis. Thus, FITC conjugate of Annexin V was used, allowing determination of apoptosis by flow cytometry. Propidium iodide was used to distinguish between viable, early apoptotic and necrotic or late apoptotic cells. Since membrane permeabilization is observed in necrosis, necrotic cells bind Annexin V-FITC and hence stain with propidium iodide, while propidium iodide will be excluded from viable (FITC negative) and early apoptotic (FITC positive) cells. Final stages of apoptosis involve necrotic-like disintegration of the total cell, thus cells in late apoptosis will be labeled with both FITC and propidium iodide (Figure 3.3.18.1).

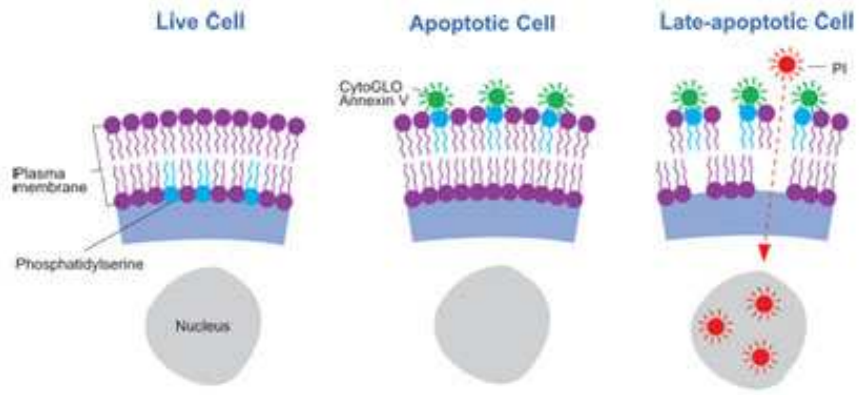


Figure 3.3.18.1. Scheme of Annexin-V/PI binding to cell components. Image from http://www.imgenex.com/apop_kits_list.php?id=76.

For detection of apoptotic SMCs, cells were incubated in 24-well plates ($1.5 \cdot 10^6$ cell/ well) in the presence or absence of the indicated agonists in 0.5 mL of RPMI 1640. After 72 h, cells were stained for measurement of apoptosis using "Annexin V-FITC Apoptosis Detection Kit" according to manufacturer's instructions. Briefly, the cells were collected and washed in microcentrifuge tubes (centrifuged at 1000 × g for 5 min at room temperature). After washing, the cells were resuspended in binding buffer provided by the kit and Annexin-V was added. Cells were then incubated in the dark at room temperature for 15 min. Then, samples were washed and resuspended in 0.5 mL of binding buffer, provided with the kit. PI was added in the last step and the samples were transferred to flow cytometry tubes in order to be analyzed by FACSCalibur (BD Biosciences) using CellQuest software (Becton Dickinson), according to the manufacturer's instructions.

3.3.19. Statistical analyses

Statistical analyses were performed using two-tailed Student's t-test, with the level of significance set at $p < 0.05$.

Significancy and used symbols:

SYMBOL	SIGNIFICANCY
n.s	**p>0.05**
*	**p<0.05, significant**
**	**p<0.01, significant**
***	**p<0.001, significant**

symbol has been used instead of * symbol to compare inhibitor-treated cells versus C1P-treated cells.

Chapter 1

4. CHAPTER 1

Ceramide 1-phosphate induces MCP-1 release and macrophage chemotaxis.

1. Introduction of Chapter 1

Chemokines (chemotactic cytokines) are a family of small secreted proteins (8-12 kDa) that function as intercellular messengers to control migration and activation of specific subsets of leukocytes. In addition, chemokines contribute to the regulation of gene expression in target cells and help to control cell proliferation and apoptosis.

The interest on these proteins was first provoked by the observation of their elevated levels in several inflammatory diseases including rheumatoid arthritis, arteriosclerosis and asthma. Although it is not clear whether excessive chemokine production might be the cause or the consequence of these diseases, it has been reported that neutralizing endogenous chemokines reduces symptoms in autoimmune diseases, chronic inflammation and cancer treatment (reviewed in [85]). A wide range of clinically important diseases are associated with chemokines and their receptors, motivating the appearance of many studies to understand the molecular details of the chemokine function.

Chemokines exert their cellular effects by activating seven transmembrane–domain G-protein–coupled receptors (GPCR). Whether a leukocyte responds to a particular chemokine is determined by its complement of chemokine receptors. The nomenclature of the chemokine receptors follows the notation used for the chemokine subfamilies and they are termed CCR1－10 (CC chemokine receptor 1－10), CXCR1－6, XCR1 and CX3CR1.

The CC chemokine receptors (CCRs) are thought to predominantly signal via Gi/o-coupled heterotrimeric G proteins to inhibit the activity of adenylyl cyclase (AC) and to regulate Ca^{2+} flux. However there is increasing evidence that CCRs are able to couple

with a wider spectrum of G proteins to potentially influence the activity of numerous intracellular signalling pathways and gene transcription events (Figure 1.2).

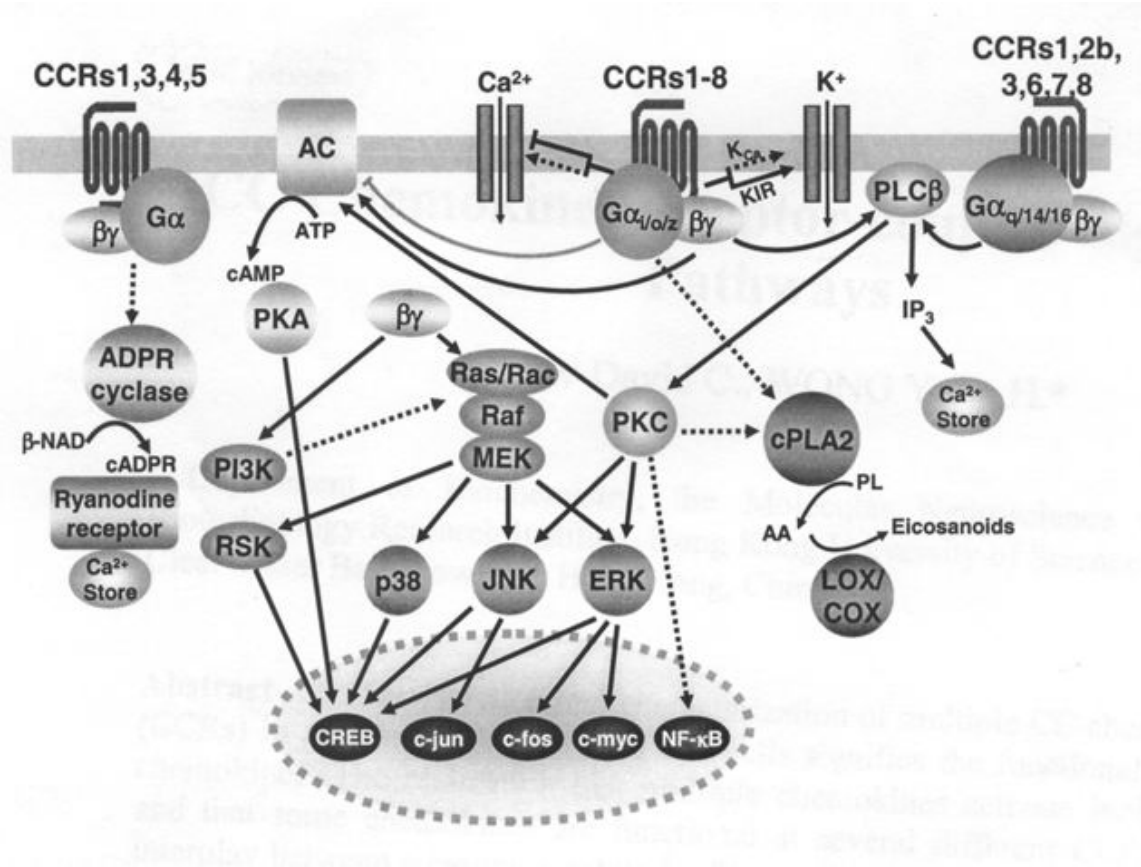


Figure 1.2 Intracellular signalling pathways activated by CC chemokine receptors, taken from [86].

Chemokines are secreted in response to signals such as proinflammatory cytokines which play an important role in selectively recruiting monocytes, neutrophils, and lymphocytes toward the chemokine source.

1.1 MCP-1/CCL2

Monocyte chemoattractant protein-1 (MCP-1 or CCL2) is the first discovered human CC chemokine. Human MCP-1 is composed of 76 amino-acids and is 13 kDa in size. However, it can acquire different molecular mass forms because of O-glycosylations. Glycosylation of MCP-1 has been shown to slightly reduce its chemotactic potency [87].

CCL2 is produced either constitutively or after induction by oxidative stress, cytokines, or growth factors. Although it can be produced by many cell types, including endothelial cells, fibroblasts, epithelial cells, smooth muscle cells, astrocytes, monocytes and microglial cells, the major MCP-1 source was found to be monocytes/macrophages [88].

CCL2 mediates its effects through its receptor CCR2, and, unlike CCL2, CCR2 expression is relatively restricted to certain types of cells. There are two alternatively spliced forms of CCR2, namely, CCR2A and CCR2B, which differ only in their C-terminal tails. CCR2A is the major isoform expressed by mononuclear cells and vascular smooth muscle cells, whereas monocytes and activated NK cells express predominantly the CCR2B isoform [87].

1.2. CELL MIGRATION

Cell migration is part of the inflammatory response. It is a complex process that requires strict coordination of the following steps: cell polarization, protrusion and cell adhesion. These processes are regulated by complex signalling networks initiated by integrins and other receptors. The regulation occurs through local, transient signals that retain polarity of the cell and drive local remodeling, like actin polymerization, adhesion, actomyosin bundling and contraction, and microtubule dynamics. Actin filaments, microtubules, and cycling lipid vesicles span the cell and contribute to integrate the processes that mediate migration.

Cell migration is a key component for the homeostasis of the adult individual. Therefore, any cell migration failure, or inappropriate migratory movements, can result in severe defects such as immunosuppresion, autoimmune diseases, defective wound repair or tumour dissemination.

Understanding the mechanisms that support cell migration is crutial for development of new strategies for inflammation and cancer therapy.

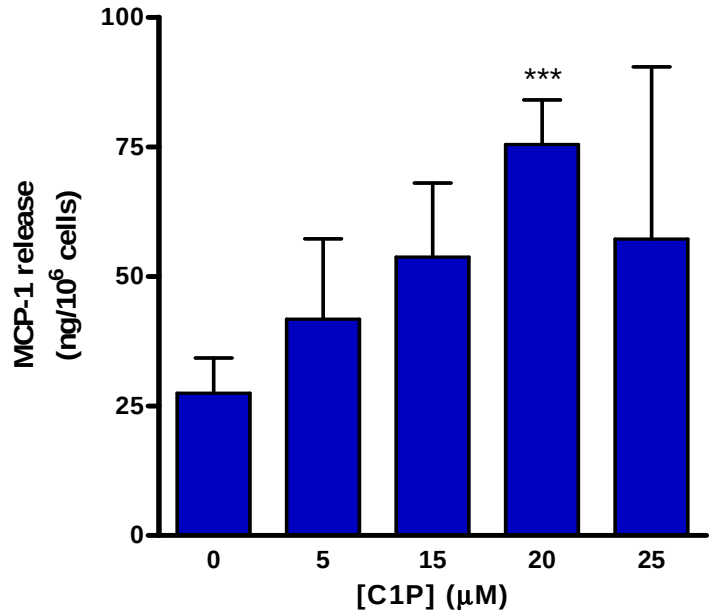
2. Results of Chapter 1

2.1. Ceramide 1-phosphate induces MCP-1 release in J774A.1 cells

We have previously reported that C1P is a potent chemoattractant agent for macrophages and that C1P exerts this action through interaction with a putative receptor for C1P [58]. Due to the importance of Monocyte Chemoattractant Protein-1 (MCP-1) in inflammatory responses and immunity, it was important to know whether C1P was able to induce MCP-1 release in macrophages.

To test this hypothesis, we cultured J774A.1 macrophages and measured the MCP-1 concentration in the culture medium after incubation with different C1P concentrations (Figure 2.1.1A) and after different incubation times with the optimum concentration of C1P (Figure 2.1.1B). These data suggest that C1P significantly stimulates MCP-1 release in a concentration- and time-depending manner.

A.



B.

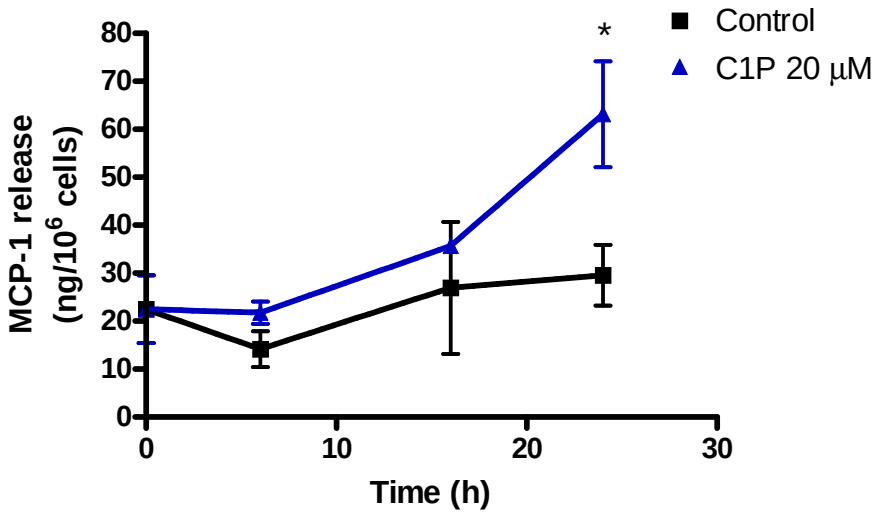
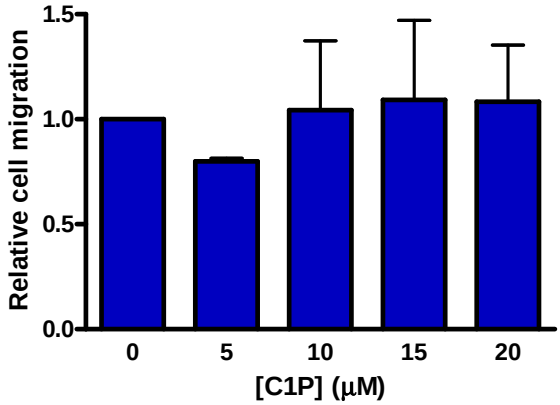


Figure 2.1.1. C1P induces MCP-1 release in J774A.1 macrophages. A. Cells were seeded (10^4 cells/well) in 24-well plates and incubated overnight in DMEM supplemented with 10% FBS. The next day, the cells were washed and the medium was replaced by DMEM supplemented with 1% FBS. After 2 h of incubation, the cells were further incubated with the indicated concentrations of C1P for 24 h. Then, the medium was collected and centrifuged. MCP-1 concentrations were measured using ELISA kits, as indicated in the *Materials and Methods* section. **B.** The cells were treated as in panel A. They were then incubated with 20 µM C1P for the indicated periods of time and the MCP-1 concentration in the medium was determined. MCP-1 values were normalized to the total cell number and the results are expressed as the mean ± SEM of 4 independent experiments performed in duplicate (*$p<0.05$; ***$p<0.001$).

2.2. C1P induces cell migration in a concentration- and time-dependent manner

Because MCP-1 is a well-known and potent chemoattractant cytokine, experiments were performed to test whether C1P was a chemoattractant molecule for J774A.1 cells. Figures 2.2.1A and 2.2.1B show that C1P stimulates cell migration after 24 h of incubation with different concentrations of C1P but not after 4 h, time that was sufficient for C1P to stimulate migration of Raw macrophages [58].

A.



B.

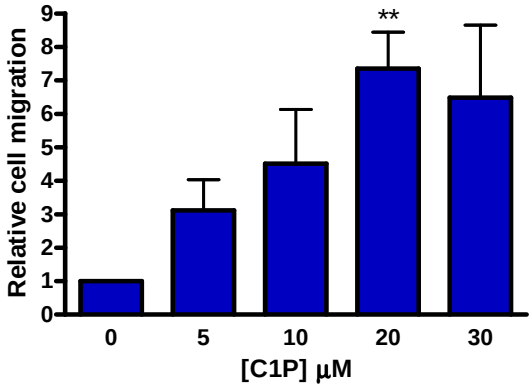


Figure 2.2.1. C1P induces cell migration in J774A.1 cells. Macrophage migration was measured using the Boyden chamber-based cell migration assay. Cells ($5 \cdot 10^4$ cells/well) were plated in the upper wells of 24-well chambers coated with fibronectin, and pre-incubated for 1 h. **A.** C1P was added to the lower wells at the indicated concentrations for 4 h. Data are expressed as cell migration relative to the number of cells migrated in the control wells. Results are the mean ± SD of 2 independent experiments. **B.** Cells were incubated with the indicated C1P concentrations for 24 h. Data are expressed as cell migration relative to the number of cells migrated in the control chamber and are the mean ± SEM, n=3, except for the 20 μM point, which is the mean of 6 independent experiments performed in duplicate (**$p<0.01$).

2.3. MCP-1 induces cell migration in J774A.1 cells

Subsequently, the ability of MCP-1 to stimulate cell migration was studied. After incubating cells with different concentrations of MCP-1 for 24 h, we confirmed that MCP-1 is a potent chemoattractant for macrophages. Maximal migration was attained at 150 ng/mL MCP-1 (Figure 2.3.1).

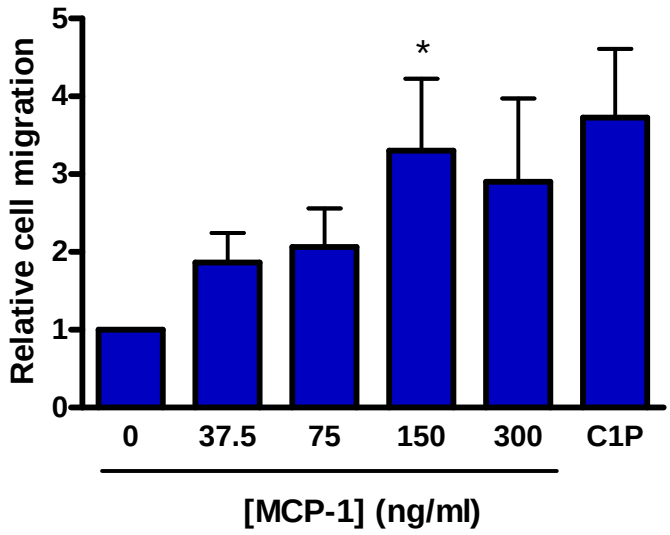


Figure 2.3.1. MCP-1 induces cell migration in J774A.1 in a concentration-depending manner. Macrophage migration was measured using the Boyden chamber-based cell migration assay. Cells ($5 \cdot 10^4$ cells/well) were plated in the upper wells of 24-well chambers coated with fibronectin, and pre-incubated for 1 h. MCP-1 was added to the lower wells at the indicated concentrations and 20 µM C1P was used as a positive control. Cells were incubated for 24 h. Results are expressed as the number of migrated cells relative to the number of cells migrated in the control chamber and are the mean ± SEM of 8 independent experiments (*$p<0.05$).

2.4 MCP-1 and C1P have additive effects on cell migration

Taking into account that C1P and MCP-1 are chemoattractant molecules, we wondered if both molecules could act through the same signalling mechanisms. In order to clarify this question we incubated the cells with the optimum concentrations of both molecules simultaneously. It was observed that their effects on cell migration were additive (Figure 2.4.1).

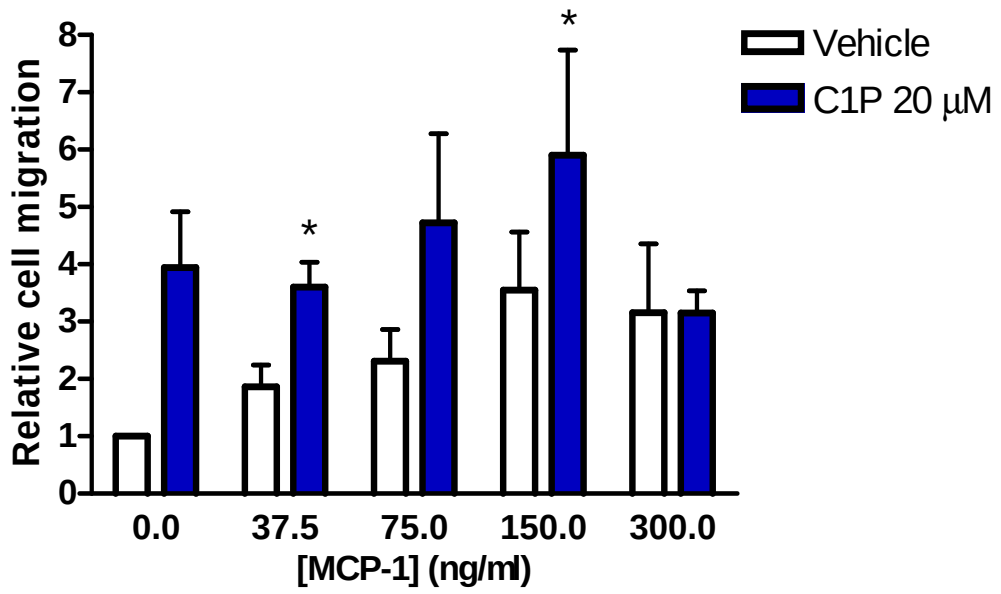


Figure 2.4.1. Effect of MCP-1 and C1P on cell migration. Macrophage migration was measured using a Boyden chamber-based cell migration assay. Cells ($5 \cdot 10^4$ cells/well) were plated in the upper wells of 24-well chambers coated with fibronectin, and pre-incubated for 1 h. The indicated MCP-1 concentrations were added to the lower chamber, with (solid bars) or without (empty bars) 20 µM C1P and cells were further incubated for 24 h. Results are expressed as the number of migrated cells relative to the number of cells migrated in the control chamber and are the mean ± SEM of 4 independent experiments (*$p<0.05$ MCP-1-stimulated migration value with C1P versus MCP-1 concentration without C1P).

The enhanced effect on cell migration observed by incubating the cells with optimum concentrations of both agonists suggests that they may act through different signalling pathways.

2.5. Neutralization of MCP-1 impairs C1P-stimulated macrophage migration

Although the effect of C1P and MCP-1 on cell migration is additive it was not clear to which extent C1P depended on MCP-1 release or other factors to achieve this action. Therefore, experiments were aimed at elucidating this point. For this, a specific antibody to neutralize the effect of MCP-1 was used.

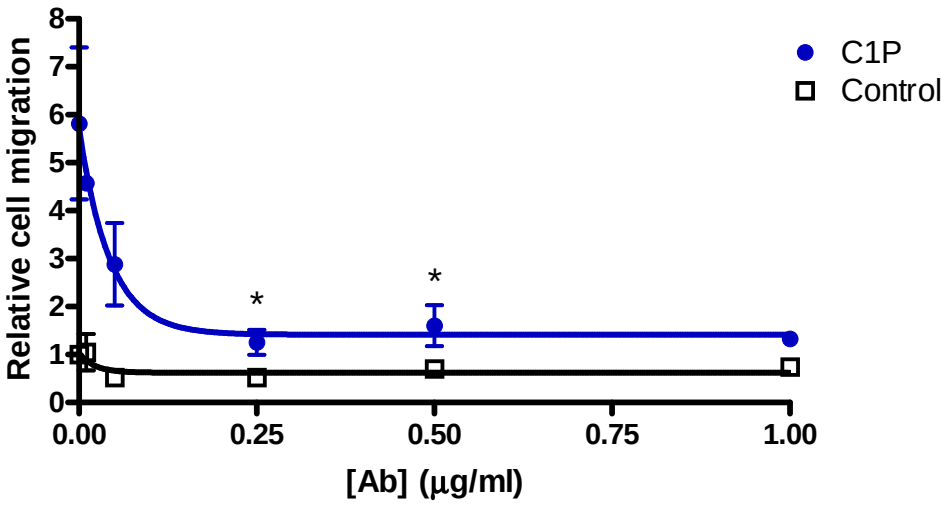


Figure 2.5.1. C1P-stimulated cell migration can be inhibited using a specific anti-MCP-1 antibody which neutralizes the actions of released MCP-1. Macrophage migration was measured using a Boyden chamber-based cell migration assay. Cells ($5 \cdot 10^4$ cells/well) were plated in the upper wells of 24-well chambers coated with fibronectin, and pre-incubated for 1 h in the presence of the indicated concentrations of anti-MCP-1 antibody. Lower wells were filled with the same antibody concentrations, with (solid circles) or without (empty squares) 20 µM C1P. Results are the mean ± SEM of 4 independent experiments (*$p<0.05$).

Figure 2.5.1 shows that the specific anti-MCP-1 antibody decreased C1P-induced cell migration significantly. However, there was still a remaining effect of C1P on cell migration that could not be inhibited by the MCP-1 antibody, even when the concentration of this antibody was markedly increased (Figure 2.5.1). These results suggested that MCP-1 release was an important part of C1P-stimulated cell migration, but that there were other mechanisms involved in this action.

2.6. GPCRs are essential for C1P-induced MCP-1 release and cell migration. Role of a putative C1P receptor

As mentioned before, our group previously demonstrated that C1P stimulates cell migration through interaction with a putative specific C1P receptor, which was found to be a Gi Protein Coupled Receptor (GPCR) [58]. A reagent widely used to characterize the involvement of heterotrimeric Gi-proteins in cell signalling processes is Pertussis Toxin (Ptx). Ptx is a protein secreted by *Bordetella pertussis* and is a member of the

family of ADP-ribosylating bacterial toxins. In order to test whether GPCRs are involved in C1P-stimulated MCP-1 release and cell migration, experiments were performed using Ptx. It was observed that stimulation of MCP-1 release and cell migration by C1P was highly sensitive to treatment with this toxin. Also, it was remarkable to see that very low amounts of Ptx were needed to inhibit C1P-stimulated MCP-1 release and cell migration. These data suggest that GPCRs are involved in the stimulation of macrophage migration by C1P (Figure 2.6.1 and 2.6.2).

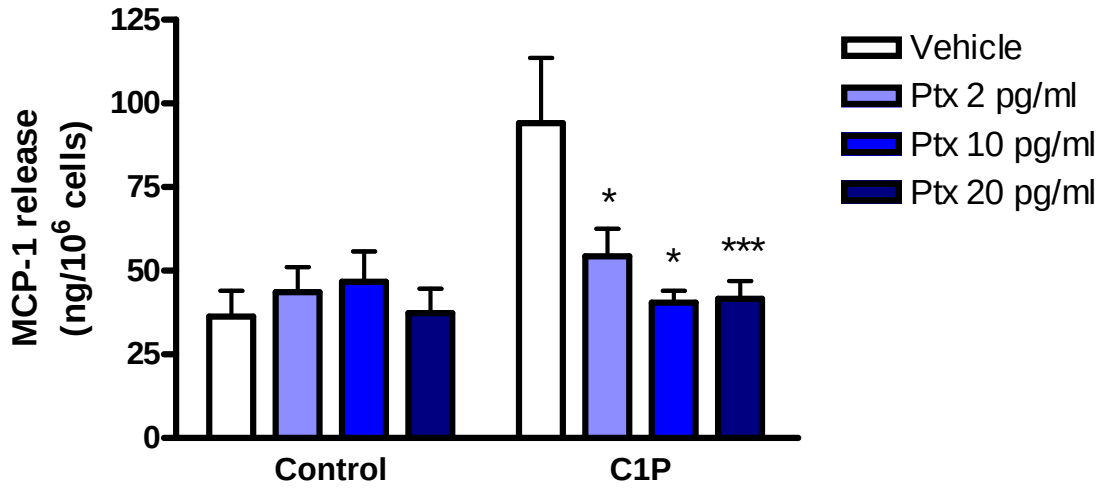


Figure 2.6.1. Ptx completely blocks C1P-stimulated MCP-1 release. Cells were seeded (10^4 cells/well) in 24-well plates and incubated overnight in DMEM supplemented with 10% FBS. The next day, the cells were washed and the medium was replaced by DMEM supplemented with 1% FBS. After 2 h of incubation, the cells were incubated overnight (for about 16 h) with the indicated concentrations of Ptx before C1P addition. Then, macrophages were incubated for 24 h with 20 µM C1P. The medium was collected and analyzed in order to measure the MCP-1 concentration as described in the *Materials and Methods* section. MCP-1 values were normalized to the total cell number and the results are expressed as the mean ± SEM of 6 independent experiments performed in duplicate (*$p<0.05$;***$p<0.001$).

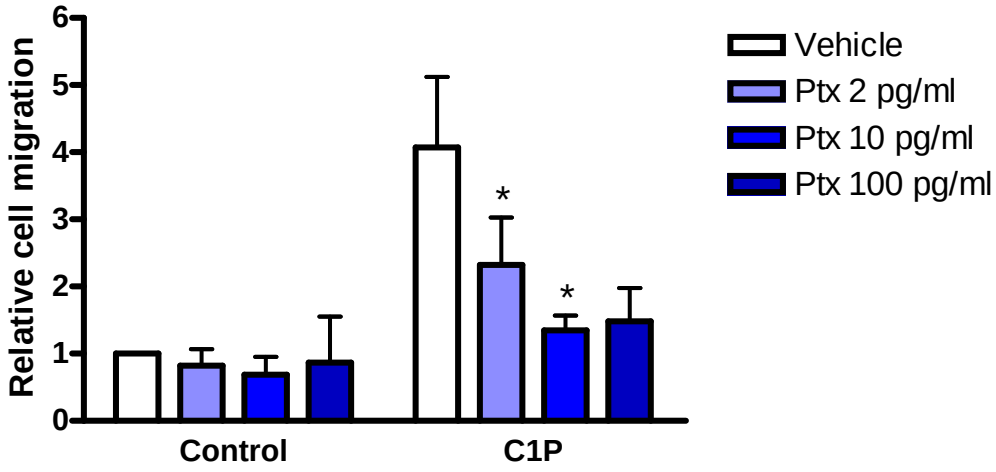


Figure 2.6.2. Ptx blocks C1P-stimulated cell migration. Macrophage migration was measured using a Boyden chamber-based cell migration assay. Cells ($5 \cdot 10^4$ cells/well) were plated in the upper wells of 24-well chambers coated with fibronectin, and pre-incubated for 4 h in the presence of the indicated concentrations of Ptx. Then, Ptx was added to the lower chamber at the indicated concentrations, with or without 20 µM C1P and the cells were further incubated for 24 h. Results are the mean ± SEM of 4 independent experiments (*$p<0.05$).

Since like C1P, MCP-1 also acts through interaction with a Gi protein coupled receptor, experiments were aimed at elucidating the extent of participation of each of these receptors in the stimulation of cell migration by C1P. There are two subtypes of MCP-1 receptors, CCR2, A and B, with the CCR2B receptor isoform being about five-fold more sensitive to induction of chemotaxis by MCP-1 than CCR2A.

Therefore, we performed migration experiments using RS 102895, a selective inhibitor of CCR2B (Figure 2.6.3).

