Investigating the role of Sphingosine Kinase 1 pathway in cancer cell-monocyte interactions

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Statement of Originality

I hereby declare that this submission is my own work, or if not, it is clearly stated and fully acknowledged. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person.

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Abstract

Strong evidence suggests that the tumour microenvironment is inflammatory and that activation of the innate immune system plays a role in cancer progression, therefore targeting the multiple interactions of tumour cells with other cell types within the tumour microenvironment may lead to development of new cancer therapies. Sphingosine kinase (SPHK1) is a tumour-associated enzyme whose over-expression has been linked to patient mortality in many types of cancer. Here I investigate whether activation of the SPHK1 pathway, with a known involvement in inflammatory responses, is a signal transduction component of the tumour-monocyte/macrophage cellular interaction and a key element in inflammation-related cancer progression. Using a co-culture model, this study shows that the presence of monocytes increases cancer cell proliferation, an effect abrogated by knockdown of SPHK1 in cancer cells. Both monocytes and cancer cells showed a transient increase in SPHK1 activity and mRNA expression levels together with an increase in MCP-1 and IL-6 secretion. Silencing of SPHK1 in cancer cells abrogated SPHK1 activation in monocytes and pharmacological inhibition of SPHK1 in monocytes cells decreased monocyte induced-SPHK1 expression in cancer cells. Mechanistically, activation of AKT was observed in cancer cells upon co-culture with monocytes, an effect that was abrogated when cancer cells were pre-treated with siRNA for SPHK1. Moreover, the increase of phospho-AKT, ERK1/2 and NF-KB in monocytes by cancer cells was also reduced by RNAi-mediated knockdown of SPHK1 in cancer cells. My data show that STAT1 can bind to SPHK1 promoter or coding region and may be involved in SPHK1 transcriptional regulation in cancer cells upon monocyte stimulation, however its role still remains unclear as it acts as a transcriptional repressor of SPHK1. Monocytes induced cancer cell chemoprotection via a SPHK1-dependent mechanism, and reduced the inhibitory effect of docetaxel on cancer cell proliferation. Accordingly, increased AKT and ERK1/2 phosphorylation in monocytes were also affected by siRNA targeting of SPHK1 in docetaxel treated cancer cells. Altogether I show for the first time that selective inhibition of SPHK1 in tumour cells can affect their interaction with surrounding cells through the modulation of signal transduction pathways (ERK, PI3K, NF-kB) and cytokine exchange (IL-6, MCP-1 and potentially S1P, GM-CSF, GROα, IL-32 and ICAM-1). SPHK1-mediated increase in proliferation and chemoresistance of cancer cells conferred by monocytes renders this enzyme a promising target for future cancer therapies.

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Dedication

I would like to dedicate this work to my Grandparents

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List of Abbreviations

ABC	Adenosine triphosphate-binding cassette
AKT	Protein Kinase B (PKB)
AP-2	Activator protein-2
AR	Androgen receptor
ATC	Apoptotic tumour cell
ATP	Adenosine triphosphate
BDM	Blood-derived monocytes
Bcl-2	B-cell lymphoma 2
BPH	Benign prostatic hyperplasia
BRCA2	Breast cancer susceptibility gene-2
BSA	Bovine serum albumin
CCL	Chemokine ligand
CD	Cluster of differentiation
CD40L	Cluster of differentiation 40-ligand
cDNA	Complementary deoxyribonucleic acid
CHX	Cycloheximide
COX2	Cyclooxygenase-2
CSF-1	Colony stimulating factor-1
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
DRE	Digital rectal examination
DTT	Dithiothreitol
EDG	Endothelial differentiation genes
EDTA	Ethylenediaminetetraacetic acid
EGF	Endothelial growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinases 1 and 2
FACS	Fluorescence activated cell sorting
FGF	Fibroblast growth factor
FT720	Fingolimod
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF-1	Granulocyte-macrophage colony stimulating factor
GPCR	G protein coupled receptor
GROα	Growth related oncogene alpha
HEK293	Human embryonic kidney 293 cell line
HeLa	Henrietta Lacks human cervical cancer cell line
HGF	Hepatocyte growth factor
HKG	Housekeeping genes
HMGB1	High mobility group box-1
HRP	Horseradish peroxidise
IFN-γ	Interferon-gamma
IGF-1	Insulin growth factor
IL	Interleukin
iNOs	Inducible nitric oxide synthase
LNCaps	Androgen-sensitive human prostate adenocarcinoma cells
LPS	Lipopolysaccharide
M1	Classically activated macrophages

M2	Alternatively activated macrophages
MAPK	Mitogen-activated protein kinase
MCF-7	Michigan cancer foundation-7 human breast cancer cell line
MCP-1	Monocyte chemotactic protein-1
MIF	Macrophage migration inhibitory factor
MIP-1α	Macrophage inflammatory protein-1
mRNA	Messenger ribonucleic acid
MSP	Macrophage stimulating protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa beta
NK	Natural killer
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PC-3	Human prostate cancer cell line
PCa	Prostate cancer
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E2
PI	Propidium iodide
PIN	Prostate intraepithelial neoplasia
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride (serine protease inhibitor)
PSA	Prostate-specific antigen
PTEN/MMAC1	Phosphatase, tensin homologue/mutated in multiple advanced cancers-1
RAC	Ras-related C3 botulinum toxin substrate
RAW	Mouse leukaemic monocyte macrophage cell line
Rho	Ras homolog gene family
ROCK	Rho-associated protein kinase
ROI	Reactive oxygen intermediates
S1P	Sphingosine 1 phosphate
S1PR	Sphingosine-1-phosphate receptor
SDS	Sodium dodecyl sulphate
siCAM-1	Soluble intercellular adhesion molecule-1
siRNA	Small interfering ribonucleic acid
Sphk	Sphingosine kinase
SPP	Sphingosine-1-phosphate phosphatise
STAT	Signal Transducer and Activator of Transcription
TAM	Tumour associated macrophage
TGF	Tumour growth factor
Th1/2	T helper cell type 1 or 2
THP-1	Human acute monocytic leukemia cell line
TNF-α	Tumour necrosis factor alpha
TRAF-2	Tumour necrosis factor receptor-associated factor-2
U937	Human leukemic monocyte lymphoma cell line
VEGF	Vascular endothelial growth factor
YWAZH	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein

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1 Introduction

1.1 Prostate Cancer

Prostate cancer (PCa) is the most common cause of cancer-related death and the most commonly diagnosed cancer in men in the UK [1](Figure **1.1**). Current data suggest in the UK that more than 10,000 men die from prostate cancer every year and one in eight men will develop prostate cancer during their lifetime [2]. The vast majority of patients diagnosed with PCa are aged over 50 years, however new epidemiological data suggest that the incidence of advanced, highly aggressive prostate carcinomas is increasing in younger men [3, 4]. When PCa is locally confined the survival rate increases considerably to approximately 100% up to 5 years in comparison with non-localised PCa. The high mortality of PCa arises from its metastatic potential; in men with metastatic PCa the 5 year survival rate decreases to 30% [5], with the main metastases occurring in bone in approximately 90% of all men diagnosed with the disease. Other tissue sites affected by the dissemination of metastatic PCa include lung (46%), liver (25%), pleura (21%) and adrenals (13%) [6].

1.2 The prostate

The prostate is an exocrine gland forming part of the male reproductive system. This male gland is involved in the production of prostatic fluid and ejaculation of this through the contraction of prostate smooth muscle cells. The prostatic fluid is a component of the seminal fluid that is released during ejaculation with an important role in the protection and motility of the sperm. It is an alkaline fluid and contains among other components, prostate-specific antigen (PSA), kallikrein enzymes such as KLK2, citric acid, zinc and proteases [7].

Prostate gland growth and development requires hormones such as testosterone, a male sex hormone produced in the testis from 6 weeks of gestation. This process is particularly important *in utero* and during puberty where the development of male reproductive systems including the prostate gland occurs. The prostate gland stops growing upon reaching full development and only grows when dysregulated, a common event during PCa.

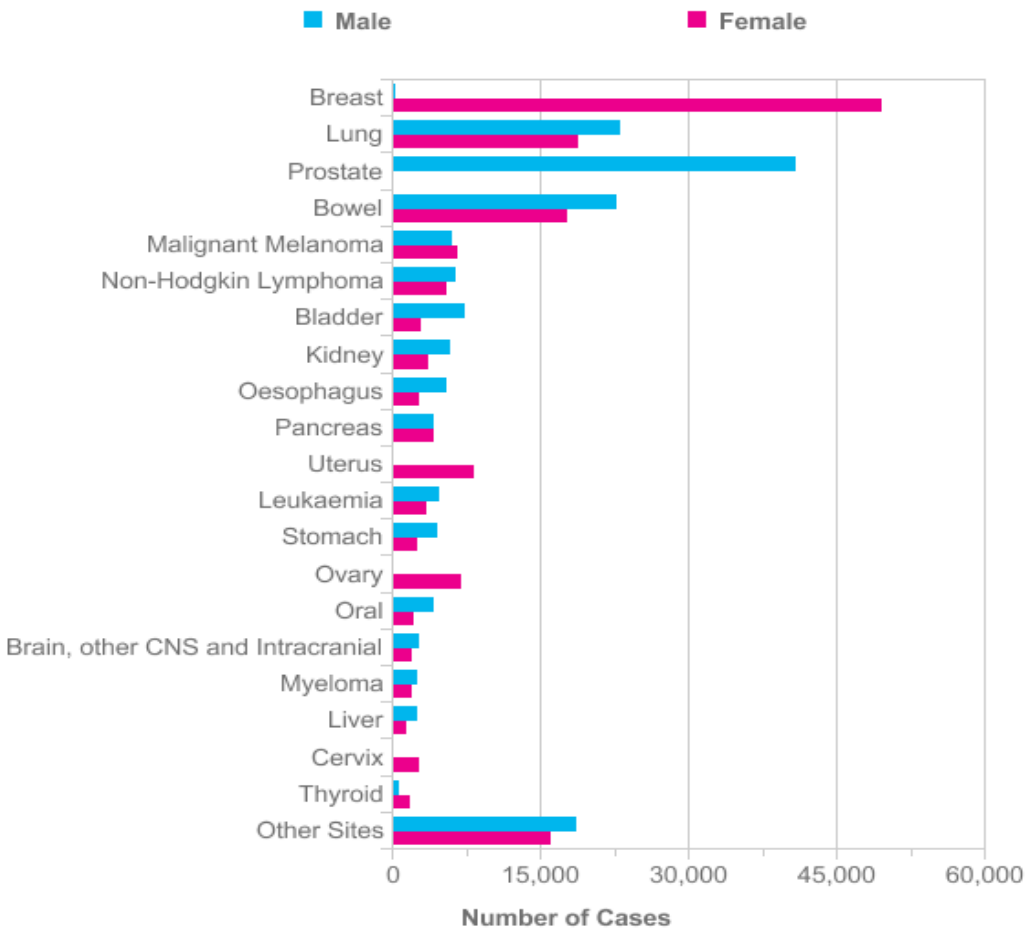

Figure 1.1: The 20 most commonly diagnosed cancers excluding non-melanoma skin cancer, UK, 2009.
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http://www.cancerresearchuk.org/cancer-info/cancerstats/incidence/commoncancers/

1.3 Prostate Cancer Development

Prostate cancer can be classified as adenocarcinoma (more than 90%) or glandular carcinoma, and is attributed to a deregulation in the proliferation of epithelial cells in the prostate that leads to the development of a primary malignant tumour [8]. As well as genetic lesions resulting in the incidence of PCa, it has been hypothesised that PCa could also be derived from the lesions that follow prostate tissue injury [9][9]. Following injury, epithelial prostate cells may lead to the development of prostate intraepithelial neoplasia (PIN), which is suggested to be an early pre-malignant stage of PCa progression [10]. The steroid hormones, androgens and oestrogens, and growth factors such as epidermal growth factor (EGF), transforming growth factor (TGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and insulin growth factor (IGF-1) are implicated in the transition of PIN to malignant PCa and therefore identified as contributory factors in PCa development [11]. As previously mentioned, genetic modifications also play a crucial role in the development of PCa, with the induction of oncogenes such as RAS, Bcl-2 or c-MYC, or the ablation of tumour suppressor genes such as p53, PTEN and MXI1 prime examples of the manner in which gene regulation can affect prostate cancer development (Hanahan and Weinberg 2000, Karan, Lin et al. 2003. Epigenetic DNA modifications such as hyper-methylation have been shown to increase with age and are found at a high incidence in prostate cancer tissue [12-14]; GSTP1 was found to be methylated in 90% of PCa and more than 70% of high-grade PIN [15]. These modifications could derive from a genetic predisposition or from microenvironmental pressures. Indeed, the microenvironment can act as an ideal “partner” for tumour development by behaving in a paracrine manner or by helping tumour cells to migrate from the primary site and form metastases [13]. Prostate cancer may remain latent for several years and in some cases can be successfully treated with current therapies such as radical prostatectomy or radiotherapy. However, PCa can relapse and may develop into life threatening metastatic disease, resistant to current therapies.

Metastatic PCa is the main cause of disease morbidity and treatment options are limited. Metastasis is defined as the process of cancer cells escaping from the primary tumour and establishing new growth at a secondary tissue site [16], with metastasising cells

needing to overcome several physical and gradient barriers in order to establish micrometastases at this secondary site. After the initial neoplastic transformation of PCa cells, neoangiogenisis and lymphogenesis are necessary for tumour growth and proliferation. The loss of cell adhesion and detachment from other tumour cells facilitates local invasion through the extracellular matrix and intravasation into the vascular and lymphatic systems. The tumour cells will encounter and need to circumvent the immune cell response and traverse the turbulent physical environment of the circulatory system before they can extravasate and enter the secondary site where tumour establishment can occur [16]. It has been estimated that only 2% of cancer cells are able to form metastases [17] and only 1% will form vascularised macrometastases [18]. As previously stated, metastasis is currently the main cause of prostate cancer morbidity and to date there remains an unmet clinical need with regards to identifying a suitable treatment.

1.4 Androgen dependency and independency

Androgens are required for the normal homeostasis, maintenance and growth of the prostate gland. The most abundant male hormone is testosterone, which can be converted by 5α-reductase to 5α dihydrostestosterone, a ligand with high affinity for androgen receptor (AR) [19]. AR is a member of the nuclear receptor superfamily which, in normal prostate cells, plays a pivotal role in driving the differentiation of luminal epithelial cells and regulating the transcription of several genes (e.g PSA) involved in the normal development and function of the prostate gland [20]. The activation of AR signalling is correlated with the increased growth and survival of prostate tumour cells [21, 22]. This is mostly a reflection of the ability of AR to regulate several cell cycle genes (e.g Cyclin D1) leading to an increase in survival and growth [23, 24].

Since PCa is also initially androgen dependent for growth, targeting of AR signalling is the standard treatment for the majority of advanced localised or metastatic PCa [25]. This may be as a component of androgen depravation therapy (ADT), involving chemical castration with or without AR antagonist treatment, the current gold standard of which is bicalutamide.

ADT shows an initial clinical response in approximately 80% of patients; however in the majority of cases patients will relapse and develop a highly aggressive PCa within two years [26, 27]. At this stage androgen ablation is no longer effective and PCa is commonly referred to as androgen-independent, hormone refractory or (more currently accepted) castrate-resistant PCa (CRPC). Indeed there is evidence that in CRPC, where the levels of circulating testosterone and DHT are reduced, AR still plays a role in PCa development. Immunohistological studies indicated AR protein expression was higher in CRPC patients in comparison with patients with benign PCa [28, 29]. In support, AR mRNA levels were also found to be significantly higher in CRPC tumours [30, 31].

The activation of AR in PCa patients treated with ADT could result from many factors: AR gene amplification [32, 33], AR mutations (20-50%) [34, 35] or alternative splicing [36, 37]; alterations of AR co-regulators or co-repressors [38, 39]; activation of signalling pathways that modulate AR function [40, 41]. Consequently, there is growing evidence that AR still remains an important regulator in CRPC and therefore future therapies directly targeting AR should be explored.

1.5 Symptoms and risk factors

1.5.1 Symptoms

PCa symptoms are often not evident at early stages of the disease; most often PCa symptoms are similar to those observed with benign prostatic hyperplasia (BPH), which comprises the enlargement of the prostate gland during ageing. BPH affects approximately 50% of men by the age of 50 years and 75% by the age of 80 years [42]. To date, there is no firm evidence that BPH increases the risk of PCa or that it is a precursor of PCa. The most common symptoms for PCa or BPH are related to urinary problems, such as urgency and inability to retain flow, difficulty passing urine and an increased frequency in urinary passing. Pain during urination or blood in the urine could also be symptoms but are rare. If PCa has spread from the primary site, symptoms such as pain in the bone, back or hips could be

associated with its metastatic site. Loss of appetite, weight loss, tiredness and erectile dysfunction are also symptoms of PCa, more associated with patients of advancing age.

1.5.2 Risk factors

1.5.2.1 Age

Age is one of the major risk factors for PCa. A recent report from the American Cancer Society indicated that at the time of diagnosis more than 60% of PCa patients are older than 65 years and that 97% occur in men aged 50 or older in the US [43]. In the UK, 75% of cases were diagnosed in men aged 65 years or over in 2010. Moreover, men aged between 55-59 showed an incidence of 163 per 100,000 men, this rate triples in men aged 65-69 and is approximately five times higher in the 75-79 years age group [1].

1.5.2.2 Genetic predisposition

Genetic predisposition is another important risk factor for PCa, in which it is estimated that around 9% of malignancies are correlated with an inherited increase in the likelihood of disease onset [44]. It is thought that this predisposition could be due to an existence of mutations within or inhibition of multiple tumour suppression genes (e.g. PTEN/MMAC1, BRCA2) [45, 46]. A 2003 study by Zeegers *et al.* revealed that men are 2.5 times more likely to develop PCa if they have a first-degree family member with PCa, rising to approximately 3.5 times more likely if a brother was afflicted with the disease [47]. Single nucleotide polymorphisms (SNPs) have also been associated with PCa, with SNPs in chromosomes 7, 8, 10 and 19 reported to increase PCa risk [48, 49]. Using whole-genome mapping, chromosomal region 8q24 was associated with a highly increased risk for PCa [50].

1.5.2.3 Ethnicity and diet

Over the last decade, the incidence of PCa in countries such as China and Japan has increased; according to some authors this was accompanied by an increase in the intake of red meat, animal fat and low fibre diets over the same period of time [51]. Moreover, second and third-generation Chinese emigrants in the US are showing a higher incidence of PCa in comparison with the same age population in their native countries [52].

Epidemiological studies indicate that mortality rates from PCa are twice as high in African American compared to Caucasian men; with the lowest rates of mortality observed in Hispanic and Asian populations [53, 54]. In the UK, a study between 2002 and 2006 indicated that African and Caribbean men under 65 years had a three-fold higher incidence than Asian men and a six-fold higher incidence than Chinese men. Over 65 years of age, African and Caribbean males had a three times higher likelihood of disease onset than both Asian and Chinese men [55].

Although dietary habits, such as a high fat and red meat rich diet [56], have been suggested to account for these differences more recent studies cannot confirm this correlation [57]. Nonetheless diet remains as a risk factor for PCa, more specifically a high intake of red meats and saturated fat is correlated with a higher risk of developing PCa [58, 59]. Diets rich in fish, vegetables and produce such as tomatoes, tea or vitamin D have been reported to be inversely correlated with the risk of PCa, although there is still some debate as to how effective they are in repressing the development of the disease [60].

1.6 Screening and Therapies

Screening for PCa is crucial as it could allow the detection of the malignancy in an early stage of development, which increases the chance to effect a cure and/or prevent the incidence of metastasis. The combination of digital rectal examination (DRE) and measuring prostate-specific antigen (PSA) levels in serum are the most commonly used methods for initial PCa screening [61, 62]. DRE was the most commonly used method in the 1990's, whereby the diagnosis is determined by the detection of palpable abnormalities in patients

[63]. PSA is a serine protease that is produced by prostatic epithelial cells and its physiological function is to lyse seminal vesicle proteins into smaller polypeptides resulting in liquefaction of the seminal coagulum [64]. Increased PSA levels have been shown to correlate with PCa; although PSA production is only increased insofar as more epithelial cells may be present, a disruption of the prostate by PCa or benign prostatic hyperplasia (BPH) can result in the leakage of this PSA into the bloodstream causing a surge in serum PSA levels [65-67]. Serum PSA levels can be influenced by several factors, including the presence of tumour cells, prostatitis or BPH, which involves continuous prostatic growth and is reported to be associated with increased PSA levels. In the indication of PCa, a biopsy is taken and graded following the Gleason score system, which aims to predict the aggressiveness of the disease and the likelihood that the tumour will be metastatic; the higher the score the more aggressive the tumour tends to be [68, 69]. According to the Gleason score and clinical stage there are different strategies for PCa treatment. If PCa is localised the most current therapies involve the removal of the entire prostate gland (radical prostatectomy) or radiation-based therapy [70]. These procedures could be combined with ADT if the recurrence risk is intermediate or high or cancer spreads outside the prostate. ADT can also include castration, AR antagonist and combined therapy as described before. There are also other possibilities such as watchful waiting, external-beam radiation therapy, brachytherapy and cryotherapy [71]. The chosen treatment will depend on patient characteristics, such as general health, body condition and age, as well as personal feelings regarding the available therapies [72]. Despite the initial treatment, PCa can recur and in most cases with a more aggressive and mestastatic phenotype.