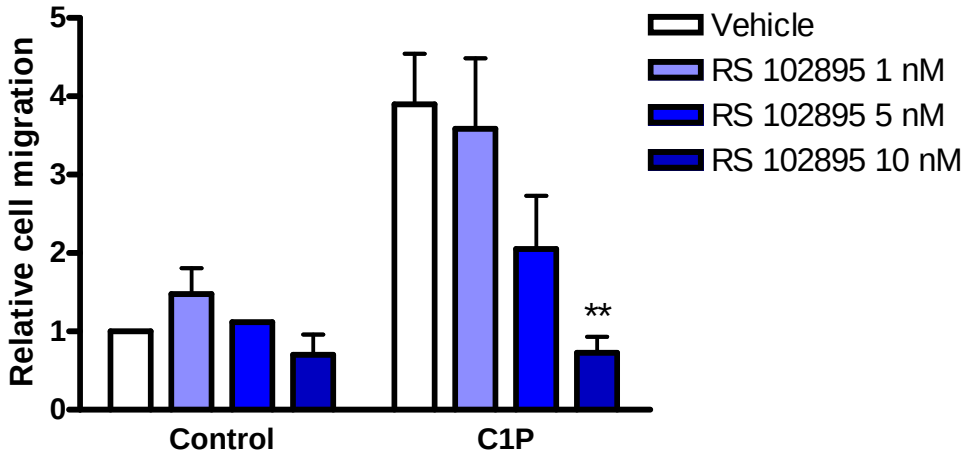


Figure 2.6.3. Inhibition of CCR2B blocks C1P-stimulated cell migration. Macrophage migration was measured using a Boyden chamber-based cell migration assay. Cells ($5 \cdot 10^4$ cells/well) were plated in the upper wells of 24-well chambers coated with fibronectin, and pre-incubated for 1 h in the presence of indicated concentrations of RS 102895. Then, the indicated concentrations of RS 102895 were added to the lower chamber, with or without 20 µM C1P. Cells were incubated for 24 h before the determination of the cell migration. Results are the mean ± SEM of 4 independent experiments (**$p<0.01$).

As expected from the results shown in Figure 2.5.1, preincubation of the cells with the selective inhibitor of CCR2B (RS 102895) at 10 nM potently decreased C1P-stimulated cell migration. Hence, it can be concluded that the MCP-1 receptor is a key factor for this action.

Up to now, little is known about the putative C1P receptor. We reported that it is a GPCR but its sequence and structure are still unknown at present. Nonetheless, it was observed that the putative receptor is specific for C1P, as other related sphingolipids, including ceramide, dihydroceramide, sphingosine, S1P or SM did not bind to it, nor did they have any significant effect on macrophage migration [58]. In additon, we have now found that phosphatidic acid (PA) is an antagonistic molecule, as it binds tightly to the receptor and blocks C1P-stimulated cell migration (Figure 2.6.4). PA is a phospholipid structurally related to C1P but does not stimulate cell migration by its own [58].

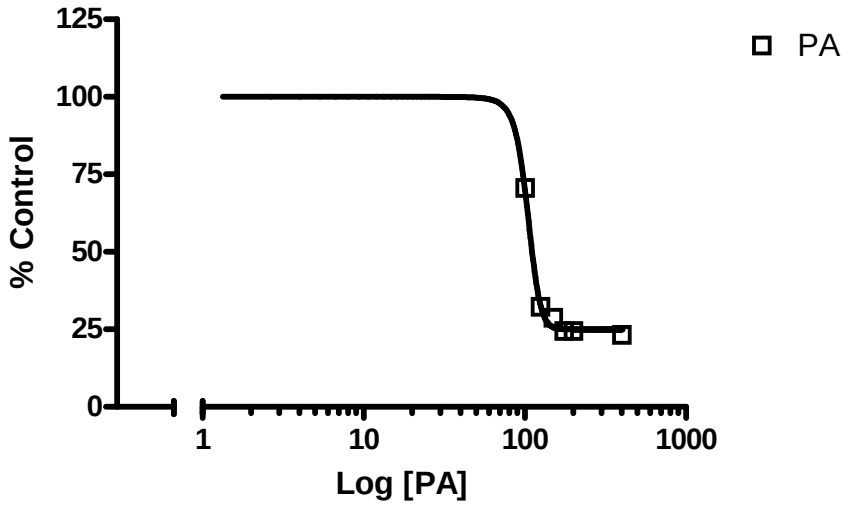


Figure 2.6.4. PA displaces [^{33}P]-C1P binding to macrophage cell membranes. Binding experiments were performed with increasing concentrations of PA using 8 μM of [^{33}P]-C1P. Non-specific binding was performed in the presence of 400 μM C1P. The radioactivity of filter-bound radionuclide was quantified by liquid scintillation counting and non-specific values were subtracted from every value. Results are the mean of three independent experiments performed in duplicate (Figure taken from the Doctoral Thesis of Dr. Maria Granado, 2009).

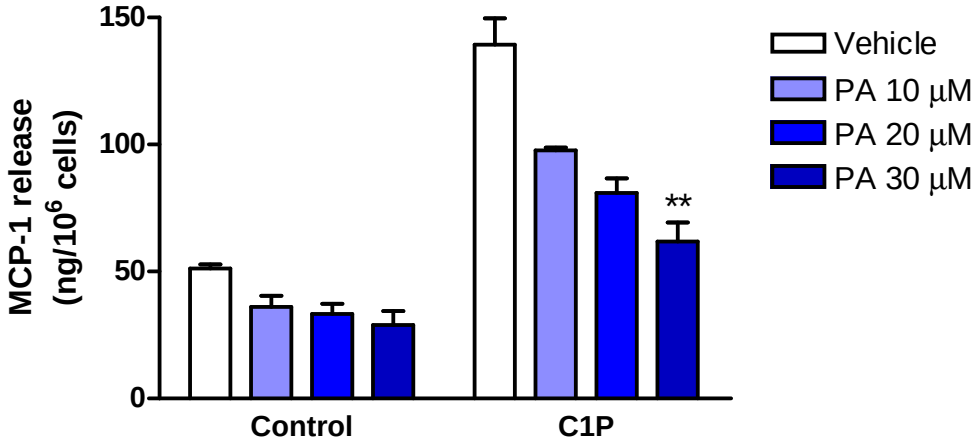


Figure 2.6.5. Pre-incubation with PA diminishes C1P-stimulated MCP-1 release. Cells were seeded (10^4 cells/well) in 24-well plates and incubated overnight in DMEM supplemented with 10% FBS. The next day, the cells were washed and the medium was replaced by DMEM supplemented with 1% FBS. After 2 h of incubation, J774A.1 cells were pre-incubated with the indicated concentrations of PA for 30 min before 20 μM C1P addition, and cells were further incubated for 24 h. The medium was collected and analyzed to measure MCP-1 concentration. MCP-1 values were normalized to the total cell number and the results are expressed as the mean ± SEM of 4 independent experiments performed in duplicate (**p<0.01).

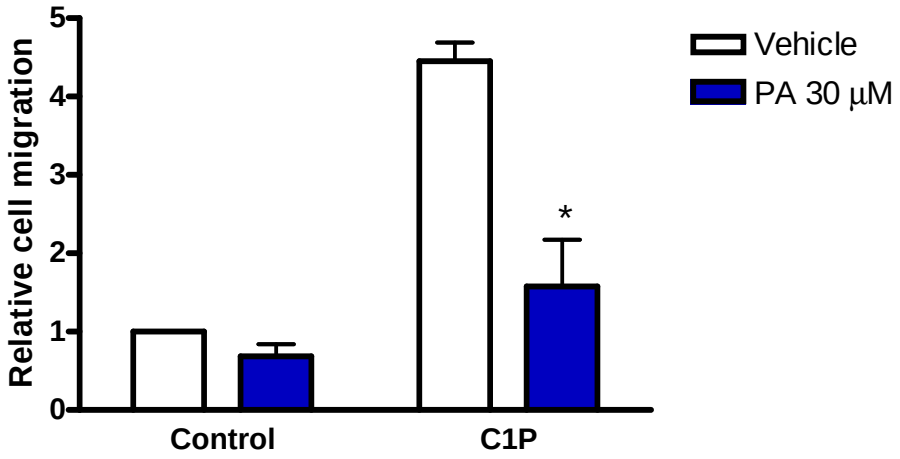


Figure 2.6.6. Effect of PA on C1P-stimulated cell migration. Macrophage migration was measured using a Boyden chamber-based cell migration assay. Cells ($5 \cdot 10^4$ cells/well) were plated in the upper wells of 24-well chambers coated with fibronectin, and pre-incubated for 1 h in the presence of the indicated concentrations of PA. Then, the same concentrations of PA were added to the lower chamber, with or without 20 µM C1P. Cells were incubated for 24 h before determination of cell migration. Results are the mean ± SEM of 3 independent experiments performed in duplicate (*$p<0.05$).

Figures 2.6.5 and 2.6.6 show that PA potently reduced C1P-stimulated MCP-1 release and cell migration.

Apart from these experimental approaches, additional experiments were performed using siRNA to silence specific genes that are related to this C1P action. Treatment of the macrophages with siRNA to silence MCP-1 or CCR2 shows that C1P-stimulated cell migration was potently blocked (Figure 2.6.7).

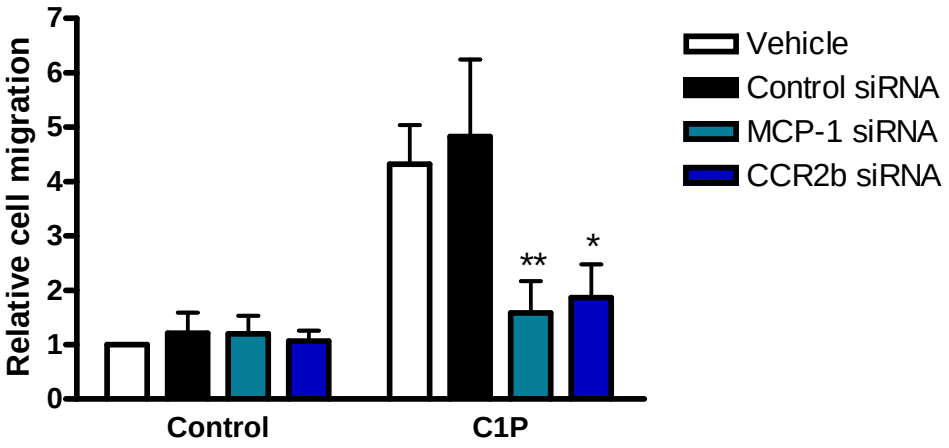


Figure 2.6.7. MCP-1 and CCR2 siRNAs reduce C1P-stimulated cell migration. Cells ($1.5 \cdot 10^5$) were seeded in 60 mm plates and the siRNAs were applied as mentioned in the *Materials and Methods* section. After the siRNA treatment, cells were scraped and counted in order to be plated ($5 \cdot 10^4$ cells/well) in the upper wells of the Boyden chambers coated with fibronectin. Cells were incubated for 1 h to allow cell adhesion and then 20 µM C1P or vehicle were added to the lower chambers. Cells were incubated for 24 h. Data are expressed as cell migration relative to the number of cells migrated in the control chamber. Results are the mean ± SEM of 4 independent experiments performed in duplicate (*$p<0.05$; *$p< 0.01$).

Our group previously demonstrated that C1P activates the PI3K/PKB, and MEK/ERK1-2 pathways in BMDM leading to NF-κB activation, and that both of these pathways are involved in the stimulation of cell migration by C1P [64]. Therefore, we have now examined the possible implication of these pathways in C1P-stimulated MCP-1 release.

2.7. The PI3K/PKB (Akt) pathway is involved in the stimulation of MCP-1 release by C1P

Phosphoinositide 3-kinases (PI3K) have been linked to an extraordinarily diverse group of cellular functions, including cell growth, proliferation, differentiation, motility, survival and intracellular trafficking. When PI3K is activated it produces PIP2 and PIP3, which are located in the inner layer of the plasma membrane. Akt translocates to the plasma membrane where it becomes phosphorylated by phosphoinositide-dependent protein kinase 1 (PDK1). This phosphorylation partially activates Akt which can subsequently activate other enzymes, such as mTOR [69]. Due to the importance of this pathway in the regulation of cell motility, we tested its possible implication in C1P-induced MCP-1 release. For this, we used selective inhibitors of PI3K and PKB as well

as specific siRNA to silence the genes encoding for these two enzymes. Selective inhibitors of both PI3K and PKB blocked C1P-stimulated MCP-1 release thereby underscoring the importance of these enzymes in the regulation of this C1P action (Figure 2.7.1).

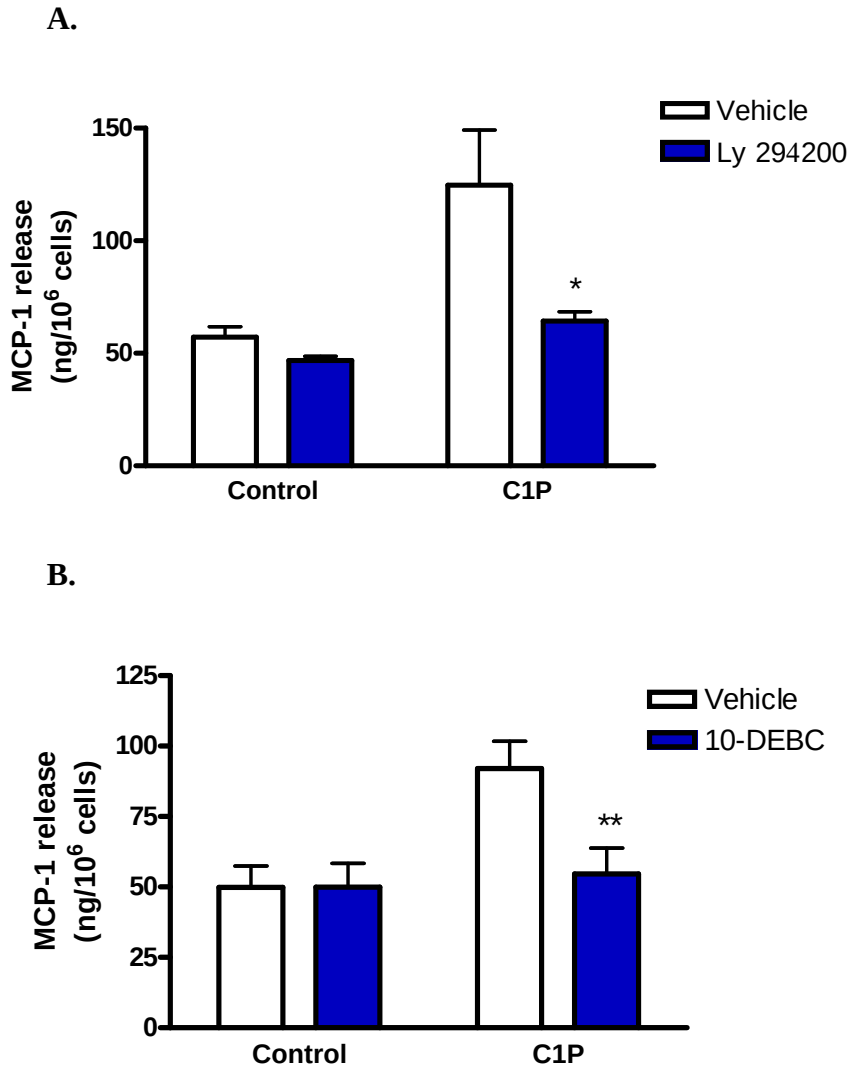


Figure 2.7.1. PI3K/PKB (Akt) pathway is implicated in C1P-stimulated MCP-1 release in J774A.1 macrophages. Cells were seeded (10^4 cells/well) in 24-well plates and incubated overnight in DMEM supplemented with 10% FBS. The next day, the cells were washed and the medium was replaced by DMEM supplemented with 1% FBS. After 2 h of incubation, the cells were treated with different agonists **A.** Cells were pre-incubated with 1 µM Ly 294002 (the PI3K inhibitor) for 30 min prior to the addition of 20 µM C1P. Cells were incubated for 24 h and the culture medium was collected. **B.** Cells were pre-incubated with 1 µM 10-DEBC (Akt inhibitor) for 30 min and then macrophages were incubated for 24 h with 20 µM C1P. After this incubation, the medium was collected and the presence of MCP-1 was measured by ELISA. The values were normalized to the total cell number and the results are expressed as the mean ± SEM of 5 independent experiments performed in duplicate (*p<0.05; **p<0.01; ***p<0.001).

The implication of the PI3K/PKB pathway in C1P-stimulated MCP-1 release was further studied using specific siRNA to PI3K and PKB. Silencing of the genes encoding for these kinases also blocked the release of MCP-1 stimulated by C1P (Figure 2.7.2).

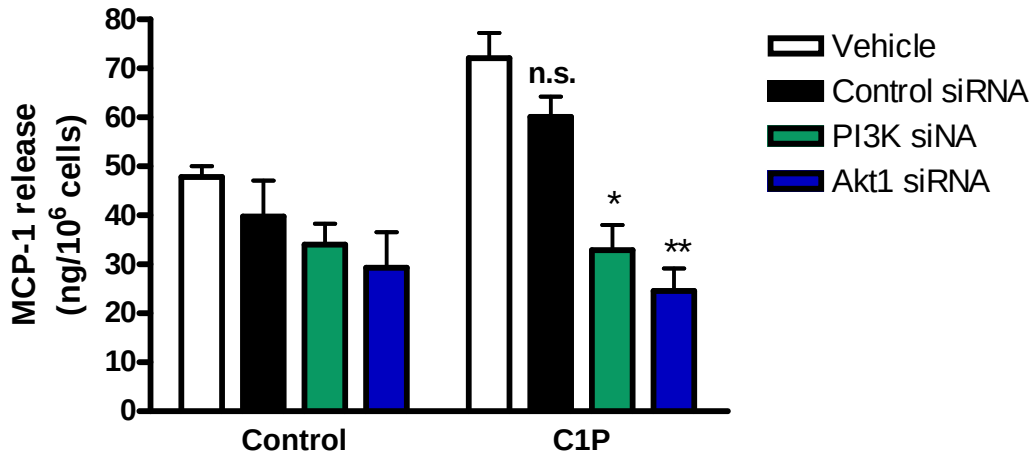


Figure 2.7.2. PI3K and Akt 1 siRNAs inhibit C1P-induced MCP-1 release. Cells were seeded in 60 mm dishes ($1.5 \cdot 10^5$ cells/well) and the siRNA treatment was performed as described in *Materials and Methods* section. Cells were then scraped and counted in order to be seeded (10^4 cells/well) in 24-well plates. The next day, cells were washed and the medium was replaced by DMEM supplemented with 1% FBS. After 2 h of incubation, the cells were further incubated with or without 20 µM C1P for 24 h. The culture medium was collected and the MCP-1 content was determined by ELISA. MCP-1 concentration was normalized to the total cell number and data are expressed as the mean ± SEM of 4 independent experiments (n.s. $p>0.05$; *$p<0.05$; **$p<0.01$).

2.8. Involvement of MAP kinases in C1P-stimulated MCP-1 release

Our group previously showed that C1P can activate MAP kinases. Here, we have studied the implication of these enzymes in C1P-stimulated MCP-1 release.

2.8.1. MAPK/ERK pathway

Extracellular signal-regulated kinases (ERKs) are classical MAP kinases that are widely expressed and can be activated by many different stimuli, including growth factors, cytokines, virus infection, ligands for heterotrimeric G protein-coupled receptors,

transforming agents and carcinogens. We tested to see whether C1P was able to activate MCP-1 through stimulation of these kinases. For this, we pre-incubated the cells with PD98059 as a selective inhibitor of the MAP kinase that phosphorylates ERK1-2, named MAP kinase kinase, or MEK. Figure 2.8.1.1 shows that PD98059 blocks C1P-induced MCP-1 release suggesting that MEK/ERK1-2 is inlvolved in this process. The phosphorylation of ERK1-2 induced by C1P is shown below.

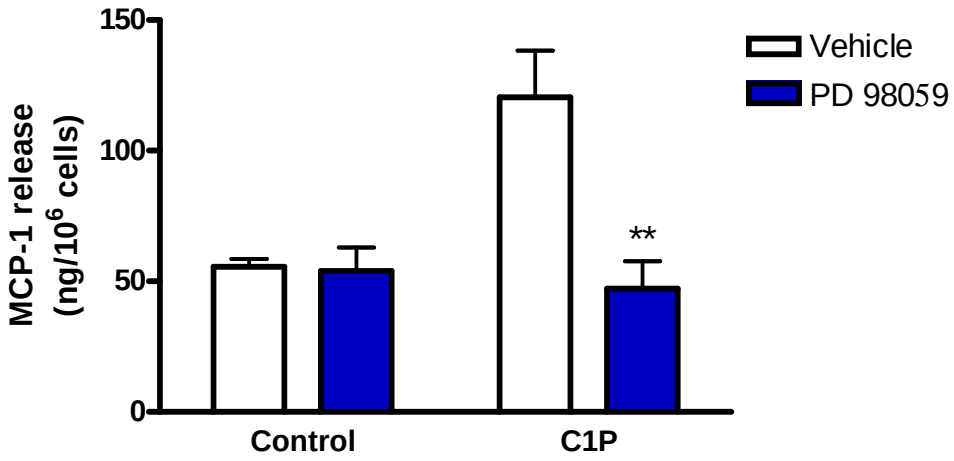


Figure 2.8.1.1. The MEK/ERK pathway is involved in the stimulation of MCP-1 release by C1P. Cells were seeded (10^4 cells/well) in 24-well plates and incubated overnight in DMEM supplemented with 10% FBS. The next day, the cells were washed and the medium was replaced by DMEM supplemented with 1% FBS. After 2 h of incubation, cells were pre-incubated with 10 μM PD 98059 prior to be incubated with 20 μM C1P for 24 h. MCP-1 in the medium was measured by ELISA and the results are the mean ± SEM of 6 independent experiments performed in duplicates (**$p<0.01$).

This observation was confirmed by using ERK siRNA. Figure 2.8.1.2 shows that C1P-stimulated MCP-1 release was inhibited after silencing of the genes encoding for ERK.

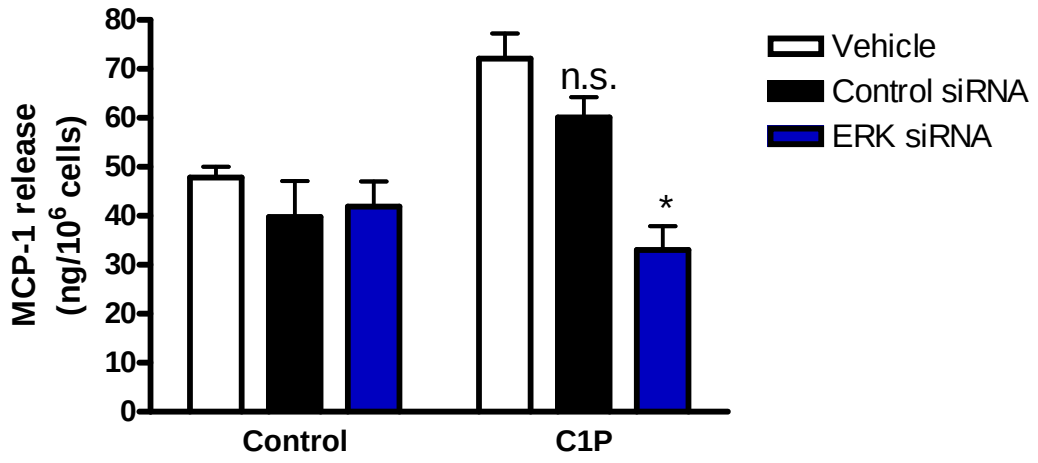


Figure 2.8.1.2. ERK siRNA inhibits C1P-induced MCP-1 release. Cells were seeded in 60 mm dishes ($1.5 \cdot 10^5$ cells/well) and the siRNA treatment was performed as described in the *Materials and Methods* section. Cells were then scrapped and counted in order to be seeded in 24-well plates (10^4 cells/well). The next day, cells were washed and the medium was replaced by DMEM supplemented with 1% FBS. After 2 h of incubation, the cells were further incubated with or without 20 µM C1P for 24 h. The culture medium was collected and the MCP-1 concentration was determined by ELISA. The MCP-1 concentration was normalized to the total cell number and data are expressed as the mean ± SEM of 5 independent experiments (*$p<0.05$).

2.8.2. p38 mitogen-activated protein kinases

p38 mitogen-activated protein kinases are a class of kinases that are activated by a variety of cellular stresses including osmotic shock, inflammatory cytokines, lipopolysaccharides (LPS), ultraviolet light and growth factors. They are involved in a signalling cascade controlling cellular responses to cytokines and stress.

We found that treating the macrophages with SB 202190, a selective inhibitor of p38, C1P-induced MCP-1 release was reduced potently, which indicates that this kinase is also involved in this process (Figure 2.8.2.1). Phosphorylation of p38 by the action of C1P is shown below (Section 2.10).

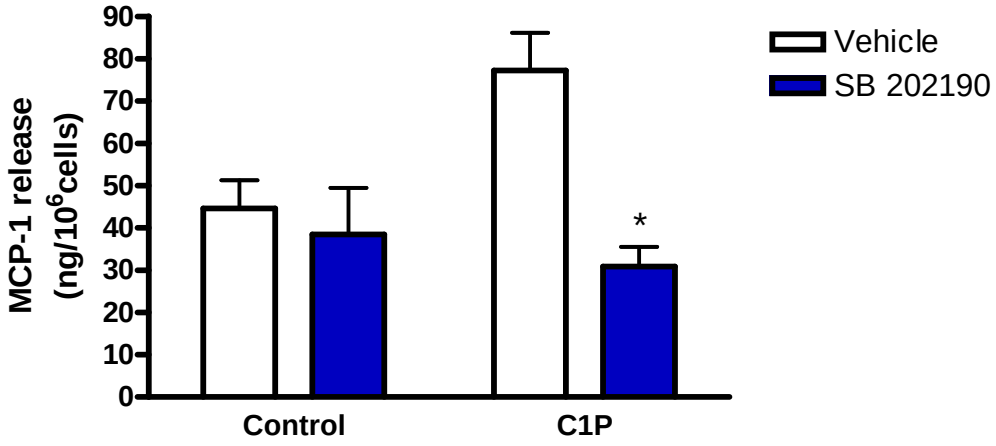


Figure 2.8.2.1. p38 MAPK is implicated in C1P stimulated MCP-1 release. Cells were seeded (10^4 cells/well) in 24-well plates and incubated overnight in DMEM supplemented with 10% FBS. The next day, the cells were washed and the medium was replaced by DMEM supplemented with 1% FBS. After 2 h of incubation, cells were pre-incubated with 1 μM SB 202190 prior to be incubated with or without 20 μM C1P for 24 h. MCP-1 in the medium was measured by ELISA and normalized to the total cell number. Results are the mean ± SEM of 3 independent experiments (*$p<0.05$).

2.8.3. JNK pathway

c-Jun N-terminal kinases (JNKs) were originally identified as kinases that bind and phosphorylate c-Jun on Ser-63 and Ser-73 within its transcriptional activation domain. They also belong to the mitogen-activated protein kinase family, and are responsive to stress stimuli such as cytokines, ultraviolet irradiation, heat shock and osmotic shock. It has been suggested that this signalling pathway contributes to inflammatory responses in mammals. To assess the possible implication of this kinase in C1P-stimulated MCP-1 release we performed some experiments with SP600125, a novel and selective inhibitor of JNK (Figure 2.8.3.1).

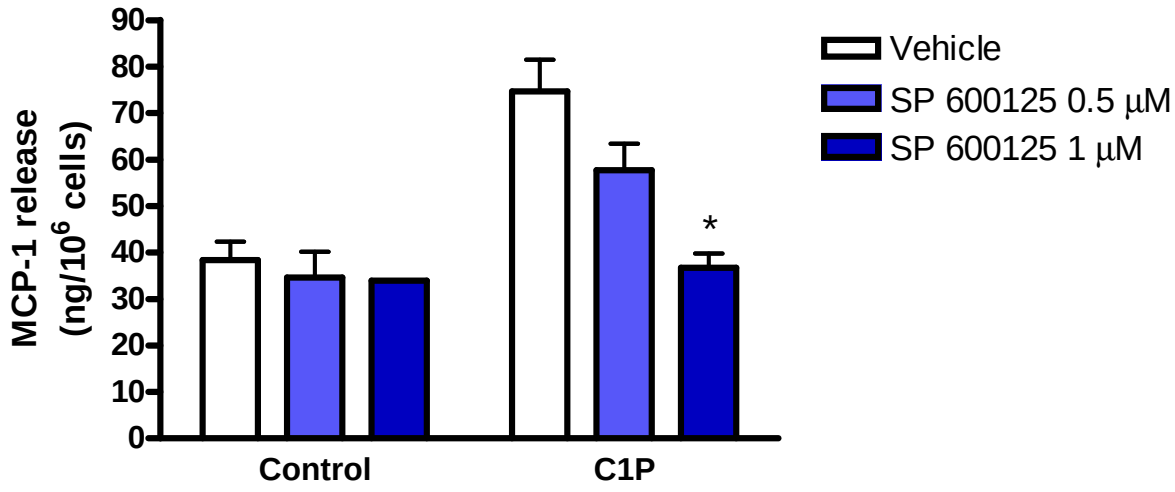


Figure 2.8.3.1. SP600125 reduces C1P-stimulated MCP-1 release. J774A.1 cells were seeded in 24-well plates (10^4 cells/well) and incubated overnight in DMEM supplemented with 10% FBS. The next day, the cells were washed and the medium was replaced by DMEM supplemented with 1% FBS. After 2 h of incubation, the cells were pre-incubated with the indicated concentrations of SP 600125 for 30 min and then 20 µM C1P was added. After 24 h, the medium was collected and centrifuged. The MCP-1 concentration was measured by ELISA and normalized to the total cell number, as indicated in the *Materials and Methods* section. Results are expressed as the mean ± SEM of 3 independent experiments performed in duplicate (*$p<0.05$).

2.9. Implication of NF-κB and STAT-3 in C1P-stimulated MCP-1 release

The PI3K/PKB and MEK/ERK have been described to lead to activation of transcription factors. Because of their relationship with expression of inflammatory cytokines, we studied the possible implication of two transcription factors: nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 3 (STAT3). The implication of these transcription factors in C1P-stimulated MCP-1 release is discussed below.

2.9.1. NF-κB

NF-κB signalling plays an important role in inflammation, the innate and adaptive immune response and stress. Dysregulated signalling can occur in inflammatory and autoimmune diseases. NF-κB is important in regulating cellular responses because it is present in cells in an inactive state and does not require new protein synthesis to be activated. Thus, it can be activated rapidly and participates in a variety of cell responses such as cell proliferation, protection of apoptosis, immune system regulation, inflammation and cell migration. The implication of this transcription factor in C1P-stimulated MCP-1 release was studied using SC-514, an ATP-competitive IKK-2 inhibitor. This inhibitor significantly reduced C1P-stimulated MCP-1 release (Figure 2.9.1.1) suggesting its involvement in this process. The activation of NF-kB by C1P was evaluated by determining its translocation to the nucleus, as discussed below (Section 2.11).

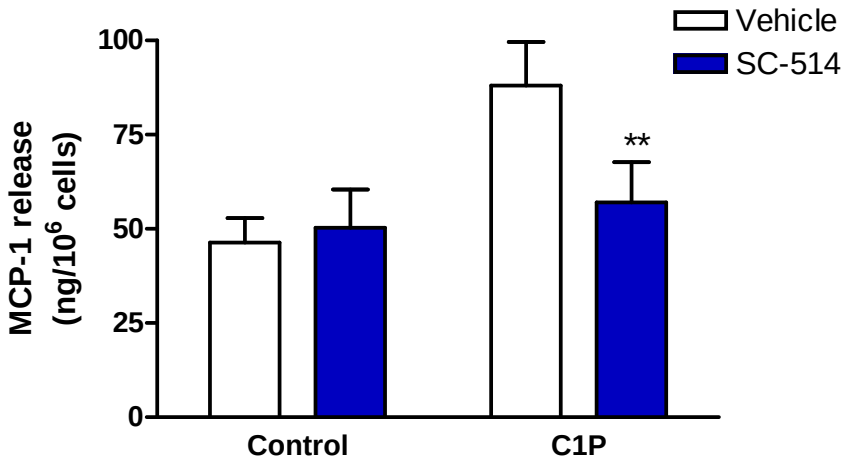


Figure 2.9.1.1. NF-κB is implicated in C1P-stimulated MCP-1 release. J774A.1 cells were seeded in 24-well plates (10^4 cells/well) and incubated overnight in DMEM supplemented with 10% FBS. The next day, the cells were washed and the medium was replaced by DMEM supplemented with 1% FBS. After 2 h of incubation, the cells were pre-incubated with 25 µM SC-514 (IKK-2 inhibitor) for 30 min prior to be incubated with 20 µM C1P. After 24 h MCP-1 was measured by ELISA and normalized to total cell number. Results are the mean ± SEM of 5 independent experiments (**$p<0.01$).