Chemotherapeutic intervention is used when the cancer spreads outside of the prostate gland and the tumour cells become unresponsive to androgen deprivation. Docetaxel (taxotere), belonging to the taxane class of drugs, is the current standard of care for CRPC, resulting in a survival benefit of up to three months over other chemotherapeutics such as mitoxantrone [10, 73, 74]. More recently Cabazitaxel, a tubulin-binding taxane drug, was shown to have beneficial effects in CRPC patients with resistance to docetaxel. Clinical trials indicate a 2.4 month increase in survival for men with docetaxel-pretreated metastatic CRPC receiving cabazitaxel (with prednisone) in comparison with mitoxantrone (with prednisone) [75]. Abiraterone, a potent inhibitor of key enzymes involved in the

testosterone synthesis pathway, is another second-line drug for metastatic prostate cancer that was recently approved by the FDA. A clinical trial study showed that treatment with abiraterone afforded a further 3.9 month increase in survival in docetaxel-treated men with CRPC compared with the placebo group [76]. Enzalutamide (MDV3100), an AR antagonist, was also recently approved for the treatment of CRPC post-chemotherapy; patients treated with this drug had an overall survival increase of 4.8 months over the placebo group [77]. An immunotherapy-based drug, sipuleucel-T, whose mechanism of action uses autologous peripheral-blood mononuclear cells (PBMCs) to activate an immune response, has shown a 4.1 month improvement in median survival and an improvement in the rate of 3-year survival over control groups (31.7% for patients receiving sipuleucel-T, as compared with 23.0% for those receiving placebo).

Despite recent advances in CRPC drug therapies, it is clear that the overall survival rate is still low and new drugs are required to overcome this deathly pathology. Recently our group has shown that docexatel therapy together with the inhibition of sphingosine kinase 1 (SPHK1) sensitises metastatic PCa cells to docetaxel, which suggests that SPHK1 regulation is a potential new target for PCa therapy [78].

1.7 Prostate Cancer Biomarkers

Currently there are few markers for diagnosis of early stages of PCa, with PSA an indicator although not specific to PCa. In clinical practice PSA is the most commonly used biomarker for diagnosis and response to treatment of prostate cancer. PSA is detected in the blood (serum), however its limitations as a biomarker arise from the fact that PSA increase is not a PCa specific event. Non-cancer related pathologies, such as BPH and prostatitis, also increase serum PSA levels. A recent review indicated that less than 50% of men undergoing biopsy, following an increase in PSA levels, were diagnosed with PCa [79]. It is therefore important to identify and characterise new biomarkers for detection of PCa to augment or replace screening by PSA levels. Current research for new biomarkers includes analysis of amino acids, proteins or nucleic acids. Promising biomarkers include GSTP1 and RASSF1A, urine-based DNA markers that were shown to be hyper methylated in patients with PCa [80]. GSTP1 (glutathione-S-transferase P1) hypermethylation was detected in more than 90% of

prostate tumours (no detection was found in BPH), whereas RASSF1A was found to be hypermethylated in 60%–74% of prostate tumours (18.5% in BPH samples).

Prostate cancer antigen 3 (PCA3, also known as DD3), a urine-based RNA marker that has recently been made commercially available, is another very promising biomarker for PCa diagnosis as its expression was not found in any other normal human tissues. PCA3 was highly over-expressed in more than 95% of primary and metastatic PCa [81]. It is believed to function as a non-coding RNA due to its multiple stop codons across the reading frames and a non-extended open reading frame. Despite its specificity in detection of PCa, its prognostic value for aggressiveness has been controversial. Some reports indicate no correlation between PCA3 score and more established prognostic parameters such as Gleason score, tumour size and stage [82, 83]. In contrast, earlier studies found a positive correlation between high PCA3 scores, Gleason score ≥7 and tumour volume [84, 85]. Due to its specificity and high expression in PCa tissues, PCA3 is currently the most specific gene product available in PCa diagnosis and could prove fundamental in selecting patients for active surveillance. The potential for PCA3 as a suitable predictor of PCa aggressiveness still remains unclear.

α-methylacyl-CoA racemase protein (AMACR, also known as P504S) is another potential biomarker that has been found to be consistently upregulated in PCa. Studies revealed that 88% of PCa cases (CRPC and untreated metastases) had a high expression of AMACR [86]. Moreover, immunohistochemical data showed that AMACR expression in needle biopsies had a 97% sensitivity and a 100% specificity for PCa detection [87]. AMACR is currently used as a biomarker for PCa biopsies and is regarded as an improvement over the serum PSA test [88], however efforts are ongoing to develop a body fluid-based assay for AMACR, with its detection in urine specimen being the most promising test to date [89].

A broader approach for PCa diagnosis could comprise the use of a multiplex panel of possible prostate cancer biomarkers. One study tested simultaneous analysis of several genes such as PCA3, TMPRSS2-ERG, Annexin A3, Sarcosine, and urine PSA, with the assay proving to have a higher accuracy when compared with the individual markers alone [90]. Another similar assay, including AMACR, ERG, GOLPH2, PCA3, SPINK1, TFF3, and TMPRSS2-ERG, using sedimented urine, outperformed PCA3 or serum PSA for the early detection of

prostate cancer [91]. In the future the diagnosis of PCa could be determined by these multiplex analyses, however further optimisations and validations are needed.

Recently our group has shown that Sphingosine-1-phosphate (S1P) was inversely correlated with the incidence of PCa. Circulating S1P levels were an early marker of PCa progression to hormonal unresponsiveness and correlated with prostate-specific antigen levels and lymph node metastasis. Interestingly, the decrease in circulating S1P in PCa patients was accompanied by a decrease in SPHK1 activity in comparison with healthy patients, suggesting a potential role of S1P and SPHK1 activity in erythrocytes as possible biomarkers for early diagnosis of PCa [92].

1.8 Sphingolipids

Sphingolipids belong to the lipids family and are characterised by a long chain base or sphingoid (long-chain aliphatic amines, containing two or three hydroxyl groups) that are bonded to fatty acids via amide bonds. They are mainly located in the plasma membrane where they can act as an anchoring site for extracellular proteins, or have a structural function by conferring stability and chemical resistance to the outer layer of the lipid bilayer [93]. Two key sphingolipids, S1P and ceramide, have attracted researchers attention due to their pleiotropic effects on cellular processes such as growth, apoptosis, survival, angiogenesis and immune cell trafficking, with implications in human diseases such as cancer, inflammation, and immune and metabolic disorders [94-96].

1.8.1 Biosynthesis of S1P

In sphingolipid metabolism ceramide is the central metabolite of sphingolipids biosynthesis as it is the only precursor for sphingosine metabolism (Figure **1.2**). Ceramide can be produced *de novo* by the condensation of serine and palmitate, often of dietary origin, or by the hydrolysis of sphingomyelin, glucosylceramide or galactosylceramide [97]. Ceramidases catalyse the conversion of ceramide into sphingosine, which is then phosphorylated by SPHK1 or SPHK2 into sphingosine-1-phosphate. S1P is the only exit point of the sphingosine pathway, it can either be dephosphorylated by S1P phosphatases (SPP1

or SPP2) into sphingosine or converted into the non-sphingolipid ethanolamide phosphate by S1P lyase [96]. Ceramide and sphingosine are important mediators of stress responses and are inducers of apoptosis and cell cycle arrest [93, 98, 99]. On the contrary, S1P has a pro-survival role, where it can abrogate apoptosis and induce cell proliferation, growth and survival [96, 98]. The opposite roles of ceramide/sphingosine *versus* S1P lead to the concept of the "sphingolipid rheostat" whereby the dynamic balances between these metabolites can determine cell fate [98].

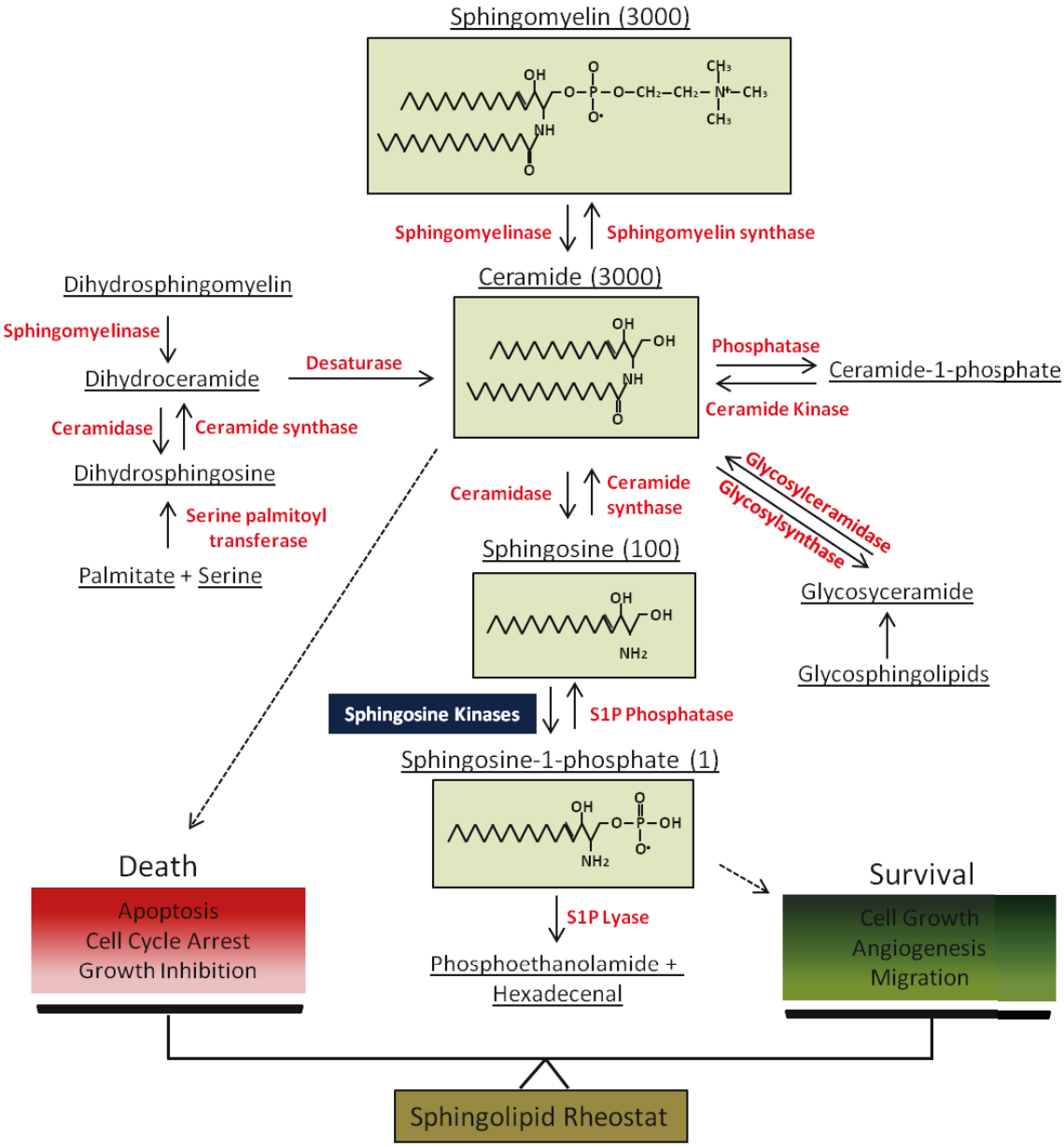


Figure 1.2: Sphingolipid metabolism. The structure of the key sphingolipids and their metabolic cycle are shown, the enzymes involved at each step are shown in red. Ceramide can be synthesised by the hydrolysis of Sphingomyelin by sphyngomyelinase, or by the degradation of Glycosphingolipids. Alternatively, ceramide can be produced by palmitate and serine condensation and further converted into dihydrosphingosine and dihydroceramide, which in turn can also be synthesised from dihydrosphingomyelin. Ceramide is then converted into sphingosine at a ratio of 30/1 by ceramidase. Sphingosine can be phosphorylated into Sphingosine-1-phosphate (S1P) by sphingosine kinases at a ratio of 100/1. S1P phosphate converts S1P to sphingosine whereas S1P lyase converts S1P into a non-sphingolipid phosphoethanolamide and hexadecenal. The degradation of S1P is the only exit from the Sphingolipid pathway. The balance between S1P (pro-survival) and ceramide/sphingosine (apoptotic inducers) levels is known to influence cell fate and led to the concept of the "Sphingolipid Rheostat".

1.8.2 S1P secretion and transport

S1P in the plasma is associated with high-density lipoproteins (HLP) and albumin, and concentration varies between 0.2 to 1µM, whereas in serum it ranges from 0.4 to 1.1µM [100]. S1P levels in the blood and other bodily fluids are higher than in tissues, which suggests that there is a secretion mechanism for S1P [101], with the platelets, red blood cells and epithelial cells being the major sources of plasma S1P [102, 103].

S1P is constitutively expressed and its regulation is affected by the balance between SPHKs and S1P lyases [96]. S1P can act as an intracellular second messenger where it is involved in calcium mobilisation, preventing apoptosis and inducing cell growth [104, 105], however its mechanisms of action have not been fully elucidated. The presence of a polar head group prevents S1P migration across the plasma membrane without a dependency on transmembrane transporters. In this context, ATP-binding cassette (ABC) transporter family members (ABCC1 and ABCG2) and sphingolipid transporter spinster homolog 2 (SPNS2) have been described to be involved in S1P export, as indicated in figure **1.3**. ABCC1 was shown to be important for S1P secretion in mast cells, the inhibition of ABCC1 by small interference RNA or by specific inhibitors markedly decreased S1P export from these cells [106]. A similar finding was reported in rat uterine leiomyoma cells (ELT3) when S1P secretion was abolished by ABCC1-siRNA, interestingly no effect was observed when ABCA1 or ABCB1 transporters were inhibited [107]. The importance of the ABCA1 transporter in S1P secretion has been characterised in astrocytes [108] and platelets [109] and in endothelial cells both ABCC1 and ABCA1 were shown to be of equal importance [110]. In MCF-7 breast cancer cells, estradiol-induced S1P export via ABCC1 and ABCG2 transporters has been reported [111]. Some studies in zebrafish myocardial precursors and CHO cells indicate that the export of S1P from cells requires SPNS2 [112, 113]. A schematic representation of S1P secretion and transport is shown in figure **1.3**.

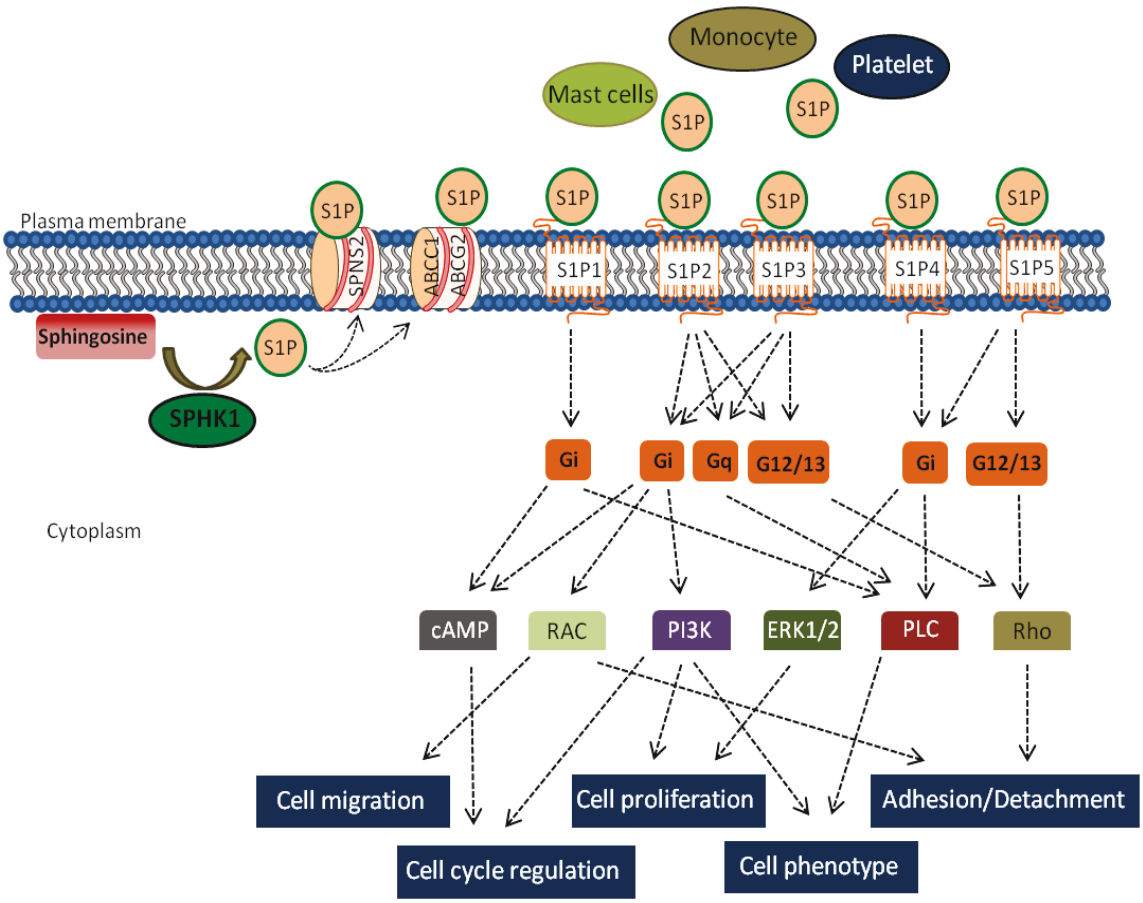


Figure 1.3: S1P receptor signalling. Sphingosine Kinase 1 (SPHK1) catalyses the phosphorylation of sphingosine into sphingosine-1-phosphate. This reaction occurs in the plasma membrane where sphingosine is located. Upon production, S1P can be secreted into the extracellular space where it can act in an autocrine or paracrine manner. S1P secretion can be mediated by an ATP-binding cassette (ABC) transporter family (ABCC1 or ABCG2) or by sphingolipid transporter spinster homolog 2 (SPNS2). S1P can bind to a family of 5 G-Protein coupled receptors named S1P1-5 that, in turn, interact with different G proteins (Gi, Gq and G12/13). These proteins are involved in the activation of several important signalling molecules (cAMP, RAC, PI3K, ERK1/2, PLC and Rho) which in turn regulate several important cellular processes.

1.8.3 S1P receptors signalling

Extracellular S1P binds specifically to a family of G-protein coupled receptors (GPCRs) named $S1P_{1-5}$, encoded by endothelial differentiation genes (EDG). Through these it can mediate both autocrine and paracrine actions [114]. $S1P_{1-5}$, as the receptors are now known, are historically referred to as their EDG family names of EDG1, 5, 3, 6 and EDG8 respectively. Upon activation, S1P receptors (S1PRs) are known to couple to different G proteins which in turn are involved in several downstream signalling pathways. S1P1 receptor was shown to bind exclusively to G_i whereas S1P2 and S1P3 coupled with G_i, G_q and G_{13} [115]. S1P4 can couple to G_i, and $G_{12/13}$ [116], while S1P5 couples with G_i and G_{12} [117]. A schematic representation of the binding patterns can be seen in figure **1.3**.

S1P receptors are ubiquitously expressed on all cells, however their relative expression levels vary by cell type. S1P1, S1P2 and S1P3 are widely expressed in most mammalian tissues, S1P4 is mainly present in hematopoietic and lymphoid tissue whereas S1P5 is highly present in brain tissue [118]. S1P activation of S1P1 stimulates motility through activation of the GTPases Rac1 and Cdc42 in fibrosarcoma cells [119], whereas in ovarian cancer cells S1P1 and S1P2 are involved in cell invasion and calcium mobilisation [120].