2.9.2. STAT3

STAT3 mediates the expression of a variety of genes in response to cell stimuli, and plays a key role in many cellular processes such as cell growth and apoptosis. STAT family members are phosphorylated by receptor-associated kinases and then form homo- or heterodimers that translocate to the cell nucleus, where they act as transcription activators.

It has been reported that STAT3 is implicated in cell migration and invasion processes. Therefore, the possible involvement of this transcriptional factor in C1P-induced MCP-1 release was tested. Stattic is a potent inhibitor of STAT3. This inhibitor blocks the binding of tyrosine-phosphorylated peptide motifs to the STAT3 SH2 domain thereby blocking STAT3 activation, dimerization and nuclear translocation. Stattic was able to significantly reduce C1P-induced MCP-1 release (Figure 2.9.2.1), suggesting its implication in this process. The activation of STAT3 by C1P was evaluated by determining its translocation to the nucleus, as discussed below (section 2.11).

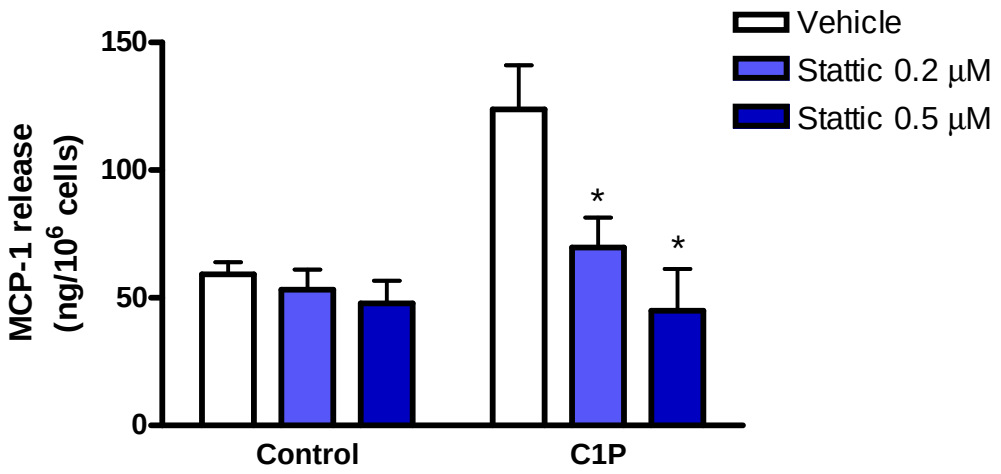


Figure 2.9.2.1. C1P-stimulated MCP-1 release is reduced by the STAT3 inhibitor Stattic. J774A.1 cells were seeded in 24-well plates (10^4 cells/well) and incubated overnight in DMEM supplemented with 10% FBS. The next day, the cells were washed and the medium was replaced by DMEM supplemented with 1% FBS. After 2 h of incubation, the cells were pre-incubated with the indicated concentrations of Stattic (pharmacological irreversible inhibitor of STAT3 activation) for 30 min before C1P addition. Cells were incubated for 24 h, with or without 20 μM C1P. The medium was collected for MCP-1 determination, as described in the *Materials and Methods* section. Results are expressed as the mean ± SEM of 4 independent experiments (*$p<0.05$).

STAT transcription factors are usually activated by phosphorylation of a specific tyrosine residue in the STAT protein which promotes the dimerization of STAT monomers via their SH2 domain. The phosphorylation of STAT3 is carried out by JAK3. To examine the implication of JAK3 we used a selective inhibitor of this tyrosin kinase, ZM 39923. We observed that this inhibitor also blocked MCP-1 release.

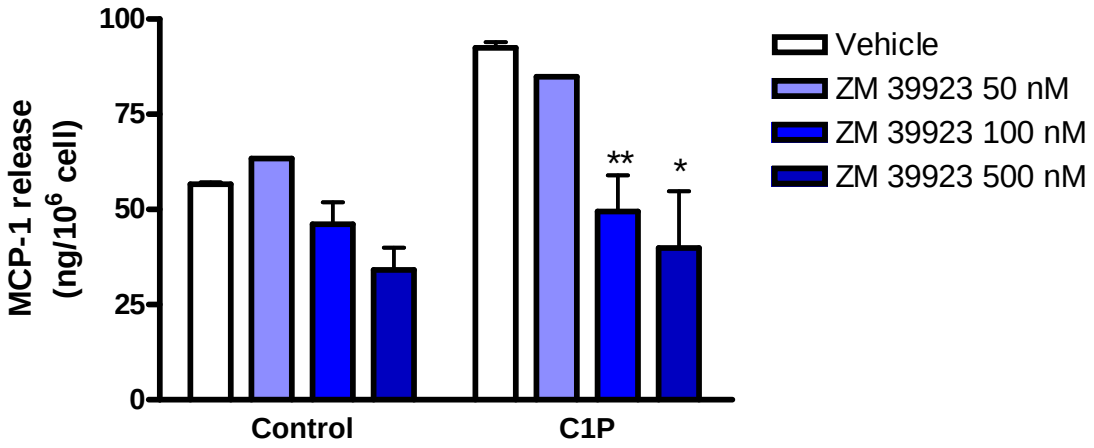
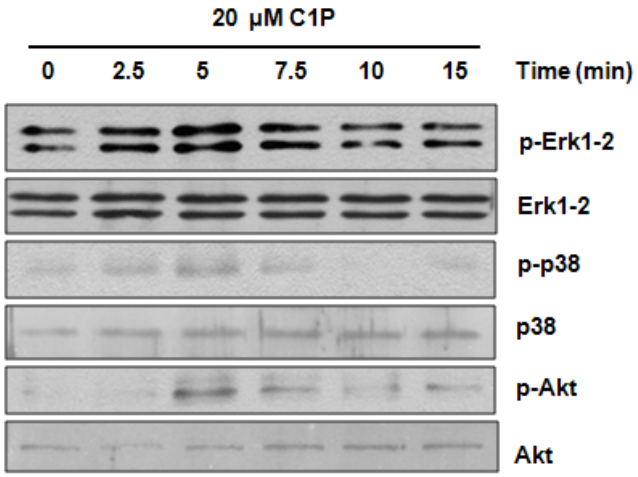


Figure 2.9.2.2. The JAK3 inhibitor, ZM 39923, reduces C1P-stimulated MCP-1 release. J774A.1 cells were seeded in 24-well plates (10^4 cells/well) and incubated overnight in DMEM supplemented with 10% FBS. The next day, the cells were washed and the medium was replaced by DMEM supplemented with 1% FBS. After 2 h of incubation, the cells were pre-incubated with the indicated concentrations of ZM 39923 for 30 min before C1P addition. The cells were incubated for 24 h with 20 μM C1P and the medium was collected for the MCP-1 determination. Results are expressed as the mean ± SEM of 3 independent experiments (*$p<0.05$).

2.10. C1P induces phosphorylation of ERK1-2, p38 MAPK and Akt

As expected from the above results, C1P caused phophorylation of ERK1-2, p38 and Akt in J774A.1 cells (Figure 2.10.1).

A.



B.

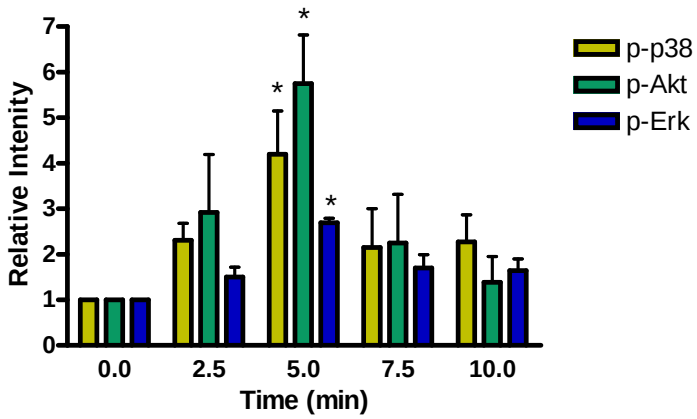


Figure 2.10.1. C1P induces the phosphorylation of ERK, p38 and Akt. Cells were seeded in 60 mm dishes ($2.5 \cdot 10^5$ cells/dish) and incubated overnight in DMEM supplemented with 10% FSB. The next day the cells were washed and the medium was replaced with 1% FBS. After 2 h, 20 µM C1P was added and the cells were harvested after the indicated periods of time. **A.** The presence of phosphorylated protein was detected by Western blotting using specific polyclonal antibodies to each phosphorylated protein. Equal loading of protein was monitored using a specific antibody to total protein of each kinase. Similar results were obtained in each of 4 replicate experiments. **B.** Results of scanning densitometry of the

exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SEM of 4 independent experiments (*p<0.05).

2.11. C1P induces translocation of STAT3 and NF-κB to the nucleus

The results shown in figures 2.9.1.1. and 2.9.2.1 show the involvement of STAT3 and NF-kB in C1P-stimulated MCP-1 release. The following figures show that C1P activated these transcription factors efficiently. NF-kB is inactive in the cytosol and becomes activated when translocated to the nucleus. In the cytosol, the p65 subunit interacts with the IkB inhibitor to maintain NF-kB inactive. Phosphorylation of IkB leads to its degradation and releases NF-κB so that it can reach the nucleus. The possible activation of NF-κB by C1P was studied by Western blotting using different cell fractions. Our results show that, C1P increases p-STAT3 and p-p65 translocation to the nucleus (Figure 2.11.1A and 1B) in a time-dependent manner. The maximal translocation was attained at 3 h of incubation with 20 μM of C1P for both transcription factors.

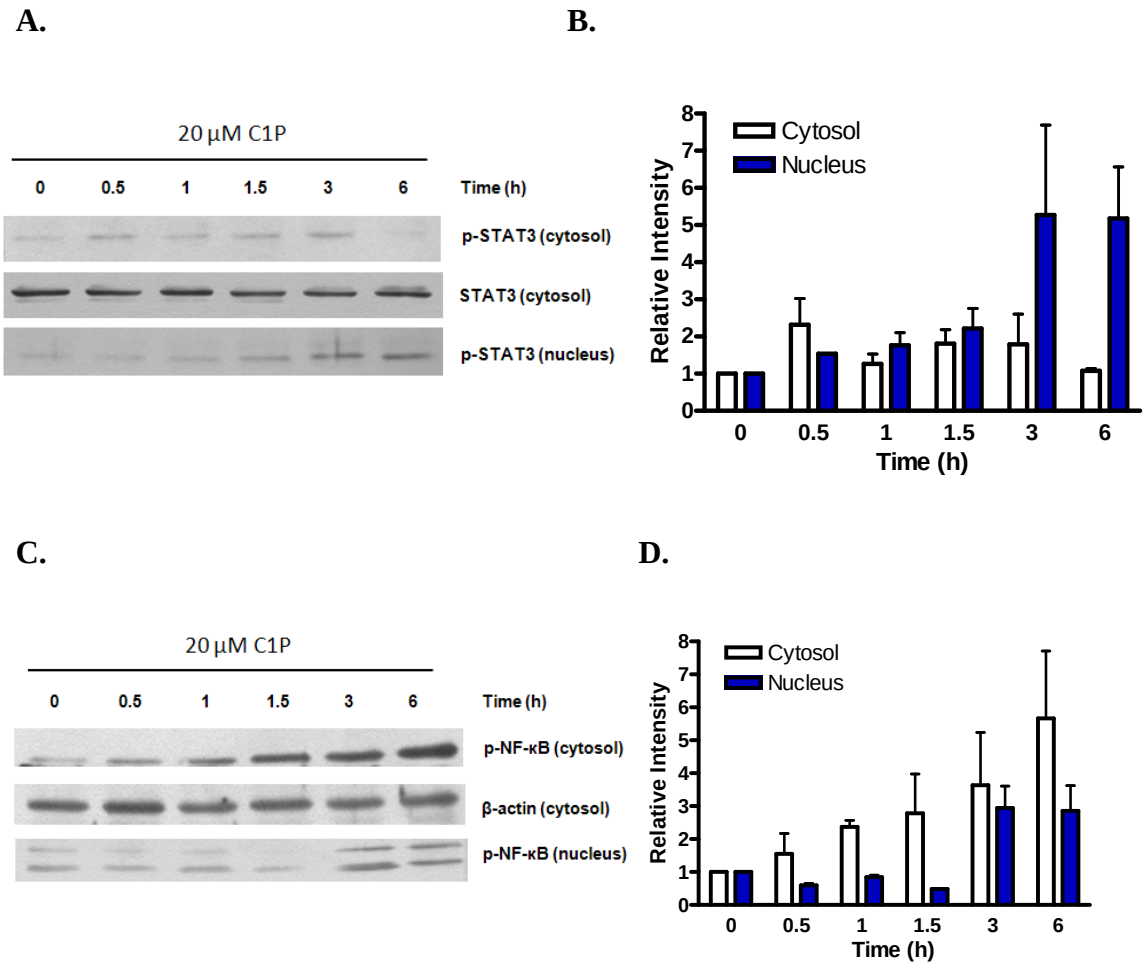


Figure 2.11.2. C1P induces translocation of p-STAT3 and p-p65 to the nucleus. Cells were seeded in 60 mm of diameter dishes ($2.5 \cdot 10^5$ cells/dish) and incubated overnight in DMEM supplemented with 10% FSB. The next day, the cells were washed and the medium was replaced with 1% FBS. After 2 h, 20 µM C1P was added and after each time point, the cells were collected. The nuclear extraction was performed as described in *Materials and Methods* section. **A**. The presence of p-STAT3 in the nucleus and cytosol was detected by Western blotting using a specific antibody to p-STAT3. Equal loading of protein was monitored using a specific antibody to total STAT3. **B.** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SEM of 3 independent experiments. **C.** The presence of phosphorylated p65 protein was detected in the nucleus and cytoplasm by western blotting using a specific antibody to p-p65. Equal loading of protein was monitored using a specific antibody to β-actin. **D**. Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SEM of 3 independent experiments.

2.12. The pathways involved in C1P-stimulated MCP-1 release are also necessary for cell migration

The above results showing that MCP-1 release is important for the stimulation of macrophage migration by C1P, suggest that the pathways involved in this process are essential for stimulation of cell migration. This was confirmed in the following experiments using selective inhibitors of these pathways (10 µM PD 98059 to inhibit MEK, 1 µM 10-DEBC to inhibit Akt, 10 µM SB 202190 to inhibit p38 and 1 µM SP 600125 to inhibit JNK). All of these compounds dramatically reduced C1P-stimulated cell migration (Figure 2.12.1).

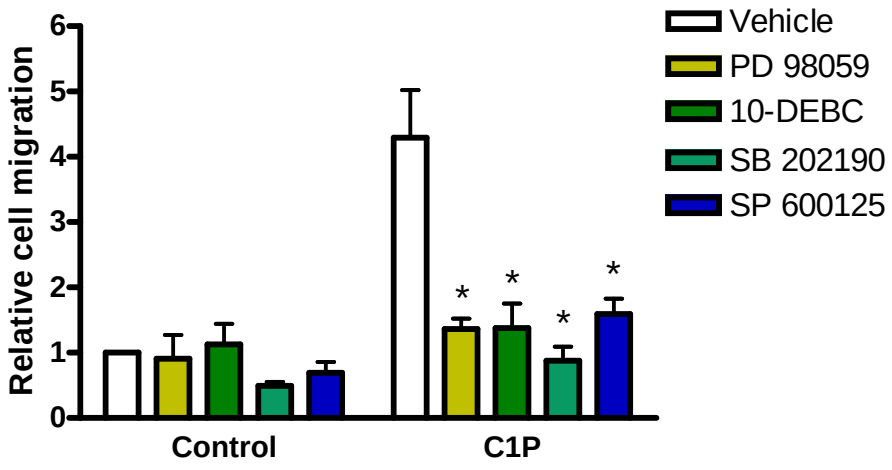


Figure 2.12.1. PI3K/Akt and MAP kinases signalling pathways are involved in C1P-stimulated cell migration. Macrophage migration was measured using a Boyden chamber-based cell migration assay. Cells ($5 \cdot 10^4$ cells/well) were plated in the upper wells of 24-well chambers coated with fibronectin, and pre-incubated for 1 h in the presence of 10 µM PD 98059, 1 µM 10-DEBC, 10 µM SB 202190 or 1 µM SP 600125. Then, the same inhibitor concentrations were added to the lower chambers, with or without 20 µM C1P. Cells were incubated for 24 h before determination of cell migration. Results are the mean ± SEM of 4 independent experiments (*$p<0.05$).

Similar experiments using inhibitors for the transcription factors NF-kB and STAT3 were used to test their implication in this process (we used 25 µM SC-514 to inhibit NF-κB and 0.5 µM Stattic to inhibit STAT3 activation). The results shown in Figure 3.12.2 indicate that both transcription factors are important for C1P-induced cell migration.

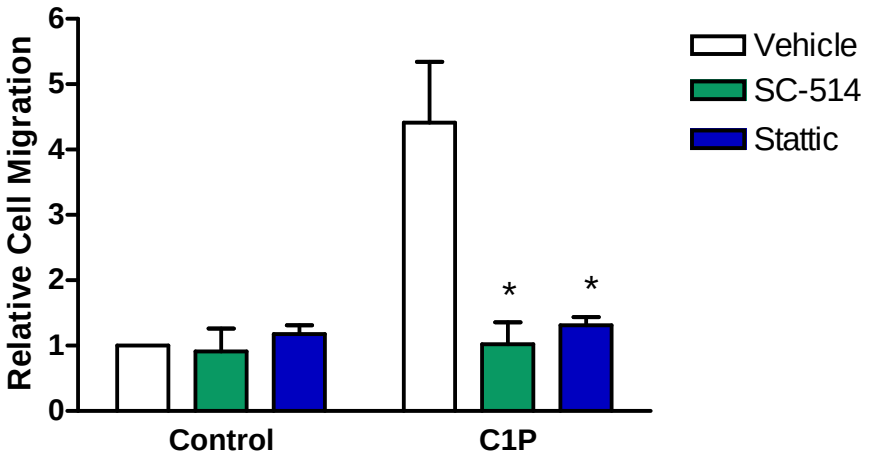


Figure 2.12.2. STAT3 and NF-κB are implicated in C1P-stimulated cell migration. Macrophage migration was measured using a Boyden chamber-based cell migration assay. Cells ($5 \cdot 10^4$ cells/well) were plated in the upper wells of 24-well chambers coated with fibronectin, and pre-incubated for 1 h in the presence of vehicle, 25 µM SC-514 or 0.5 µM stattic. Then, the same inhibitor concentrations were added to the lower chambers, with or without 20 µM C1P. The cells were incubated for 24 h before determination of the cell migration. Results are the mean ± SEM of 4 independent experiments (*$p<0.05$).

2.13. C1P increases the expression of CD11b and ICAM-1. Involvement in C1P-stimulated cell migration

In order to migrate from blood vessels to the endothelium, monocytes need to attach firmly to endothelial cells. This attachment to the endothelium is attained by interaction of integrins or adhesion molecules with their ligands, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [89]. Also, it is well established that ligation of chemokine receptors, including CCR2, induces surface expression and activation of integrins [90, 91].

The β2 integrin heterodimer CD11b/CD18 (Mac-1, CR3) is a multifunctional receptor with significant and diverse roles in immunity and inflammation. The migratory processes are mediated by interacting sets of cell adhesion molecules, including the CD11b/CD18-ICAM-1 pair, which has been experimentally implicated in atherosclerosis and other inflammatory conditions. It is abundantly expressed in neutrophils and monocytes, and CD11b/CD18 plays a role in their migration to sites of xtravascular inflammation. These adhesive interactions are tightly regulated. CD11b/CD18 activation can be induced upon stimulation of other surface receptors, such as chemotactic receptors [92] or TLRs [93].

Due to the importance of cell adhesion in cell migration, we hypothesized that C1P could enhance the expression of CD11b and/or ICAM-1. This was tested by analyzing CD11b and ICAM-1 expression using flow cytometry. The macrophages were incubated for different periods of time with 20 μM C1P and the changes in CD11b and ICAM-1 expression were determined, as indicated in the *Materials and Methods* section. C1P enhanced the cell surface expression of CD11b and ICAM-1 in the macrophages (Figure 2.13.1).

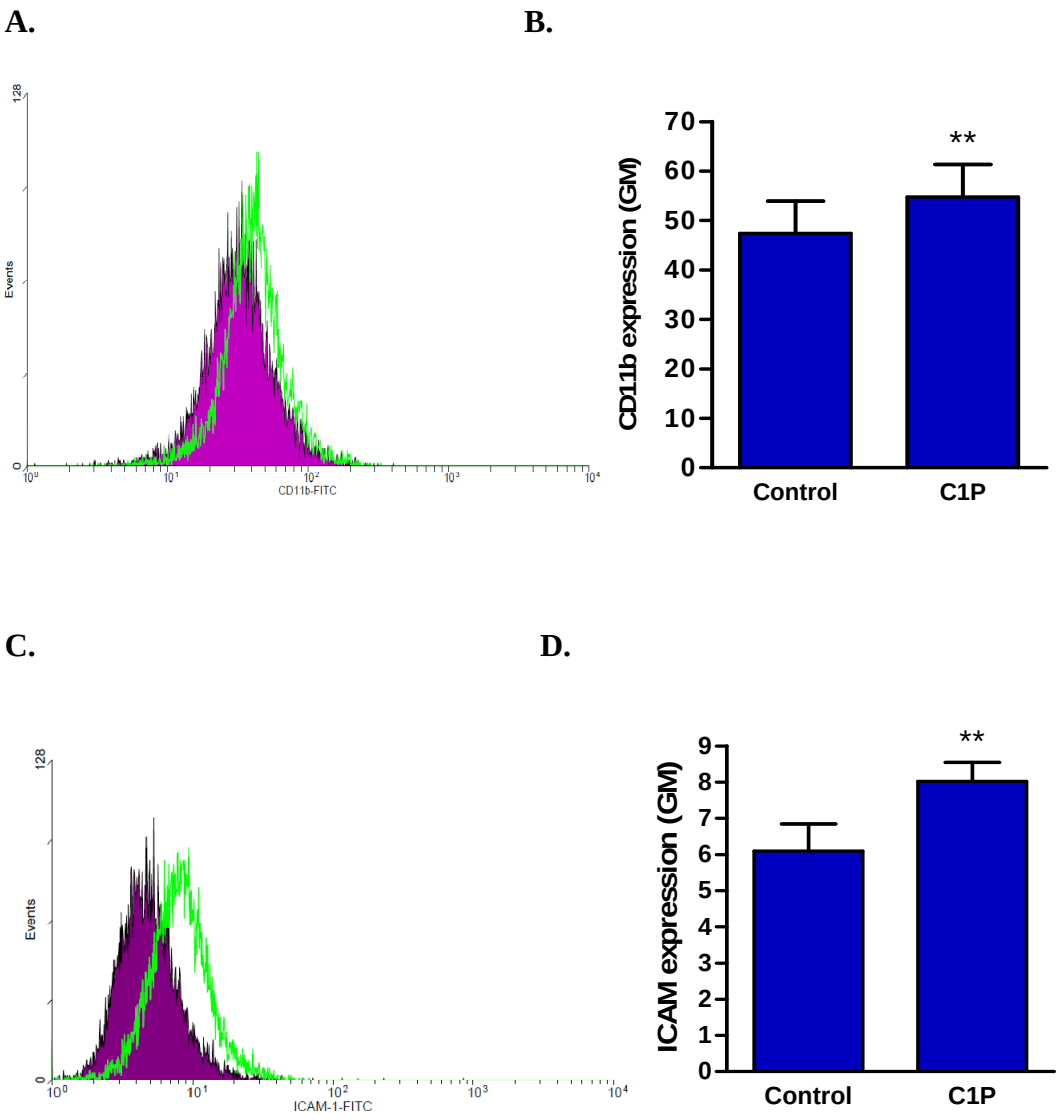


Figure 2.13.1. C1P enhances cell surface expression of CD11b and ICAM-1. Macrophages were seeded at $0.25 \cdot 10^6$ cells/60 mm plate and incubated in DMEM supplemented with 10% FBS overnight. They were then washed and further incubated for 24 h in DMEM containing 1% FBS with or without 20 µM C1P. After incubation, the cells were washed and scrapped and they were stained as described in the *Materials and Methods* section. The samples were analyzed by flow cytometry. **A.** A representative histogram obtained with CD11b expressing cells with (green line) or without (solid purple area) 20 µM C1P treatment. Similar results were obtained in 5 independent experiments. **B.** Results are expressed as the CD11b-FITC GeoMean of fluorescence intensity (GM) ± SEM of 5 independent experiments. **C.** A representative histogram obtained with ICAM-1 expressing cells with (green line solid) or without (purple area) 20 µM C1P treatment. Similar results were obtained in 5 independent experiments. **D**. Results are expressed as the ICAM-1-FITC GeoMean of fluorescence intensity (GM) ± SEM of 5 independent experiments (**p<0.01).

3. Discussion of Chapter 1

The regulation of cell migration is a complex process involving hundreds of molecules. It is necessary for tissue homeostasis, and is crucial for regulation of vital biological processes including embryogenesis, organogenesis or regeneration. In pathology, production of abnormal migratory signals may induce the migration of the wrong cell type to the wrong place, which may have catastrophic effects in overall health. Some examples include autoimmune syndromes or the process of metastasis, in which tumour cells abandom the primary tumour and migrate to distant tissues where they generate secondary tumours. Cancer cell migration is typically regulated by integrins, matrix-degrading enzymes, and cell-cell adhesion molecules. Several cytokines and growth factors have been shown to stimulate invasion and to be upregulated in a variety of tumour types. For instance, it has been reported that MCP-1 recruits CCR2 expressing inflammatory monocytes to lung and facilitates breast-tumour metastasis in mice [94].

Our group previously reported that C1P is a chemoattractant molecule for leukemic Raw246.7 macrophages [58] but the mechanisms involved are not well known. We have now demonstrated that C1P also stimulates migration of a different population of macrophages, J774A cells. Of interest, these two populations of macrophages responded differently to treatment with C1P. In particular, whilst Raw 264.7 macrophage migration occurred after 4h of incubation with C1P, J774A.1 macrophages required longer incubation time (24 h) to be stimulated. In addition, the extent of cell migration was greater in the Raw 264.7 cells than in J774A.1 following treatment with C1P. The reasons for these differences are not known at present, but it is possible that different signalling pathways are responsible for the regulation of cell migration in different cell types.

In this thesis, we demonstrate that C1P is able to induce the release of MCP-1, which is a potent chemoattractant for macrophages. We found that neutralization of MCP-1 using a specific antibody to MCP-1 inhibited C1P-stimulated cell migration. In addition, silencing of the gene encoding for MCP-1 with specific siRNA also abolished the migration of J774A.1 macrophages that was stimulated by C1P. These results suggest that MCP-1 plays a key role in the regulation of cell migration by C1P. Moreover,

experiments using CCR2 siRNA and a CCR2B specific inhibitor demonstrated that this protein takes part of the signalling cascade leading to macrophage migration.

We next evaluated whether the stimulation of macrophage migration by C1P was elicited by interaction with a putative C1P receptor (that we previously reported to exist [58]). From the work of Granado and coworkers, we knew that the putative C1P receptor is couppled to Gi proteins, and it is sensitive to inhibition by PA in a specific manner. Using PA as a C1P receptor antagonist we were able to demonstrate the implication of this receptor in C1P-stimulated MCP-1 release. It seems clear that C1P causes the release of MCP-1 through activation of its specific receptor, and that this is a major part of the mechanism by which C1P stimulates macrophage migration.

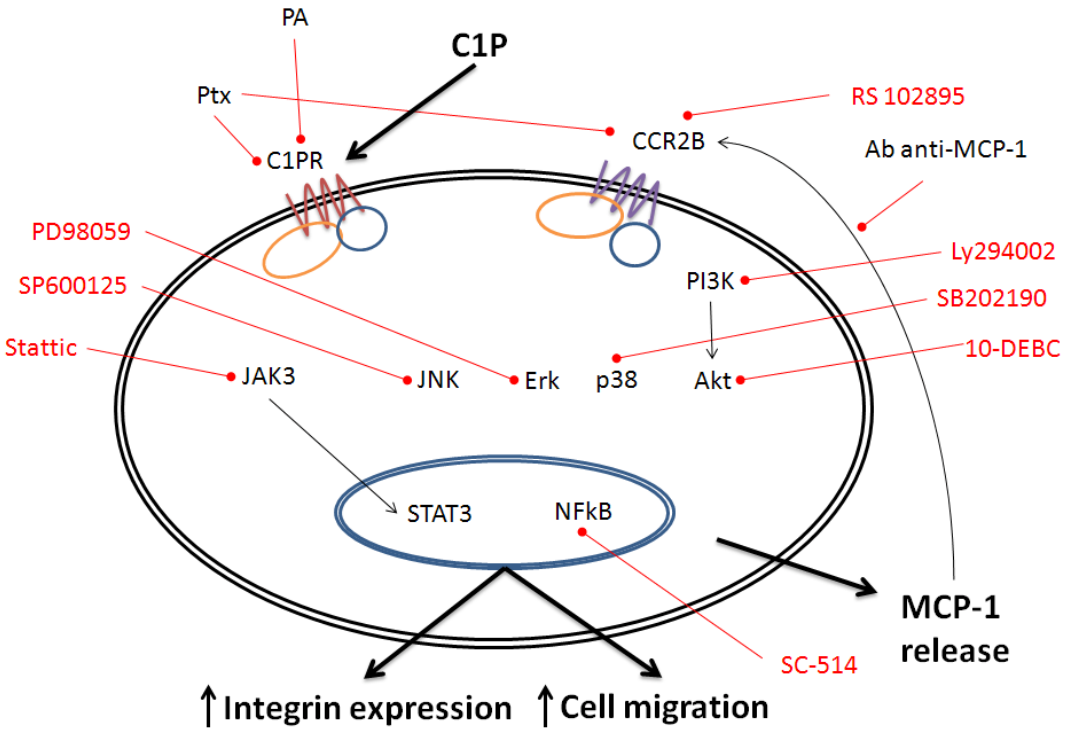
A typical MCP-1 release-inducing molecule is tumour necrosis factor-α (TNF-α). It has been reported that TNF-α increases MCP-1 expression through activation of p38, ERK and JNK MAPK pathways and that the PI3K/PKB pathway is also involved [95-97]. Also, C-reactive Protein (CRP) is a well-known MCP-1 secretion enhancer and it has been demonstrated that ERK1-2 is also involved in this action [98]. Here we show that p38, ERK and PI3K/PKB are implicated in C1P-stimulated MCP-1 release and the subsequent stimulation of J774A.1 macrophage migration.

MCP-1-induced cell migration has been tightly related to integrin expression and activation. It has been demonstrated that molecules like CRP or LDL overexpress and activate integrins in order to stimulate cell migration [98, 99]. In this work, we demonstrate that C1P significantly increases the surface expression of CD11b and ICAM-1 after 24 h of incubation. Although ICAM-1 has generally been related to endothelial cells, changes in the surface expression of ICAM-1 in monocyte/macrophages has also been reported [98, 100, 101]. The C1P-stimulated increase in surface expression of ICAM-1 is more pronounced than that for CD11b. Presumably, this occurs because CD11b expression in control macrophages is already high and differences between control and C1P-stimulated macrophages are harder to detect.

NF-κB induces several cellular alterations associated with tumourigenesis including self-sufficiency in growth signals, insensitivity to growth inhibition, resistance to apoptotic signals, immortalization, angiogenesis, tissue invasion and metastasis. STAT3, which is a point of convergence for numerous oncogenic signalling pathways,

is constitutively activated both in neoplastic and in immune cells in the tumour microenvironment and plays a role in oncogenesis by both promoting cell transformation and inhibition of apoptosis [102]. In C1P-treated J774A.1 macrophages we have observed activation of both NF-κB and STAT3 transcription factors. Although our experimental conditions do not emulate cancer microenvironment conditions, it would be interesting to analyze the activity of these transcription factors in tumour microenvironment conditions.

Summarizing, the scheme shown below emphasizes the signalling pathways involved in C1P-stimulated MCP-1 release and the subsequent stimulation of J774A.1 macrophage migration.



Chapter 2

5. CHAPTER 2

Ceramide 1-phosphate induces VEGF secretion in Raw 264.7 cells

1. Introduction

Vascular endothelial growth factor (VEGF) is a signal protein that stimulates vasculogenesis and angiogenesis. When blood circulation results inadequate, cells secrete this angiogenic factor in order to supply oxygen to tissues. VEGF induces the creation of new blood vessels during embryonic development, after injury or physical exercise and new vessels (collateral circulation) to bypass blocked vessels.

There are different types of VEGF proteins. The most important member is VEGF-A, but there are also others, such as, placenta growth factor (PlGF), VEGF-B, VEGF-C and VEGF-D. VEGF-C and VEGF-D regulate the lymphatic endothelial cells via their receptor VEGFR-3. VEGF-A is commonly referred to as VEGF.

VEGF family members bind their corresponding receptors VEGFR-1, VEGFR-2 and VEGFR-3, found in the vascular endothelium. VEGF can bind VEGFR-1 and VEGFR-2 and, although the binding affinity of VEGF towards VEGFR-2 is lower than that for VEGFR-1, selective activation of VEGFR-1 and VEGFR-2 has shown that VEGFR-2 is the primary receptor transmitting VEGF signals in endothelial cells [103].

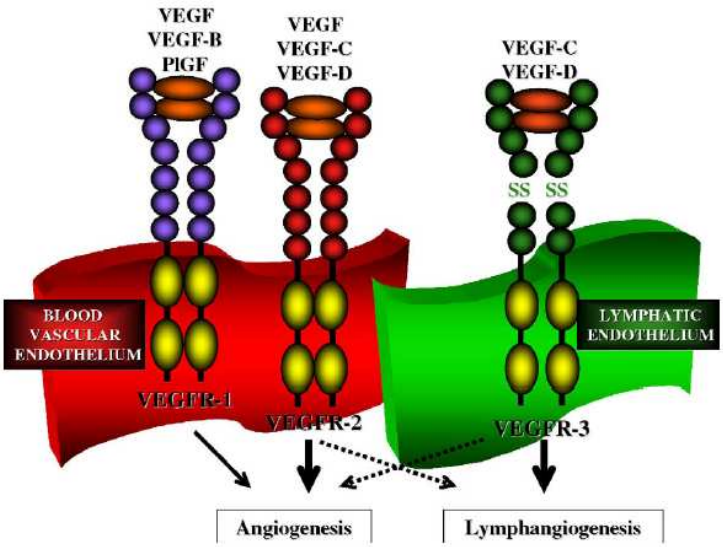


Figure 1.1. The VEGF family ligands and their receptors. VEGF, VEGF-B and PlGF bind VEGFR-1 and VEGFR-2 on the blood vascular endothelium. VEGF-C and VEGF-D primarily bind VEGFR-3 on lymphatic endothelium [104].

Although VEGF acts mostly on endothelial cells, it has been shown to also bind VEGF receptors on hematopoietic stem cells, monocytes, osteoblasts and neurons [105]. VEGF induces proliferation, sprouting, migration and tube formation of endothelial cells [105]. VEGF is also a potent survival factor for endothelial cells during physiological and tumour angiogenesis and it has been shown to induce the expression of antiapoptotic proteins in the endothelial cells.

When VEGF is overexpressed, it can contribute to disease. For instance, solid cancers cannot grow beyond a limited size without an adequate blood supply; cancers that can overexpress VEGF are able to grow and metastasize. Angiogenesis is stimulated by angiogenic factors released by tumour cells, though other cells, such as tumour-associated macrophages (TAMs), also contribute towards increasing the angiogenic process.

1.1. ANGIOGENESIS

Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels. It is a complex multistep process of growth and remodeling involving degradation of the extracellular matrix (ECM), cell migration and

proliferation, as well as tube formation. Under normal conditions, this process requires a balance between pro-angiogenic factors and anti-angiogenic factors. Angiogenesis also requires the activation of many receptors by their each cognate ligand. These ligands include vascular endothelial growth factors (VEGF), placental growth factor (PlGF), fibroblast growth factors (FGF-1 and -2), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), angiopoietins (Ang-1 and -2), epidermal growth factor/transforming growth factor-α (EGF/TGF-α) and others. VEGFs are known to play the most important role in angiogenesis.

2. Results of Chapter 2

2.1. C1P does not stimulate VEGF secretion in J774A.1 cells

We first studied the possible induction of VEGF secretion by C1P using J774A.1 macrophages. However, incubating these macrophages with different C1P concentrations and for different periods of time did not result in induction of VEGF release in these cells (Figure 2.1.1).

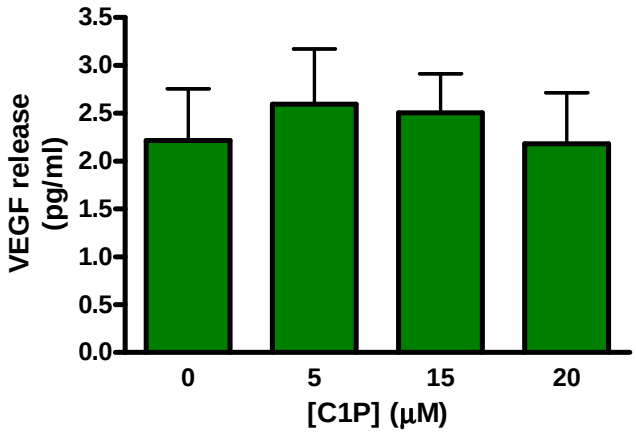


Figure 2.1.1. C1P does not stimulate VEGF secretion in J774A.1 cells. Cells were seeded (10^4 cells/well) in 24-well plates and incubated overnight in DMEM containing 10% FBS. The next day, the medium was replaced by DMEM supplemented with 1% FBS and after 2 h C1P was added at the indicated concentrations. After 24 h, the medium was collected and centrifuged. The VEGF concentration in the medium was measured using a "Mouse VEGF Quantikine" ELISA kit, as described in *Materials and Methods*. Results are expressed as the mean ± SEM of three independent experiments, performed in duplicate.

To evaluate whether the lack of effect of C1P to stimulate VEGF secretion was universal or might be dependent on macrophage type, we used Raw 264.7 cells. In Section 2.2, the effect of C1P on VEGF secretion is discussed.

2.2. C1P induces VEGF secretion in Raw 264.7 cells

We tested whether C1P could induce VEGF secretion in Raw 264.7 macrophages by incubating these cells with different concentrations of C1P for 24 h. It was observed that C1P did stimulate the release of VEGF in these cells (Figure 2.1.1).

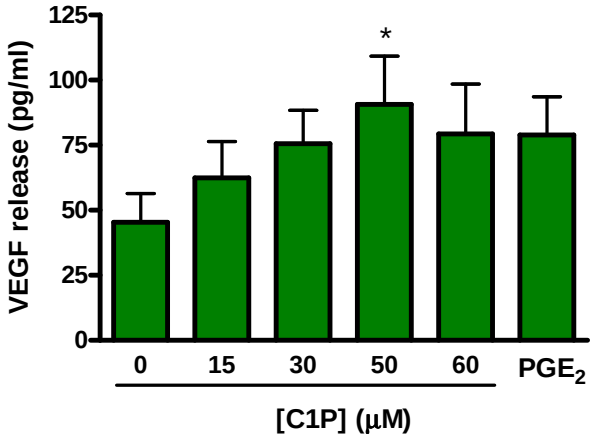


Figure 2.2.1. C1P stimulates VEGF secretion in a concentration-depending manner in Raw 264.7 cells. Cells were incubated in 6-well plates ($0.75 \cdot 10^6$ cells/well) overnight in DMEM supplemented with 10% FBS. The medium was then removed and the cells were incubated in DMEM supplemented with 1% FBS. After 2 h, cells were further incubated with the indicated concentrations of C1P or 100 ng/mL PGE_2 for 24 h. The medium was collected and centrifuged. The VEGF concentration in supernatants was measured and the results are expressed as the mean ± SEM of 4 independent experiments performed in duplicate (*$p<0.05$).

2.3. Involvement of PI3K in C1P stimulated VEGF release

To study the possible signalling cascades involved in C1P-induced VEGF release, pharmacological inhibitors were first used. The Akt inhibitor 10-DEBC was not inhibitory, whereas the general inhibitor of PI3K, Ly 294002, and ERK1-2 inhibitor (PD 98059) completely blocked C1P-stimulated VEGF secretion (Figure 2.3.1).

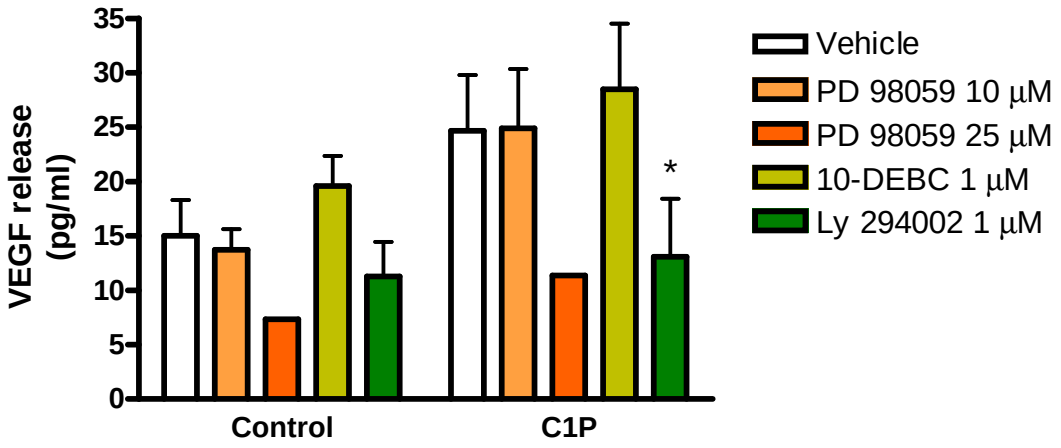


Figure 2.3.1. PI3K is involved in C1P-stimulated VEGF secretion Raw 264.7 cells. Cells were incubated in 6-well plates ($0.75 \cdot 10^6$ cells/well) overnight in DMEM containing 10% FBS. The next day, the medium was replaced by fresh DMEM supplemented with 1% FBS and the indicated inhibitors were added. After 30 min 50 µM C1P was added and the cells were further incubated for 24 h. The next day, the culture medium was collected and centrifuged and the cells were harvested in lysis buffer in order to measure protein concentration. The VEGF concentration in supernatants was measured and the results are expressed as the mean ± SEM of 4 independent experiments performed in duplicate (*$p<0.05$).

2.4. PI3K and ERK2 siRNAs inhibit C1P-stimulated VEGF release

In addition to pharmacological inhibition, we used siRNA technology to specifically silence the genes encoding for ERK (2), PKB (Akt 1 and 2) or PI3K. It was observed that ERK 2 siRNA, Akt 2 and PI3K siRNA significantly decreased C1P-stimulated VEGF release, whereas Akt 1 siRNAs had no effect, in agreement with the results observed using pharmacological inhibitors (Figure 2.4.1).

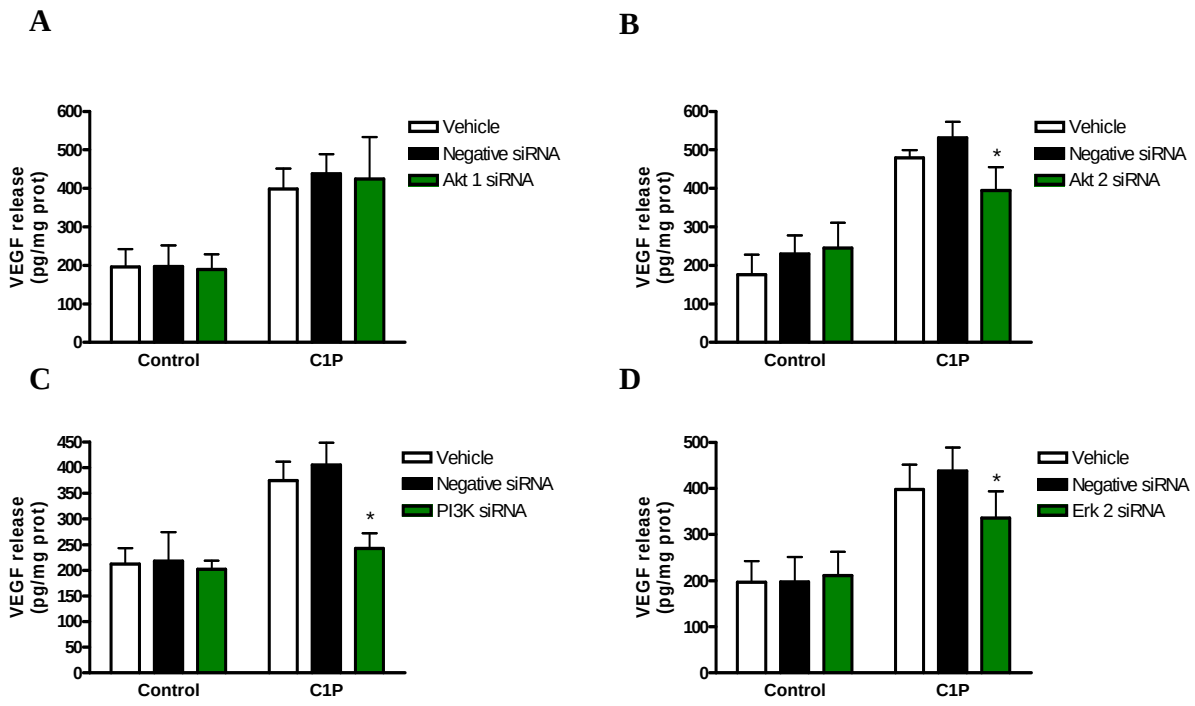
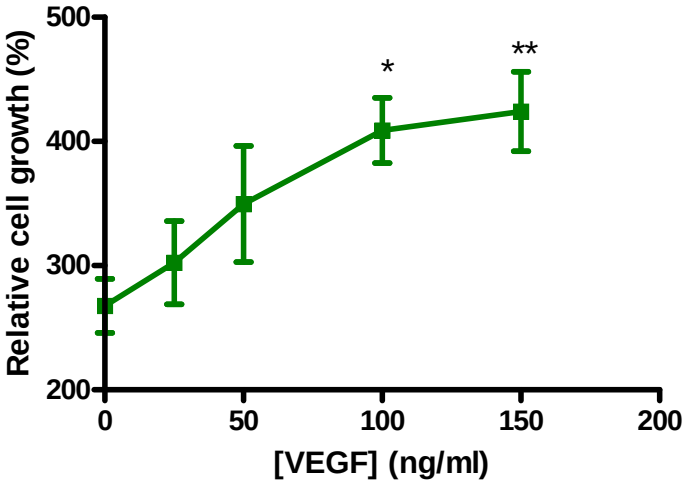


Figure 2.4.1. PI3K, Akt 2 and ERK 2 siRNA, but not Akt 1 siRNA, inhibit C1P-stimulated VEGF secretion in J774A.1 cells. Cells were seeded in 6-well plates (10^5 cells/well) and the siRNA treatment was performed as indicated in the *Materials and Methods* section. Cells were then treated with Akt 1 siRNA (panel A), Akt 2 siRNA (panel B), PI3K siRNA (panel C) or ERK2 siRNA (panel D) as well as with the control siRNA and the vehicle. After treatment with siRNA, the cells were incubated with fresh DMEM supplemented with 10% FBS for 24 h. Then, the medium was replaced by DMEM containing 1% FBS and after 2 h, the cells were treated with or without 50 µM C1P. After 24 h, the medium was collected and centrifuged and VEGF secretion was measured by ELISA, as mentioned before. Results are expressed as the mean ± SEM of 4 independent experiments (n.s.*>0.05; *p<0.05).

2.5. VEGF induces cell proliferation

Besides promoting angiogenesis, VEGF can exert many other functions. For instance, it has been reported that VEGF stimulates cell proliferation and migration in different cell types, including macrophages [106]. Therefore, we tested to see whether VEGF could induce proliferation of Raw 264.7 cells under our experimental conditions. Figure 2.4.2 shows that VEGF does stimulate proliferation of these cells in a manner that was concentration dependent.



2.5.1. VEGF induces Raw 264.7 macrophage proliferation. Cells were seeded (10^4 cells/well) in 96-well plates with DMEM supplemented with 10% FBS and they were incubated overnight. Then, the medium was replaced by DMEM containing 0% FBS with the indicated concentrations of VEGF. After 48 h, cell growth was determined using the MTS-formazan assay. Results are expressed as fold stimulation relative to the control value at 0 h and are the mean ± SEM of 6 independent experiments performed in triplicate (*$p < 0.05$; **$p<0.01$).

2.6. C1P induces VEGFR-2 phosphorylation

It is known that VEGF up-regulates the expression of VEGFR-2 in endothelial cells [107]. Therefore, we examined whether C1P could also increase the expression of VEGFR-2 in our cell system. We analyzed VEGFR-2 expression by Western blotting and observed that C1P did not induce a significant increase in VEGFR-2 expression at the indicated periods of time (Figure 2.6.1). Nonetheless, we also studied the activation of this receptor measuring the phosphorylation of the protein. Following its binding to VEGF, VEGFR-2 dimerizes and undergoes autophosphorylation on tyrosine residues within its cytoplasmic portion. Specifically, we studied the phosphorylation in Tyr 1175 residue, which is known to initiate the activation of the MAP kinase/ERK pathway that regulates cell proliferation [108]. We observed that C1P significantly increases the phosphorylation level of this residue (Figure 2.6.1).

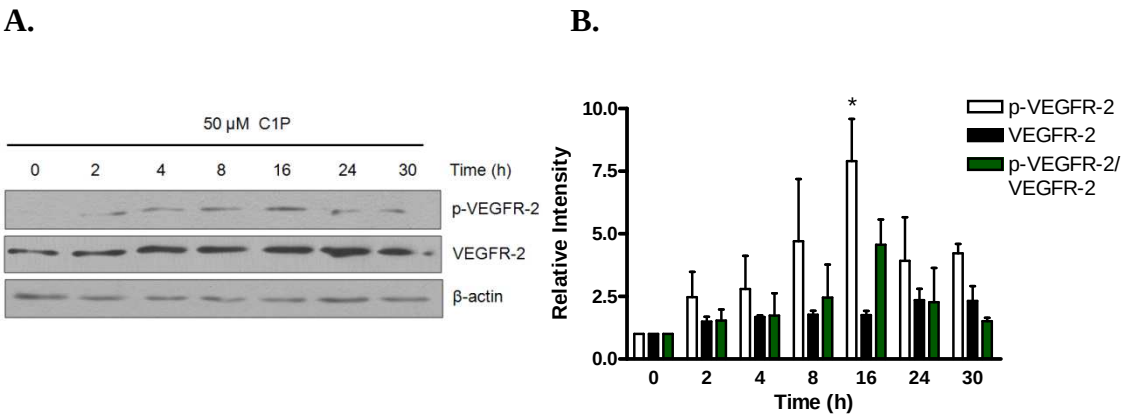


Figure 2.6.1. C1P induces VEGFR-2 phosphorylation. Cells were seeded (10^6 cells/plate) in 60 mm diameter plates with DMEM supplemented with 10% FBS and they were incubated overnight. Then, the medium was replaced by DMEM containing 1% FBS and the cells were incubated in the presence of 50 µM C1P for the indicated periods of time. **A.** Cells were then harvested in homogenization buffer and samples were analyzed by Western blotting. The presence of p-VEGFR-2 and VEGF was determined using a specific antibody to p-VEGFR-2 and VEGFR-2, respectively. Equal loading of protein was monitored using a specific antibody to β-actin. **B.** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity of the phosphorylated protein, the total protein and the ratio of phosphorylated and total protein. Data are expressed as the mean ± SEM of 3 independent experiments (*$p<0.05$).

2. Discussion of Chapter 2

Angiogenesis is essential for the growth of solid tumours because without stimulating new vessel formation, oxygen and nutrients cannot be supplied to the cells inside the tumour. VEGF is a key factor for the stimulation of angiogenesis, but it is also a potent stimulator of cell growth. Therefore, apart from improving nutrient uptake for tumour cells, it can also contribute to survival of malignant cells. In this connection, C1P can also contribute to cell survival but it has never been implicated in angiogenetic processes.

Here we demonstrate that C1P can induce VEGF secretion in Raw 264.7 cells, but not in J774A.1 macrophages, suggesting that this action is cell specific. Furthermore, we demonstrate that C1P-stimulated VEGF secretion is mediated through activation of PI3K. Also, Akt2 and ERK 2 seem to be involved in this action since silencing these genes significantly decreases C1P-stimulated VEGF secretion. However, the fact that

siRNA to Akt1 is not inhibitory and that siRNA to Akt2 only inhibits C1P-stimulated VEGF secretion partially suggests the existence of other Akt isoforms involved in this process.

Moreover, we have observed that VEGF induces cell proliferation in Raw 264.7 cells and that C1P stimulates the phosphorylation of the Tyr1175 from the VEGFR-2 receptor. When this residue is phosphorylated, it triggers the activation of the MAP kinase/ERK pathway which leads to cell proliferation in endothelial cells. Thus, it seems that there could be a link between C1P-stimulated cell proliferation and VEGF secretion in Raw 264.7 macrophages.

Chapter 3

6. CHAPTER 3

Generation of Reactive Oxygen Species (ROS) is a key factor for stimulation of macrophage proliferation by ceramide 1-phosphate

1. Introduction

Reactive oxygen species (ROS) are molecules with unpaired valence electrons that can oxidize many cell components with high reactivity. ROS are generated in a wide variety of biological contexts, both physiological and pathological, and in nearly all tissues and organs of multicellular organisms, including animals, plants, and even microbes. ROS are being constantly produced in various normal cellular events and aerobic respiration conforms their major source. ROS produced during these events are generally counteracted by several antioxidant compounds, including proteins. A large amount of ROS can also be produced by inflammatory processes, ionizing radiation, and many chemotherapeutic drugs. If the production of ROS exceeds the capacity of the antioxidant proteins, this may cause the so-called “oxidative stress”; in a biological sense, the oxidative stress may be broadly defined as an imbalance between oxidant production and the antioxidant capacity of the cell to prevent oxidative injury.

While severe oxidative stress is associated with the activation of cell repair mechanisms, apoptosis and sometimes necrosis, smaller perturbations in oxidant burden appear to be involved in growth factor or other receptor-mediated signalling pathways (Figure 1.1).

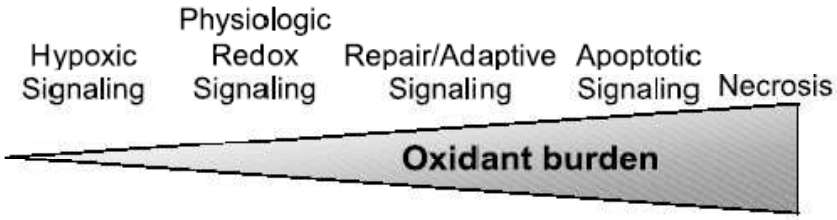


Figure 1.1. The relationship of oxidant burden to biological response (from [109]).

Oxidative stress is known to be implicated in many human diseases, including atherosclerosis, neurodegenerative diseases, aging and cancer. However, there is still a debate on whether oxidative stress is a cause or a consequence of these diseases, mainly due to a lack of our understanding of the mechanisms by which ROS function in both physiological and pathological conditions.

ROS play an important role maintaining cellular homeostasis and cell signalling processes because they can activate a wide variety of cell responses, including cell proliferation and apoptosis.

There are several potential sources of ROS in most cells, including the mitochondria, cytochrome P450-based enzymes, xanthine oxidase, uncoupled NO synthases and NADPH oxidases (nicotinamide adenine dinucleotide phosphate-oxidase) (also known as NOX). Among these, the NADPH oxidases appear to be especially important for modulating the activity of diverse intracellular molecules and signalling pathways. In macrophages, several enzymes are now recognized as being potentially able to produce ROS, perhaps the most important of these is NADPH oxidase [110]. NADPH oxidase is a membrane-bound multicomponent enzyme that catalyzes the electron transfer from the substrate (NADPH) to O_2, leading to generation of superoxide anions (Figure 1.2).

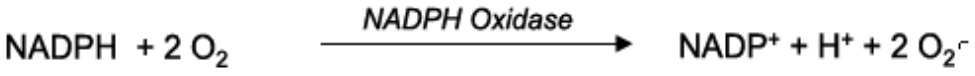


Figure 1.2. The enzymatic reaction catalyzed by NADPH oxidase.

There are seven different NADPH oxidases in the NOX family: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2. In macrophages, the most expressed member of NOX family is NOX2. This enzyme consists of a membrane-associated cytochrome that comprises two subunits, gp91*phox* ('phox' being derived from phagocytic oxidase) and p22*phox*. Besides these, there are at least three cytosolic subunits (p47*phox*, p67*phox* and p40*phox*) and a low-molecular-weight G protein (Rac2). Upon cell stimulation, the cytosolic subunits form a complex which migrates to the plasma membrane where it binds to the membrane-associated subunits. Only after the assembling of all subunits, the enzyme is catalytically active (Figure 1.3).

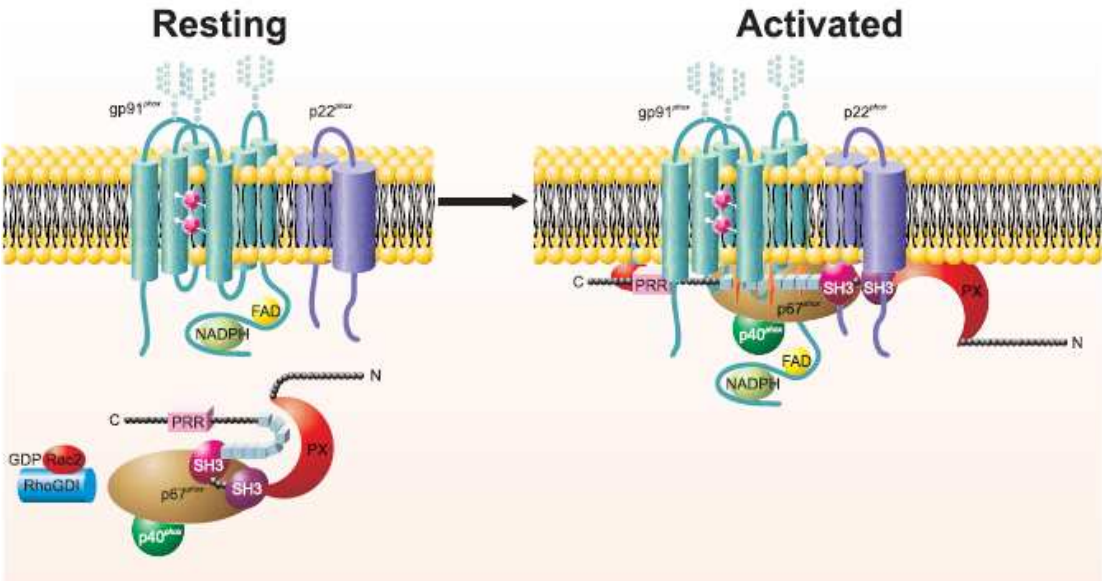


Figure 1.3. Assembly and activation of the phagocyte NADPH oxidase. In resting phagocytes, heterodimeric gp91phox-p22phox resides in the membrane, whereas the complex of p47phox-p67phox-p40phox is cytosolic. Agonist-triggered phosporylation of the autoinhibitory domain of p47phox (series of small boxes) releases a conformational restriction, making interactive protein motifs, including the PX domain, Src homology 3 regions (SH3), and proline-rich regions (PRR), in p47phox accessible to associate at the membrane to mediate oxidase assembly [111].

It has been reported that some proinflammatory cytokines and growth factors, such as TNF-α, IL-1β, TGF-β, M-CSF, PDGF, EGF, generate ROS as second messengers in the corresponding signalling pathways [112]. However, growth factor independent stimulation of cell proliferation by ROS has also been demonstrated [113]

Macrophage colony-stimulating factor (M-CSF) is a secreted cytokine that can induce the differentiation of hematopoietic cells into macrophages. This cytokine is also essential for the survival of BMDM in vitro. Besides, it has been reported that M-CSF induces ROS generation in human monocytes and that ROS activate the MAPK/ERK pathway leading to monocyte proliferation [114].

2. Results of Chapter 3

2.1. C1P induces ROS generation in BMDM

A major goal of this study was to determine whether ROS production was related to the mitogenic effect of C1P. This hypothesis was tested using primary bone marrow-derived macrophages (BMDM) as the biological system. Primary cells are preferentially used over immortalized cell lines to study cell proliferation because the latter are usually genetically modified and so long-term treatments with agonists may render unrealistic results.

Figure 2.1.1 shows that C1P stimulates ROS production in a concentration- (panel A) and time-(panel B) dependent manner at concentrations previously found to stimulate macrophage growth (20-50 μM).

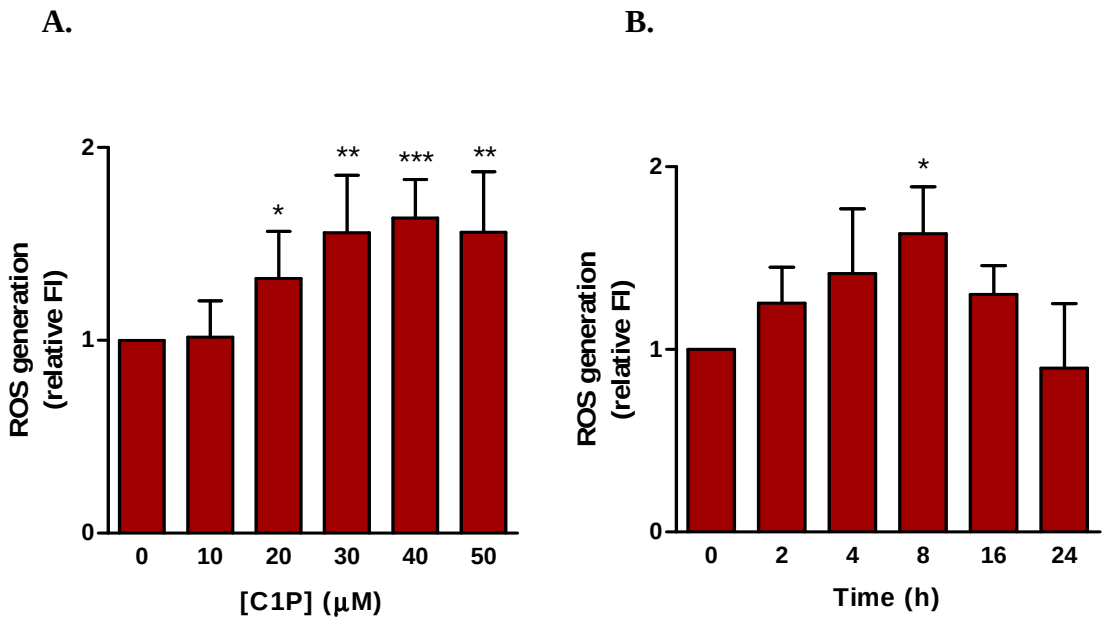


Figure 2.1.1. Intracellular ROS generation induced by C1P. BMDM were pre-incubated for 16 h in 1.5% L-cell conditioned medium, as the source of M-CSF. **A**. BMDMs were incubated with C1P at the indicated concentrations for 8 h. After incubation, ROS production was determined by measuring the fluorescence intensity (FI) of CM-H2DCFDA. Data are expressed as the fluorescence intensity (FI) relative to the control value and are the mean ± SEM of 6 replicate experiments. **B.** Cells were incubated with 40 μM C1P for the indicated periods of time. ROS generation was measured as indicated above. The results are shown as fluorescence intensity relative to control value and are the mean ± SEM of 4 independent experiments performed in duplicate (*p<0.05; **p < 0.01; ***p< 0.001).