S1P1 is also crucial for immune-cell trafficking, more specifically the migration of hematopoietic cells and lymphocytes into blood, lymph and tissues [121, 122]. S1P1 knockout mice were found to die prenatally as a consequence of incomplete vascular maturation which caused vascular leakage, indicating a role of S1P1 in angiogenesis and vascular maturation [123]. In CHO cells, S1P-induced activation of S1P1 receptors lead to ERK1/2 activation in a Ras-dependent manner, simultaneously there was an inhibition of cyclic AMP accumulation [124].

S1P, via **S1P2** receptor, inhibits cell motility in glioblastoma and astrocytoma cells [125], whilst S1P2 deletion in MEF cells increased their migration towards serum or PDGF. The authors also found that this effect was dependent upon SPHK1 activity and S1P1 [126]. In glioma cells S1P2 is involved in increasing invasiveness via cytoskeletal rearrangements [127]. S1P2, but not S1P1 or S1P3, decreased doxorubicin-induced apoptosis in cardiomyocytes via ERK1/2 and STAT3 [128]. *In vivo* studies in S1P2-deficient mice showed no anatomical or physiological defects during birth, however spontaneously lethal seizures

occurred within the first seven weeks of life. The authors proposed that S1P2 was involved in the development and mediation of neuronal excitability [129].

S1P3 is highly expressed in heart, lung, kidney and spleen, however its absence (as investigated in $S1P3^{-/-}$ mice) does not induce any phenotype modifications. S1P3 signalling via ROCK and Rho is important for the regulation of vascular permeability and chemotactic migration of cancer cells towards growth factors [127, 130]. The role of S1P3 in endothelial barrier function is critically important for immune responses [131] and more recently S1P3 was suggested as a biomarker for acute lung injury as a result of its increased levels in circulation during inflammatory lung states [132].

S1P4 is involved in cytoskeletal rearrangement, calcium mobilisation and cell motility [116]. Furthermore, in $S1P4^{-/-}$mice, megakaryocytes showed atypical and reduced formation of proplatelets indicating S1P4 involvement in megakaryocyte terminal differentiation. S1P4 involvement in T-cell cytokine production has been proposed; the receptor was shown to mediate immunosuppressive effects of S1P through the inhibition and enhancement of the secretion of cytokines [133]. In MDA-231 cells S1P4 was crucial for ERK1/2 activation via HER2 (human epidermal growth factor receptor 2) upon S1P stimulation, with the authors proposing S1P4 as a possible target for breast cancer therapy [134]. A more recent study showed that high levels of S1P4 expression were associated with shorter disease-free periods for breast cancer patients [135].

In prostate cancer cells **S1P5** is required for an S1P-induced effect in increased autophagy activity in starvation conditions [136]. In MEK cells S1P5 was shown to localise with SPHK1 and SPHK2 in centrosomes, indicating a possible role of S1P5 in cell division [137]. In brain endothelial cells S1P5 is required for modulation of endothelial inflammatory processes by maintaining an immunoquiescent state in the brain [138]. Furthermore, natural killer (NK) cell trafficking and mobilisation to inflamed organs was affected in S1P5 deficient mice [139].

1.8.4 S1P signalling as a therapeutic target

It is clear that S1P receptors play a pivotal role in modulating S1P responses upon binding interactions. The immunosuppressant drug FTY720 (Fingolimod), a sphingosine analogue, has been described to decrease autoimmune disease severity in animal models [140, 141]. Interestingly, FTY720 actions appear to be mediated through S1P receptors, in particular S1P1 where it induces receptor internalisation and degradation. S1P-induced migration of lymphocytes was blocked by FTY720 in an S1P1-dependent manner; another study showed S1P1 activation by FTY720 reduced lymphocyte transmigration by increasing endothelial barrier function [121, 142]. Indeed, FTY720 immunosuppressive properties against T cell migration could contribute to tumour tolerance and development by inhibiting lymphocyte recruitment from lymph nodes into efferent lymphatics and blood [143].

FTY720 was also shown to have anticancer properties; FTY720 treatment induced prostate cancer apoptosis, reduced tumour growth and increased radiotherapeutic sensitivity. Interestingly, the FTY720 inhibition of SPHK1 was crucial for its pro-apoptotic actions [144]. This potent inhibitory effect has been reported in other types of cancer such as breast [145], lung [146] and pancreatic [147]. FTY720 phosphorylation is an important step for its activation, however the mechanism underlying this process is still in debate. SPHK1 and SPHK2 are natural candidates, with a SPHK2 knockout mouse model displaying impaired FTY720 effects in reducing the number of lymphocytes in the blood [148]. In contrast, a similar study using a knockout SPHK1 mouse model did not reduce FTY720 effects on lymphocyte numbers in the blood, indicating that SPHK1 may not be involved in FTY720 phosphorylation. The molecular mechanisms of FTY720-induced cancer growth arrest and apoptosis include activation of caspases [149], mitochondrial damage [150] and inhibition of cyclin D1 , Akt and ERK1/2 [151]. FTY720 has been approved by the FDA for the treatment of multiple sclerosis [152] however due to its anti-cancer effect it is reasonable to assume that an additional indication as a possible therapy for cancer may be considered.

1.8.5 S1P internalisation

Upon activation, S1P receptors can either be transported to lysosomes for degradation or can be recycled back to the plasma membrane (re-sensitisation). The lysosome pathway is considered a way to down-regulate receptor expression and consequently to abrogate its intracellular signalling [153]. At the moment the causes underpinning this difference in receptor fate remain unclear, with some reports indicating that ubiquitination status could play a major role. Studies on S1P1 receptor showed that S1P and FTY720-P induce different receptor conformations, most likely due to the fact that S1P induces mono-ubiquitination whereas FTY720-P induces poly-ubiquitination and consequent degradation [154]. Internalisation of S1P receptors is important not only due to their capacity to activate nuclear signalling pathways but also due to the capacity to directly interact with important signalling molecules. In this context S1P1 has been shown to be phosphorylated by Akt and GPCR kinases (GRKs) on serine and threonine residues [155, 156]. Furthermore, in endothelial cells S1P induced a rapid S1P1 internalisation and translocation from the plasma membrane to the nucleus where it was involved in the transcription of Cyr61 and CTGF, two growth factors involved in the regulation of vasculature [157]. In mast cells activation of SPHK1 leads to an increase in intracellular S1P which in turn promotes S1P1 and S1P2 internalisation; the mechanism underlying the internalisation of these receptors still remains unclear [158]. Contrary to S1P1, the S1P3 internalisation event seems to be independent of phosphorylation [159]; interestingly S1P3 internalisation appears to be quicker than for S1P1 receptors. S1P and FTY720 are both known to induce a concentration-dependent effect on S1P3-receptor internalisation [159, 160]. In contrast with the other S1P receptors, S1P5 is constitutively active and is mainly located in intracellular compartments, believed to be as a consequence of constitutive internalisation. Nonetheless the remaining plasma membrane fraction is regulated and internalised by S1P and FTY720 ligand binding. S1P5 translocation to the centrosomes indicates a possible role in cell division [137].

1.8.6 S1P signalling independently of S1P receptors

Recent reports indicate a nuclear epigenetic regulation by S1P binding to histone deacetylases (HDAC1 and HDAC2) [161]. In support, *in vivo* work performed in S1P lyase-deficient mice showed elevated intracellular levels of S1P and enhanced Ca2+ storage, whilst nuclear accumulation of S1P caused a reduction in HDAC activity. This was accompanied by an increase in acetylation of histone 3-Lys9 and the HDAC-regulated gene p21 cyclin-dependent kinase inhibitor, leading to a dysregulation of Ca2+ homoeostasis [162]. Moreover, S1P was shown to form complexes with TNF receptor-associated factor 2 (TRAF2), resulting in the activation of NF-KB signalling [105].

The multifaceted roles of S1P in cell survival, proliferation, migration, angiogenesis and immune system regulation including the pro-inflammatory response, identify S1P as a key target in the treatment of cancer and inflammatory disease, upon which potential new therapies could be focused [163]. Consequently S1P regulators such as SPHKs could also be targeted in the same clinical setting.

1.9 Sphingosine Kinase

Cellular S1P formation is mediated by the action of sphingosine kinases (SPHKs). Two kinases have been identified, SPHK1 and SPHK2, the genes for which are located on different chromosomes; SPHK1 is present at 17q25.2 whereas SPHK2 is found at 19q13.2. In terms of size, SPHK1 encodes a protein of 43KDa whilst a 65KDa protein is encoded by SPHK2. There is high homology between the sequences of the two enzymes, with N-terminal sequences displaying 47% similarity whereas C-terminal regions share 43% identical amino acid sequence. There are five highly conserved regions evolutionarily present within all known sphingosine kinases (C1-C5). Their catalytic domain is located within C1 and C3 whereas the ATP-binding site for S1P phosphorylation of sphingosine is situated within the C2 domain. To date, three isoforms of human SPHK1 and two isoforms of human SPHK2 have been identified, a schematic representation of which can be seen in figure **1.4**.

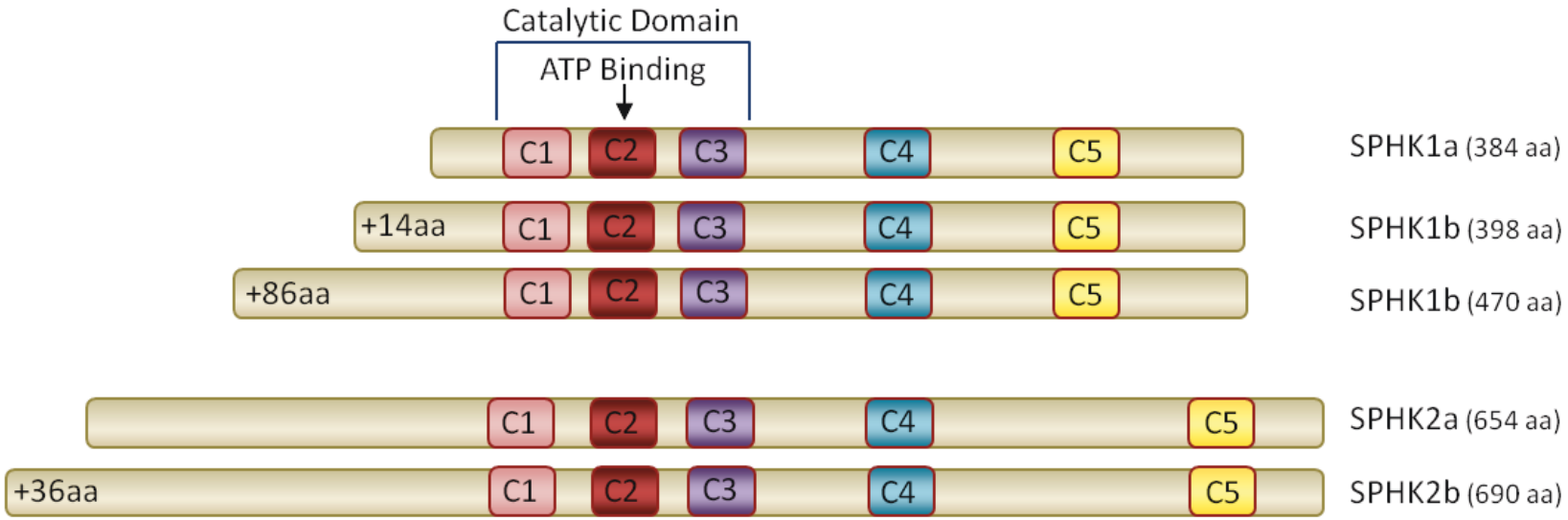


Figure 1.4: Human Sphingosine Kinase isoforms. Schematic representation of both human SPHKs and their respective isoforms. Both kinases have five highly conserved regions (C1-C5), with the catalytic site situated within the C2 domain. SPHK2 is larger than SPHK1; these differences in size are due to an additional region at the N-terminus and another within the conserved domains C4 and C5. So far three SPHK1 splice variants have been described (SPHK1a, -b and –c) that vary at the N-terminus regions with SPHK1b having 14 more amino acids and SPHK1c having 86 more amino acids than SPHK1a. SPHK2 has two splice variants (SPHK2a and –b); SPHk2b is expressed more abundantly and possesses 36 more amino acids than SPHK2a. Some reports indicate that these two splice variants arise from alternative start codon usage.

There are three splice isoforms of SPHK1 (SPHK1a, b and c) that differ only in their N-terminal region. There are an additional 14 amino acids in SPHK1b (RefSeq NM_021972) and a further 86 in SPHK1c (RefSeq NM_182965) in comparison with SPHK1a (RefSeq NM_001142601). The longer sequence of SPHK2 is mainly due to its additional regions at the N-terminus and between the conserved C4 and C5 regions (**Fig.1.4**). To date there are two SPHK2 isoforms reported and these appear to arise from alternative start codons, SPHK2a (GenbankTM accession number AF245447) and SPHK2b (RefSeq NM_020126). SPHK2b has an additional 36 amino acids in the N-terminus region and appears to be a more dominant form in cell lines and tissues [164]. This region contains nuclear localisation and export signals that are not present in SPHK1 [165].

1.9.1 Localisation of SPHK1 and SPHK2

Celullar localisation of SPHKs appears to be important for their function. SPHK1 is mainly cytosolic whereas upon activation it translocates to the plasma membrane where it produces S1P. Importantly, this translocation event is only observed for the wild-type form of SPHK1; phosphorylation-deficient forms are unable to translocate and subsequently unable to induce an oncogenic cascade. However, both forms of SPHK1 retain intrinsic activity; the basal levels of phosphorylation-deficient forms are indicative of an ability to maintain a housekeeping role independent of its signalling role. The phosphorylation and subsequent translocation of SPHK1 to the plasma membrane are therefore critical factors involved in the oncogenic effect of this protein [166]. Supporting its subcellular localisation in the plasma membrane, phosphatidic acid [167] and filamin [168] have been implicated in SPHK1 function. Calcium and integrin-binding protein 1 (CIB1) interacts with SPHK1 in a calcium-dependent manner and functions as a Ca(2+)-myristoyl switch that enables SPHK1 translocation to the plasma membrane. Inhibition of CIB1 by siRNA or by dominant-negative CIB1 blocked SPHK1 translocation upon PMA stimulation [169]. It is to be noted that the authors suggest that CBI1 binding to SPHK1 is not dependent on Ser225, instead they believe that CIB1/SPHK1 binding to the plasma membrane is calcium dependent and that S225 phosphorylation is important in retaining SPHK1 in the plasma membrane by enabling the binding to acidic phospholipids such as phosphatidylserine.

The localisation of SPHK1 within the plasma membrane plays an important role in the regulation of its signalling function; the location of SPHK1 in lipid rafts, highly organised membrane microdomains implicated in signalling molecule assembly and membrane protein trafficking, induces pro-survival and proliferative effects [170]. In contrast, another study indicated that an SPHK1-derivative tagged with a myristoylation sequence (50-fold increase in SPHK1 activity in crude membrane) had a strong inhibitory effect on cell proliferation but an increased protection from apoptosis induced by serum withdrawal. SPHK1 containing a false myristoylation sequence and consequently less present in the lipid rafts did not protect cells from apoptosis, suggesting a different role for SPHK1 according to its location [169].

SPHK2 is mainly located in the nucleus and cytoplasm; however, studies have reported changes in SPHK2 location under different conditions and cell type. In HEK293 cells it is present in the plasma membrane, mitochondria, endoplasmic reticulum (ER) and cytosol [163], whilst in HeLa and MCF-7 cells it is predominantly found in the nucleus [165]. Upon serum deprivation SPHK2 was found to be highly present in the ER where it is believed to be involved in ceramide-mediated pro-apoptotic signalling by metabolising S1P [171]. Protein kinase D (PKD) activation induces a post-translational regulation of SPHK2 leading to its export from the nucleus in HeLa cells. It has been shown that PKD phosphorylates serine residues in the nuclear export signal of SPHK2 both *in vivo* and *in vitro* [172]. SPHK2b was found to be secreted from apoptotic cells in a caspase-1 dependent event involving cleavage at its N-terminus [173]. Similarly, the catalytically active form of SPHK1 is shed in vesicles from tumour cells. The presence of sphingosine within the membrane of the shed vesicles provides an appropriate substrate for the SPHK1, leading to the concept that the vesicles could be a site for S1P production in the extracellular medium and also within other cells if endocytosis occurs [174]. A schematic representation of SPHK1 and SPHK2 localisation is shown in figure **1.5**.

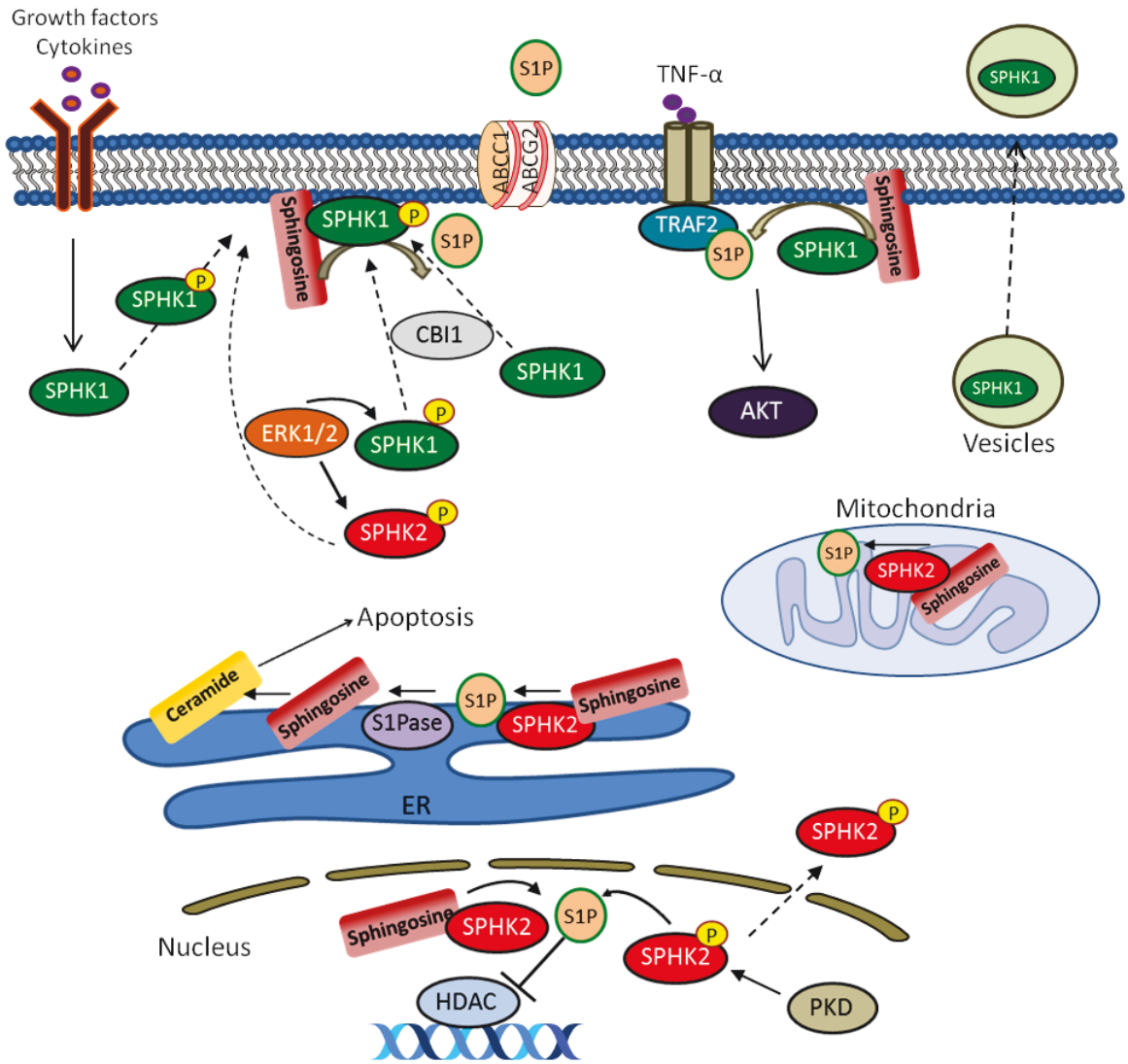


Figure 1.5: Cellular location of sphingosine kinase 1 and 2. SPHK1 is mainly located in the cytoplasm; upon activation SPHK1 translocates to the plasma membrane to catalyse the phosphorylation of sphingosine into S1P. SPHK1 activation can be mediated by ERK1/2 phosphorylation or through receptor signalling upon binding of growth factors (e.g EGF, TGF-β) or other cytokines (e.g TNF-α). Transport to the membrane can be mediated by calcium and integrin-binding protein 1 (CIB1) in a calcium-dependent manner. Catalytically active SPHK1 could also be shed into vesicles and secreted from the cell. SPHK2 is predominantly present in the nucleus where it can be phosphorylated by protein kinase D (PKD) and exported from the nucleus, or it can produce S1P. In the nucleus S1P acts as a repressor of histone deacetylases (HDAC). In the cytoplasm SPHK2 can be directly phosphorylated by ERK1/2 and translocated to the plasma membrane, where it produces S1P. In the endoplasmic reticulum (ER) SPHK2 is involved in apoptotic signalling through S1P, which in turn, is degraded by S1P phosphatases (S1Pase) to sphingosine and ceramide. SPHK2 was also found to be present in the mitochondria, where it produces S1P.