2.2. NADPH oxidase is the major ROS source in macrophages treated with C1P

There are several sources of ROS generation in cells. The principal source in vivo results from leakage of electrons from the mitochondrial respiratory chain (*i.e.* from intermediate electron carriers onto molecular oxygen) during oxidative metabolism to generate ATP. However, examination of ROS generation in mitochondria-free cell homogenates obtained from C1P-treated macrophages revealed that ROS production was not dependent upon the presence of mitochondria in these cells (Fig. 2.2.1).

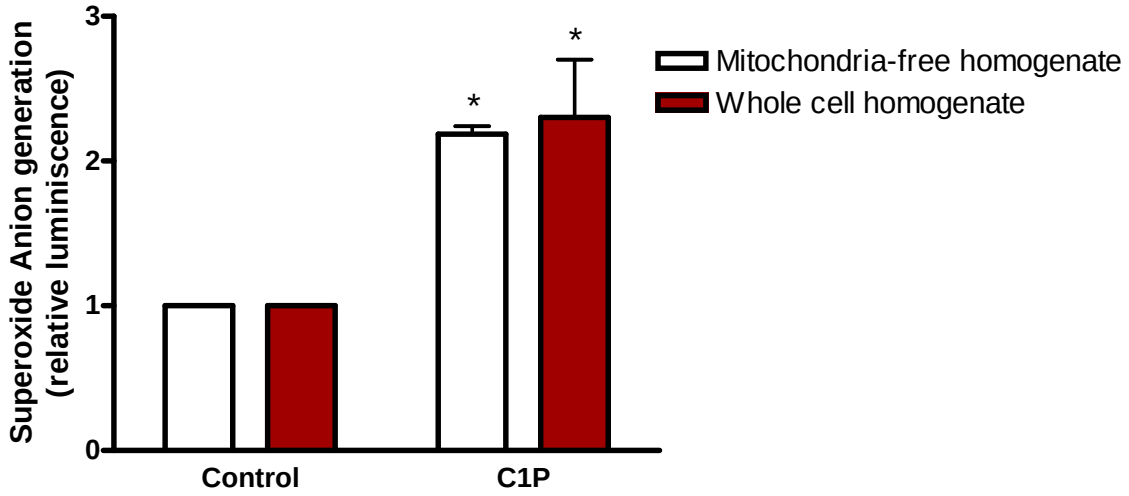


Figure 2.2.1. C1P induces superoxide anion generation. BMDMs were incubated as indicated above and treated with or without 40 μM C1P for 8 h. After treatment the cells were lysed with a Dounce homogenizer and the generation of superoxide anions was detected with the "superoxide anion assay" kit, according to the manufacturer's instructions. In order to measure the generation of superoxide anions in a mitochondria-free system (empty bars), cell homogenates were centrifuged at 10.000 × g for 30 min. Results are expressed as relative luminescence and are the mean ± SEM of 4 independent experiments performed in duplicate (*$p<0.05$).

Furthermore, rotenone (an inhibitor of the mitochondrial complex I respiratory chain), and thenoyltrifluoroacetone (TTFA, an inhibitor of the mitochondrial complex II respiratory chain) did not alter C1P-stimulated ROS production in the macrophages (Figure 2.2.2.). Other major sources of ROS generation result from the action of specific enzymes, including xanthine oxidases, nitric oxide synthases, or cytochrome P450. However, inhibitors of these enzymes such as allopurinol (an inhibitor of xanthine oxidase), NG-

nitro-L-arginine methyl ester (L-NAME, an inhibitor of nitric oxide synthase) or 1-aminobenzortriazole (ABT, an inhibitor of cytochrome P450 enzymes), did not affect C1P-stimulated ROS production (Figure 2.2.2).

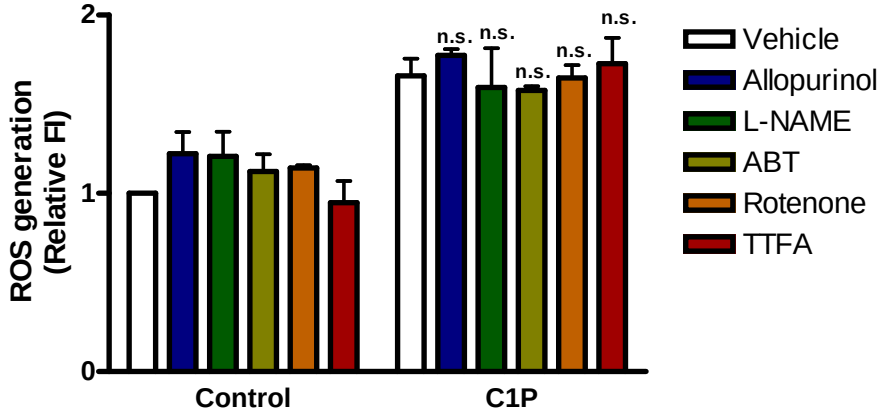


Figure 2.2.2. Xanthine oxidase, iNOS, cytochrome P450 and mitochondrial electron transport chain complex I and II do not seem to be implicated in C1P-stimulated ROS generation. Cells were pre-incubated with vehicle, 10 µM allopurinol, 0.5 mM L-NAME, 50 µM ABT, 0.5 µM rotenone or 10 µM TTFA for 30 min and then treated with C1P (40 µM). ROS generation was determined after 8 h of incubation. Results are indicated as the fluorescence intensity relative to the control value and are the mean ± SEM of 3 independent experiments performed in duplicate (n.s. $p > 0.05$).

Although all of the former enzymes are critical for the generation of ROS in different cell types, the first known example of regulated ROS generation in mammalian cells was through the respiratory burst of phagocytic cells by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [110, 115]. For this reason, we studied the possible implication of this enzyme in C1P-stimulated ROS generation. As mentioned before, this is a multicomponent enzyme which requires the assembly of all subunits to be activated. Key events for activating NADPH oxidase are phosphorylation of the p40phox subunit and translocation of p47phox from the cytosol to the plasma membrane. Therefore, we examined whether these two actions were elicited by C1P. First, we observed a time-dependent increase of the p47phox presence in the membranes, which is consistent with translocation of this subunit from the cytosol (Figure 2.2.3).

A. **B**

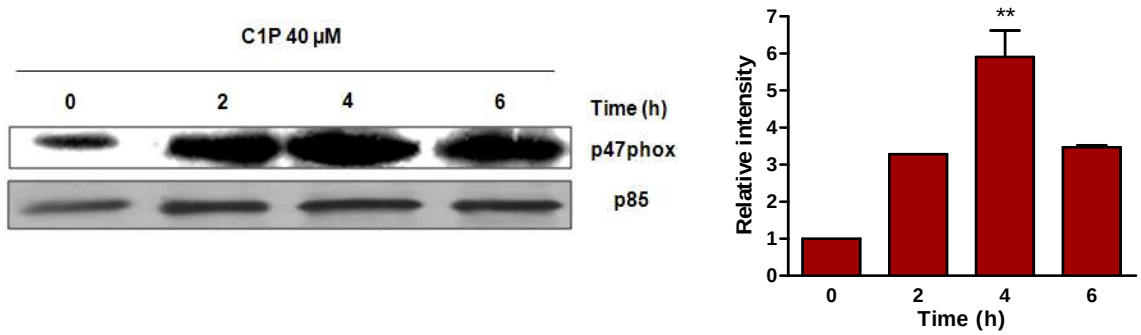


Figure 2.2.3. C1P promotes the translocation of p47phox subunit into the microsomal fraction. BMDMs were pre-incubated for 16 h in 1.5% L-cell conditioned medium. **A.** BMDM were treated with 40 µM C1P for the times that are indicated. The presence of p47phox in the microsomal fraction was determined by Western blotting using an antibody specific to this subunit. Equal loading of protein was monitored using a specific antibody to p85. **B.** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SEM of 3 replicate experiments, except for the 4 h time point, which is the mean ± SEM of 4 replicate experiments (**$p < 0.01$).

Using a similar approach we also observed that p40phox was phosphorylated after incubation of BMDM with 40 µM C1P (Figure 2.2.4).

A. **B.**

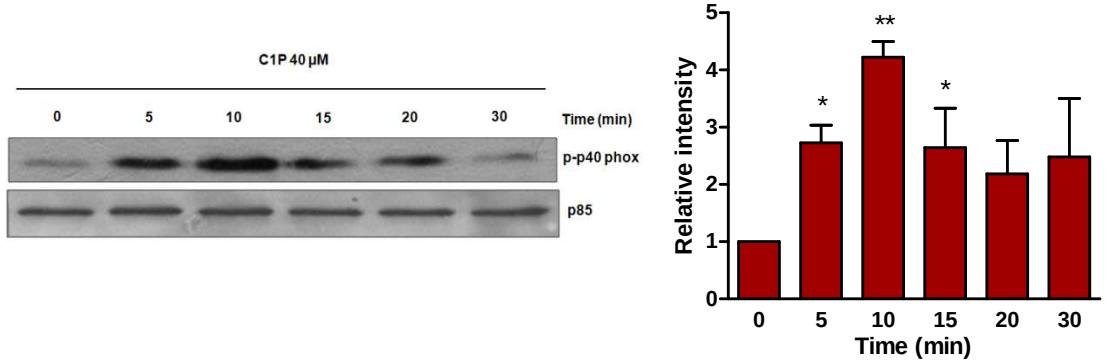


Figure 2.2.4. C1P induces phosphorylation of the p40phox subunit in a time-depending manner. BMDMs were pre-incubated for 16 h in 1.5% L-cell conditioned medium. **A.** BMDMs were treated with 40 µM C1P for the indicated period of time. The presence of p-p40phox was determined by Western blotting using an antibody specific to this subunit. Equal loading of protein was monitored using a specific antibody to p85. **B.** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SEM of 4 replicate experiments (*$p < 0.05$; **$p < 0.01$).

To evaluate whether NADPH oxidase is the major mechanism for the production of ROS by the macrophages we used different inhibitors of this activity. We found that inhibition of NADPH oxidase with the selective inhibitors DPI or apocynin [116, 117] completely blocked C1P-stimulated ROS production (Figure 2.2.5).

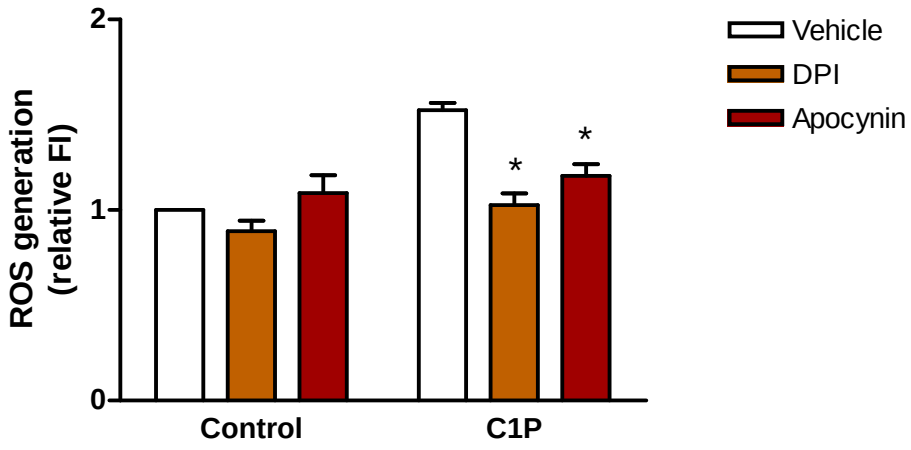


Figure 2.2.5. NADPH oxidase inhibitors reduce C1P-stimulated ROS production. BMDMs were pre-incubated with 0.5 mM apocynin or 0.5 µM DPI for 30 min. Then 40 µM C1P was added and ROS production was determined after 8 h of incubation by measuring the fluorescence intensity of CM-H2DCFDA. The results are expressed as fold of stimulation over the control value and are the mean ± SEM of 3 independent experiments performed in duplicate (*$p<0.05$).

2.3. C1P-stimulated ROS production is essential for the proliferative effect of C1P

Although primary cultures are considered to be an appropriate model to study the mechanisms involved in macrophage proliferation, transfection of these cells is highly inefficient [118]. Therefore, we used chemical inhibitors to study the contribution of NADPH oxidase-derived ROS to macrophage growth. NADPH oxidase inhibitors DPI and apocynin, as well as the general antioxidant NAC, completely blocked C1P-stimulated DNA synthesis, as determined by the incorporation of [^{3}H]thymidine into DNA (Figure 2.3.1A). Furthermore, macrophage growth, as measured by using the MTS-formazan assay [57, 69], was also inhibited (Figure 2.3.1B).

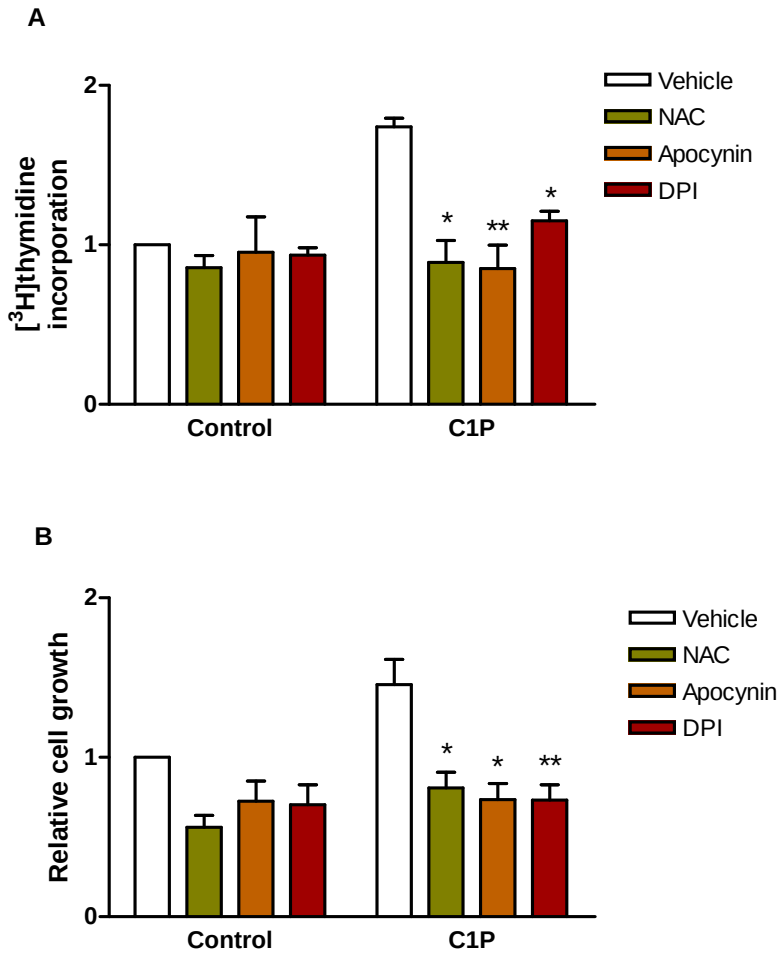


Figure 2.3.1. C1P-stimulated cell proliferation was blocked by ROS inhibitors. BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium **A**. Cells were pre-incubated with 0.5 mM NAC, 0.5 mM apocynin or 0.5 µM DPI for 30 min before C1P addition. Then, cells were stimulated with 40 µM C1P for 24 h. The incorporation of [^{3}H]thymidine into DNA was determined as indicated in the *Materials and Methods* section. Results are expressed as fold stimulation relative to the control value without inhibitor, and shown as the mean ± SEM of 3 independent experiments performed in triplicate. **B.** Cells were pre-incubated with indicated inhibitors for 30 min and then stimulated with 40 µM C1P for 24 h. Cell growth was determined using the MTS-formazan assay. Results are expressed as fold the stimulation relative to the control value and are the mean ± SEM of 6 independent experiments performed in triplicate (*$p<0.05$; **$p < 0.01$).

It is well established that a major type of ROS produced by phagocytic cells is hydrogen peroxide (H_2O_2), which is originated mainly from enzymatic dismutation catalyzed by superoxide dismutase (SOD) [119]. Several lines of evidence have demonstrated that exposure of cells to low (non-toxic) levels of H_2O_2 can exert a stimulatory effect on cell proliferation [120-122]. In agreement with this, we found that incubation of macrophages

with low H_2O_2 concentrations (within the nanomolar range) resulted in a significant increase of DNA synthesis in BMDM (Figure 2.3.2).

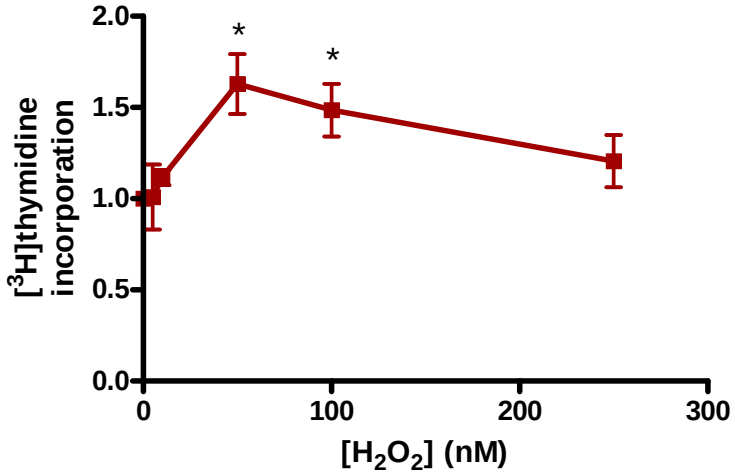


Figure 2.3.2. Hydrogen peroxide induces cell growth in BMDM. BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium. BMDMs were then treated with indicated concentrations of hydrogen peroxide. DNA synthesis was determined by measuring the incorporation of [3H]thymidine into DNA as described in the *Materials and Methods* section. Results are expressed as fold stimulation relative to the control value (without inhibitors) and shown as the mean ± SEM of 3 independent experiments performed in triplicate (*$p<0.05$).

Taken together, these results suggest that the NADPH oxidase/ROS axis is essential for the stimulation of cell proliferation by C1P in primary macrophages.

2.4. cPLA$_2$α is upstream of NADPH oxidase activation in the stimulation of cell proliferation by C1P

It was reported previously that C1P activates cPLA$_2$α potently in A549 lung adenocarcinoma cells [71] and NR8383 alveolar macrophages [123], and that this phospholipase is important for endothelial cell cycle progression and proliferation. Besides, it was described that cPLA$_2$α is implicated in the translocation of p47phox into the plasma membrane [124], which is an indication of NADPH oxidase activation.

We studied the phosphorylation state of cPLA$_2$α after incubation of macrophages with C1P. Phosphorylation of cPLA$_2$ was detected after 1 h of incubation and it was maintained up to at least 5 h (Figure 2.4.1).

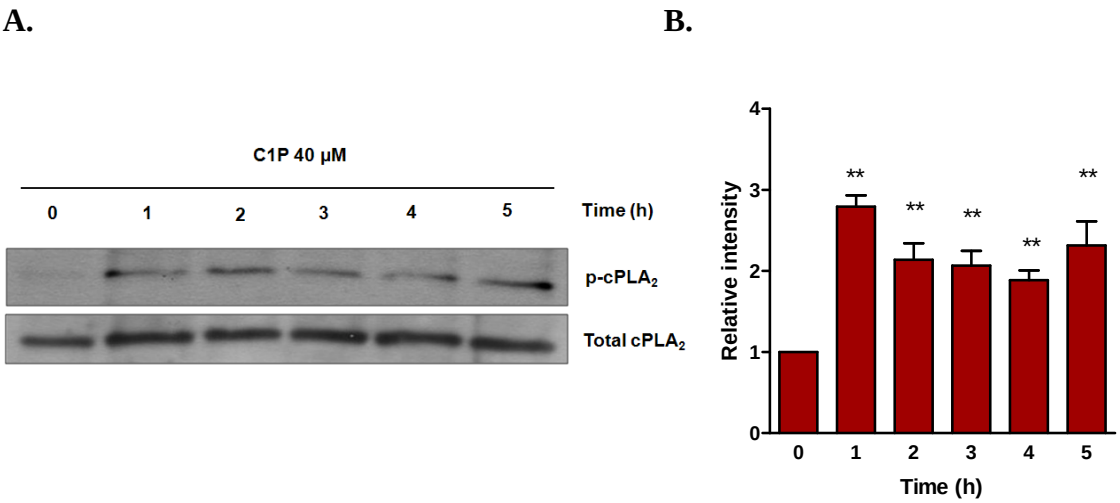
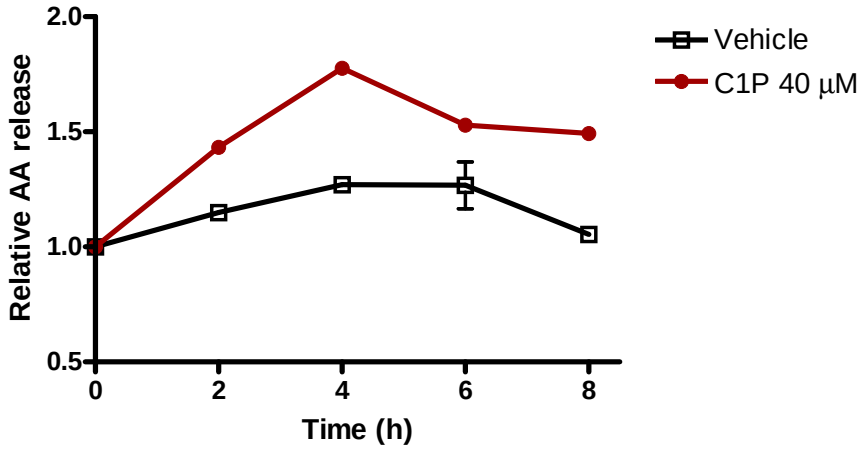


Figure 2.4.1. **C1P stimulates cPLA2α phosphorylation.** BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium **A**. Cells were then incubated with 40 µM C1P for indicated period of time and harvested in homogenization buffer. The presence of phosphorylated cPLA$_2$α in cell homogenates was analysed using an antibody specific to phospho-cPLA2α. Equal loading of protein was monitored using a specific antibody to total cPLA$_2$α. Similar results were obtained in each of 4 replicate experiments. **B**. Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and results are the mean ± SEM of 5 independent experiments (**p < 0.01).

cPLA$_2$α phosphorylation is necessary for the activation of NADPH oxidase. In addition, it has been reported that C1P is a direct agonist of cPLA$_2$α; therefore, we tested to see whether C1P could induce arachidonic acid (AA) secretion to the extracellular medium in our macrophage cell system.

C1P induced a significant release of AA after 4 h of incubation with 40 µM C1P in the macrophages and this was time and concentration dependent (Figure 2.4.2.A and B).

A.



B.

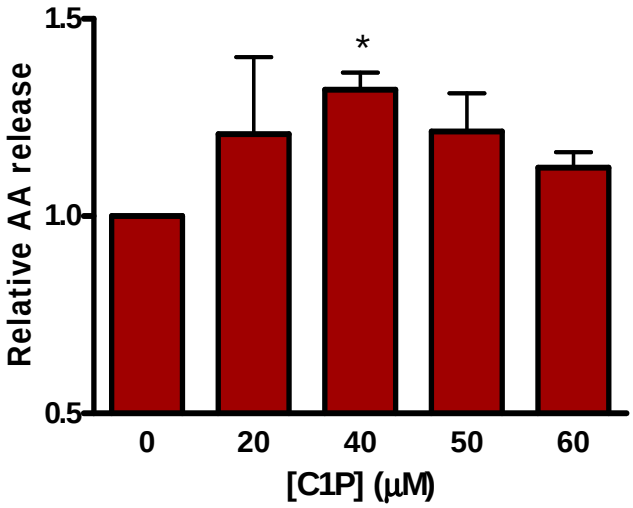
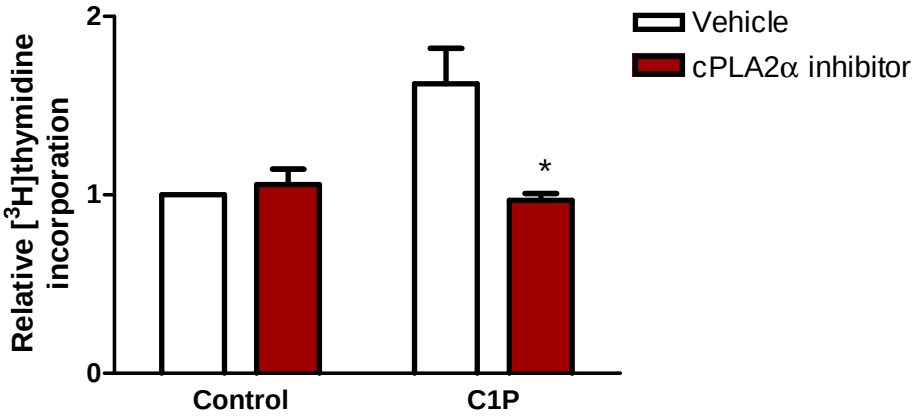


Figure 2.4.2. C1P induces the release of arachidonic acid in BMDM. BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium. Cells were then incubated overnight with 0.25 µCi [3H]AA/well. **A.** Cells were incubated with (red circles) or without (empty squares) 40 µM C1P for the indicated incubation times. The medium and the cells were harvested and the AA release was determined as indicated in the *Materials and Methods* section. Results are expressed as fold of AA release relative to the control value at 0 h, and are the mean ± SEM of 3 independent experiments performed in triplicate. **B.** Cells were incubated for 4 h with the indicated concentrations of C1P. The cells and the culture medium were collected separately and the AA release was determined as indicated in the *Materials and Methods* section. Results are expressed as fold of released AA relative to the non-treated control and are the mean ± SEM of 3 independent experiments performed in triplicate (*$p<0.05$).

To study whether cPLA2α activation was important for the mitogenic effect of C1P, we used a selective cPLA2α inhibitor, pyrrolidine-2. Figure 2.4.3A and B show that this inhibitor completely blocked C1P-stimulated DNA synthesis and cell growth, indicating that cPLA$_2$α is critical for the stimulation of macrophage proliferation by C1P.

A.



B.

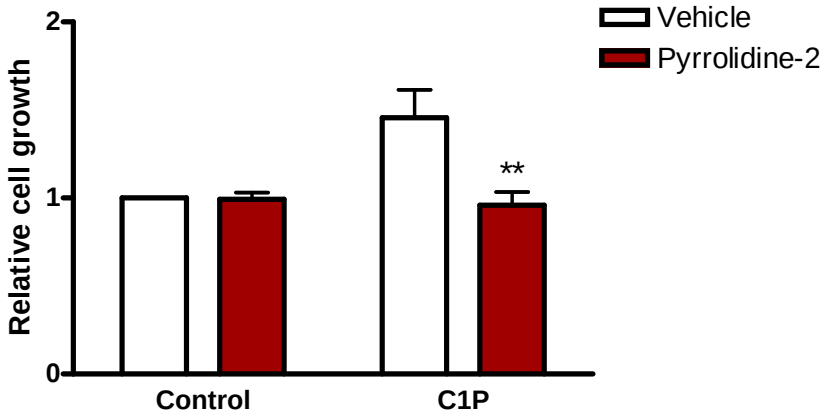


Figure 2.4.3. C1P-stimulated macrophage proliferation is inhibited by the selective cPLA$_2$α inhibitor, pyrrolidine-2. BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium. Macrophages were then pre-incubated with 2.5 μM pyrrolidine-2 for 30 min before C1P addition. Then, cells were stimulated with 40 μM C1P for 24 h. **A**. The incorporation of [^{3}H]thymidine into DNA was determined as indicated in the *Materials and Methods* section. Results are expressed as fold stimulation relative to the control value without inhibitor, and showed as the mean ± SEM of 3 independent experiments performed in triplicate. **B.** Cell growth was determined using the MTS-formazan assay. Results are expressed as fold stimulation

relative to the control value and are the mean ± SEM of 6 independent experiments performed in triplicate (**p < 0.01).

Since both ROS generation and cPLA$_2$α activation are involved in the mitogenic effect of C1P, we sought to determine if there is a link between these two events. Pyrrolidine-2 completely blocked C1P-induced ROS generation (Figure 2.4.4), suggesting that cPLA$_2$α activation is also a relevant factor in this process. This observation also indicates that cPLA$_2$α activation is upstream of ROS production in the cascade of events triggered by C1P in BMDM.

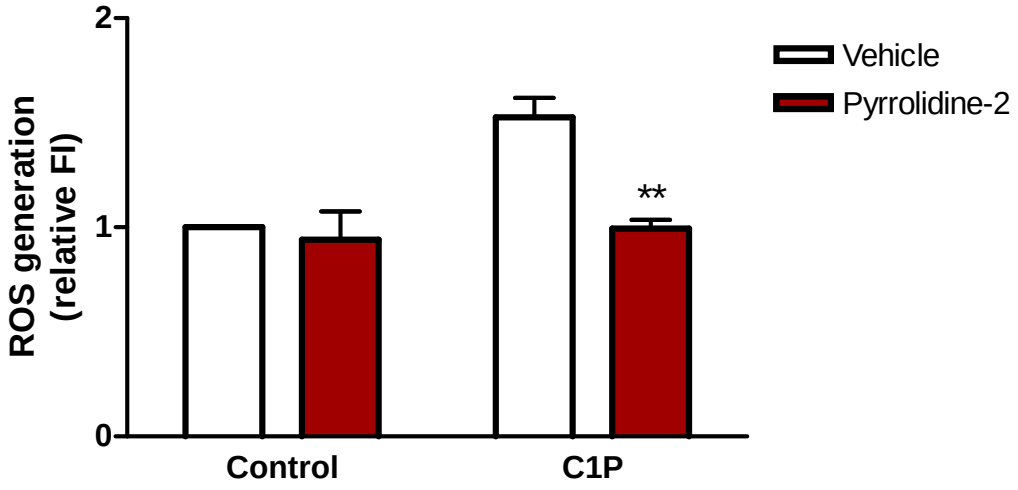


Figure 2.4.4. The cPLA$_2$α selective inhibitor pyrrolidine-2 reduces C1P-stimulated ROS generation. BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium. Cells were then pre-incubated with 2.5 µM pyrrolidine-2 for 30 min and then treated with 40 µM C1P. ROS generation was determined after 8 h of incubation. Results are indicated as the fluorescence intensity relative to the control value and are the mean ± SEM of 3 experiments performed in duplicate (**p < 0.01).

In addition to this, exogenously added AA significantly increased intracellular ROS generation obtaining ROS generation values to a similar extent to that of C1P (Figure 2.4.5). This result suggests that the enzymatic product of cPLA$_2$α, AA, might be implicated in ROS generation.

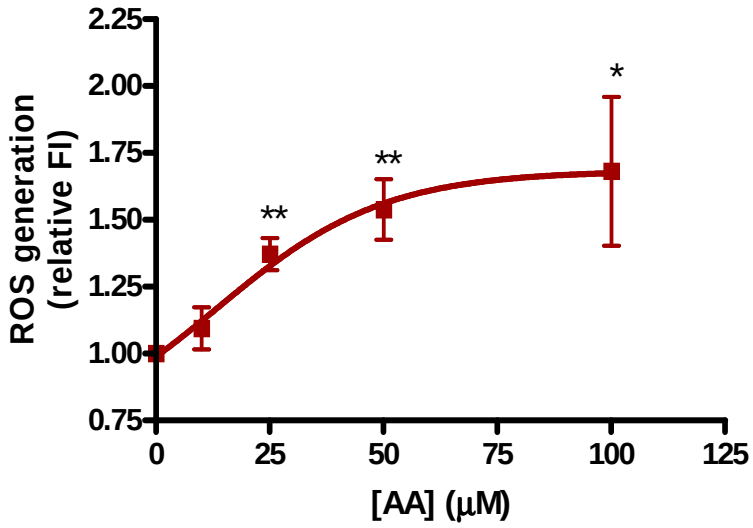


Figure 2.4.5. Exogenously added AA induces ROS generation. BMDMs were pre-incubated for 24 h in RPMI 1640 supplemented with 10% FBS and 1.5% L-conditioned medium for 24 h. AA was added exogenously at the indicated concentrations and after 6 h ROS generation was measured as indicated before. Results are expressed as fluorescence intensity relative to the control value and are the mean ± SEM of 7 independent experiments performed in duplicate (*p<0.05; **p<0.01).

2.5. PKCα is implicated in C1P-stimulated ROS generation

It has been reported that PKCα activates cPLA$_2$α [125]. Therefore, studies were conducted to determine whether PKCα might be implicated in the generation of ROS by C1P.

There are many PKC isoforms and therefore, there are also many PKC inhibitors. However, the phorbol ester 4b-phorbol 12-myristate 13-acetate (PMA) is known to downregulate conventional PKC isoforms very efficiently after prolonged incubations (16-24 h) [126-129]. We tested the inhibitory action of this compound by measuring PKC activity in the membrane and cytosolic fraction. We observed that PKC activity decrease after PMA overnight incubation, both in the cytosolic and in the microsomal fractions (Figure 2.5.1).

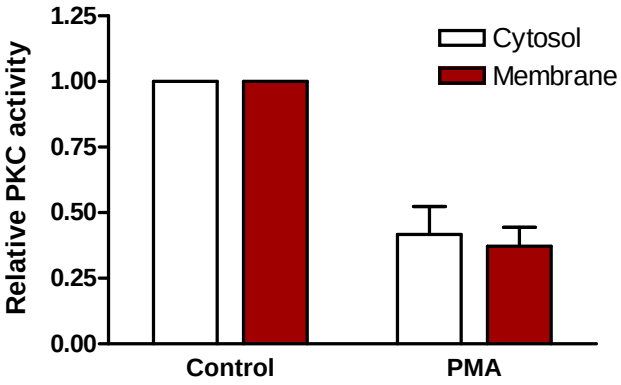


Figure 2.5.1. Overnight incubation with 100 nM PMA reduces PKC activity in BMDM. BMDMs were incubated overnight in the presence or absence of PMA (100 nM). Cells were harvested and fractioned as mentioned in *Materials and Methods* section. PKC activity was determined using a commercial kit from Calbiochem according to the manufacturer's instructions. Results are expressed as the fold of stimulation relative to control value and are the means ± SEM of three independent experiments performed in duplicate.

Therefore, PMA was used as a general inhibitor for conventional PKC and Go6976 or dequalinium were used as selective inhibitors of PKCα. It is known that the most abundant PKC isoform in cells is PKCα, so we used the selective inhibitors Go6976 and dequalinium to inhibit this enzyme activity.

We observed that C1P-stimulated ROS generation was completely inhibited by Go6976 (Figure 2.5.2A) and this was also the case when the macrophages were incubated for 24 h in the presence of 100 nM PMA to downregulate PKC (Figure 2.5.2B).

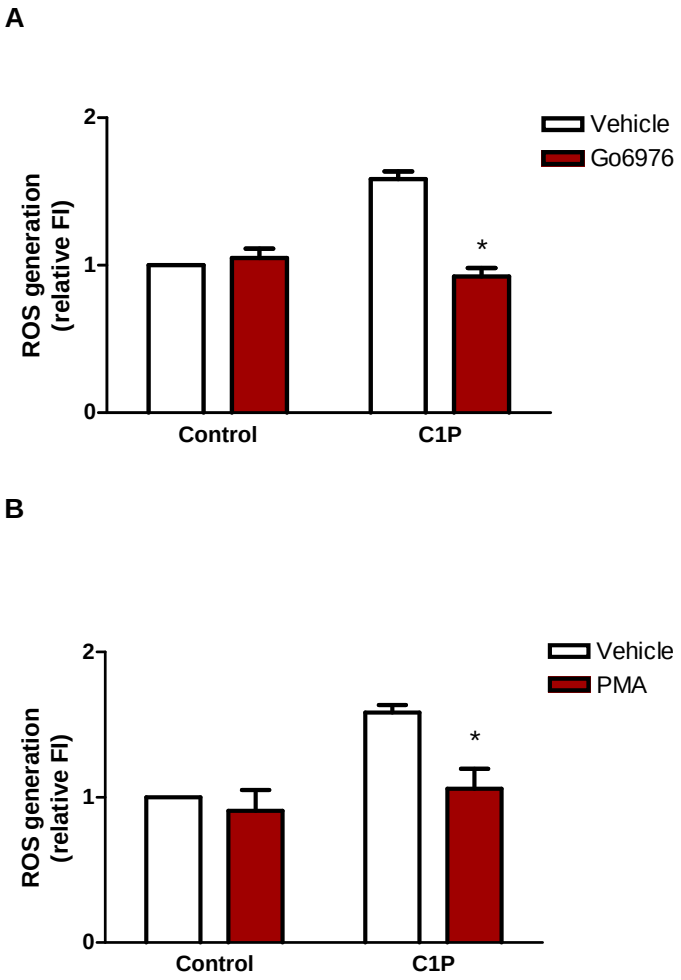


Figure 2.5.2. C1P-induced ROS generation is blocked by PKC inhibitors. BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium. **A.** Cells were pre-incubated for 30 min with 50 nM Go6976 and ROS generation was measured after 8 h of incubation with 40 µM C1P. Results are indicated as the fluorescence intensity relative to the control value and are the mean ± SEM of 5 independent experiments performed in duplicate. **B.** Cells were pre-incubated for 16 h with 100 nM PMA and then 40 µM C1P was added. After 8 h, ROS generation was determined as indicated above. Results are the mean ± SEM of 3 experiments performed in duplicate (*$p<0.05$).

2.6. cPLA$_2$α and PKCα are implicated in C1P-induced NADPH oxidase activation

The above data led us to hypothesize that intervention of cPLA$_2$α and PKCα might be critical factors for the activation of NADPH oxidase by C1P. This was further studied using the selective inhibitors of these enzymes on p47phox translocation and p40phox phosphorylation.

Inhibition of cPLA$_2$α or PKCα blocked C1P-stimulated p47phox translocation (Figure 2.6.1) as well as phosphorylation of p40phox (Figure 2.6.2).

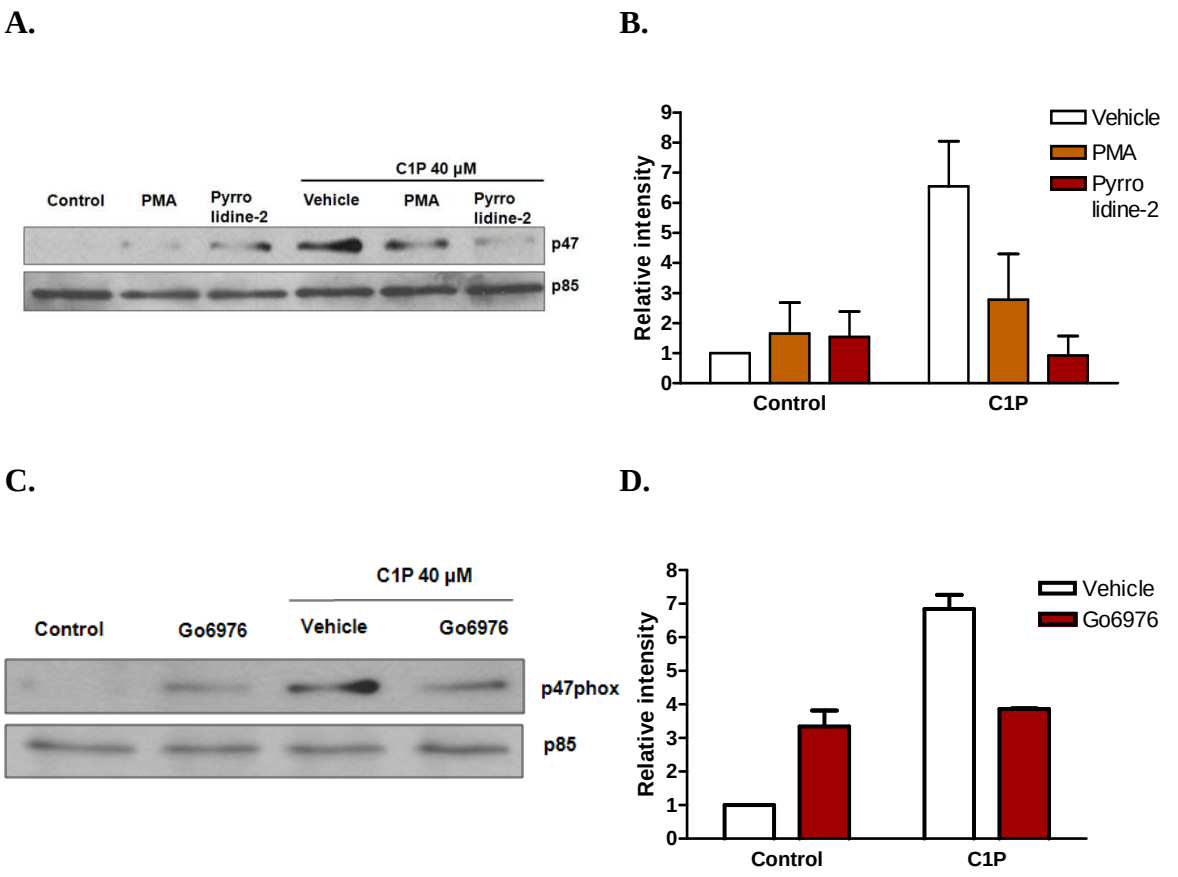


Figure 2.6.1. Inhibition of p47phox translocation by pyrrolidine-2 and inhibitors of PKC. A. Cells were incubated for 30 min with vehicle, 100 nM PMA (for 16 h) or 2.5 μM pyrrolidine-2 prior to C1P addition and after 4 h of incubation with 40 μM C1P, the cells were harvested. Cell lysates were ultracentrifuged and the microsomal fraction was collected in order to analyse the presence of p47phox by Western blotting. Equal loading of protein was monitored using a specific antibody to p85. Similar results were obtained in each of 2 replicate experiments. **B.** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SD of 2 replicate experiments. **C.** Cells were pre-incubated with 50 nM Go6976 (for 30 min) and then 40 μM C1P was added. After 4 h of incubation, the cells were collected, lysed and ultracentrifuged. The presence of p47phox in the microsomal fraction was analysed by Western blotting and equal loading of protein was monitored using a specific antibody to p85. **D.** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SD of 2 replicate experiments.

A. **B.**

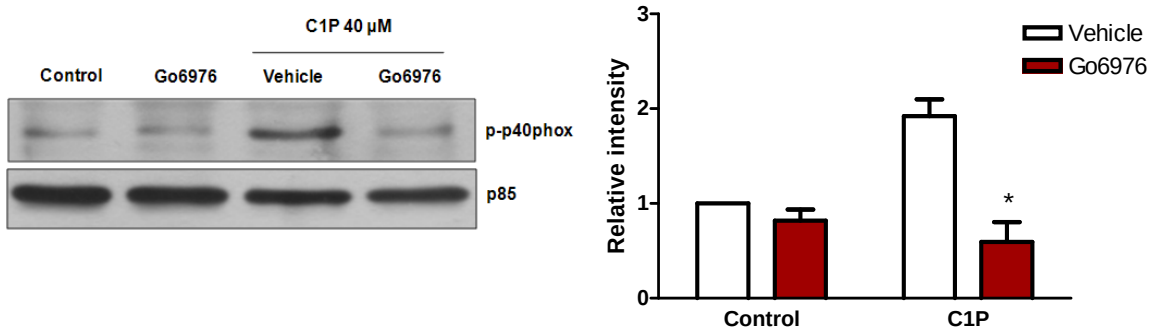


Figure 2.6.2. C1P-stimulated p40phox phosphorylation is inhibited by the PKC selective inhibitor, Go6976. A. Cells were pre-incubated with 50 nM Go6976 for 30 min and then incubated with 40 μM C1P for 10 min. Then, cells were collected and lysed. The presence of phosphorylated-p40phox was determined by Western blotting using an antibody specific to phospho-p40phox. Equal loading of protein was monitored using a specific antibody to total p40phox. **B.** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SEM of 3 replicate experiments (*$p<0.05$).

These results suggest that PKCα and cPLA$_2$α are implicated in C1P-stimulated NADPH oxidase activation.

2.7. PKCα is upstream of cPLA$_2$α in C1P-induced NADPH oxidase activation

In order to test which is the relationship between cPLA$_2$α and PKCα, we studied cPLA$_2$α activity using PKCα inhibitors (Go6976 and PMA overnight incubation). Most likely, activation of PKCα is upstream of cPLA$_2$α stimulation, since the PKCα inhibitor Go6976 was able to completely block cPLA$_2$α activity (Figure 2.7.1).

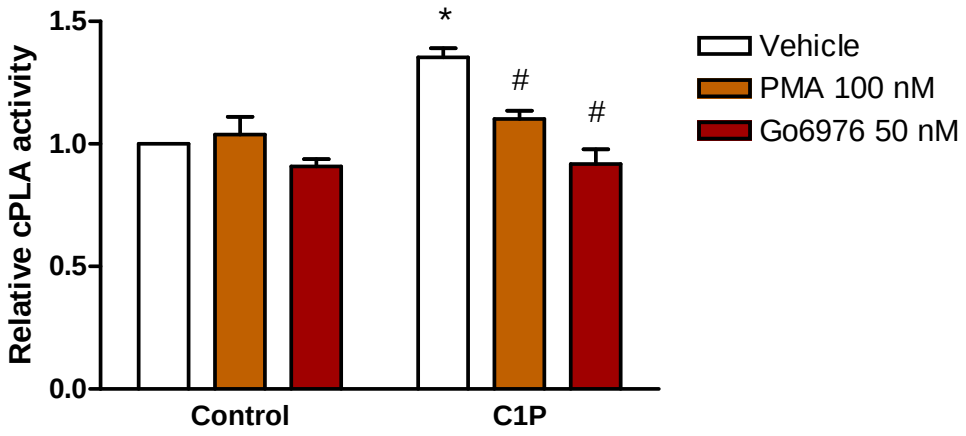


Figure 2.7.1. **C1P-stimulated cPLA activation was inhibited by PKC inhibitors**. Cells were pre-incubated with 100 nM PMA for 16 h or with 50 nM Go6976 for 30 min prior to C1P addition. After 4 h of incubation with 40 µM C1P, the cells were washed and harvested in cold buffer and cPLA activity was measured as indicated in *Materials and Methods* section. The cPLA activity in the cells without treatment was 5.12 µmol/min/mg protein ± 0.49 SEM. Results are expressed as fold of stimulation relative to the control value and shown as the mean ± SEM of 4 independent experiments. (*$p<0.05$ C1P-treated cells versus control value; #$p<0.05$ PMA- or Go6976-treated cells versus C1P-treated cells).

2.8. C1P induces PKCα translocation and increases PKC activity

Previous results indicated us that PKCα was implicated in C1P-stimulated cell growth, so that we studied PKCα activation with optimal C1P for cell proliferation, 50 µM [57].

PKCα is a serine/threonine kinase and a member of the conventional (classical) PKCs (cPKCs), which have four conserved (C1 to C4) regions. This ubiquitously expressed PKC isoform is activated in response to many different kind of stimuli and it translocates from the cytosol to specialized cellular compartments including the nucleus, caveolae, or the plasma membrane where it is presumed to be activated. Activation of PKCα has been implicated in a variety of cellular functions including proliferation, apoptosis, differentiation, motility, and inflammation [130, 131].

Because PKCα must be translocated to the membrane compartments in order to exert its function, we first studied this possibility. We observed that C1P significantly stimulated PKCα translocation to the microsomal fraction (Figure 2.8.1).

A. **B.**

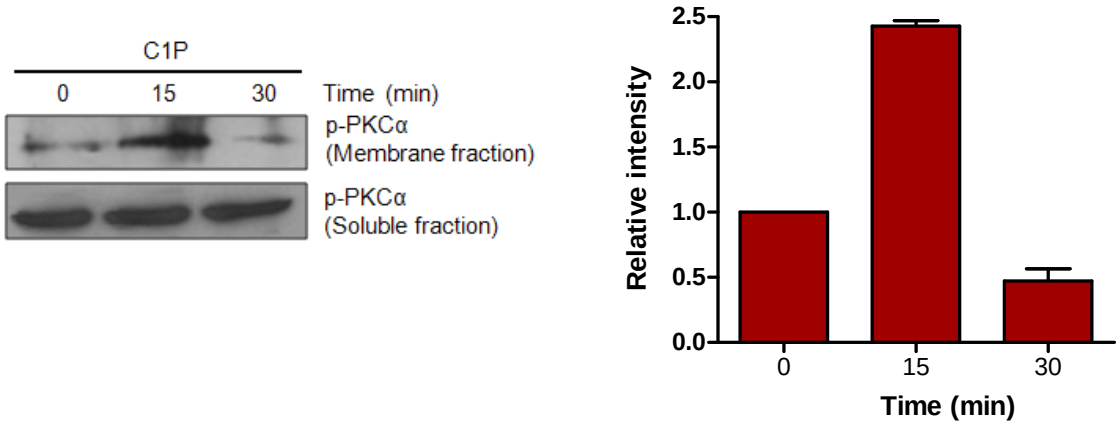


Figure 2.8.1. C1P stimulates PKCα translocation to the microsomal fraction. **A.** BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium prior to treatment with C1P (50 µM) for the indicated times and the microsomal fraction was obtained as mentioned in the *Materials and Methods* section. The presence of p-PKCα in the microsomal fraction was determined by Western blotting using an antibody specific to p-PKCα. Results are representative of one experiment and were confirmed in an additional experiment performed in triplicate. **B.** Results of scanning densitometry of the exposed films of the microsomal fraction. Data are expressed as arbitrary units of intensity relative to control value and are the means ± SD of two replicate experiments.

Although the increase in PKCα was obvious in the microsomal fraction, this was not the case for the cytosolic fraction. The reason for this might be that PKCα is very abundant in the cytosol, and small changes in its mass may not be easily detected. Therefore, to ensure that PKCα also decreased in the cytosolic fraction we determined its enzymatic activity. We found that C1P significantly decreased PKC activity in the cytosolic fraction, and this was in agreement with the increased activity of PKCα observed in the membrane fraction (Figure 2.8.2). This effect of C1P was reproduced by a short incubation (15 min) with PMA, a well-known activator of PKC.

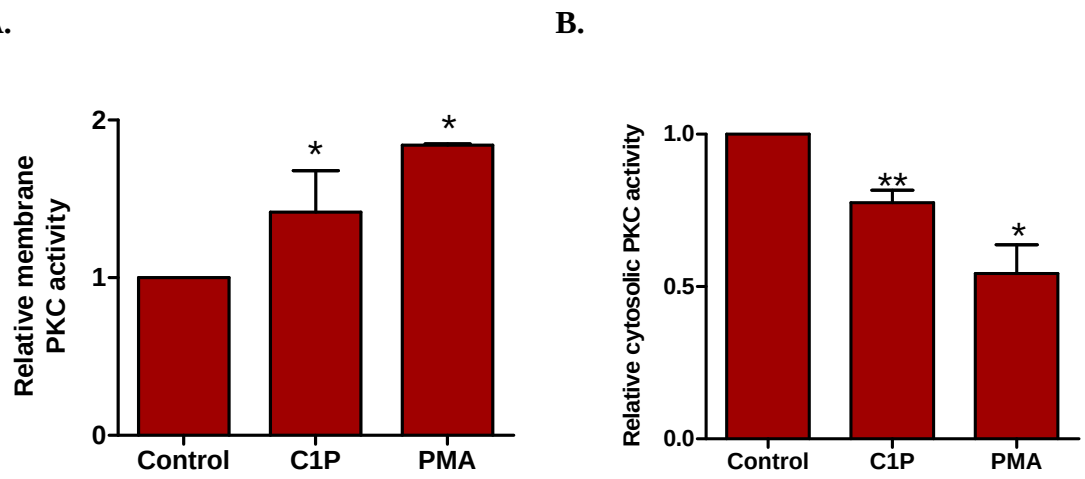


Figure 2.8.2. C1P enhances PKC activity in the microsomal fraction while this activity is reduced in the cytosolic fraction. BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium and treated with 50 µM C1P or 100 nM PMA for 15 min. The microsomal fraction (**A)** and the soluble (cytosolic) fraction (**B)** were separated and the PKCα activity was measured using a commercial kit from Calbiochem according to manufacturer's instructions. Results are expressed as the fold of stimulation relative to the control value and are the mean ± SEM of 3 independent experiments performed in duplicate for microsomal fraction (A) and mean ± SEM of 5 independent experiments performed in duplicate for the cytosolic fraction (B) (*$p < 0.05$; **$p < 0.01$).

It is well established that optimal catalytic output of PKC isoforms requires phosphorylation on their activation loops. In particular, Ser 657 is a critical site for phosphorylation of PKCα because it controls the accumulation of phosphate at other sites on PKCα [132]. Therefore, we tested to see whether C1P was able to induce phosphorylation of this important site of PKCα. We observed that C1P promoted phosphorylation of PKCα at Ser 657 in a time dependent manner, with maximal effect attained at 10–15 min of incubation (Figure 2.8.3.).

A. **B.**

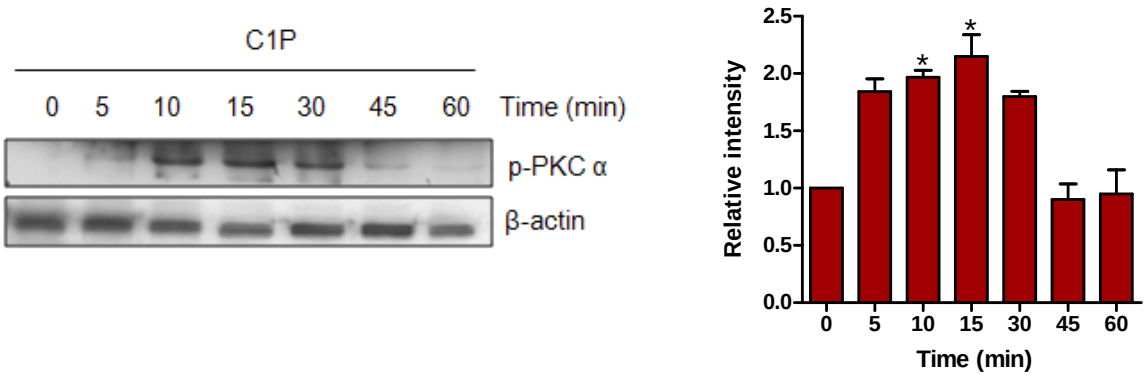


Figure 2.8.3. C1P stimulates PKCα phosphorylation. A. BMDMs were treated with 50 µM C1P for the indicated times. The phosphorylation of PKCα was examined by Western blotting. Equal loading of protein was monitored using a specific antibody to β-actin. Similar results were obtained in each of three replicate experiments. **B.** Results of scanning densitometry of the exposed films. Data are expressed as arbitrary units of intensity relative to control value and are the mean ± SEM of three independent experiments (*p< 0.05) (This experiment was performed in collaboration with Dr. Patricia Gangoiti).

As expected, phosphorylation of PKCα was sensitive to inhibition by dequalinium, a selective inhibitor of this PKC isoform (Figure 4.8.4).

A. **B.**

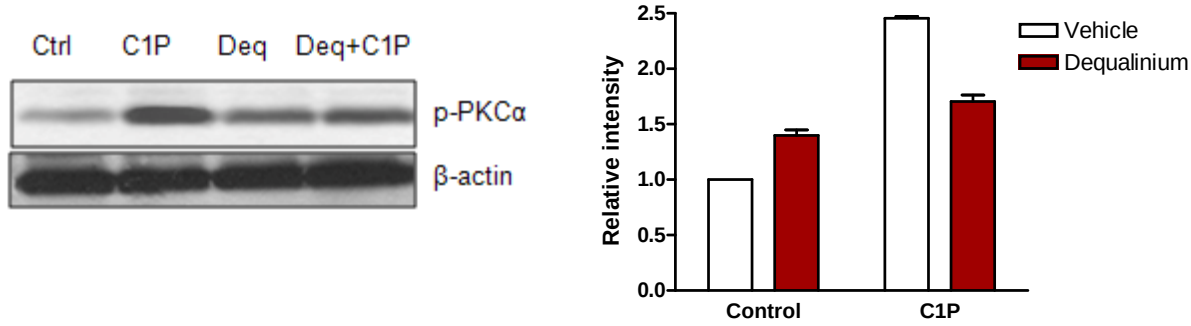


Figure 2.8.4. Dequalinium inhibits C1P-stimulated PKC-a phosphorylation. A. BMDMs were treated with vehicle (empty bars) or 1 µM dequalinium (deq) for 30 min (filled bars) in the presence or absence of 50 µM C1P for 10 min. The phosphorylation of PKCα was examined by Western blotting. Equal loading of protein was monitored using a specific antibody to β-actin. Similar results were obtained in each of three replicate experiments. **B.** Results of scanning densitometry of the exposed films. Data are expressed as

arbitrary units of intensity relative to the control value and are the mean ± SEM of three replicate experiments (*p< 0.05).

2.9. Phosphatidylinositol specific phospholipase C (PI-PLC) is not implicated in C1P-stimulated cell proliferation

It is well established that diacylglycerol (DAG) is the physiological activator of conventional and novel PKCs, including PKCα. DAG can be produced after stimulation of a variety of signal-activated enzymes, including different phospholipases. A major phospholipase involved in PKC activation is phosphatidylinositol specific phospholipase C (PI-PLC), which generates DAG and inositol phosphates directly by degrading phosphatidylinositol (PI). However, inhibition of this enzyme with the selective inhibitor U73122 did not significantly alter C1P-stimulated DNA synthesis (Figure 2.9.1). In addition, intracellular Ca^{2+} levels remained unchanged after treatment with C1P, as previously reported [57].

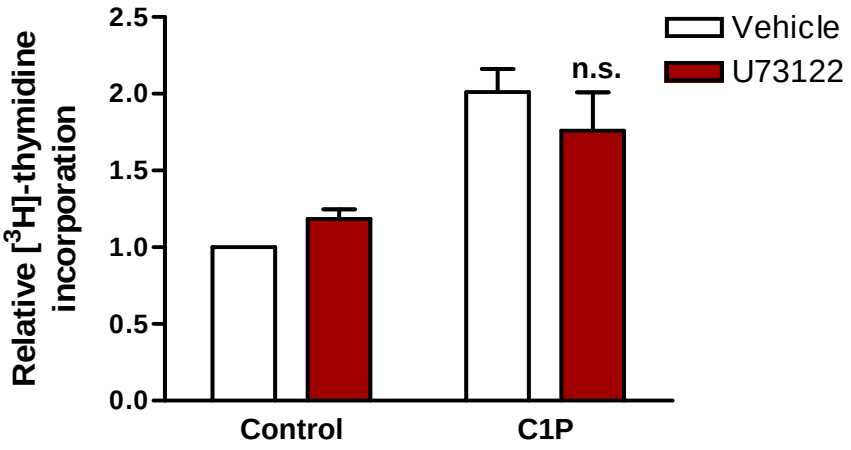


Figure 2.9.1. **PI-PLC is not implicated in C1P-stimulated DNA synthesis.** BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium, and then treated with or without 1 μM U73122 for 30 min prior to addition of vehicle or 50 μM C1P for 24 h. The incorporation of [^{3}H]thymidine into DNA was determined as mentioned in the *Materials and Methods* section. Results are expressed as the fold of stimulation relative to the control value without inhibitor, and shown as the mean ± SEM of four independent experiments performed in triplicate (n.s.p> 0.05).

2.10. C1P increases sphingomyelin synthase (SMS) activity. SMS is implicated in C1P-stimulated cell proliferation

Another enzyme capable of generating DAG is sphingomyelin synthase (SMS), which catalyzes the transfer of phosphocholine from phosphatidylcholine (PC) to ceramide forming sphingomyelin (SM). Interestingly, C1P increased SM levels after 10 min of incubation (Figure 2.10.1).

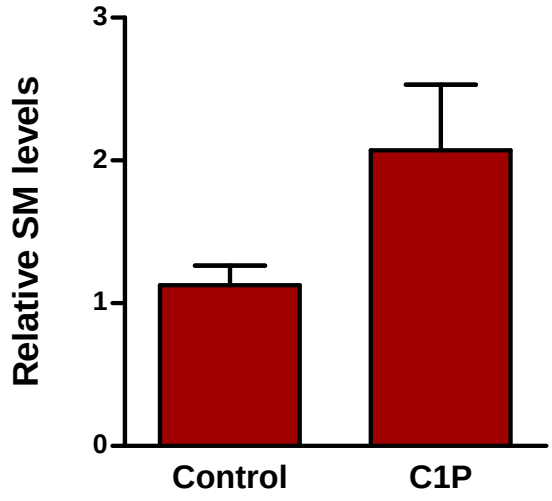


Figure 2.10.1. C1P increases SMS level in BMDM. BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium, and then 50 µM C1P was added for 10 min. SM levels were measured as indicated in the *Materials and Methods* section. Results are expressed as the fold of stimulation relative to the control value, and are the mean ± SEM of 3 independent experiments performed in triplicate. Basal SM levels were 7358 ± 793 dpm (mean ± SEM, n = 3) per million cells.

To evaluate whether this enzyme activity was involved in the stimulation of cell proliferation by C1P we used tricyclodecan-9-yl-xanthogenate (D609), a compound initially shown to inhibit PC-PLC but that is a potent inhibitor of SMS [133, 134]. D609 completely blocked the incorporation of [^{3}H]thymidine into DNA and the phosphorylation of PKCα that were stimulated by C1P (Figure 2.10.2).