1.9.2 Sphingosine kinase activation and regulation

Sphingosine kinases have intrinsic catalytic activity, this allows the generation of basal levels of S1P to occur independently of any post-translational modifications in both kinases [175]. In SPHK1 this intrinsic activity can be further increased via stimulation by several soluble molecules such as platelet-derived growth factor (PDGF), fetal calf serum (FCS) [176], nerve growth factor (NGF) [177], endothelial growth factor (EGF) [178], vascular endothelial growth factor (VEGF) [179], agonists of GPCRs, vitamin D [180], transforming growth factor-beta (TGF-β) or pro-inflammatory cytokines such tumour necrosis factor-alpha (TNF-α) [181].

ERK1/2 (extracellular signal-regulated kinases 1 and 2) is an important regulator of SPHK1 activity as it has the ability to further increase SPHK1 activity. Pitson *et al*. showed that in HEK293 cells ERK1/2 phosphorylates SPHK1 on Ser225 leading to an increase in SPHK1 activity and translocation to the plasma membrane [182]. SPHK1 binding specificity to membrane acid phospholipids is increased by conformational changes induced by its phosphorylation at Ser225, suggesting that these conformational changes expose the lipid binding domain [183]. Phorbol 12-myristate 13-acetate (PMA) induces SPHK1 up-regulation via the activation of activator protein-2 (AP-2) binding motifs; an effect that is abrogated by ERK1/2 and protein kinase C (PKC) inhibition. These findings suggest that ERK1/2 and PKC mediated transcriptional factors that bind AP-2 motifs regulate SPHK1 transcription [184, 185]. SPHK1 activation is reported to regulate Ca^{2+} signalling, which in turn regulates SPHK1 translocation and subcellular distribution, however Ca^{2+}/calmodulin is not critical for SPHK1 activity [186]. Other signalling molecules such as PI3K/AKT2 and mTOR have also been shown to be involved in SPHK1 regulation in imatinib-resistant chronic myeloid leukemia cells [187]. K-RAS, an upstream regulator of the MAPK pathway, increased S1P production via an SPHK1 dependent manner; it also increased SPHK1 plasma membrane localisation leading to a decrease in cytoplasmic levels [187]. Cytokines have also been shown to play a role in the activation of SPHK1; TNF-α activates SPHK1 via a TNF-associated factor 2 (TRAF2)-dependent mechanism, leading to activation of the Akt signalling pathway in glioblastoma cells [188]. The aforementioned multiple signalling molecules that are associated with SPHK1 regulation and activation are important mediators

in oncogenic events; this clearly underpins SPHK1 as a focal point in such events and the far reaching implications of targeting SPHK1 in potential cancer therapies.

1.9.3 The role of SPHK2

The role of SPHK2 in a pro- or anti-apoptotic manner is still debatable; early reports indicate its main mechanisms of action to be via inhibition of DNA synthesis, suppression of cell growth and proliferation through the activation of cell cycle inhibitors [161]. Moreover SPHK2 has BH3 (Bcl-2 homology 3)-binding domain activity that can inhibit the anti-apoptotic protein BCL2L1 and induce apoptosis [189], however contrasting publications implicate SPHK2 in a pro-survival role. SPHK2 knockdown increases sensitivity of glioblastoma, breast, colon and lung tumour cells to chemotherapy [190, 191]. In support, xenograft tumours (MCF-7 cells) showed a reduction in growth when SPHK2 was subject to knockdown [192], whilst similar findings were reported in breast adenocarcinoma, hepatocellular carcinoma and renal xenograft tumours following SPHK2 inhibition [193-195]. *In vitro*, SPHK2 chemical inhibition induced autophagic cell death in kidney, prostate and breast tumour cell lines and also a decrease in cell proliferation [195]. Overall, it is clear that the role of SPHK2 varies in different settings, with further studies needed to elucidate and clarify its potential as a pro- or anti-tumorigenic factor.

SPHK2 has also been shown to play a role in the proliferation of synovial fibroblasts in rheumatoid arthritis [196]. In addition it was also shown that SPHK2 is both ubiquitously and highly expressed in limb buds, branchial arches and eyes, indicating a possible role for SPHK2 in their development [197]. The genetic ablation of SPHK2 or SPHK1 in a mouse model did not result in abnormalities, however when both SPHKs were simultaneously deleted it proved to be embryonically lethal due to severe bleeding [198]. The author also showed abnormalities in angiogenesis and neurogenesis suggesting a role for SPHKs in these critical physiological processes.

1.9.4 The role of SPHK1

SPHK1 is a multi-faceted molecule with wide-ranging implications in the promotion of cellular survival, proliferation and transformation, stimulation of angiogenesis and apoptosis. As a consequence, its role in tumour progression and chemoresistance has been studied, with significant findings outlined below.

1.9.4.1 The role of SPHK1 in cancer

SPHK1 is over-expressed in several solid tumours and is associated with a poor prognosis in patients with glioblastoma [199] and breast cancer [200]. High levels of SPHK1 were detected in more than 60% of colorectal cancer tumours [201] and in colon cancer SPHK1 expression was higher in carcinomas than in benign lesions [202]. Importantly, SPHK1 expression was crucial for tumour growth and aggressiveness in azoxymethane-induced colon carcinomas [202], whilst in breast cancer SPHK1 expression is estimated to be 4-fold higher than in normal breast tissue [203]. In both ER positive and ER negative tumours, SPHK1 expression was correlated with a poor outcome and increase in metastatic incidence during a five year period [200]. In prostate cancer SPHK1 activity was correlated with PSA levels, tumour volume, gleason score and disease recurrence in patients who underwent radical prostatectomy [204]. Moreover, SPHK1 is reported to have a ten-fold higher basal activity in androgen independent metastatic PC-3 than in androgen-dependent, non-metastatic LNCaP cells; interestingly androgen deprivation increases SPHK1 levels in LNCaP cells in a PI3K/AKT dependent manner. The authors propose that SPHK1 could act as a compensatory mechanism to androgen deprivation that could ultimately lead to androgen-independence [205].

The mechanism underlying SPHK1 over-expression in PCa still remains unclear, however a recent study showed for the first time that SPHK1 is regulated by prostate tumour inducer-1 (PTI-1) which can induce neoplastic cell transformation. PTI-1 is a truncated isoform of eEF1A1 (elongation factor 1A) that was previously shown to activate and interact with SPHK1 [206]; indeed these actions appear crucial for PTI-1 induced neoplastic transformation. SPHK1 inhibitors or the over-expression of dominant-negative SPHK1 abrogate the

carcinogenic effect of PTI-1, indicating SPHK1 may be a crucial partner in prostate cancer oncogenesis [207].

SPHK1 has also been shown to induce cancer cell migration. In the MCF-7 breast cancer cell line estradiol-induced migration was accompanied by an increase in SPHK1 activity and inhibited by the use of siRNA-SPHK1 [208]. In gastric cancer cells SPHK1 expression and protein levels were increased after LPA (Lysophosphatidic acid) stimulation, this was concomitant with an increase in migration. Inhibition of SPHK1 or EGF receptor attenuated LPA-stimulated migration and invasion of MNK1 cells [130]. In ML-1 thyroid carcinoma cells SPHK1 over-expression increased migration, with the authors identifying the autocrine S1P-induced ERK1/2 and PKC activation loop as crucial for this migration [209]. Expression of PAI-1 and uPAR, two known regulators of cell migration and adhesion, have also been linked with SPHK1 expression by increasing adhesion and invasion of U373 glioblastoma cells [210].

Angiogenesis is a critical and vital process in tumour growth, with SPHK1/S1P signalling reported to be involved. S1P can induce VEGF expression and promote endothelial cell growth [211]. Tumour cells can secrete S1P into peripheral blood or lymph flow to attract different cells, such as vascular endothelial cells, into the tumour site [212]. In murine models S1P1 expression is strongly induced in tumour vessels whereas its ablation by siRNA abrogates angiogenesis and decreases tumour growth [213]. VEGF-induced blood vessel formation and endothelial cell migration was inhibited by S1P antibody in several cancer cell lines. Proangiogenic cytokines such as IL-8 and IL-6 were also reduced by the presence of an anti-S1P antibody [214]

1.9.4.2 The role of SPHK1 in chemoresistance

SPHK1 over-expression increases resistance to chemotherapeutic agents such as doxorubicin in MCF-7 breast cancer cells; conversely, SPHK1 inhibition sensitises these cells to the drug [215]. The synergistic effect of SPHK1 inhibition with other chemotherapy agents such as camptothecin [216] and radiotherapy have also been reported [144]. SPHK1 over-

expression inhibited apoptosis in MCF-7 breast cancer cells and HL-60 acute myeloid cells upon doxorubicin and etoposide treatment [217, 218], whilst the SPHK1 inhibitor N,N-dimethylsphingosine (DMS) proved very effective in overcoming radio and chemo-resistance in acute myeloid leukaemia cells [219]. An increase in sensitivity to cisplatin, carboplatin and doxorubicin was observed in HEK293 and A549 lung cancer cells expressing human S1P lyase [220], whilst high levels of SPHK1, S1P1 and S1P3 were associated with the development of tamoxifen resistance in ER positive breast cancer patients [221]

Previous work by Pchejestki *et al*. showed that over-expression of SPHK1 in LNCaP and PC-3 cells increased their resistance to docetaxel treatment and that by inhibiting SPHK1 using siRNA this protective effect was abrograted [78, 222]. This mechanism was also confirmed in an *in vivo* setting; increases in tumour volume and docetaxel resistance were observed following SPHK1 over-expression in PCa cells when compared to empty vector controls [222].

1.10 Tumour microenvironment

1.10.1 Cancer and Inflammation

There is strong evidence to suggest that the tumour microenvironment is inflammatory and that activation of the innate immune system plays a role in the progression of cancer. The identification of this phenomenon has led to the development of new strategies that target the multiple interactions of tumour cells with other cell types within the tumour microenvironment [223-225].

The inflammatory tumour microenvironment contains an abundance of cytokines (e.g. CSF-1 (Colony stimulating factor-1), GM-CSF-1 (Granulocyte-macrophage colony stimulating factor), IL-8, IL-6 and TNF-α), chemokines (e.g. CCL1, CCL2, CCL3 and CCL7) and growth factors (e.g. TGF-β, EGF, VEGF) that are secreted by tumour cells and stromal cells (e.g. macrophages, fibroblasts and endothelial cells) creating a favourable microenvironment for tumour survival, growth and progression [226-228]. Likewise, inflammatory diseases such as Crohn's and ulcerative colitis, viral infections such as Hepatitis B and C, or bacterial

infections have been associated with an increased risk of developing cancer [226, 229, 230]. Harmey *et al.* have used *in vitro* and *in vivo* models to demonstrate that lipoplysaccharide (LPS), a cell wall component of Gram-negative bacteria, promotes metastatic growth, angiogenesis and an increase in vascular permeability [231].

1.10.2 Monocyte/Macrophage differentiation: Th1 and Th2 involvement in cancer

Myeloid progenitor cells in the bone marrow are the precursors of monocytes which in turn, after entering the blood stream, have the ability to migrate into different tissues. This migration is modulated by chemotactic stimuli such as chemokines or growth factors, and once in the site (or still in circulation) monocytes can be differentiated to macrophages by different growth factors including GM-CSF or M-CSF [232](Figure **1.6**). As part of their natural functions monocytes and macrophages are among the first cells of the immune response to arrive at sites of infection or wound healing. Recent studies reveal that direct communication between macrophages and tumour cells is critical to invasion and egress of tumour cells into blood vessels [233]. Tumour cells also have the ability to recruit myeloid cells by secreting a variety of different growth factors including GM-CSF, CSF-1 and macrophage stimulating protein (MSP) or chemokines such as monocyte chemotactic protein-1 (MCP-1), macrophage migration inhibitory factor (MIF) or macrophage inflammatory protein-1 alpha (MIP-1α) [233]. Blood circulating and tissue resident monocytes are attracted to tumour sites, where according to the stimulus, they can be differentiated into two main macrophage phenotypes, classically activated macrophages (M1) or alternatively activated macrophages (M2) [234] as shown in figure **1.6**. LPS or IFN-γ are known inducers of M1 activation which is characterised by a high capacity to present antigen, high levels of IL-12, IL-23, nitric oxide (NO) and reactive oxygen intermediates (ROI), along with an activation of Th1 immune responses, including the activation of T-cells and other cell types such as NK cells [235, 236]. Th1 responses are known to have an anti-cancer activity, it is thought M1 type macrophages are capable of inducing lysis in cancer cells due either by the secretion of high amounts of superoxide anions, oxygen radicals, and nitrogen radicals; or by expressing high levels of MHC class I to facilitate complement-mediated

phagocytosis. Their ability to interact with T-Cells though MHC class I and present the antigen to CD8+ T-cell leads to a cytotoxic effect of these cells towards cancer cells [237].

Alternatively activated macrophages, M2, and Th2 immune responses are induced by IL-4, IL-10, IL-13, inhibition of Th1 responses by production of immunosuppressive mediators such as TGF-β or PGE2, an increase in the production of IL-6 and VEGF, and the production of chemokines (e.g. CCL17 or CCL22) capable of recruiting Th2 cells [227, 238].

Tumour associated macrophages (TAMs) are phenotypically similar to M2 macrophages and it has been shown that a high amount of TAMs are associated with a poor clinical prognosis [239]. TAMs are linked to tumour progression, intravasation and the initiation of the metastatic process [238-240]. Interestingly, Chen *et al.* showed that high mobility group box 1 protein (HMGB1) is secreted from necrotic cells and acts as a strong chemotactic factor for macrophages [241]. Conversely, apoptotic tumour cells (ATCs) can release anti-inflammatory cytokines such as IL-10 and TGF-β to promote an M2 phenotype. The macrophage phenotype is also affected by phagocytosis of ATCs resulting in an inhibition of the pro-inflammatory cytokines IL-8, TNF-α and GM-CSF, leading to an increase in TGF-β and PGE2 [242]. Mantovani *et al.* also showed that conditioned media from ATCs can induce a TAM phenotype in macrophages [243].

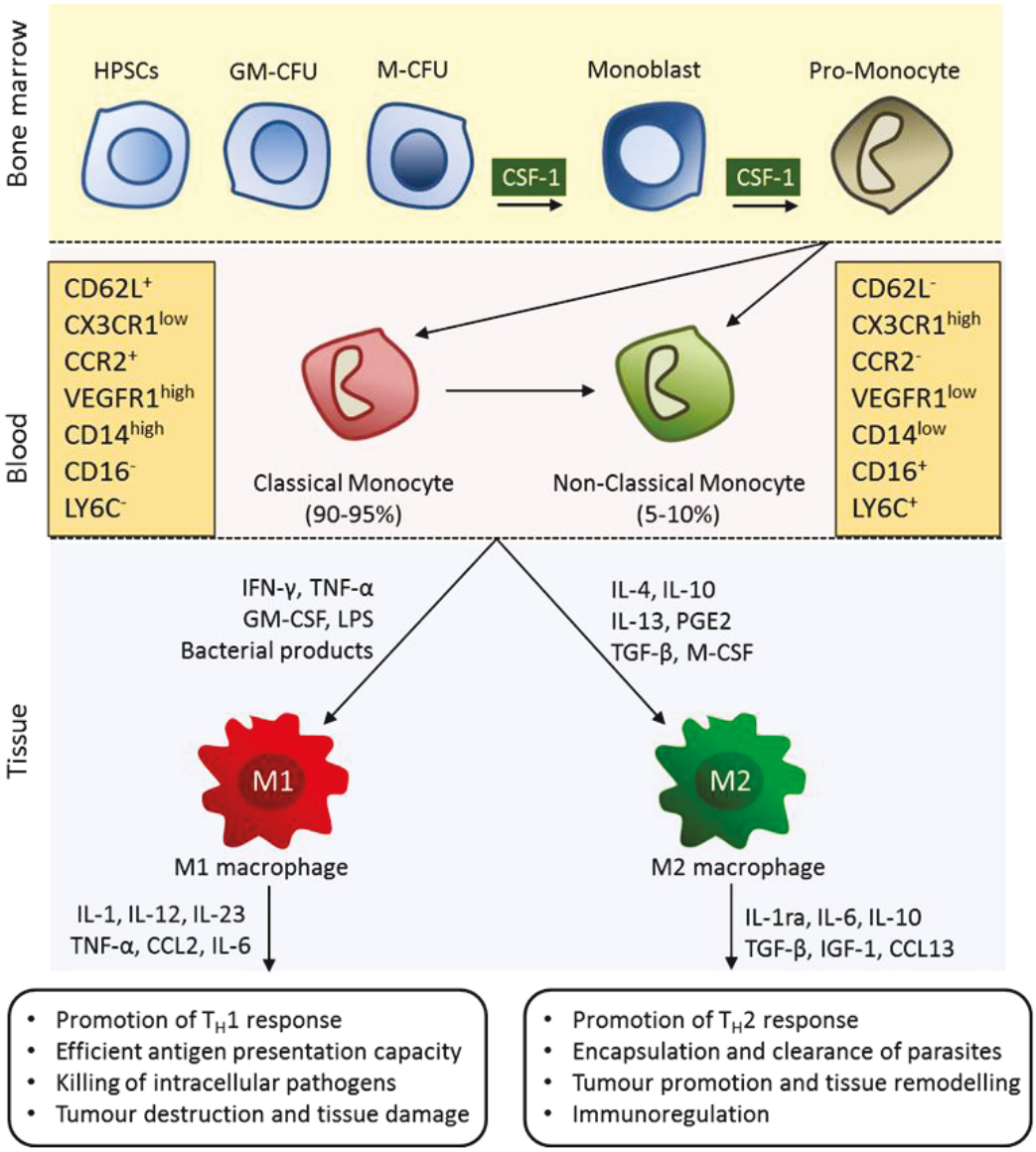


Figure 1.6: Monocyte and macrophage diversity. Both monocytes and macrophages derive from hematopoietic stem cells (HPSCs). HPSCs are produced in the bone marrow where they can be differentiated into pro-monocytes through various multipotent progenitor stages involving granulocyte/macrophage colony-forming unit (GM-CFU) to monocyte colony-forming unit (M-CFU). The presence of colony-stimulating factor 1 (CSF-1) induces M-CFU differentiation into monoblasts and subsequently into pro-monocytes. At this point cells enter the blood stream and monocytes are produced in two main groups; the classical monocytes which comprise approximately 90-95% of total monocytes in an adult, and non-classical monocytes (5-10%). These two sets of monocytes express different surface markers and receptor expression profiles which may determine not only their function but also their migration patterns. Monocyte migration into the tissues leads to their differentiation into either M1 or M2 macrophages depending on the microenvironment signalling profile. Once activated, macrophages secrete an array of cytokines and growth factors commonly accepted as pro-inflammatory (M1) and anti-inflammatory (M2) responses. The interaction with T-cells is different for each subset of macrophages; TH1 (M1 macrophage) and TH2 (M2 macrophage) resulting in different T-Cell responses.