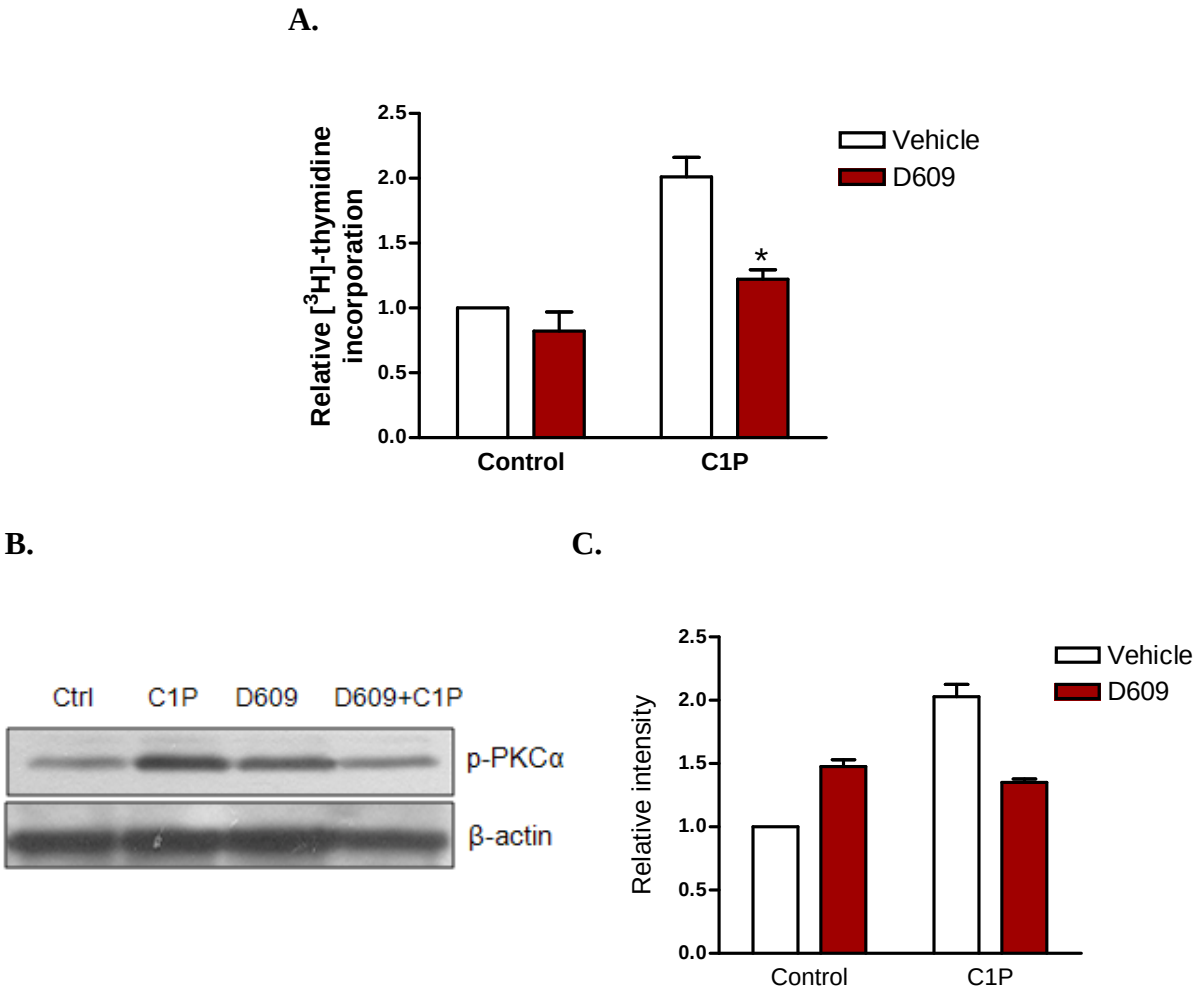


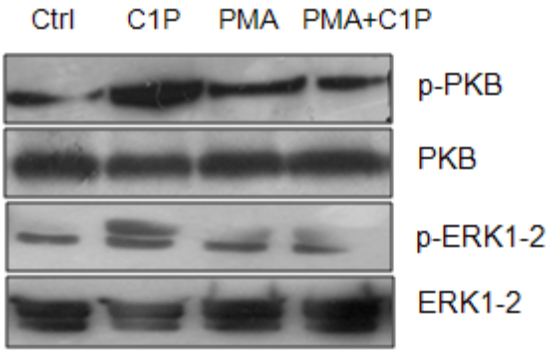
Figure 2.10.2. Involvement of SMS in C1P-stimulated DNA synthesis and PKCα phosphorylation. A. BMDMs were treated with or without 20 µM D609 for 30 min prior to addition of vehicle or 50 µM C1P for 24 h. The incorporation of [^{3}H]thymidine into DNA was determined as indicated in the *Materials and Methods* section. Results are expressed as the fold of stimulation relative to the control value without inhibitor, and are the means ± SEM of 4 independent experiments performed in triplicate. **B.** Involvement of SMS in C1P-induced PKCα phosphorylation. BMDMs were treated as in panel A. Phosphorylation of PKCα was examined by Western blotting using a specific antibody to p-PKCα. Equal loading of protein was monitored using a specific antibody to β-actin. Similar results were obtained in each of 3 independent experiments. **C.** Results of scanning densitometry of the exposed films. Data are expressed as arbitrary units of intensity relative to the control value and are the mean ± SEM of 3 replicate experiments (*p< 0.05).

2.11. C1P-stimulated PKCα activation is upstream of PKB and ERK1-2 activation

We have previously reported that C1P exerts its proliferative effect through ERK and PKB pathways [57]. Therefore, we tested to see whether PKCα could be upstream of ERK and

PKB pathways. To answer this question, we used PKC inhibitors (prolonged incubation with PMA or dequalinium, a selective PKCα inhibitor) as well as the SMS inhibitor, D609. We observed that inhibitors of both enzymes decreased C1P-stimulated phosphorylation of PKCα (Figure 2.11.1).

A.



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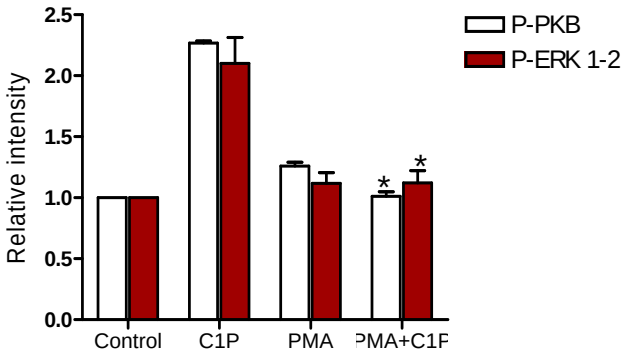
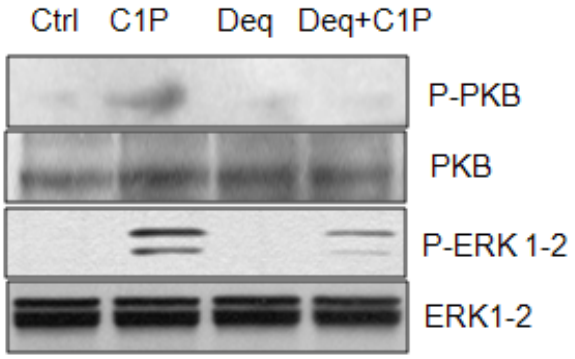
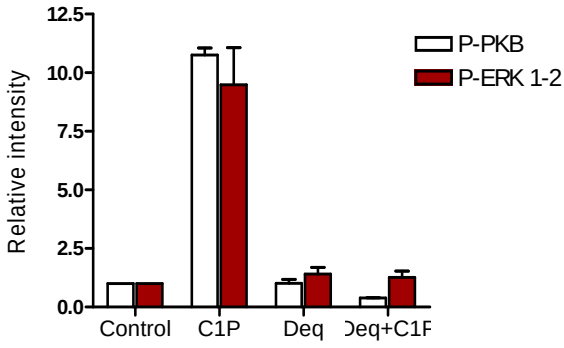


C.



D.



E. **F.**

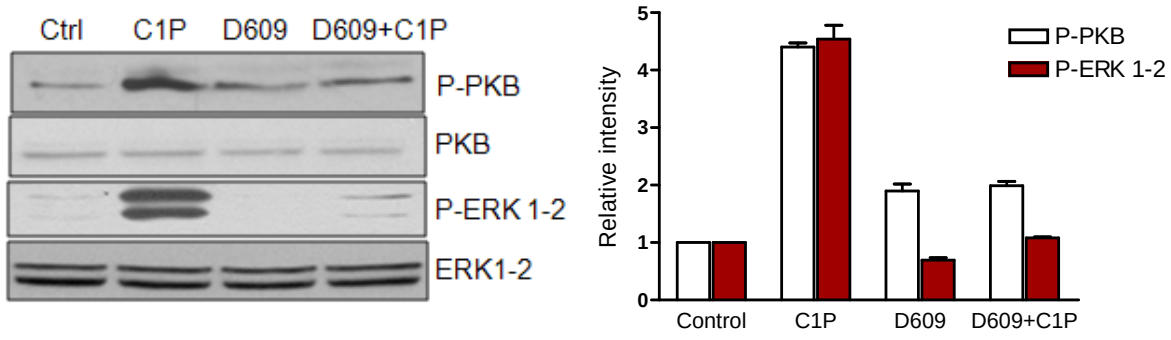


Figure 2.11.1. PKC and SMS are upstream of C1P-stimulated ERK1-2 and PKB phosphorylation. BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium. **A.** BMDM were treated for 16 h with or without PMA (100 nM) prior to addition of 50 µM C1P for 10 min. Phosphorylation of ERK1-2 and PKB was examined by Western blotting as described in the *Materials and Methods* section. Equal loading of protein was monitored using specific antibodies to total ERK1-2 and PKB. Similar results were obtained in each of 3 replicate experiments. **B.** Results of scanning densitometry of the exposed films. Data are expressed as arbitrary units of intensity relative to the control value and are the mean ± SEM of 3 replicate experiments. **C.** BMDMs were treated for 30 min with or without 1 µM dequalinium prior to addition of 50 µM C1P for 10 min. Phosphorylation of ERK1-2 and PKB was examined by Western blotting as described above. Equal loading of protein was monitored using specific antibodies to total ERK1-2 and PKB. Similar results were obtained in each of 2 replicate experiments. **D.** Results of scanning densitometry of the exposed films. Data are expressed as arbitrary units of intensity relative to the control value and are the mean ± SD of 2 replicate experiments. **E.** Cells were treated with or without 20 µM D609 for 30 min and phosphorylation of ERK1-2 and PKB were examined by Western blotting as described above. Equal loading of protein was monitored using specific antibodies to total ERK1-2 and PKB. Similar results were obtained in each of 2 replicate experiments. **F.** Results of scanning densitometry of the exposed films. Data are expressed as arbitrary units of intensity relative to the control value and are the mean ± SD of 2 replicate experiments (*$p < 0.05$) (Experiments in panel A and B were performed in collaboration with Dr. Patricia Gangoiti).

2.12. ERK and PI3K are upstream of ROS generation

Considering these results and taking into account the stimulation times (10-15 min) for PKB and ERK we hypothesized that PI3K/PKB and MAPK/ERK pathways could be also upstream of ROS generation. To test this hypothesis we measured ROS generation in the presence of PI3K and ERK inhibitors (Ly 290042 and PD98059, respectively). We

observed a significant decrease in C1P-stimulated ROS generation with both inhibitors (Figure 2.12.1).

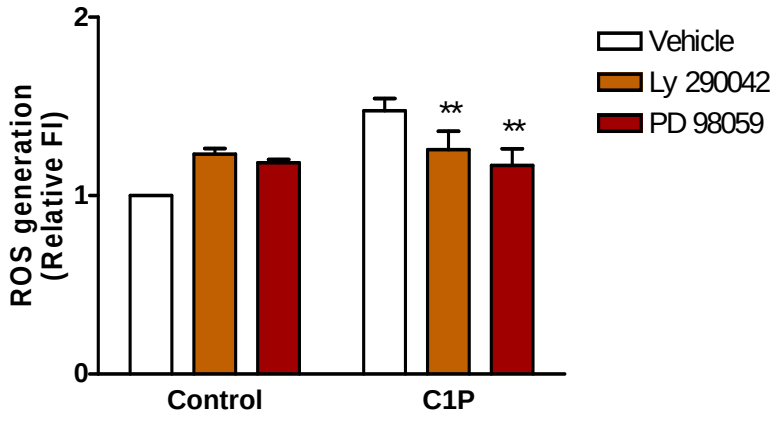


Figure 2.12.1. PKB and ERK inhibitors, Ly290042 and PD98059, decreased C1P-stimulated ROS generation. BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium. Cells were pre-incubated for 30 min with vehicle, 5 µM Ly 290042 or 10 µM PD 98059 and ROS generation was measured after 8 h of incubation with 40 µM C1P. Results are indicated as the fluorescence intensity relative to the control value and are the mean ± SEM of three independent experiments (**p<0.01).

2.13. The putative C1P receptor is not implicated in C1P-stimulated cell proliferation

Our previous work demonstrated that C1P can act intracellularly to stimulate cell growth [69, 80] and to inhibit apoptosis [53, 64], or through interaction with a putative Gi protein-coupled (Ptx-sensitive) plasma membrane receptor to stimulate cell migration [58].

Therefore, studies were aimed at defining whether C1P-stimulated ROS production was dependent or independent of receptor interaction. We found that Ptx, at concentrations known to inhibit G_i proteins (50 – 100 ng/mL) in these same cells [135], did not affect C1P-stimulated ROS generation (Figure 2.13.1), suggesting that C1P acts intracellularly rather than through interaction with its receptor.

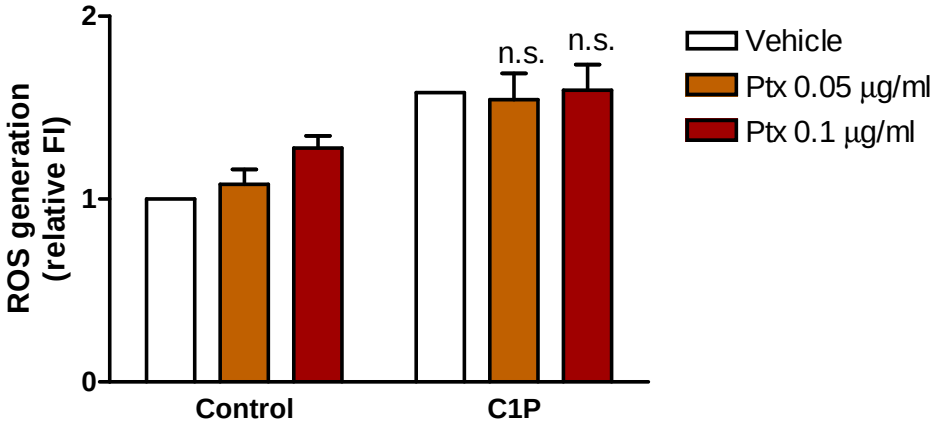


Figure 2.13.1. The C1P-specific receptor is not implicated in C1P-stimulated ROS generation. Pertussis toxin does not impair C1P-stimulated ROS generation. Cells were incubated overnight with indicated concentrations of pertussis toxin (Ptx) and then incubated with or without 40 µM C1P for 8 h. ROS generation was determined as mentioned before and the results are expressed as the fluorescence intensity relative to the control value and are the mean ± SEM of 3 independent experiments performed in duplicate (n.s.p>0.05).

2.14. Stimulation of cell proliferation by C1P is independent of receptor interaction. Role of intracellularly generated C1P

The above observation was confirmed using a novel caged C1P analogue, named 4-bromo-5-hydroxy-2-nitrobenzhydryl C1P (BHNB-C1P), which was previously found to be plasma membrane permeable and bypass surface receptors of BMDM [80]. The bioactive lipid C1P is released in the cytosol upon photolysis using visible light, that does not damage cellular components (wavelength > 360 nm). The caging/uncaging strategy allowed us to conclude that only intracellular C1P was responsible for stimulation of cell growth in BMDM [80]. In addition, concentrations as low as 2.5 µM BHNB-C1P were almost as effective as 40 µM of exogenous C1P in stimulation of BMDM proliferation, which is consistent with our previous observation that exogenously added C1P (in the form of liposomes) is only poorly taken up by cells [43].

Treatment of BMDM with BHNB-C1P in the dark (conditions in which the C1P remains in the caged form) failed to stimulate ROS production, whereas BHNB-C1P-treated cells (at 1-2.5 µM) that were exposed to 400-500 nm light for 60 min in order to release C1P into the cytosol stimulated ROS generation as potently as 40 µM of exogenous C1P (Figure 2.14.2).

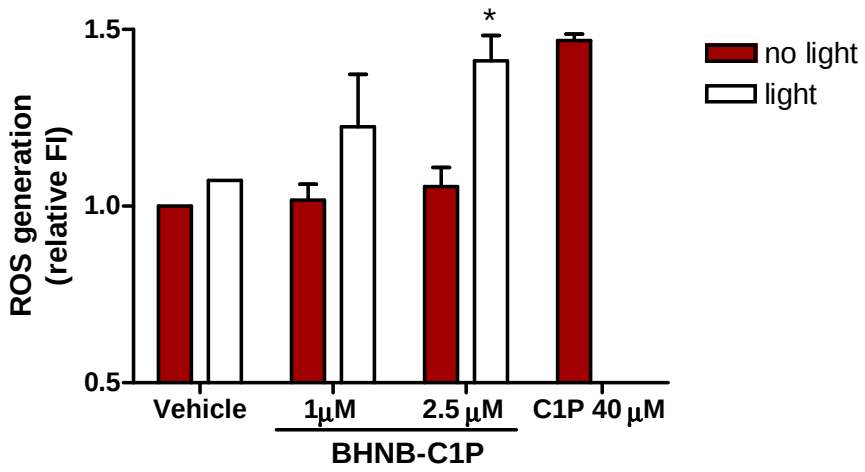


Figure 2.14.2. ROS are generated by C1P acting intracellularly. Macrophages were incubated with vehicle, indicated concentrations of BHNB-C1P or 40 μM C1P. The cells were then exposed to 400-500 nm light for 1 h using a transilluminator (open bars) or incubated in the dark (filled bars). Intracellular ROS generation was measured after 8 h of incubation. The results are expressed as the mean ± SEM of 3 independent experiments performed in duplicate, except for the point at 1μM BHNB-C1P, which is the mean ± SD of 2 independent experiments (*$p<0.05$).

3. Discussion of Chapter 3

ROS production by macrophages has been commonly related to host defense and oxidative stress. Nevertheless, several lines of evidence suggest that ROS generation could be part of intracellular signalling pathways leading to different cell responses, including cell proliferation.

Although there are reports relating ROS generation to cell damage and DNA injury, their implication in cell growth stimulation has also been demonstrated for some growth factors or agonists [112]. In addition, ROS generation has been involved in the promotion of angiogenesis and cancer development [12].

In this thesis we demonstrate that C1P stimulates ROS generation through activation of NADPH oxidase. Although the increase in ROS levels by C1P was not very high its impact in cell biology is likely to be highly relevant, because relatively low concentrations of ROS have been mainly associated to stimulation of cell growth and survival [136-141], whereas

high ROS levels are cytotoxic and can contribute to a number of diseases, including type 2 diabetes, atherosclerosis, arthritis, neurodegenerative disorders, and cancer [122, 142-144].

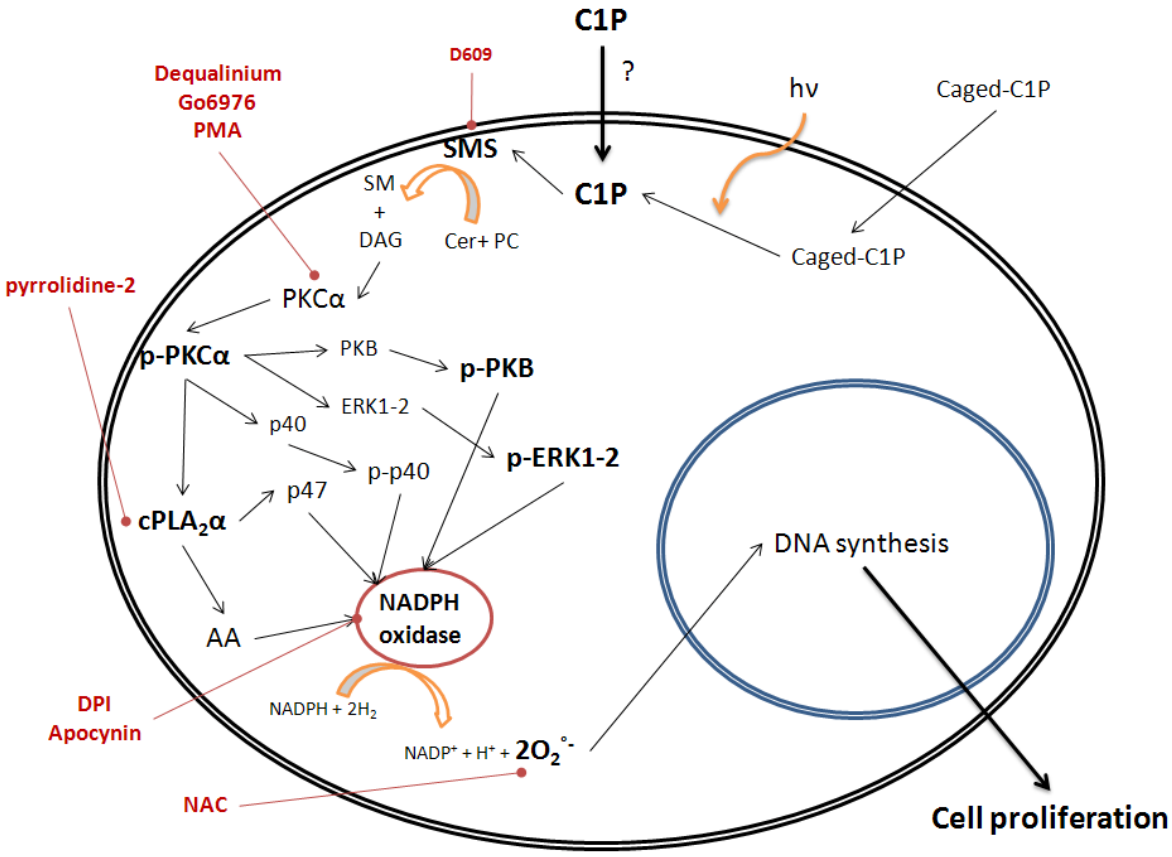
That ROS formation is essential for stimulation of cell growth by C1P is clear from the experiments using ROS scavengers, which we found to block cell proliferation completely. In addition, there are many papers in the literature showing that increases of ROS levels similar to the increases observed in this work are associated with stimulation of cell growth [136, 140, 141].

Because of their high toxicity, it is possible that once generated these radicals are rapidly metabolized and eliminated by the cells, and this may be reason why ROS do not accumulate to high levels inside cells. In fact, several defense systems have been evolved within the cells to impede ROS increments. They include non-enzymatic molecules (*e.g.*, glutathione, vitamins A, C, and E and flavonoids) as well as enzymatic scavengers of ROS (*e.g.*, superoxide dismutases, catalase, and glutathione peroxidase). To these well-known defense systems, peroxiredoxins, glutaredoxins, sulphiredoxs and thioredoxins have recently been added [138].

A major action of C1P is activation of cPLA$_2$α [123], as indicated above. Interestingly, apart from being involved in the control of inflammatory responses, cPLA$_2$α has been suggested to also be involved in the regulation of cell proliferation [145]. Our results suggest that C1P activates NADPH oxidase through stimulation of cPLA$_2$α activation, which is also in agreement with previous work reporting that AA increases p47phox translocation to the microsomal fraction [124] and that C1P can activate cPLA$_2$α [123]. Therefore, activation of cPLA$_2$α may be a major mechanism by which C1P stimulates growth in macrophages. In an attempt to study the events upstream of cPLA$_2$α activation we found that SMS activity was increased by C1P. As mentioned before, this enzyme catalyzes the synthesis of SM by transfering phosphocholine from PC to ceramide. This reaction generates free DAG, which is a well-known activator of PKC. So, we examined this possibility and we found that PKCα is an upstream activator of cPLA$_2$α, in agreement with other works [146, 147]. In subsequent experiments we were able to demonstrate that PKCα activation preceded the stimulation of PI3K/PKB and ERK1-2 by C1P. Taken together, these observations indicate that SMS, PKCα, cPLA$_2$α, ERK, PKB, PKCα and NADPH oxidase are essential components of the cascade of events leading to ROS

production and the subsequent stimulation of macrophage proliferation by C1P. There is a cartoon below summarizing these findings.

In conclusion, we have demonstrated that ROS production is a key factor for stimulation of macrophage proliferation by C1P. The enzyme responsible for C1P-stimulated ROS generation in BMDM is NADPH oxidase. This enzyme lies downstream of cPLA2α, which in turn, is activated by phosphorylation by PKCα in this pathway.



Chapter 4

7. CHAPTER 4

Ceramide 1-phosphate activates spleen mononuclear cells (SMCs).

1. Introduction

Immune system cells are constantly tracking the body searching for foreign agents. In order to distinguish between the body cells or the foreign pathogens, immune cells have developed a complex recognition system. All cells have different subsets of receptors in their surface called surface markers and these receptors can be used for recognition.

Thanks to these surface receptors immune cells can recognize, apart from foreign pathogens, other immune cells and cells from the body that are altered, such as virus infected cells or cancer cells.

When the immune system recognizes any of these signals it becomes activated and attacks the source of the signal. Hence, the proper recognition is critical for the accurate immune function, because reaction against self cells or tolerance to pathogens can be dangerous for the organism.

Apart from being useful for the immune system recognition, there are a number of cell surface markers that are used in clinical laboratories to distinguish subpopulations of lymphocytes. The cluster of differentiation (often abbreviated as CD) is a protocol used for the identification and investigation of cell surface molecules present on cells, providing targets for immunophenotyping them. The CD system allows cells to be defined based on which molecules are present on their surface. These markers are often used to associate cells with certain immune functions. While using one CD molecule to define populations is uncommon (though a few examples exist), combining markers has allowed a very specific definition of most of the cell types within the immune system (Table 1.1 and Figure 1.1).

Type of cell	CD markers
stem cells	CD34+, CD31-
all leukocyte groups	CD45+
Granulocyte	CD45+, CD15+
Monocyte	CD45+, CD14+
T lymphocyte	CD45+, CD3+
T helper cell	CD45+,CD3+, CD4+
Cytotoxic T cell	CD45+,CD3+, CD8+
B lymphocyte	CD45+, CD19+ or CD45+, CD20+
Thrombocyte	CD45+, CD61+
Natural killer cell	CD16+, CD56+,CD3-
Natural killer T cell	CD16+, CD56+,CD3+

Table 1.1. Definition of some hematopoietic cells based on the cell surface expression of CD receptors.

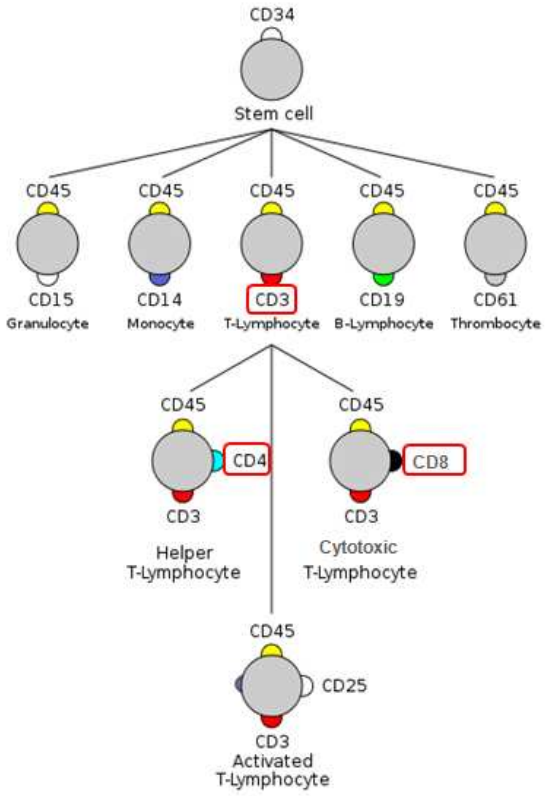


Figure 1.1. Schematic representation of some hematopoietic cell sorting depending on the expressed CD.

1.1. CELL SURFACE MARKERS RELATED TO IMMUNE SYSTEM ACTIVATION

The immune system works as a complicated network of many cell surface receptor cross-links which lead to activation and inhibition of signalling cascades. When activation signals overcome inhibitory ones, immune cells become activated. This activation is translated into changes in cell distribution and in the synthesis of new proteins in order to attack and suppress the signal that has activated the immune response.

During this response, immune cells also change the expression of cell surface receptors, in order to execute their function (effector or regulatory function). These cell surface markers can be used to detect the activation of different immune cells.

1.1.1. CD69

The activation inducer molecule (AIM/CD69) is a phosphorylated disulfide-linked 27/33-kDa transmembrane homodimeric glucoprotein. It is also called as “early activation marker” because of its rapid expression. The CD69 molecule is expressed on the surface of T-lymphocytes upon *in vitro* activation with a wide variety of agents, including anti-CD3/T cell receptor and anti-CD2 antibodies and activators of protein kinase C [148]. Similarly, the expression of CD69 is inducible on the surface of Natural Killer (NK cells), B-lymphocytes and eosinophils [149, 150]. Although a physiologic ligand for CD69 has not been identified so far, experiments with specific antibodies indicate that this antigen functions as a signal-transmitting receptor. Signals triggered by specific antibodies to CD69 in T-lymphocytes include an increase in the intracellular calcium concentration and result in the synthesis of different cytokines and their receptors, enhancement of the expression of c-*myc* and c-*fos* protooncogenes, and cell proliferation. In NK cells and platelets, CD69 also acts as a triggering molecule, being involved in the redirected target cell lysis by interleukin- 2-activated NK cells and in the induction of platelet aggregation, Ca^{2+} influx, and hydrolysis of arachidonic acid.

The molecular cloning of cDNAs encoding human and mouse CD69 revealed that this antigen is a member of the Ca^{2+} dependent (C-type) animal lectin superfamily of type II transmembrane receptors [151, 152]. This superfamily includes the human NKG2, rat and mouse NKR-P1, and mouse Ly-49 families of NK cell-specific antigens as well as

the low avidity IgE receptor (CD23), the Kupffer cell receptor, and the hepatic asialoglucoprotein receptor. The CD69 antigen is variably detectable on peripheral blood lymphocytes; however, it is expressed at high levels by the majority of T cells in the inflammatory cell infiltrates of several human diseases, such as rheumatoid arthritis and chronic viral hepatitis, suggesting that inflammatory cytokines may be involved in CD69 expression.

It is known that the constitutive and inducible expression of the CD69 molecule is regulated at the transcriptional level. In addition, it has been demonstrated that TNFα is capable of inducing the expression of the CD69 antigen and that this induction is mediated by an increase in the CD69 promoter activity and that this promoter is transactivated by members of the NF-κB/Rel family, specially by RelA (also known as p65) [153].

1.1.2. CD25

CD25 is the alpha chain of the IL-2 receptor (IL-2R). It is a type I transmembrane protein present on activated T cells, activated B cells, some thymocytes, myeloid precursors and oligodendrocytes.

Its expression has been associated to lymphocyte differentiation and activation/proliferation. This receptor when highly expressed in CD4+ T lymphocytes identifies the subpopulation of T regulatory cells (Treg). When activated, they express the Foxp3 marker and produce TGF-β, a cytokine able to inhibit the activity of other immune cells (*e.g.* NK, CTL)

1.1.3. NKG2-D (CD314)

NKG2-D is a member of NK Cell Lectin-Like Receptor Subfamily expressed in mice. Although it was supposed to be expressed only in NK cells, it has been reported that T lymphocytes also express this receptor. NKG2-D is implicated in regulating immune responses to infections and neoplasms. It has an extracellular C terminus and a C-type lectin domain. NKG2-D binds to a diverse family of ligands that include MHC class I chain-related A and B proteins and UL-16 binding proteins. Ligand-receptor interactions can result in the activation of NK and T cells. The surface expression of NKG2-D is important for the recognition of stressed cells by the immune system, and thus this protein and its ligands are therapeutic targets for the treatment of immune

diseases and cancers. For instance, it has been recently reported that hepatitis-C virus downregulates NKG2-D expression as a strategy to evade NK-cell mediated immune responses [154].

2. Results of chapter 4

Given the importance of the CD69 marker in immune cells, we undertook some studies to evaluate whether C1P might be able to alter the expression of this marker in spleen mononuclear cells (SMC) and to define the mechanism(s) involved in this process.

2.1. C1P increases CD69 expression in SMC

Most of the work demonstrating that C1P is a regulator of cell growth, survival and migration was performed by our group using macrophages. In this thesis, we hypothesized that C1P could also activate other cells involved in immune reponses. In order to test this possibility, we first studied whether C1P could stimulate immune cell activation by analyzing CD69 expression. As mentioned before, CD69 is an early activation marker and its expression can increase very quickly. As positive control, we used 10 nM *N*-Acetyl-D-glucosamine-coated polyamidoamine dendrimer (GN8), a synthetic molecule that has been reported to activate immune system responses [81, 155, 156].

We observed that 30 min of incubation with C1P was sufficient to increase CD69 expression significantly in NK cells, NKT cells, CTL and Th cells in a concentration-depending manner (Figure 2.1.1). Thus, it seems clear that C1P can stimulate the immune system.

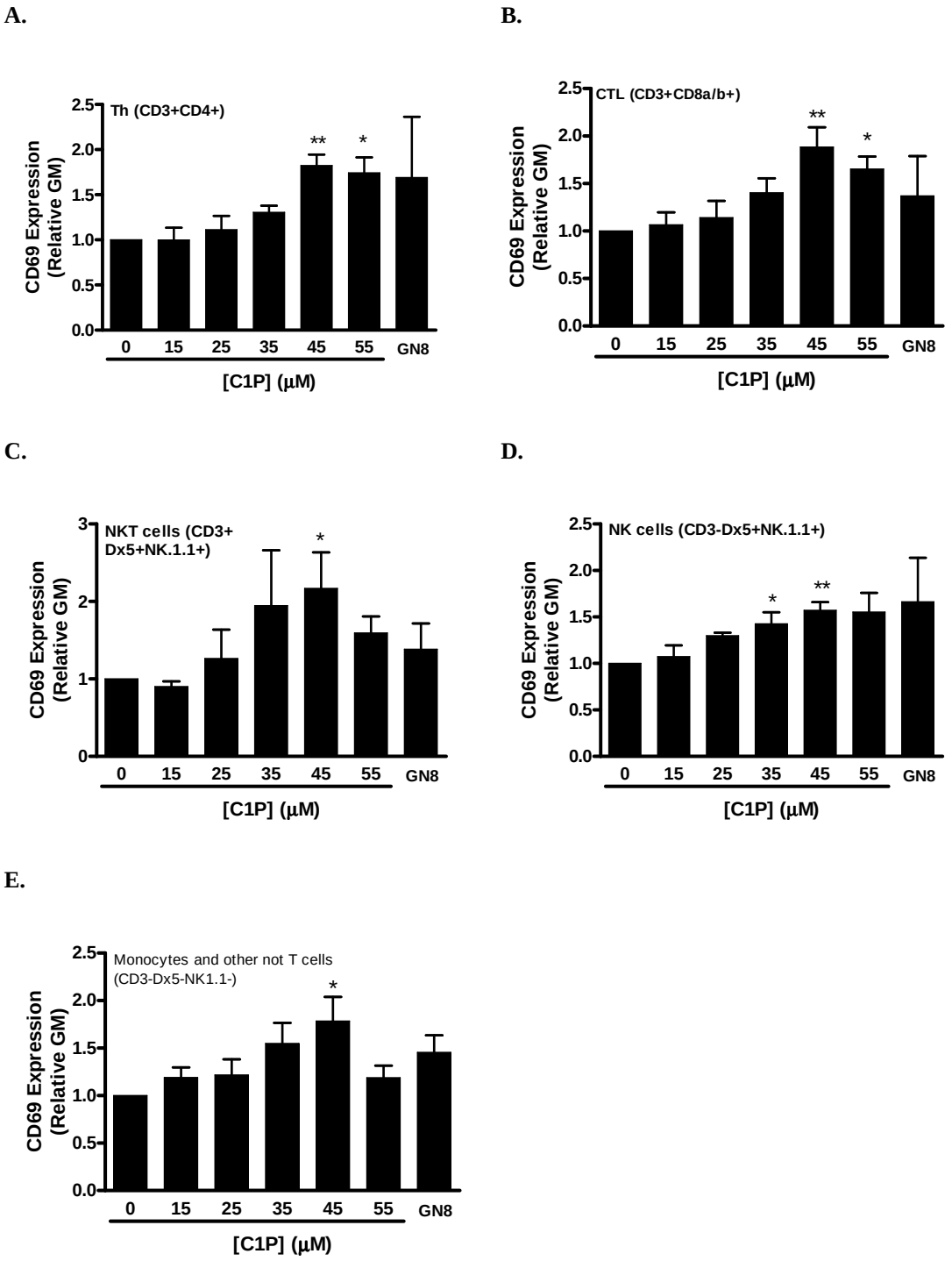


Figure 2.1.1. C1P increases CD69 expression in SMC. Cells were incubated with indicated concentrations of C1P or with GN8 for 30 min. Surface staining was performed as described in the *Materials and Methods* section. **A.** Cells corresponding to CD3+/CD4+/CD8a- gate. **B.** Cells from CD3+/CD4-/CD8a+. **C.** Cells correnspondingto CD3+/Dx5+/NK.1.1+. **D.** Cells with CD3-/Dx5+/NK.1.1+ staining. **E.** Cells from gate CD3-/Dx5-/NK1.1-. Results are expressed as the relative GeoMean for CD69+ staining and are the mean ± SEM of 5 independent experiments (*$p<0.05$; **$p<0.01$).

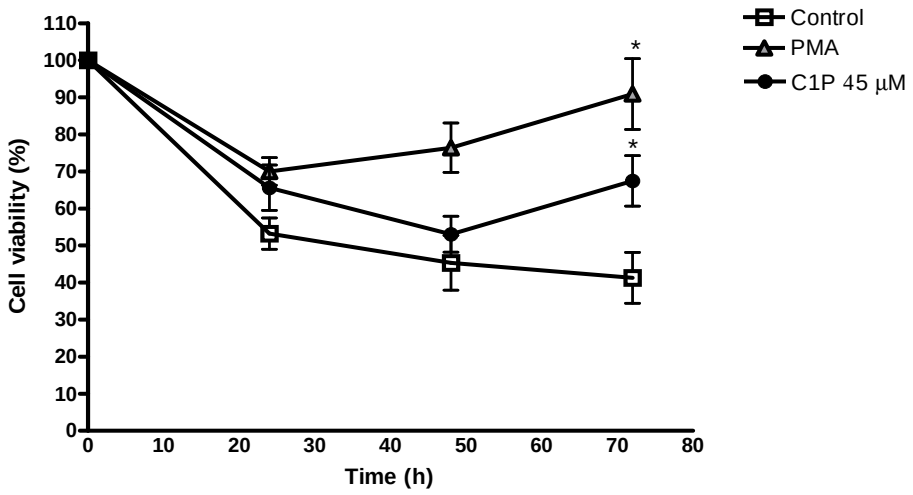
CD69 expression was increased in all of the cell subtypes in a similar pattern. Optimum stimulations were attained at 45 µM C1P in all cases. Thus, to simplify the number of subsequent experiments we decided to study C1P-induced increases of CD69 expresion in samples containing all SMC, without separating cell subtypes.

2.2. C1P inhibits apoptosis in SMC

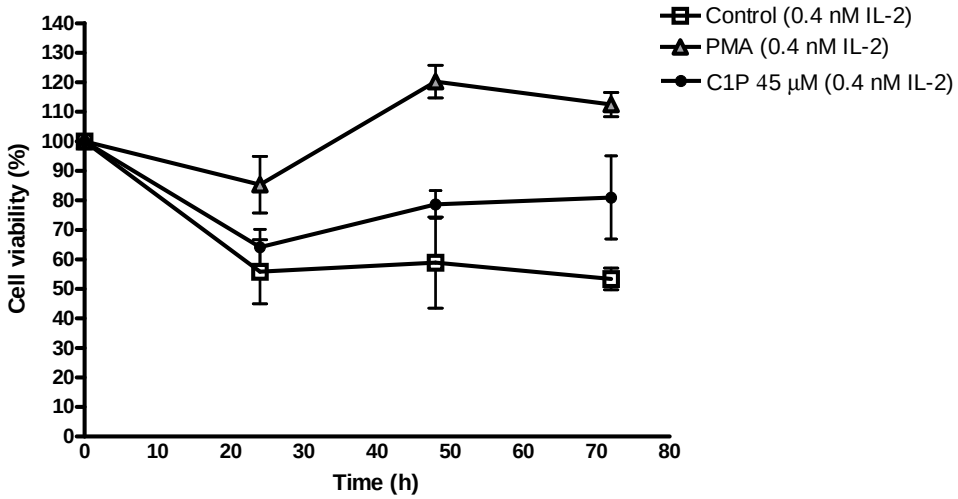
Due to the existing relationship between increased CD69 expression and lymphocyte proliferation [148] and taking into account that C1P is a proliferative and antiapoptotic signal, we performed MTS experiments to examine whether C1P was involved in the regulation of cell growth or survival in SMC.

For this purpose we incubated cells at different concentrations of IL-2, which is known to be a growth and survival factor for lymphocytes maintained in culture [157, 158] and it is widely used to the expansion of human peripheral mononuclear cells [159]. The cell survival seemed to augment in an IL-2 dependent-manner (Figures 2.2.1A, B, C and D) and treating SMC with 1.6 nM IL-2, around the 80% of viability was maintained up to 76 h. We observed that under apoptotic conditions (incubation without IL-2), C1P acted as a pro-survival factor thereby increasing cell survival significantly after 76 h of incubation (Figure 2.2.1A). This effect seemed to be more pronounced when the cells were treated with 0.4 nM or 0.8 nM IL-2 and 45 µM C1P. However, cells incubated under non-apoptotic conditions (cell culture containing 1.6 nM IL-2) were not sensitive to stimulation by C1P. By contrast, 100 nM PMA (a well-known activator of lymphocyte proliferation) significantly increased cell viability in all cases. These results suggest that C1P plays an anti-apoptotic role without affecting SMC proliferation (Figure 2.2.1D).

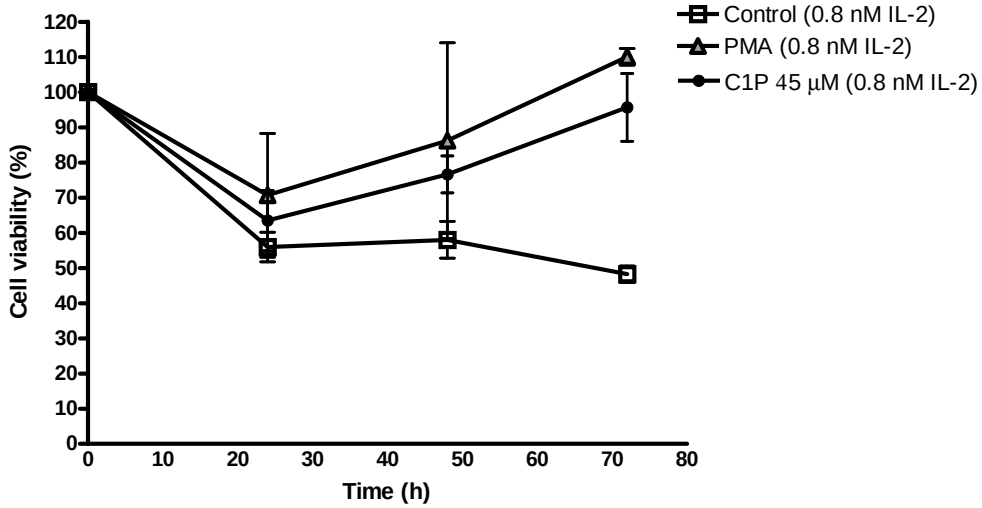
A.


Control
PMA
C1P 45 µM
Cell viability (%)
Time (h)
0
10
20
30
40
50
60
70
80
90
100
110
*
*

B.


Control (0.4 nM IL-2)
PMA (0.4 nM IL-2)
C1P 45 µM (0.4 nM IL-2)
Cell viability (%)
Time (h)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140

C.



D.

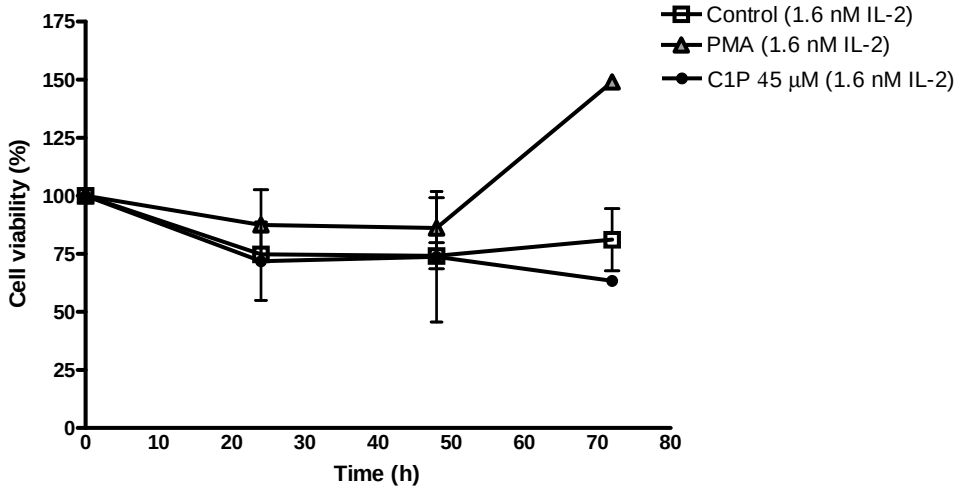


Figure 2.2.1. C1P inhibits apoptosis in SMC. Cells were seeded in 96-well plates (10^5 cells/well). **A.** Cells were incubated in RPMI 1640 supplemented with 10% FBS with vehicle (□), 100 nM PMA (▲) or 45 µM C1P (●). **B.** Cells were incubated in RPMI 1640 supplemented with 10% FBS and 0.4 nM IL-2 with vehicle (RPMI with 10% FBS, □), 100 nM PMA (▲) or 45 µM C1P (●). **C.** Cells were incubated in RPMI 1640 supplemented with 10% FBS and 0.8 nM IL-2 with vehicle (RPMI with 10% FBS, □), 100 nM PMA (▲) or 45 µM C1P (●). **D.** Cells were incubated in RPMI 1640 supplemented with 10% FBS and 1.6 nM IL-2 with vehicle (RPMI with 10% FBS, □), 100 nM PMA (▲) or 45 µM C1P (●). After the indicated incubation times, MTS/PMS was added and after 2h absorbance was measured in each well, as described in the *Materials and Methods* section. Results are expressed as cell viability relative to the control value at 0 h (100%) and are the mean ± SEM of 3 independent experiments performed in triplicate (*$p<0.05$).

To confirm that C1P plays an anti-apoptotic role in SMC, we analyzed the rate of cell apoptosis in SMC using apoptotic conditions in the presence or absence of PMA or C1P. Cells were stained with Annexin-V/PI and apoptosis was determined by flow cytometry. We observed that C1P and PMA increased the percentage of viable cells and diminished that of apopotic cells significantly (Figure 2.2.2).

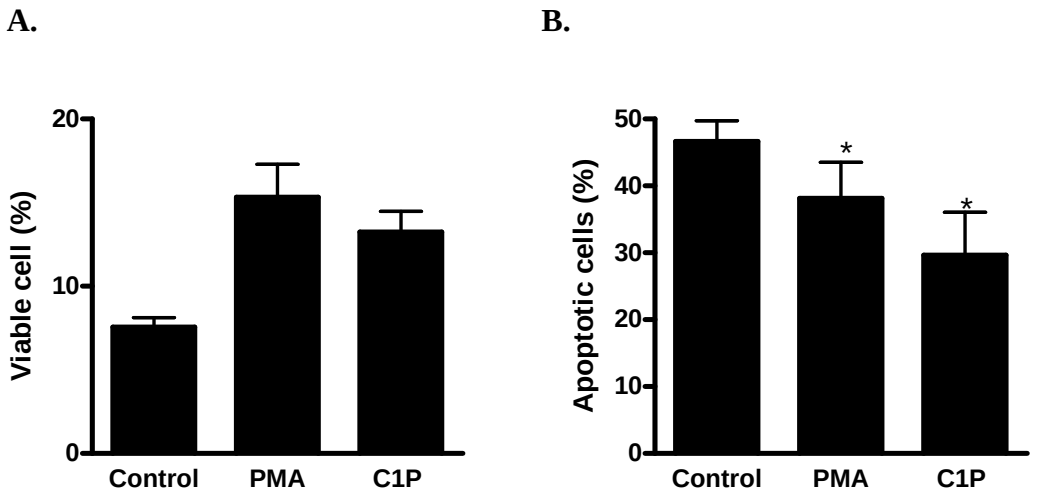


Figure 2.2.2. C1P reduces the percentage of apoptotic cells. Cells were seeded in 12-well plates (10^6 cell/well) and incubated in RPMI 1640 supplemented with 10% FBS. The cells were treated with vehicle, 100 nM PMA or 45 µM C1P for 76 h. The cells were stained with Annexin-V/PI as described in the *Materials and Methods* section. Fluorescence was measured by flow cytometry also as described in the *Materials and Methods* section and the results were processed using CellQuest sofware. **A.** The cells gated in the AnnexinV-/PI- quadrant were considered as viable cells. **B.** The cells gated in the Annexin-V+/PI- quadrant cells were considered apoptotic cells. Results are expressed as the percentage of cells and are the mean ± SEM of four independent experiments (*$p<0.05$).

2.3. C1P stimulates NF-κB phosphorylation

In previous work, we observed that NF-κB is implicated in the antiapoptotic effect of C1P in macrophages [64]. Therefore we tested to see whether C1P could stimulate NF-κB in SMC. For this, we measured NF-κB phosphorylation after challenging the cells with 45 µM C1P. Figure 2.3.1 shows that C1P stimulates NF-κB phosphorylation, with the maximal effect attained after 30 min of incubation with 45 µM C1P.

A. **B.**

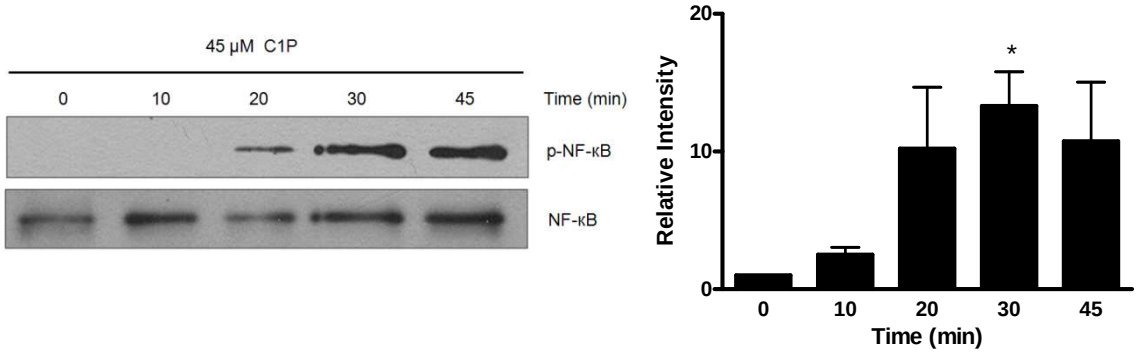


Figure 2.3.1. C1P stimulates NF-κB phosphorylation in SMC. A. SMCs were incubated with 45 µM C1P for the indicated periods of time. Cells were collected in homogenization buffer and phosphorylation of NF-κB was analyzed by Western blotting using a specific antibody to p-NF-κB. Total amount of protein was monitored using a specific antibody to total NF-κB. Similar results were obtained in each of 3 replicate experiments. **B.** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SEM of 3 independent experiments (*$p<0.05$).

2.4. C1P stimulates ERK phosphorylation

Subsequent studies were aimed at defining whether C1P could stimulate activation of other signalling kinases known to be involved in cell survival. We observed that C1P activated ERK1-2 phosphorylation in a time-dependent manner (Figure 2.4.1).

A. **B.**

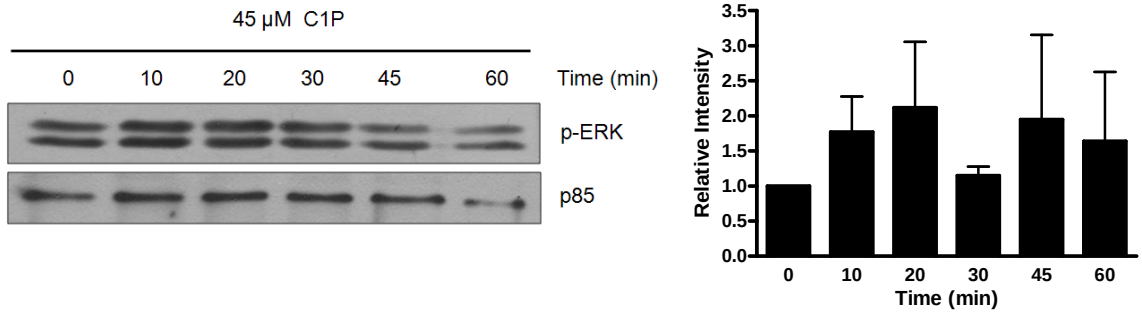


Figure 2.4.1. C1P stimulates ERK1-2 phosphorylation in SMC. A. Cells were incubated with 45 µM C1P for the indicated periods of time. Cells were collected in homogenization buffer and ERK phosphorylation was analyzed by Western blotting using a specific antibody to p-ERK1-2. Total amount of protein was monitored using a specific antibody to total ERK1-2. Similar results were obtained in each of 2 replicate experiments. **B.** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SD of 2 independent experiments.

2.5. C1P-stimulated NF-κB phosphorylation is inhibited by the MEK inhibitor PD 98059

Our group previously demonstrated that C1P-stimulated phosphorylation of ERK1-2 was upstream of NF-κB activation in macrophages [57].

To evaluate the implication of ERK1-2 in C1P-stimulated NF-κB phosphorylation in SMC, we tested to see whether PD 98059 could inhibit this effect in these cells. Figure 2.5.1 shows that preincubation of cells with 10 µM PD 98059 for 30 min abrogated C1P-stimulated NF-κB phosphorylation.

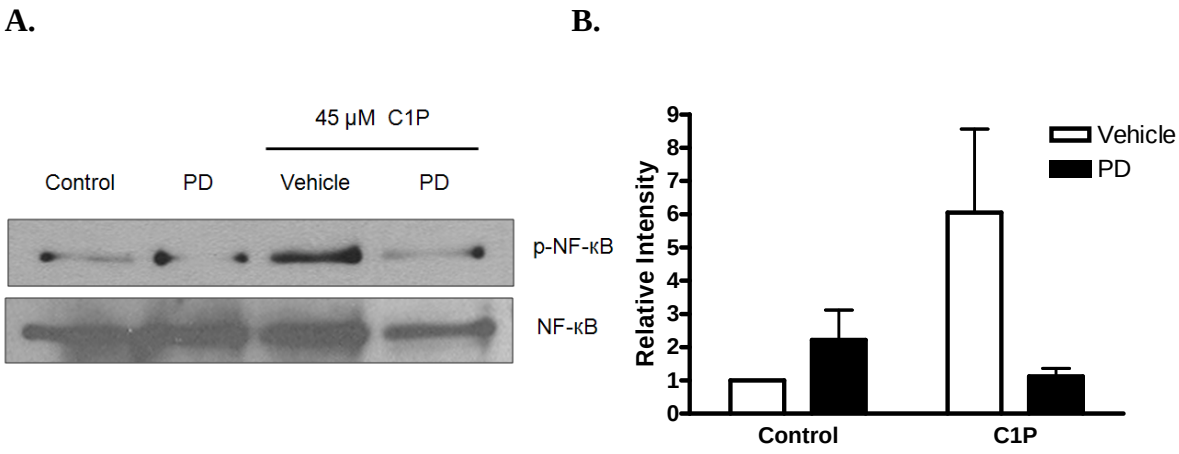


Figure 2.5.1. C1P-stimulated NF-κB phosphorylation is blocked by the MEK inhibitor PD 985059. Cells were pre-incubated with 10 µM PD 98059 for 30 min and then treated with 45 µM C1P for additional 30 min. They were then lysed with homogenization buffer and phosphorylation of NF-κB was analyzed by Western blotting using a specific antibody to p-NF-κB. Total amount of protein was monitored using a specific antibody to total NF-κB. Similar results were obtained in each of the 2 replicate experiments. **B.** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SD of 2 independent experiments.

2.6. ERK1-2, but not NF-κB, is implicated in C1P-induced CD69 expression

Since C1P can activate both ERK1-2 and NF-κB, we tested to see whether these two effectors were involved in the induction of CD69 by C1P. For this, we used selective inhibitors of ERK1-2 and NF-κB. The cells were pre-incubated for 30 min with PD98059 or SC-514 to inhibit ERK1-2 or NF-κB, respectively, prior to stimulation with C1P. Figure 2.6.1 shows that PD 98059 significantly reduced C1P-induced CD69

expression. However, SC-514 failed to inhibit the increase of CD69 expression that was elicited by C1P.

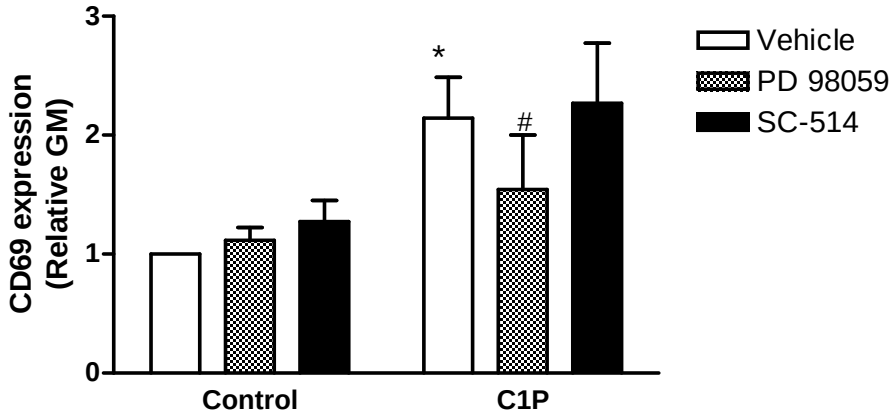


Figure 2.6.1. PD 98059, but not SC-514, inhibit C1P-stimulated increase of CD69 expression. SMCs were seeded (10^6 cells/mL) and pre-incubated with 10 µM PD 98059 or 25 µM SC-514 for 30 min. Then, 45 µM C1P was added and the cells were further incubated for 30 min. SMCs were stained as indicated in the *Materials and Methods* section and the CD69 expression was measured by flow cytometry. Results are expressed as relative GeoMean and are the mean ± SEM of 3 independent experiments (*$p<0.05$ C1P-treated cells versus control value; #$p<0.05$ PD 98059-treated cells versus C1P-treated cells).

Therefore, we can conclude that ERK1-2 is upstream of CD69 expression and NF-κB seems to be not implicated in the increase of CD69 expression. Presumably, NF-κB is downstream CD69 expression or they are two independently activated pathways.

3. Discussion of chapter 4

These preliminary data suggest that C1P can activate the cell surface expression of CD69 in all SMC in a concentration-depeding manner. CD69 is a broadly expressed cell activation marker and it has been reported to have a conservative cell function in all hematopoietic cells [160]. CD69 is widely used used to detect immune cell activation, and this is why we decided to study the activation signalling pathway by which C1P stimulated CD69 expression.

Interestingly, GN8 increases CD69 expression in NK cells and NKT cells. These SMC subpopulations express NK1.1 receptors and it has been reported that GN8 binds directly to NK1.1 receptors and that this cross-link activates NK cells [81, 155]. However, C1P induces CD69 expression in all SMC subtypes, more or less with the same pattern. These results suggest us that C1P-induced CD69 expression is not specific to NK cells.

Typically, immune cell activators, such as, LPS, PHA, IL-2 or TNFα, lead to cell proliferation and survival [161, 162]. Our results suggest that after activating SMCs, C1P can significantly decrease cell death when cells are cultured in apoptotic conditions. This is in accordance with some reports which suggest that inhibition of A-SMase can rescue NK cells from apoptosis [73], because it has been reported that C1P can inhibit A-SMase [53]. Thus, this could be one possible mechanism by which C1P could inhibit apoptosis in SMC.

Besides, our results suggest that ERK/MAPK is implicated in CD69 expression induction, as well as in NF-κB activation. In addition, we conjecture that ERK is upstream of NF-κB activation in SMC. These pathways have been implicated in C1P-stimulated cell survival and proliferation in macrophages [57, 64, 69, 70], therefore, it is not surprising the implication of NF-κB in the anti-apoptotic effect of C1P in SMC.

Hence, with these preliminary results, we conclude that C1P activates all subtypes of SMC in the same pattern and that this activation leads to cell survival.

Conclusions

8. CONCLUSIONS.

From the results obtained in this Thesis, the following conclusions can be drawn:

1. Concerning the regulation of cell proliferation, this Thesis has contributed to the elucidation of three major mechanisms by which C1P exerts its mitogenic actions:

1.1. Stimulation of VEGF secretion in Raw 264.7 macrophages. This action involves activation of the PI3K/Akt pathway.

1.2. Induction of translocation and activation of PKCα

1.3 Activation of NADPH oxidase and the subsequent formation of reactive oxygen species (ROS)

2. C1P stimulates the release of MCP-1 in J774A.1 macrophages, an action that turned out to be essential for the induction of macrophage migration. This effect requires the interaction of C1P with a putative C1P receptor, and the subsequent activation of the PI3K/Akt, MAPK/ERK1-2, p38 and JNK signalling pathways.

3. C1P increases CD69 expression in spleen mononuclear cells and protects these cells from entering apoptosis. A major pathway involved in this action is the MEK/ERK1-2/ NF-κB pathway.

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Appendix

APPENDIX

Some of the results obtained during the development of this Doctoral Thesis have been published in scientific journals or they have contributed to the following conferences:

1. PUBLICATIONS:

1. Gangoiti P, Granado MH, Arana L, Ouro A, Gomez Muñoz A. Activation of protein kinase C-alpha is essential for stimulation of cell proliferation by ceramide 1-phosphate. *FEBS Lett.* 2010 Feb 5; 584(3):517-24.

2. Arana L, Gangoiti P, Ouro A, Rivera IG, Ordoñez M, Trueba MA, Lankalapalli RS, Bittman R, Gomez-Muñoz A. Generation of Reactive Oxygen Species (ROS) is a key factor for stimulation of macrophage proliferation by ceramide 1-phosphate. *Exp Cell Res,* In press.

2. CONTRIBUTIONS TO CONFERENCES:

1. Gangoiti P, Granado M, Arana L, Ouro A, Gómez-Muñoz A. Implication of PKC-α in the mitogenic effect of ceramide 1-phosphate. Poster. "5th International Charleston Ceramide Conference" March 11-14, 2009. Charleston, California.

2. Arana L, Ouro A, Gangoiti P, Gomez-Muñoz A. Ceramide 1-phosphate stimulates reactive oxygen species (ROS) formation. Implication in macrophages growth. Oral communication. "Sphingolipid Club 8th Annual Meeting" April 21-23, 2010. Glasgow, UK.

3. Arana L., Ouro A, Gangoiti P, Gómez-Muñoz A. Stimulation of cell proliferation by ceramide 1-phosphate involves formation of reactive oxygen species. Poster. "51st International Conference on the Bioscience of Lipids" September 7-11, 2010. Bilbao, Basque Country, Spain.

4. Arana L., Ouro A, Gangoiti P, Trueba M, Gómez-Muñoz A. Ceramide 1-phosphate-stimulated macrophage proliferation requires ROS production. Poster. “XXXIV Congreso de la Sociedad Española de Bioquímica y Biología Molecular” Barcelona, September 5-8, 2011.

5. Arana L., Ouro A, Gangoiti P, Trueba M, Gómez-Muñoz A. Generation of reactive oxygen species is a key factor for promotion of macrophage proliferation by ceramide 1-phosphate. “IX Sphingolipid Club Meeting” Sept 28th-Oct 1st, 2011 Favignana, Sicilly.Italy.