1.10.3 The effect of the tumour microenvironment on cancer development

Inflammatory cells such as monocytes and macrophages have been suggested to play a role in the early stages of cancer development where they appear to be present in areas of hyperplasia and atypia [233, 244]. Importantly, Lin *et al.* reported that mice lacking CSF-1, a strong chemoattractant for monocytes and macrophages, display attenuated tumour progression and metastasis when compared to wild-type mice; an effect not related to changes in primary tumour growth [245]. Conversely, human leukemic monocyte lymphoma cell line (U937) co-inoculated with PC-3 in male athymic nude mice increased tumour growth and vascular density when compared to PC-3 tumours alone. Pre-treatment of U937 with IL-4 to promote an M2 phenotype induced a bigger increase in tumour size and was accompanied by the secretion of CCL2 (MCP-1) [246]. In support, breast cancer cells showed higher invasion and motility into a basement membrane (Matrigel) when in co-culture with U937 cells [247]. Another monocytic cell line, THP-1, enhanced the growth of colon cancer cells in an IL-1β dependent mechanism [248]; interestingly the authors showed that Vitamin D interrupted the cross-talk between tumour cells and monocytes and that it was crucial for a reduction in tumour cell proliferation. PMA-treated THP-1 cells (M2 phenotype) enhanced angiogenesis and invasion in human basal cell carcinoma through the activation of COX-2 [249], whereas in ovarian cancer the increase in angiogenesis was also accompanied by an increase in IL-8 mRNA expression levels [250]. Direct culture of blood derived monocytes with MDA-231 breast cancer cells resulted in increased expression of factors such as MMP9, PAI-1 and uPAR that are involved in cancer cell invasiveness. In this case the authors did not perform analysis on the proliferation or invasion of the cancer cells [251].

1.10.4 SPHK1/S1P regulation in immune cells

Monocytes and macrophages are part of the first line of defence against invading organisms such as bacteria. SPHK1 and S1P have been reported to be involved in antimicrobial activity in macrophages. Indeed, ingestion of non-viable *M.tuberculosis* triggers a rapid increase in SPHK1 activity in human macrophages after its ingestion; interestingly viable *M. tuberculosis* inhibited SPHK1 activity and its translocation to the nascent phagosomes [252]. Moreover, the addition of exogenous S1P activated phospholipase D which is involved in the acidification of mycobacteria-containing phagosome [253]. This study also showed that in mycobacteria-infected mice the addition of S1P reduced pulmonary tissue damage and mycobacterial growth.

As previously described for tumour cells, the SPHK1/S1P signalling pathway can also play a role in the protection of macrophages against apoptosis. S1P secreted from apoptotic cells protected macrophages from TNFα/cycloheximide-induced cell death. This effect was correlated with an increase in PI3K, ERK1/2 and calcium signalling which were shown to be activated by S1P [254]. Furthermore, in apoptotic bone marrow-derived macrophages S1P stimulation inhibited acid sphingomyelinase activity via PI3K signalling. Interestingly there was no ceramide accumulation but instead an increase in Bcl-XL expression [255]. Another study indicated that COX-2 activation by LPS was mediated by SPHK1/S1P; more interestingly S1P can mediate induction of arachidonic acid, an upstream regulator of COX-2 [256, 257]. A schematic representation of SPHK1 and S1P role in macrophages is shown in figure **1.7**.

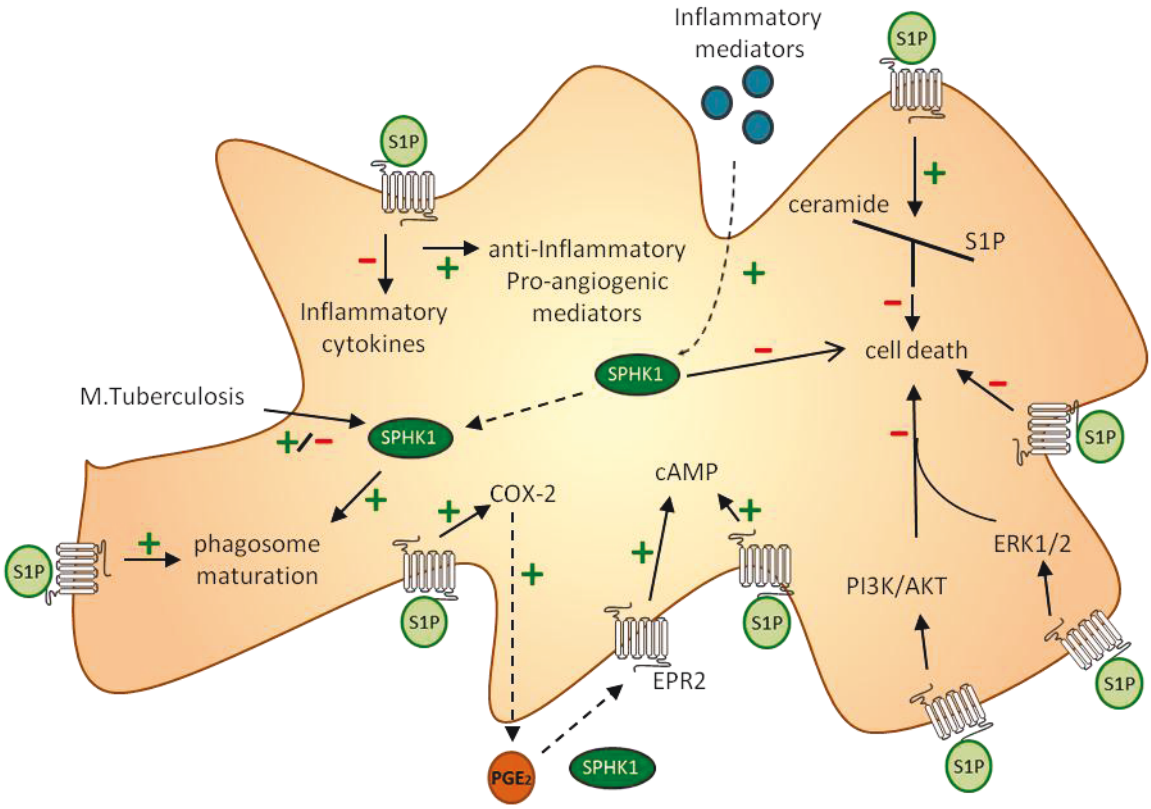


Figure 1.7: Divergent role of S1P and SPHK1 signalling in macrophages. S1P binding to S1P receptors induces anti-inflammatory responses and inhibits the production of pro-inflammatory cytokines whilst elevating the production of pro-angiogenic mediators. COX-2 expression is increased by S1P, leading to the production and secretion of PGE2 (prostaglandin), which in turn is able to induce cAMP (cyclin adenosine monophosphate) via EPR2 receptor (E prostanoid receptor). S1P can also induce cAMP production independently of PGE2 signalling. Extracellular S1P induces phagosome maturation after pathogen uptake. *M.Tuberculosis* (if non-viable) can induce the rapid recruitment of SPHK1 to the phagosome where it contributes to phagosome maturation. Ingestion of viable *M.Tubeculosis* inhibits SPHK1 recruitment to the phagosomes. S1P can also activate PI3K/AKT and ERK1/2 that mediate cell survival and inhibit apoptosis. SPHK1 activation by inflammatory mediators also contributes towards pro-survival signalling.

1.10.5 SPHK1/S1P role in immune cell trafficking

S1P is a potent regulator of immune trafficking not only due to its direct binding to membrane receptors and consequent activation of migratory pathways but also due to its differential concentrations in different tissues and in circulation [114, 258]. S1P secreted from apoptotic cells is a potent chemotactic agent for monocytes and macrophages [259]. A more recent *in vivo* study showed that S1P antibody reduced the level of macrophage influx into ischemic areas [260]. Similarly, the inhibitor FTY720 also decreased macrophage infiltration into peripheral nerves and into the central nervous system in experimental autoimmune neuritis [261] and encephalomyelitis models [262]. It is known that FTY720 blocks S1P signalling by binding to the S1P receptors (S1P1 receptor) causing its internalisation and degradation [263]. KRP-203, an FTY720 analogue and S1P1 receptor antagonist, reduced macrophage infiltration in the myocardium of rats [264].

SPHK1 involvement in the regulation of vascular cell adhesion molecules in endothelia is an important link to the recruitment of different types of leucocytes such as macrophages during inflammation [265]. In monocytes, SPHK1 activation triggers calcium release and regulates NADPH oxidase activity, both of which were shown to be mediated via the high-affinity IgG receptor (FcγRI) [266] which is a key regulator of immune responses, indicating SPHK1 as a critical molecule in immune cell intracellular signalling regulation.

In lymphocyte trafficking, S1P and S1P1 receptor are important regulators of T-cell egress from the thymus into the blood stream and their subsequent maturation and transition to a secondary lymphoid organ. In B cells the S1P/S1P1 axis regulates their position in the spleen as well as mediating the control of mature B cell emigration from the lymphoid tissue or in the case of immature B cells, the egress to the bone marrow vascular compartment and peripheral blood [267].

During mast cell degranulation, S1P was found to be one of the released factors along with histamine, cytokines and chemokines. Indeed, S1P action in mast cells can increase their migration towards antigen or induce its degranulation, indicating S1P as a mediator in the recruitment of these cells to the inflammatory sites and in inducing their degranulation as part of their immune response [158].

1.10.6 Bidirectional regulation of SPHK1 and cytokines in macrophages

A key event in inflammation-related tumorigenesis is cytokine release by TAMs, which has been associated with an up-regulation of several intracellular pathways, most notably TNF-α, PI3K/Akt, MAPK and NF-κB [188, 227, 268, 269]. In primary human monocytes SPHK1 is activated by TNF-α leading to the activation of Ca^{2+} intracellular signalling, activation of NF-kB and cytokine production (IL-1β and IL-6). Furthermore, SPHK1 inhibition by siRNA abrogates the TNF-α induced effect [268] whilst similarly in RAW macrophages, TNF-α induced PGE_2 production, which was inhibited by SPHK1 siRNA [256].

In microglia, resident macrophages of the central nervous system, LPS has been shown to induce TNF-α, IL-1β and iNOs through SPHK1 transcriptional activation, whereas its inhibition reduces cytokine secretion and iNOs levels. Moreover, the addition of exogenous S1P can restore these LPS-induced effects [270]. On the contrary another study showed that S1P induced an inflammatory response in macrophages. This study indicated that S1P inhibits LPS-induced secretion of pro-inflammatory cytokines such as TNF-α, MCP-1 and IL-12 in peritoneal macrophages isolated from S1P2 receptor-deficient mice. This effect was mediated through S1P1 receptor, as the addition of S1P1 receptor-specific agonist SEW2871 mimics the S1P inhibitory effect on LPS-induced cytokines. In addition, S1P increased Arginase I activity and decreased iNOS, indicating an alternative anti-inflammatory macrophage phenotype [271]. Unfortunately the authors did not measure SPHK1 and therefore it is not possible to establish a correlation with the previous study in order to understand if SPHK1 was playing a central role in the macrophage phenotype switch. Interestingly, Spiegel *et al*. have shown that apoptosis of Jurkat and U937 cells can induce the release of S1P via the up-regulation of SPHK1. This effect was independent of SPHK1 Inhibition, which led to the hypothesis that S1P release is a consequence of stress rather than SPHK1 inhibition.

1.11 Interleukin-6

Interleukin-6 (IL-6) is a potent cytokine that is omnipresent in inflammatory responses and most cancers [272]. IL-6 signals through a cell-surface type I cytokine receptor complex involving IL-6 receptor (IL-6R) and gp130, the signal-transducing component. IL-6 belongs to the cytokine family encompassing OSM (oncostatin M), LIF (leukaemia inhibitory factor), IL-11, CT-1 (cardiotrophin-1), CNTF (ciliar neurotrophic factor) and cardiotrophin-like cytokine (CLC) [273]. IL-6 intracellular signalling is mediated through Janus Activated Kinases 1 and 2 (JAK1 and JAK2) which in turn induces the activation of signal transducers and activators of transcription (STAT) proteins (mostly STAT3) [274], RAS/MEK/ERK [275] and PI3K/Akt signalling pathways [276] (Figure **1.8**). These IL-6 activated signalling pathways are involved in tumour proliferation, migration [277], survival and chemoresistance [272] increasing the appeal of IL-6 as a potential therapeutic target.

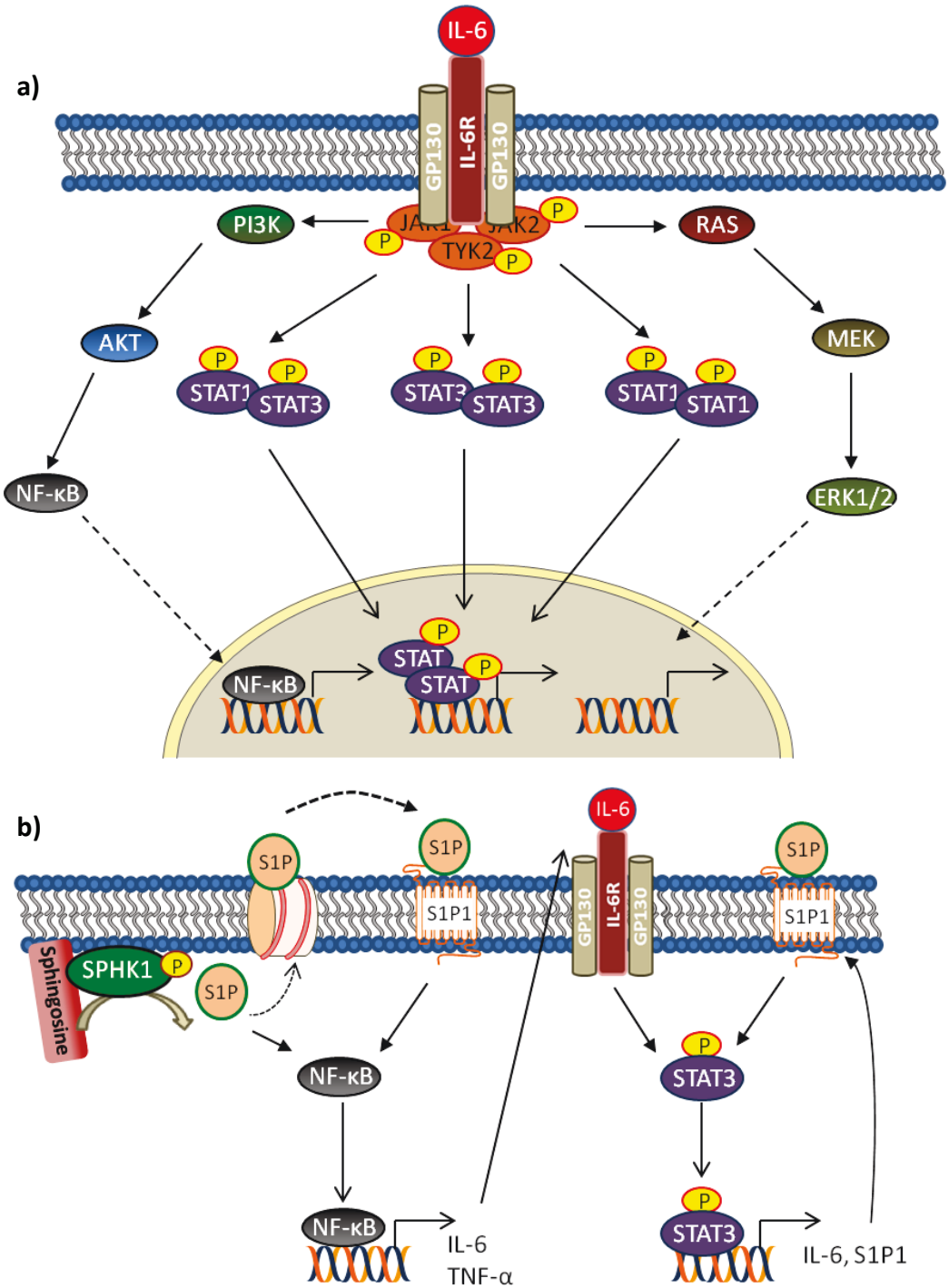


Figure 1.8: IL-6 signalling link with the SPHK1/S1P pathway. a) IL-6 receptor (IL-6r) requires the presence of signal transducer activator G130 to activate the IL-6 signalling pathway. Upon receptor activation JAKs are phosphorylated and can induce STAT1 and STAT3 phosphorylation, which in turn can result in the formation of heterodimers (STAT1/STAT3) or homodimers (STAT3 /STAT3 (most common) or STAT1/STAT1). STATs can then translocate to the nucleus and act as a transcription factor for several genes, including IL-6 itself. Alternatively, IL-6/IL-6r/gp130 signalling can lead to the activation of PI3K/AKT /NF-kB and/or RAS/MEK/ERK1/2 signalling pathways which results in the transcriptional regulation of several genes involved in pro-survival actions. **b)** Schematic showing how SPHK1/S1P signalling connects with NF-k/IL-6/STAT3. SPHK1 production of S1P induces NF-kB activation via S1P1 receptor, which in turn acts as a transcription factor for IL-6 and TNF-α. IL-6, in turn, can activate STAT3 as well as S1P/S1P1 signalling to generate a positive feed-back loop resulting in a persistent activation of STAT3.

1.11.1 IL-6 and cancer

IL-6 expression has been described in several human cancers; in colorectal cancer IL-6 expression and serum levels have been correlated with advanced stages and poor survival [278], whilst in gastric cancer elevated IL-6 expression correlates with invasive and metastatic potential. Of particular interest to my study, gastric cancer initiation is linked with chronic gastritis in which high levels of inflammatory mediators and inflammatory cells are present to promote cancer progression [279, 280]. In pancreatic cancer [281], cervical cancer [282], melanoma [283], ovarian cancer [284] and glioblastoma [285] IL-6 levels were also indicative of tumour progression and poor survival. Interestingly in ovarian cancer IL-6 was also responsible for the induction of other inflammatory mediators and angiogenesis factors including TNF-α, IL-1β, CCL2 and VEGF which can act in an autocrine and paracrine manner within the tumour microenvironment [286]

In prostate cancer high levels of IL-6 were found to be a tumour cell survival factor and mediator of chemoresistance in prostate tumours [287-289]. IL-6 has also been shown to induce androgen receptor activation and increase cell proliferation and tumour growth, whereas the use of the androgen receptor antagonist bicalutamide abrogated IL-6-induced growth [290]. IL-6 involvement in myeloid recruitment to the tumour site was shown in an androgen-independent prostate cancer xenograft model. This study correlated STAT3 and IL-6 levels with high infiltration of myeloid cells; in contrast IL-6 ablation reduced the recruitment of these cells and decreased tumour growth and angiogenesis [291].

1.11.2 IL-6 signalling and the SPHK1/S1P pathway

The SPHK1/S1P pathway and IL-6 signalling are known to be involved in various cellular processes with pro-survival effects. Evidence of their cross-regulation has been reported in several studies with particular relevance to cancer and in inflammatory diseases, with most studies to date indicating SPHK1 as a regulator of IL-6 expression [292-294]. In patients with severe acute pancreatitis, SPHK1 activity and S1P3 expression in peripheral neutrophils and lymphocytes were higher than in healthy patients, in addition the IL-6 serum levels positively correlated with SPHK1 intracellular expression. In this case no direct

regulation of SPHK1 on IL-6 or *vice-versa* was investigated [295]. TNF-α induced IL-6 in fibroblast-like synoviocytes (key contributors to rheumatoid arthritis) was blocked by SPHK1 inhibition, which also resulted in a decrease in STAT3, ERK1/2 and PGE2 [296]. In THP-1 and RAW264.7 macrophages SPHK1 was crucial for IL-6 expression upon LPS treatment [297].

In STAT3 positive tumours S1P1 receptor expression was elevated in comparison with STAT3 negative tumours. In addition, the over-expression of S1P1 receptor (S1P1R) induced STAT3 activation, IL-6 up-regulation and increased tumour growth and metastasis, whereas the absence of S1P1 receptor in either tumours or immune cells had an opposite effect. STAT3 was shown to be involved in S1P1 receptor activation, indicating a positive feed-back loop for persistent STAT3 activation in either tumour cells or immune cells [298]. This work was followed by another study indicating S1P1 receptor and STAT3 as a link between chronic intestinal inflammation and development of colitis-associated cancer (CAC). In this work it is suggested that SPHK1 is crucial for S1P production, which in turn activates STAT3, creating a positive feed-back loop (figure **1.8b**). It was also shown that intracellular S1P increases IL-6 and TNF-α expression through NF-kB. As a result, IL-6 has the capacity to activate STAT3 whereas TNF-α can induce SPHK1 activation which again creates a positive feed-back loop with persistent activation of STAT3 leading to chronic inflammation and CAC development [299]. It is believed that cancer cells recruit immune cells to produce S1P and other cytokines, such as IL-6, to activate STAT3, sustain inflammation and induce tumour development [300, 301]. Interestingly this STAT3-S1P1 axis has been implicated in the colonisation of future metastatic sites for prostate cancer cells; indeed a disruption in either STAT3 or S1P1 in myeloid cells decreased their ability to intravasate to distant sites and inhibited not only their own proliferation but also that of stromal cells [302]. A very recent study in BeWo cells indicated S1P2, and not S1P1, as a critical component of S1P induced IL-6 activation in a RhoGTPases-dependent pathway.

1.12 Monocyte chemotactic protein-1 (MCP-1)

CCL2, also known as monocyte chemotactic protein-1 (MCP-1) is a chemokine that has a strong chemoattractant effect on immune cells. It belongs to a family with three other

members (MCP-2 or CCL8, MCP-3 or CCL7 and MCP-4 or CCL4) sharing a sequence homology of 61% (MCP-2 and MCP-4) and 71% (MCP-3) with these [303]. Endothelial, epithelial, fibroblast and smooth muscle cells, monocytes and macrophages are amongst the cells that express MCP-1; this expression may be constitutive or induced by growth factors or cytokines. Its action is mediated by specific binding to the CCR2 G-Protein coupled receptor, that conversely to CCL2 is restricted to few cell types including monocytes, B cells, T cells and some tumour cells [304]. MCP-1 is involved in the migration and infiltration of monocyte/macrophages, T-lymphocytes and natural killer cells to sites of tissue injury and inflammation and in many cases to tumour sites [304]. Indeed the involvement of MCP-1 has been described in several human diseases such as rheumatoid arthritis, atherosclerosis [305], inflammatory bowel disease [306], asthma [307], diabetes (insulin resistance) [308] and in the development of many types of cancer [309].

1.12.1 MCP-1 and cancer

MCP-1 is highly expressed in several human tumours such as melanoma [310], glioma [311], ovarian carcinoma [312], breast cancer [313] and prostate cancer [314, 315], with MCP-1 expression levels in the tumour positively correlating with the recruitment of monocytes and macrophages into the tumour tissues. In addition, a study using melanoma cell lines with differential levels of MCP-1 expression indicated a strong positive association between MCP-1 expression and the recruitment of monocytes and macrophages as well as tumour size and vascularisation [316]. Studies in which MCP-1/CCR2 signalling was blocked by anti-MCP-1 antibodies or by the use of siRNA showed a delayed tumour progression and metastasis seeding, as well as a reduced recruitment of monocytes and macrophages [313, 317]. One mechanism of action of MCP-1 in monocyte recruitment and accumulation in the tumour site is through its ability to induce the up-regulation of MAC-1, which is the receptor for intracellular adhesion molecule-1 (ICAM-1) in endothelial cells [318]; this molecule can also be secreted in its soluble form as siCAM-1 acting as an autocrine or paracrine molecule in the tumour microenvironment and promoting tumour growth [319].

As previously stated, MCP-1/CCR2 expression correlates with poor clinical prognosis in prostate cancer patients [320, 321]. A Mizutani study showed that mouse monocyte

migration was higher towards conditioned media from PC-3 cells over-expressing MCP-1 (PC-3/MCP-1) than towards parental PC-3 cells. *In vivo*, tumours grew significantly quicker and formed bone metastases in transplanted PC-3/MCP-1 xenografts, an effect that was accompanied by an increase in macrophage accumulation in the tumour site [315]. In support, MCP-1 importance in prostate cancer growth was found to be through a direct regulation of macrophage infiltration into the tumour microenvironment. MCP-1 induces phenotypic changes in mouse macrophages (TAMs) whereas a neutralising MCP-1 antibody decreased macrophage infiltration to the tumour site as well as the lowering the amount of TAMs. This effect results in the retardation of tumour, decrease in microvascular density and decrease in Akt phosphorylation in tumour cells [322]. Another study showed that exogenous MCP-1 induced migration and proliferation in PC-3 and VCaP (prostate cancer cell line) in an Akt dependent manner [323].

1.12.2 MCP-1/SPHK1 interactions

SPHK1/S1P are known to play an important role in inflammation and regulation of immune cell trafficking, effects that can also be attributed to MCP-1/CCR2, suggesting that these two important mediators could have a synergistic action not only in inflammatory responses but in cancer development and the tumour microenvironment. So far most studies identify SPHK1 as an important mediator of MCP-1. In mast cells SPHK1 was critical for MCP-1 secretion whereas S1P stimulated the production and secretion of MCP-1 (TNF-α and IL-6 to a lesser extent) in a SPHK1-dependent mechanism [324]. In human aortic endothelial cells SPHK1 knockdown by siRNA was found to abrogate TNF-α-induced MCP-1 expression and secretion, whereas exposure to S1P increased MCP-1 protein secretion and mRNA expression in a p38 MAPK-dependent manner [325]. Similarly, in A549 lung carcinoma cells, MCP-1-induced expression by TNF-α and IL-1β was mediated by SPHK1 [326]. In a SPHK1 deficient mouse model, MCP-1 production was inhibited in dermal microvascular endothelial cells despite treatment with thrombin, a known inducer of MCP-1 [293]. In this study a dominant negative SPHK1 mutant and SPHK1 inhibitor (DMS and SKi-II) also inhibited the MCP-1, as well as IL-6, IL-8, and PGE(2), response to thrombin.

1.13 PI3K/AKT pathway

1.13.1 AKT activation

AKT (also known as PKB) is a down-stream target of the phosphoinositide 3-kinase (PI3K) signalling pathway. To date there are three described isoforms, namely AKT1, AKT2 and AKT3, although the elucidation of their roles, in terms of similarity between the isoforms or whether each maintains a responsibility for a specific function, remains a topic of debate [327]. These isoforms share 80% homology; all contain a hydrophobic motif at the C-terminus and a similar ATP binding region which in AKT3 contains a switch from Ala232 to Val228 in comparison with AKT1 and AKT2. AKT is initially inactive and located in the cytoplasm, its translocation to the inner membrane occurs by the activation of the growth factor receptor-associated PI3K. This activation is mediated through the receptor tyrosine kinases (RTKs) that can either induce direct binding to PI3K or act by activating scaffolding adaptors such as IRS1. Once activated, PI3K induces the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3), which serves as an anchor for the assembly of signalling proteins such as AKT, PDK1 and PTEN. The binding of PI3K occurs through the pleckstrin-homology (PH) domains. PIP3 can be reversed to PIP2 by PIP3 phosphatase PTEN (Figure **1.9**). Once in the membrane, AKT is phosphorylated at two sites in two independent phosphorylation events; AKT1 at Thr308 and Ser473, AKT2 at Thr309 and Ser474, and AKT3 at Thr305 and Ser472. PDK1, when recruited to the plasma membrane, acts as key molecule in the phosphorylation of the tyrosine residue (T-loop). This first event is crucial for AKT activation, whereas the second phosphorylation in the C-terminus enhances AKT activity by inducing conformational changes in the T-loop. mTOR complex 2 (mTORC2) is activated by the RTKs and has the ability to phosphorylate AKT in the Ser473 motif. Once activated, AKT migrates to the cytosol where it acts as a key mediator and activator of several pro-survival pathways (Figure.**1.9**) [328].

1.13.2 The role of AKT in cancer

PI3K/AKT is often dysregulated in many types of cancer and has become a pivotal target for cancer therapy due to its ability to regulate several cellular processes such as proliferation, survival, angiogenesis, chemotaxis and apoptosis [328]. Increase in AKT kinase activity is observed in several human cancers; in thyroid carcinoma 83% of tumours were shown to have increased levels of AKT1 [329], 25-55% of breast cancer tumours showed an increase [330, 331], as well as 57% of glioma [332], 40-70% of non-small cell lung carcinoma [333, 334], 78% of gastric carcinoma [335], 60-70% of pancreatic carcinoma [336, 337], 40-70% of ovarian [338, 339] and 45-55% of prostate carcinoma showed an increased activation of AKT kinase [331, 340]. In prostate cancer biopsies, AKT expression correlated with high gleason score (8-10) and 92% of cases correlated with a strong staining for AKT, in comparison with prostatic intraepithelial neoplasia and other grades of PCa where AKT was only highly expressed in 10% of the cases [340].

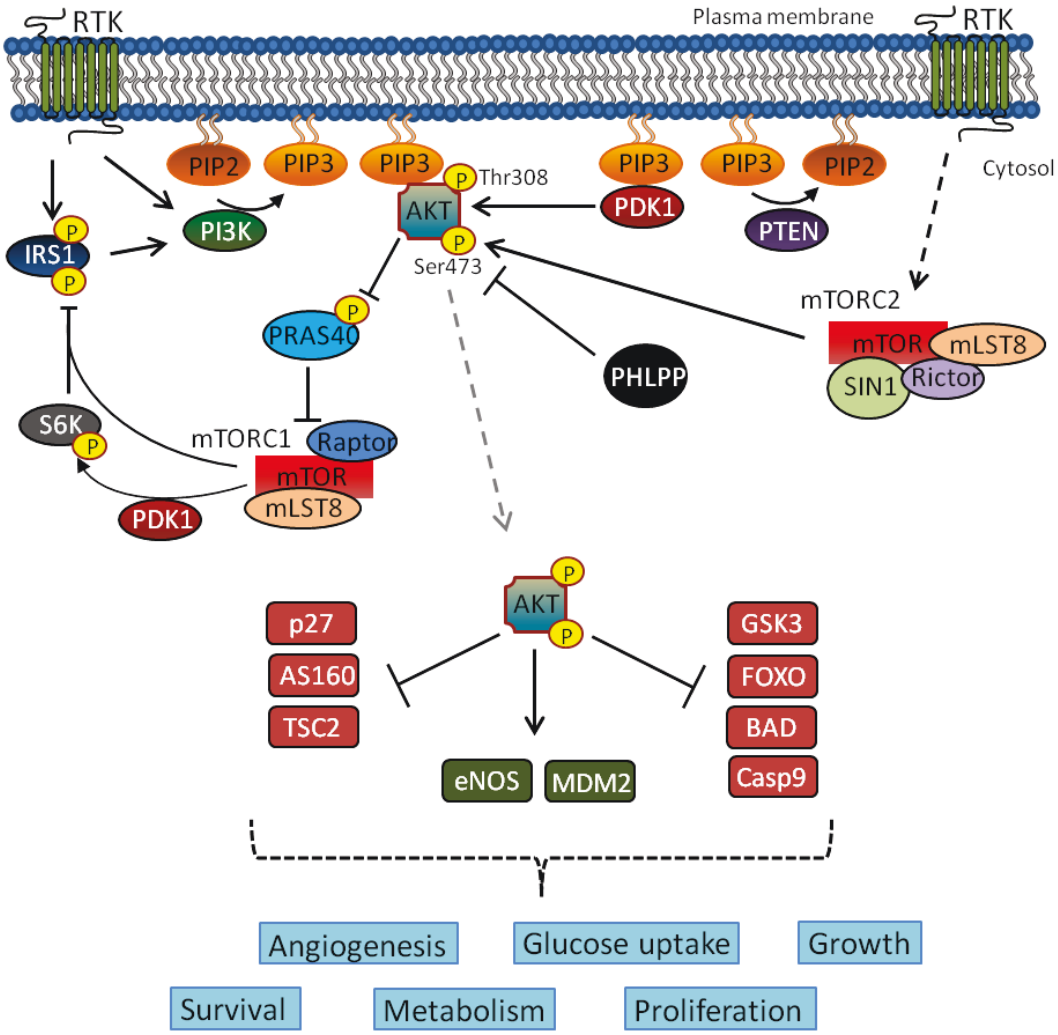


Figure 1.9: Schematic representation of AKT activation pathway. Activation of Receptor Tyrosine Kinases (RTKs) provokes activation of class I phosphatidylinositol 3-kinase (PI3K) through direct binding or through tyrosine phosphorylation of IRS1, that in turn activates PI3K. As a consequence, PI3K phosphorylates PIP2 into PIP3 allowing AKT binding through the PH domain. PTEN acts as a negative regulator of PIP3 production as it converts PIP3 into PIP2. PIP3 activation also allows PDK1 to bind and induce AKT phosphorylation at Thr308 (AKT1). A second AKT phosphorylation on Ser473 results from the mTORC2 activation induced by RTKs, which can be dephosphorylated by PHLPP. AKT activates mTOR complex 1 (mTORC1) through PRAS40 phosphorylation that disrupts the binding between mTORC1 and PRAS40, and relieves the inhibitory effect of PRAS40 in mTORC1. Activated mTORC1 phosphorylates the hydrophobic motif on the S6 kinases that, together with mTORC1, can act as a negative regulator of PI3K through the inhibition of IRS1 phosphorylation. AKT phosphorylation results in its translocation to the cytosol where it represses or activates important signalling molecules involved in several cellular processes such as survival (Caspase 9, FOXO,BAD,GSK3,MDM2), angiogenesis (eNOS,TSC2), metabolism (GSK3,FOXO), glucose uptake (AS160), growth (TSC2) and proliferation (TSC2,p27, GSK3,MDM2, FOXO).

In PCa genetic alterations of the PI3K/AKT pathway are observed in approximately 40% of primary and 70% of metastatic PCa [341]. In this context PTEN, a negative regulator of PIK3/AKT signalling, is the most commonly mutated tumour-suppressor gene in PCa [342]. PTEN loss of function is normally attributed to DNA methylation, deletions, point mutations or defects in protein stability [343]. Immunohistological data in PCa tumours indicated that a higher PTEN expression was associated with a higher relapse-free survival (85.7%) than in low PTEN-expressing tumours (16.6%) at 30 months post-surgery. Furthermore, after 36 months 100% of low PTEN-expressing tumours had a biochemical relapse compared with only 28.5% of PTEN high expressors [344]. Beside the loss of PTEN function, AKT constitutive activity in prostate cancer cells is mainly due to EGFR and PDGFR activation [345, 346]. Interestingly the ligands for these two receptors are EGF and PDGF, both of which have been reported to activate SPHK1 (Section **1.9.2**).

AKT activation is also important for epithelial-mesenchymal transition (EMT) [347], induction of metastasis, and cell invasion by activating the secretion matrix metalloproteinases (MMP-9) in ovarian cancer cells [348].

1.13.3 AKT and SPHK1 cross-talk

Involvement of both AKT and SPHK1 in anti-apoptotic and pro-survival responses in different disease states is well documented. A cross-regulation between these two potent mediators has been reported; in non-small cell lung cancer (NSCLC) SPHK1 inhibition by SKi-I inhibitor resulted in a reduction in AKT levels and increased the sensitivity to doxorubicin and docetaxel-induced apoptosis. On the contrary, SPHK1 over-expression increased AKT phosphorylation levels and the inhibition of AKT abrogated the SPHK1 anti-apoptotic effect on NSCLC [349]. Another study suggested that SPHK1 can act through the AKT/FOXO3 pathway to protect glioma cells from apoptosis [350]. Indeed, several other reports identify SPHK1 as a key mediator of AKT phosphorylation in melanoma cells [351], ovarian cancer cells [352], chronic myeloid leukaemia cells [187] and in glioblastoma cells [353].

1.14 The JAK/STAT Pathway

Janus Activated Kinase (JAK) and Signal Transducers and Activators of Transcription (STAT) signalling has been widely studied by researchers worldwide due to its fundamental importance in cell proliferation, migration, apoptosis and immune response [354, 355]. This signalling pathway links cell membrane receptors to gene expression, therefore it is of no surprise that JAK/STAT signalling pathways are currently used as targets in different disease states such as cancer and chronic inflammation. JAK/STAT signalling is activated by the binding of different molecules such as growth factors (eg. G-SCF, GM-CSF, GH), Interferons (eg. IFNα, γ, β), or interleukins (eg. IL-2, IL-4, IL-6, IL-7, IL-9, IL-10, IL-12, IL-15, IL-19, IL-20, IL-21) to their specific receptors [356]. JAKs are receptor-associated protein kinases, comprising a family of four members: JAK1, JAK2, JAK3 and TYK2 (Tyrosine Kinase 2). JAK1, JAK2 and TYK2 are ubiquitously expressed and JAK3 is mainly expressed in leukocytes [357]. JAK activation is driven by the phosphorylation of tyrosine residues releasing the blockade of the catalytic site. Upon stimulation JAKs can either form homodimers (eg. JAK1/JAK1) or heterodimers (eg. JAK1/JAK3) [358]. JAKs can also play an active role in transcriptional regulation as it has been shown that JAK2 can translocate to the nucleus and regulate the phosphorylation of tyrosine 41 on histone 3, indicating its involvement in the regulation of target genes and/or its own expression. A schematic representation of JAK/STAT pathway and their different activators is shown in figure **1.10**.

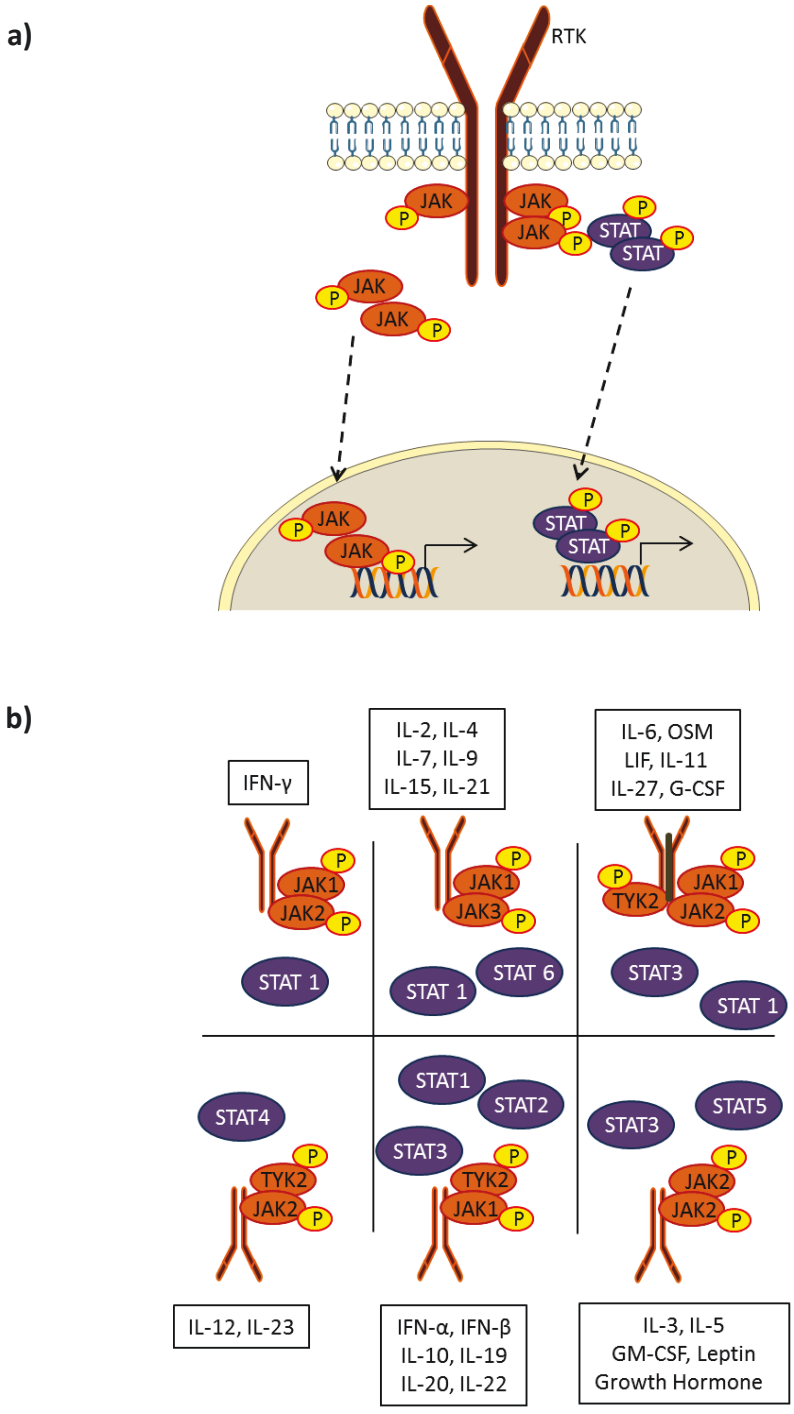


Figure 1.10: Schematic representation of STAT1 activation pathway. **a)** Activation of Receptor tyrosine kinases (RTKs) induces JAK phosphorylation, which allows the binding of multiple signalling regulators such as STATs. Upon binding, STATs can form homo or heterodimers before translocation to the nucleus were they act in the transcription of several genes. JAKs can also form homo or heterodimers and act as a transcription factor. **b)** Activation of RTKs by different agonists leads to the recruitment of different JAK family members. This in turn will lead to the recruitment of different STATs that can induce the transcription of several sets of genes.

1.14.1 STATs

At present, seven different members of the STAT family of transcription factors are known: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 [359]. They share functionally and structurally conserved domains, including an amino-terminal domain (NH2), DNA binding domain (DBD), linker domain, SH2/tyrosine activation domain and coiled–coiled domain (CCD). The carboxy-terminal transcriptional activation domain (TAD) differs between STATs and contributes to STAT specificity [360].

JAK phosphorylation induces the formation of docking sites enabling the recruitment of multiple signalling regulators such as STATs. This binding is mediated by the Src homology domain (SH2) that is present in all STAT proteins [361]. The JAK-mediated tyrosine phosphorylation of STATs promotes rapid translocation of the latter to the nucleus, where they accumulate and bind to DNA, promoting gene expression. Upon phosphorylation of the SH2 domain, STATs can form either homodimers or heterodimers, which plays an important role in their ability to act as transcription factors (Figure **1.9a**). Different STATs can be activated by several cytokines and growth factors leading to the activation of distinct sets of genes. Nuclear accumulation of STATs may take hours, however their activity decays with time as a result of down-regulation of receptors and a decrease in JAK and STAT transcriptional activity. STATs can be re-exported back to the cytoplasm, where they can be re-activated for a second round of signalling.

STATs can also be negatively regulated by phosphatases such as SHP-2 and TC-PTP that induce their dephosphorylation, or through nuclear export. The balance between the multiple nuclear export sequence (NES) and nuclear localisation sequence (NLS) elements are crucial for STATs location and consequently their activity [362]. Another negative regulation mechanism arises from SOCS (suppressors of cytokine signalling), which act as negative feedback regulators [363]. Although STAT phosphorylation correlates with nuclear accumulation, some reports indicate that unphosphorylated STATs can be shuttled from the cytoplasm to the nucleus [364]. Indeed, unphosphorylated forms of STAT1 and STAT3 were able to induce transcriptional activation of several genes, interestingly these genes differ from those activated upon stimulation [365]. Apart from phosphorylation, STATs have also been reported to be regulated by methylation and acetylation. STAT1 methylation on Arg13 increased its transcriptional activity (Mowen *et al.*, 2001), whereas acetylation at Lys410 and

Lys413 residues was crucial for NF-KB activation and subsequent increase in cell apoptosis [366].

1.14.2 STAT1: Cancer and Inflammation

Constitutive activation of STAT proteins has been observed in many human cancers. While STAT3 role in promoting cellular processes such as proliferation, migration, invasion and angiogenesis is associated with tumour growth and metastasis [367, 368], STAT1 role in cancer remains unclear as to whether it acts as a pro- or anti-cancer molecule. The vast majority of studies on STAT1 in cancer attribute a pro-apoptotic and tumour suppressor role to STAT1. This is shown in studies where the STAT1 loss of function and expression in cancer cells has been correlated with breast cancer, melanoma, leukaemia and lymphoma [354]. In support, mice with a homozygous deletion of STAT1 developed tumours more rapidly than the wild type controls [369]. These studies suggest that STAT1 not only acts as a tumour suppressor but also the loss of STAT1 signalling can further increase oncogenesis [354, 369].

With STAT1 being the main if not only mediator of IFN signalling, it has led to the hypothesis that STAT1 anti-proliferative effects are partially mediated by the presence of IFN-γ. The ability of IFN-γ to modulate tumour cell immunogenicity may lead to tumour growth advantages in IFN-/STAT1 deficient cells [370]. Moreover, IFN-γ increases HLA class I activity which, in turn, increases tumour recognition by lymphocytes leading to tumour growth reduction [371].

STAT1 knockout mice proved particularly sensitive to viral and microbial pathogens and showed reduced responses to IFN [372]. In human patients with a defective STAT1 axis a very similar effect was observed as these patients were more susceptible to mycobacterial infection [373]. In addition to its role in the response to intracellular bacteria, STAT1 target genes have been shown to promote inflammation and inhibit proliferation [362].

Despite the predominant inference of STAT1 as an anti-tumorogenic regulator some evidence indicates that STAT1 could also play a role in cell survival and/or growth. STAT1 was found to act as a tumour promoter in leukemia development [374], while in prostate cancer cells (DU-145) STAT1 expression was induced upon docetaxel treatment and its

expression was higher in docetaxel resistant DU-145 than in wild type cells. Remarkably STAT1 knockdown by siRNA re-sensitised DU-145 to docetaxel treatment. The authors did not observe any difference in STAT3 levels throughout the treatments [375]. In Wilm's tumour cells (WT), STAT1 phosphorylation on S727 was found in 90% of the analysed tumours; an inactivating mutation of S727 proved to reduce colony formation in 80% in WT cells and increase apoptosis under stress conditions [376] reinforcing the role of STAT1 as pro-survival regulator. Another study indicated that STAT1 was responsible for the activation of genes normally activated by STAT3, including Bcl2I1 and Myc in glioma cells.

1.14.3 STAT1 and SPHK1/S1P

To date, very few studies have associated the SPHK1/S1P pathway with STAT1. A Weins et al. study indicated that VEGFA secretion from macrophages upon S1P stimulation was STAT1 dependent. In addition, a second activation loop induced by VEGFA autocrine function induced formation of STAT1/STAT3 heterodimer that bound to the HO-1 promoter and consequently induced the expression of proteins such as Bcl-2, Bcl-X(L) and anti-inflammatory adenosine receptor A(2A) [377].

A more recent study correlated the levels of intracellular S1P with the amplification of infectious influenza virus. The authors conclude that the over-expression of S1P increases susceptibility to influenza virus infection, whereas over-expression of S1P lyase had an opposite effect. Also STAT1 phosphorylation increased only in S1P lyase over-expressing cells after infection with the virus, whereas blocking of STAT1 elevated the levels of replication of the virus within the same cells. This indicates that S1P lyase acts through STAT1 in controlling the host defence against influenza virus infection [378]. It is clear that, to date, STAT1 and SPHK1/S1P cross-regulation or co-dependency has not been described. Nonetheless, SPHK1/S1P regulation of or by STAT1 should not be discarded, indeed SPHK1 genomic DNA was found to contain several possible binding sites for STAT1. CHIP-Seq analysis performed on HeLA-S3 and K562 (leukaemia cell lines) after IFN-y treatment indicated that STAT1 binds to SPHK1 DNA in either the coding region or upstream of the coding region, possibly in the SPHK1 promoter region indicating that STAT1 can acts a SPHK1 transcription factor (UCSC Genome Bioinformatics, genome.ucsc.edu).

1.15 Hypothesis and aims

Inflammation is a new hallmark of cancer and several types of immune cells interplay with cancer cells to exert pro- or anti-tumour effects. Important processes involved in cancer-associated inflammation, such as cell proliferation, survival, cytokine and chemokine production are also known to be regulated by the SPHK1/S1P pathway. The ability of S1P to regulate immune cell trafficking coupled with the role of SPHK1 in the regulation of pro-inflammatory mediators suggests the potential involvement of these pathways in inflammation-related cancer progression. This has significant implications as evidence suggests that inflammatory microenvironment is pro-tumorigenic with macrophages and monocytes being important contributors to the development and maintenance of a pro-cancer inflammatory microenvironment. In this work, I hypothesise that a possible dysregulation of the SPHK1/S1P pathway may play a role in supporting cancer-related inflammatory response. The aims of this project are:

a) To elucidate whether SPHK1 is a mediator of the interaction of monocytes such as human leukemic monocyte, U937 and THP-1 cells with prostate cancer cells (PC-3 cells) and a regulator in the recruitment of these cells to the tumour site.
b) To investigate whether the SPHK1/S1P pathway is dysregulated in prostate cancer cells and/or monocytes (U937 and THP-1), study the molecular mechanisms by which tumour cells benefit from this interaction and identify the soluble mediators involved.
c) To investigate SPHK1/S1P involvement in monocyte-mediated prostate cancer cell chemoresistance

This study will provide a scientific basis for the involvement of the SPHK1/S1P pathway in tumour-mediated inflammation and its potential as a target for an anti-cancer therapy.

2. Materials and Methods

2.1 Materials

2.1.1 Mammalian cell culture

Cell Line	Cell type	Type of tumour	Origin	Medium and Additives
PC-3 [379]	Prostate	Adenocarcinoma	Bone	RPMI 1640, 2mM glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin, 10% fetal calf serum (FCS)
DU-145 [380]	Prostate	Adenocarcinoma	Brain	
MDA-MB-231 [381]	Breast	Adenocarcinoma	*Pleural effusion*	
BT549 [382]	Breast/ mammary gland	Ductal Carcinoma	Breast	
C4-2 [383]	Prostate	Adenocarcinoma	Left supraclavicular lymph (LnCaP derivative cell line)	
THP-1 [384]	Monocyte	*Acute monocytic leukemia*	Blood	
U937 [385]	Monocyte	histiocytic lymphoma	Pleura/pleural effusion	
RAW 264.7 [386]	Macrophage	Abelson murine leukemia virus-induced tumor		
PC-3/OFP	Prostate		Empty vector phmKO2	RPMI 1640, 2mM glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin, 10% fetal calf serum (FCS), 1mg/ml G418
PC-3/OFP-SPHK1	Prostate		Overexpression of SPHK1: phmKO2-SPHK1	

Table 2.1: Cell lines and respective media for mammalian cell culture

2.1.2 Antibodies used in Western blotting, ChIP, Immunohistochemistry and Flow Cytometry

Protein	Species	Clone	Manufacturer	Application	Dilution
ERK1/2 (phospho)	Mouse	9106	Cell signalling	Western Blot	1:1000
ERK1/2 (Total)	Rabbit	9102	Cell signalling	Western Blot	1:1000
AKT (phospho)	Rabbit	4051	Cell signalling	Western Blot	1:1000
AKT (Total)	Rabbit	9272	Cell signalling	Western Blot	1:1000
NF-KB (phospho)	Rabbit	3031	Cell signalling	Western Blot	1:1000
Tubulin	Mouse	T9026	Sigma-Aldrich	Western Blot	1:5000
GAPDH	Mouse	sc-69778	Santa Cruz Biotechnology	Western Blot	1:5000
PARP	Rabbit	9532	Cell signalling	Western Blot	1:1000
STAT1 (phospho)	Rabbit	9171	Cell signalling	Western Blot	1:1000
				ChiP	1:100
IgG	Rabbit	sc-2027	Santa Cruz	ChiP	1:100
STAT 1 (Total)	Rabbit	610120	BD Bioscience	Western Blot	1:1000
STAT3 (phospho)	Rabbit	9131	Cell signalling	Western Blot	1:1000
STAT 3 (Total)	Rabbit	c-20	Santa Cruz	Western Blot	1:1000
HLA ABC (Class I)	Mouse	MCA81F	Serotec	Flow Cytometry	1:20
Anti-Rabbit	Goat	7074	Cell Signalling	Western	1:2000
Anti-mouse	Goat	7076	Cell Signalling	Western	1:2000
SPHK1	Mouse/Rabbit	AP7237	Cambridge Bioscience	Immunohistochemistry	1:50

Table 2.2: Antibodies used for Western Blotting, Chromatin Immunoprecipitation, Immunohistochemistry and Flow cytometry

2.1.3 siRNA oligonucleotides

Target	Sequence Sense 5' to 3'
SPHK1	UCACGCUGAUGCUCACUGA
AKT	1.CAUCACACCACCUGACCAA 2.ACAAGGACGGGCACAUUAA 3.CAAGGGCACUUUCGGCAAG 4.UCACAGCCCUGAAGUACUC
ERK1	1.AGACUGACCUGUACAAGUU 2.CCAAUAAACGGAUCACAGU 3.CCUGCGACCUUAAGAUUUG 4.GACCGGAUGUUAACCUUUA
ERK2	1.UCGAGUAGCUAUCAAGAAA 2.CACCAACCAUCGAGCAAAU 3.GGUGUGCUCUGCUUAUGAU 4.ACACCAACCUCUCGUACAU
Non-targeting	UUCUCCGAACGUGUCACGU

Table 2.3: siRNA oligonucleotide sequences used throughout this study

2.1.4 Primers used in real-time PCR and ChIP

Target	Application	Forward (5' to 3')	Reverse (5' to 3')	Company
SPHK1 (h)	qRT-PCR	TATGAATGCCCCTACTTGGTATTG	GCCTCGCTAACCATCAATTCC	Primer Design
SPHK1 (m)	qRT-PCR	TCCAGAAACCCCTGTGTAGC	CCGCACGTACGTAGAACAGA	Invitrogen
MCP-1	qRT-PCR	ACCGAGAGGCTGAGACTAAC	AATGAAGGTGGCTGCTATGAG	Primer Design
IL-6	qRT-PCR	AAATTCGGTACATCCTCGACG	GTTGTTTTCTGCCAGTGCCTCT	Invitrogen
IRF1	qRT-PCR	CTTCGCCGCTAGCTCTACAACAG	GCTCCGGGTGGCCTCGGTTCG	Invitrogen
GAPDH	qRT-PCR	Not disclosed by the company	Not disclosed by the company	Primer Design
YWHAZ	qRT-PCR	Not disclosed by the company	Not disclosed by the company	Primer Design
UBC	qRT-PCR	Not disclosed by the company	Not disclosed by the company	Primer Design
SPHK1 (A)	ChiP	AGGTGCAGGACCCATCATT	CTCCGAGAAACAGGAACGAG	Invitrogen
SPHK1 (B)	ChiP	ACTGGCCTCCAAAGAAGTGA	GATTGGAAAGCCAAGCATGT	Invitrogen
SPHK1 (C)	ChiP	GCGAAGTTGAGCGAAAAGTT	CAAGCTCAGCCCACGTCT	Invitrogen
SPHK1 (D)	ChiP	CACGGGGCTCTGACTCAT	CGCCGTGTGACTAAGCCTAC	Invitrogen
SPHK1 (E)	ChiP	GTCAATTACCGGGGTGTTTC	CGCTGAGAAAACAAAACCAA	Invitrogen

Table 2.4: List of primers used in real-time PCR and Chromatin Immunoprecipitation

2.1.5 Recombinant proteins

Name	Product	Characteristics	Concentration	Company
S1P	62570	Stock (2mM): Reconstituted in DMSO	1µM	Avanti Polar Lipids
IL-6	200-06	Stock (100µg/ml): Reconstituted in PBS/0.1%BSA	100ng/ml	PeproTech
MCP-1	279-MC-010	Stock (100µg/ml): Reconstituted in PBS/0.1%BSA	100ng/ml	R&D systems
IFN-γ	300-02	1.0 mg/ml: Reconstituted in water	500 IU/ml	PeproTech
Docetaxel	01885	Stock (20mM): In DMSO solution	5nM or 20nM	Sigma-Aldrich
SKi-II	10009222	Stock (50mg/ml): In DMSO solution	(50mg/ml) *in vivo*	Calbiochem
		Stock (20mM): In DMSO solution	10µM	

Table 2.5: Recombinant proteins and drugs used throughout the study

2.1.6 Buffers used in Western blotting

Reagent	Components
TG solution (10x)	288g glycine, 60g Tris dissolved in 2L ddH2O
1x SDS running buffer	200ml 10xTG, 20ml SDS (20%) dissolved in 2L ddH2O
1x Transfer buffer	200ml 10xTG, 400ml methanol dissolved in 2L ddH2O
Phosphate-buffered saline-Tween 20 (PBS-T wash buffer)	0.05% Tween 20 dissolved in 1xPBS
Dried skimmed milk powder blocking buffer	5% dried skimmed milk powder dissolved in PBS-T wash buffer

Table 2.6: Western Blotting buffers

2.1.7 Components of SPHK1 buffer

Name	For 1 ml buffer take		
SPHK1 buffer	**Component**	**Concentration**	**Final Concentration**
	Tris HCl buffer pH 7.4	200µl of 0.1M	20 mM
	Glycerol	200µl neat	20%
	B-mercaptoethanol	1µl of 14.3M	1 mM
	EDTA	100µl of 10mM	1 mM
	PMSF	10µl of 0.1M	1 mM
	NaF	30µl of 0.5M	15 mM
	Leupeptin	10µl of 1mg/ml	10 µg/ml
	Aprotinine	10µl of 1mg/ml	10 µg/ml
	Soybean trypsin inhibitor	10ul of 1mg/ml	10 ug/ml
	4-Deoxypyridoxine	10 µl of 50mM	0.5mM
	B-glycerophosphate	20µl of 2M	40 mM
	Sodium orthovanadate	10µl of 0.1M	1 mM
	H_2O distilled	483µl	

Table 2.7: SPHK1 buffer components

2.1.8 SPHK1 Activity Assay

Name	Components	Company
SPHK1 buffer	Up to 170 µl/reaction	
Sphingosine	10 µl 1 mM sphingosine/assay	Sigma-Aldrich
MgCl2	2µl of20mM	Sigma-Aldrich
ATP	0.2µl of 10mM/sample	Sigma-Aldrich
(γ-32P)- ATP	1ul ATP-32P (10uCi/sample)	Perkin-Elmer
SPHK1 washing solution	per reaction: 200µl ddH2O, 20µl HCl 1M, 240µl KCl 2N, 506µl chloroform, 533µl methanol	Sigma-Aldrich
Stop solution	per reaction: 50µl 1M HCl, 800µl Chloroform/Methanol/HCl, 240µl CHCl3 and 240µl 2M KCl	Sigma-Aldrich
Dissolving Solution	per sample: 40 µl Chloroform/Methanol (2:1, v/v)	Sigma-Aldrich
Migratory Solution	200-300ml 1-butanol/ethanol/acetic acid/water (80:20:10:20, v/v)	Sigma-Aldrich

Table 2.8: Reagents used in SPHK1 activity assay

2.2 Methods

2.2.1 Mammalian cell culture

Cells were cultured and maintained in RPMI 1640 containing 10% FBS as well as 50U/ml Penicillin, 50µg/ml Streptomycin and 2mM glutamine at 37°C in a humidified atmosphere of 5% CO_2. Adherent cell lines were passaged twice a week or upon reaching 80-90% confluency. The passage consisted of removal of spent media followed by incubation with trypsin [2.5 mg/ml trypsin in 0.02% (w/v) EDTA/0.09% (w/v) NaCl] at 37°C until cells detached from the culture plate. The cells were then plated into a new culture dish, at the ratio of 1:10. Suspension cells (U937, THP-1 and RAW264.7) were passaged 2-3 times per week depending upon cell confluency. The passage consisted of removing 2ml of media (containing suspended cells) into a new culture dish with 18ml of fresh complete media. PC-3 cells transfected with phmKO2 or phmKO2-SPHK1 vector were cultured in complete media with the addition of G418 to ensure selection of transfected cells only. The cells lines used in this study were routinely tested for mycoplasma levels. PC-3 cells were authenticated for genotype phenotype.

2.2.2 Freezing and thawing of mammalian cells

A minimum of $1x10^6$ cells per vial were resuspended in RPMI media with 10% FCS (v/v) and 2mM L-glutamine, containing 10% DMSO. Cells were frozen to -80˚C in an isopropanol freezing chamber for at least 24 hours. The following day vials were transferred to liquid nitrogen. To thaw cells, frozen vials were placed on dry ice for 15 minutes and then transferred directly into a 37˚C water bath. After complete thawing, cells were transferred to a 25 ml cell culture flask containing complete RPMI medium and left incubating overnight at 37˚C in a humidified atmosphere containing 5% CO_2. The following day, media was removed in order to remove the DMSO and new media was added until the next passage.

2.2.3 Transwell Assay

PC-3 cells were seeded into the bottom chamber of the transwell at $1x10^5$ cells per well in 2ml of complete medium, as shown in the figure below (**Fig.2.1**).

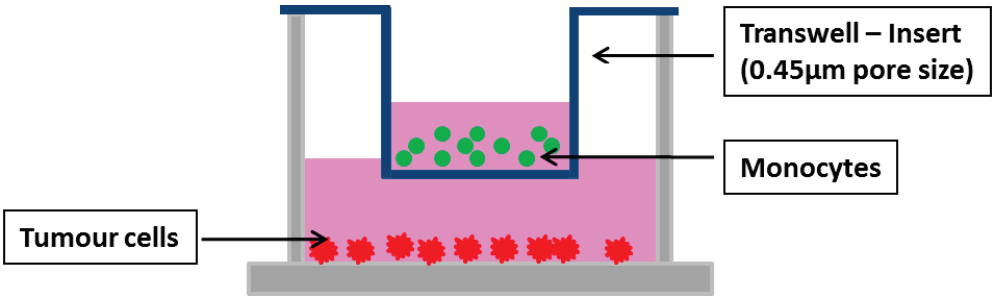


Figure 2.1 Schematic representation of the transwell model used during this study.

After 24 hours, cells were carefully washed twice with PBS before adding 1.5ml of serum-free medium for a further 24 hours at 37°C and 5% CO_2. Transwells (VWR, cat# 353493) were placed into each well of the 6-well plates for 20 minutes before the addition of U937 to the upper chamber at $1x10^6$ cells into 1ml of serum-free medium. At each of the indicated time points, U937 cells were removed from the upper chamber together with the media. Media and cells were then centrifuged at 2500rpm for 5 minutes at 4°C, supernatant was collected and transferred to a different micro-centrifuge tube and frozen at -20°C. U937 cell pellets were re-suspended in PBS to remove any traces of media and centrifuged as described previously. Cell pellets were then frozen at -20°C. U937 cells that were adherent to the transwell were washed with PBS and lysed with specific lysis buffer according to the future assay. Simultaneously, media from the bottom chamber was collected into 2ml micro-centrifuge tubes followed by centrifugation and collection of the media into a new tube. PC-3 cells were washed with PBS directly in the plates, and a specific lysis buffer was added to the cells. Lysed cells were scraped from the 6-wells, transferred to a micro-centrifuge tube and frozen at -20°C.

2.2.4 Sulphorhodamine B (SRB) Assay

Cells were placed into a 24-well plate in complete media at 40% confluency. 24 hours later, cells were washed with PBS, and serum-free medium was added for another 24 hours before the addition of $2x10^5$ U937 cells to the top chamber of the transwell. After 24, 48 and 72 hours of co-culture, 500µl of 40% (v/v) trichloroacetic acid (TCA) was added to the bottom of each well after removing the upper chamber. TCA was added and incubated at 4°C for one hour to fix the cells. Plates were then gently washed with water and air-dried overnight. 300µl of 0.4% (w/v) SRB in 1% acetic acid was added to each well and plates were incubated for one hour at room temperature. Plates were then washed four times in 1% acetic acid and left to air-dry overnight. 300µl of 10mM Tris-HCl pH8.0 was then added to each well and plates were incubated for one hour at room temperature with shaking. 100µl of dissolved SRB solution from each well of the 24-well plate was then added in duplicate to wells of a 96-well plate and absorbance was read at 492nm.

2.2.5 RNA interference

RNA interference (RNAi) was performed using short-interfering (si) RNA oligonucleotides. Cells were transfected with siRNA (5nM or 20nM) using Oligofectamine™ reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, cells were plated into 6-well plates 24 hours prior to the transfection. The specific target was down-regulated using 200pmol siRNA combined with 8µl Oligofectamine in Opti-MEM™ (Invitrogen). Cells with oligo-lipid complexes were incubated at 37°C in 5% CO_2 atmosphere for four hours and subsequently RPMI 1640 media containing 30% FCS was added. All experiments included cells transfected with a non-targeting siRNA (siNT) as a control. Protein and mRNA knockdown were assessed 48 or 72 hours post-transfection using western blotting and/or quantitative real-time PCR.

2.2.6 *In vivo* PC-3 xenograft tumour establishment

Human prostate cancer xenografts were established in nude mice by subcutaneous injection of 30µl 1:1 mixture of 10^6 PC-3 cells with Matrigel (BD Bioscience Cat# 354248) in both flanks of the mouse (Male BALBc/nude 7 week old mice). Tumours were left to establish for 11 days until visible. At this point mice were divided into two groups of five, and treated with either SKi-II (50mg/ml) or DMSO. SKi-II and DMSO were equally diluted in PBS before each injection. Mice were injected every two/three days with the final treatment one day before sacrifice (day20). In total mice were treated five times in a space of nine days. Tumours were measured using calipers and tumour volume was calculated by the following formula: (length x length)*width/2= mm^3. Relative tumour volume was calculated by normalising with the first day of treatment (day 0). The statistical significance of differences between the means of two groups was evaluated by unpaired Student's t test.

2.2.7 Immunohistochemistry

Sections of 4 µm thickness from paraffin-embedded PC-3 xenograft tumours and spleen were dewaxed by immersing sections in xylene for 5 minutes in a total of 3 times. Then sections were rehydrated by washing in 100%, 90%, 70% and 50% ethanol for 5 minutes for a total of two washes per condition. Sections were then incubated with 0.3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. After washing twice with PBS, antigen retrieval was performed by adding the sections to pre-warmed 0.01 M citrate buffer (pH 6.0) and microwaving for 20 minutes at 750W. Slides were left to cool to room temperature and washed three times with PBS for a period of 5 minutes each. Sections were then blocked to reduce primary antibody background in 10% goat serum (DaKO,cat# E0466) for one hour at room temperature. Next, slides were incubated with the primary antibody SPHK1 (1:100 in goat serum) overnight at 4^{o}C. After washing with PBS, the sections were incubated with goat anti-rabbit immunoglobulin (1:200), for 45 minutes. Slides were washed three times for 5 minutes, followed by peroxidase conjugated with streptavidin (1:100) for 30 minutes. After washing with PBS, sections were developed with activated 3,3-diaminobenzidine-tetrahydrochloride solution (Sigma-Aldrich, Dorset, UK) and 0.1%

H_2O_2 until brown, and counterstained with Cole's hematoxylin (Vector Laboratories, Burlingame, CA, USA) for one minute. Sections were then dehydrated, mounted on glass microscopy slides using Pertex mountant (CellPath, Hemel Hempstead, UK) and processed by microscopy. Sections were scored blindly by a qualified histopathologist.

2.2.8 Sphingosine kinase-1 Assay

PC-3 and U937 cell pellets were resuspended in SPHK1 buffer, sonicated for 3-5 seconds and centrifuged at 20000xg for 30 minutes at 4°C. The protein concentration of the supernatant was measured by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The enzymatic assay was performed with 50-100 µg protein. The remaining components of this reaction were prepared as followed; 10 µl of 50 mM sphingosine (Avanti Polar Lipids) in ethanol was transferred into a new 2 ml safe-lock tube, before being evaporated in a heat block at 40^{o}C for 5-10 minutes. Sphingosine was resuspended in 500µl 0.25% triton and sonicated for 15 minutes in a water bath. As a standard, two 2ml micro-centrifuge tubes containing 10µl of 50 µM sphingosine in ethanol were dried, resuspended and sonicated as described above. The volume of triton added to resuspend sphingosine was 10 µl. To each of the standards 1 or 2µl of SPHK1 was added before sonication. For each reaction, 2µl of 20mM $MgCl_2$, 0.2µl of 10mM ATP, 6.8µl of H2O were mixed with 180µl of total protein or SPHK1 buffer for the standards. 10µl of 50 mM sphingosine (resuspended in triton) were added to each mixture before the addition of 1µl 10 µCi [γ-^{32}P]-ATP (Perkin-Elmer, Waltham, MA). The mixture was incubated between 45 minutes and 1 hour at 37 °C in a 5% CO_2. To stop the reaction, solutions were added in the following order; 50µl 1M HCl, 800µl Chloroform/Methanol/HCl mix, 240µl $CHCl_3$ and 240µl KCL 2M, before being vortexed and centrifuged for 10 minutes at 2500rpm. The lower (organic) phase was transferred into a new 2ml micro-centrifuge tube with 800µl of SPHK washing solution. Samples were centrifuged as described previously and the lower phase was again transferred into a new 1.5ml micro-centrifuge tube and left to vaporise overnight. The samples were solubilized in 40µl Chloroform/Methanol (2:1 v/v) and added to a thin layer chromatography on silica gel G60 plate (Whatman, GE Healthcare, Waukesha, WI, USA). The plate was placed into a closed container with 200-300ml of 1-

butanol/ethanol/acetic acid/water (80:20:10:20 v/v) and left to migrate for a minimum of 4 hours. Plates were left to dry for another 30 minutes under the fume hood before being visualised by autoradiography. The radioactive spots were quantified using standards and ImageJ software. Sphingosine kinase specific activity was expressed as pmol of sphingosine-1-phosphate formed per minute per mg protein (pmol/min/mg). The values displayed in the results chapter are shown as percentage relative to the control or untreated sample.

2.2.9 RNA preparation

To avoid RNA degradation, all used equipment was cleaned with RNase*Zap*® (Ambion, Applied Biosystems, Foster City, CA, USA). Total RNA was isolated using the RNeasy® Mini kit from Qiagen according to the manufacturer's instructions. Briefly, cells were washed once with PBS and collected using 350µl or 700µl of RLT buffer depending on cell number. 70% ethanol was added in an equal volume to RLT buffer before being transferred to an RNeasy column. RNA bound to the column was washed with RW1 (750 µl) and twice with RPE (500 µl). RNA was eluted with 40-60µl of RNAse free water and stored at -80°C. RNA concentration was determined using the NanoDrop (ND-100-Spectrophotometer, NanoDrop Technologies). For the subsequent real-time PCR, a high quality of RNA was required. All used RNAs had high purity, meaning an OD_{260}/OD_{280} ratio of 2, and a low salt concentration, meaning an OD_{230}/OD_{260} ratio of at least 1.6 when to be used for cDNA synthesis and real-time PCR.

2.2.10 cDNA synthesis

The reverse transcription was performed according primer design protocol (Precision nanoScript TM Reverse Transcription kit, cat#RT-nanoScript). Briefly, 0.5-1µg of total RNA was mixed with 1µl of random hexamers and up to 14µl of sterile water. This reaction mix was incubated at 65°C for 10 minutes and placed on ice for 5 minutes. Then, 2µl of 10mM deoxynucleotide-triphosphate (dNTPs), 2µl of 100nM Dithiothreitol (DTT), 2µl of 10x nanoscript Buffer and 1µl reverse transcriptase were added to the mix and cDNA synthesis was carried out at 55°C for 20 minutes, followed by 15 minutes at 75°C to inactivate the

reverse transcriptase enzyme. The cDNA was stored at -20°C. ABI Thermal Cycler (Applied Biosystems) was used for all incubation steps.

2.2.11 Quantitative real time PCR (qPCR)

Quantitative PCR was performed using SYBR Green master mix (asymmetrical cyanine dye) containing ROX as a reference dye (primer design). 10µl of SYBG was added to 1µl of primer mix (containing forward and reverse primers), 4µl of nuclease free-water and 5µl of cDNA (5ng/µl) of each sample. Primers were designed and optimised by Primer Design for a product length of approximately 100bp. Each sample was analysed in triplicate. After adding the samples and the mix, the Fast Optical 96-well reaction plate was sealed with an Optical Adhesive Cover and centrifuged for 2 minutes at 3000xg. The quantitative PCR was performed using an ABI PRISM 7900 sequence detection system (Applied Biosystems). Thermal cycling conditions were as follows: 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C followed by the dissociation step: 15 seconds at 95°C, 15 seconds at 60°C and 15 seconds at 95°C. Data was analyzed using the ΔΔCT method and the expression of target genes such as SPHK1 TATGAATGCCCCTACTTGGTATTG (FWD) and GCCTCGCTAACCATCAATTCC (REV) was normalised to the expression of three housekeeping genes. The house keeping genes that were used are GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), YWAZH (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein) and UBC (Ubiquitin C).

2.2.12 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Cell lysates in 1x SDS-loading dye were boiled at 95^{o}C for 5 minutes before being loaded onto an acrylamide gel. Proteins were separated using a 5% acrylamide stacking gel [5% (v/v) acrylamide, 0.125 M Tris, pH 6.8, 0.1% (w/v) SDS, 0.075% (w/v) APS and 0.083% (v/v) TEMED], and a 7.5 or 12.5% acrylamide resolving gel [7.5-12.5% (v/v) acrylamide, 0.375 M Tris pH 8.8, 0.1% (w/v) SDS, 0.06% (w/v) APS, 0.07% (v/v) TEMED] depending on the size of proteins being analysed. Gels were run using 1x SDS running buffer at 120 V until the dye

reached the bottom of the gel. Separated proteins were transferred onto PVDF membrane (Millipore, Billerica, MA, USA) in 1x transfer buffer (table 2.1.6) at 100V for 1 hour using tank blotting system (Bio-Rad) in wet transfer conditions. In order to prevent non-specific binding of antibody, PVDF membranes were incubated with 5% non-fat dried milk powder in 0.05% Tween-20 in 1xPBS (PBST)) with gentle shaking for one to two hours at room temperature. Membranes were then washed with PBST for 2-3 times for 10 minutes before being incubated with a primary antibody. Primary antibodies were diluted in 5% (w/v) BSA/PBST containing sodium azide and incubated overnight at 4 ˚C with gentle shaking. Membranes were then washed three times for 10 minutes in PBS-T. Secondary antibodies conjugated to horseradish peroxidase (HRP) against mouse or rabbit IgG (DAKO, Glostrup, Denmark) were diluted in 5% (w/v) milk/ PBS-T and incubated for 1 to 2 hours at room temperature. Membranes were washed again 3-4 times for 5 minutes with agitation before being exposed to ECL reagents (Millipore, Watford, UK) and exposed to Kodak GRI autoradiography film. Band intensities were measured and quantified by ImageJ.

2.2.13 ELISA-*Enzyme-linked immunosorbent assay*

ELISA kits for MCP-1, IL-6, TNF-α and IL-10 were purchased from R&D Systems (Duo set). Capture antibodies were diluted in PBS according to the manufacturer's instructions and 100µl was added to each well (96-well plate) and left incubating overnight, at room temperature. Plates were then carefully washed three times with 0.05% PBS tween (PBS-T) and 300µl of 1% BSA was added to each well for a minimum of 1 hour. Plates were then washed at least 3 times with PBS-T before 100µl of media from each sample was added in duplicate and left incubating overnight at 4^{o}C. Simultaneously, recombinant protein corresponding to the specific target was diluted according to manufacturer's protocol and added into separate wells in order to obtain a standard curve (R&D DuoSet). Plates were then washed with PBS-T for at least 3 times before the addition of 100µl of the detection antibody (concentrations used according to the manufacturer's protocol) for two hours at room temperature. After washing with PBS-T, 100µl of diluted Streptavidin-HRP was added to each well and incubated for 20 minutes in the dark. This was followed by a washing step with PBS-T and the addition of 100µl of substrate solution (R&D Systems # DY999) for 20

minutes in the dark. The reaction was stopped by the addition of 50µl of 2N H_2SO_4 (R&D Systems # DY994) when samples were turning blue. The optical density of each well was measured using a microplate reader (Tecan Sunrise) set at 450nm with wavelength correction at 540nm.

2.2.14 Human cytokine profile array A

Proteome Profiler Array, Human Cytokine Array Panel A was obtained from R&D Systems (ARY005). This array consisted of 36 different molecules (i.e. cytokines, chemokines, growth factors) spotted in duplicate onto four membranes. Experiments were performed as recommended by the manufacturer's instructions. Briefly, membranes were incubated with 2ml of buffer 4 for one hour on a rocking platform. 1.5ml media of each sample was incubated with 15µl of Detection Antibody cocktail and left incubating for one hour at room temperature. This solution was then added to the membrane upon the removal of the blocking buffer and left incubating overnight at 4^{o}C on a rocking platform. Membranes were then washed in 20ml of washing buffer before the addition of 1.5ml of diluted Streptavidin-HRP (buffer 5) for 30 minutes at room temperature on a rocking platform. Washing was performed on each membrane and Chemiluminescent HRP Substrate (Millipore) was added for 1-5 minutes. X-ray film was used to visualise the positive signals. Intensity of each signal was measured using ImageJ software.

2.2.15 Sphingosine kinase inhibitor treatment

SKi-II was resuspended in DMSO to a stock concentration of 20mM, aliquoted and stored at -20°C. U937 cells (1x10^6 per well) were incubated with 10nM of SKi-II for up to 24 or 48 hours at 37°C and 5% CO_2 to test SPHK1 inhibition. In a co-culture system, cells were treated with SKi-II for 24 hours at a final concentration of 10nM. Cells were washed to remove any traces of SKi-II to avoid leaking into PC-3 cells.

2.2.16 Chromatin Immunoprecipitation (ChIP)

PC-3 cells were seeded in 6-well plates at a density of $0.5x10^6$ cells and allowed to adhere overnight. A total of four 6-well plates was used for this assay. U937 cells were added to the top chamber of the transwell for two hours to two of the 6-well plates. Cross-linking of proteins to DNA was performed by adding, drop-wise directly to the PC-3 cells, 40µl of 37% (w/v) formaldehyde per 1ml of medium for 10 minutes at RT. 50µl of 2.5M glycine/1ml of medium was added for 5 minutes at RT in order to stop the cross-linking reaction. Cells were then washed twice with ice-cold PBS and IP buffer containing protease inhibitors (Roche, Diagnostic Ltd, West Sussex, UK) was added to the cells before being scraped and collected into 50 ml tube on ice. After centrifugation at 12,000g for 1 minute, the resulted nuclear cell pellet was sonicated three times for 10 minutes in 1ml of IP buffer containing inhibitors. Fractions were centrifuged at 12,000g for 10 minutes at 4^{o}C and supernatant was divided into 1/10 (imput) and the remaining equally divided before the addition of STAT1, histone and IgG antibodies overnight at 4^{o}C. The following day, samples were centrifuged at 12,000g for 10 minutes and supernatant was incubated with protein G - Dynabeads (30µl per IP sample) three times with IP buffer protein G – Dynabeads were blocked overnight with sheared salmon sperm DNA and BSA (30µl/sample). The beads were eluted with IP buffer and incubated at 4˚C for at least 45 minutes on a rotating platform (20-30 rotations per min). Beads were washed 3-4 times with IP buffer without the inhibitors. Chelex was added to the washed beads, followed by vortexing, and boiled for 10 minutes. Tubes were centrifuged at 12,000xg for 1 minute at 4˚C. 80µl of the supernatant was collected. The DNA in the imput was precipitated with 2.5-3 volumes of ethanol and incubated overnight at 4^{o}C. After centrifugation (14,000xg for 30 minutes at 4^{o}C) the supernatant was discarded and pellet left to dry. Pellets were dissolved in 100ml 10% (w/v) Chelex 100 suspension, and boiled for 10 minutes. The same procedure was performed for the imput as to IP samples. STAT1 binding site to SPHK1 DNA were assessed by qRT-PCR using primers described in table (2.1.4). Each sample was normalised to imput and results are shown in percentage of STAT1/imput.

2.2.17 Flow cytometry

HLA Class I expression was assessed by flow cytometry as a read-out for how MCP-1, IL-6 and IFN-γ treatment would affect STAT1 activated molecules. PC-3 cells were treated with MCP-1 (100ng/µl), IL-6 (100ng/µl) and IFN—γ (500IU) for 24 and 48 hours. Cells were then washed, resuspended with ice-cold PBS and incubated with or a FITC-conjugated anti-HLA-Class I-A,-B, -C antibody for 45 minutes at 4 °C in the dark. After three washes with ice-cold PBS, cells were finally fixed in 1% (v/v) PFA in PBS and analysed on a flow cytometer FACSCanto (Becton, Dickinson and Company, Erembodegem Belgium).

2.2.18 SPHK1 plasmid construct (phmKO2_ MNLinker)

2.2.18.1 Restriction and ligation

In order to obtain a fluorescent tagged SPHK1 protein, SPHK1 cDNA was digested from pcDNA-3.1 vector and cloned into phmKO2-MNLinker vector (cat#AM-V0140) that encodes a monomeric version of the fluorescent protein *CoralHue*. Briefly, pcDNA3.1 vector was digested with BamHI and NotI respectively according to manufacturer's instructions (NEB). SPHK1 cDNA was subsequently purified using QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions. SPHK1 was then inserted into phmKO2-MNLinker previously digested with both BamHI and NotI. Ligation was performed using 100ng of digested vector with a 3-fold molar excess of insert and Quick Ligation Kit (NEB) according to the manufacturer's instructions. Vector and insert were incubated with 1µl T4 ligase for 15 minutes at room temperature.

2.2.18.2 Transformation

Plasmid was heat shock transformed into One Shot TOP10 competent cells (Invitrogen, CA, USA) according to the manufacturer's instructions. 2µl ligation mix plasmid and 50µl competent cells were incubated for 30 minutes on ice, 30 seconds at 42°C and again placed on ice. Pre-warmed S.O.C. medium (Invitrogen) was added and cells were

incubated for 1 hour at 37°C and 225rpm. The cell suspension was spread on LB agar plates supplemented with 50µg/ml Kanamycin. Positive clones were amplified and purified using QIAPrep Spin Miniprep kit (Qiagen, Hilden, Germany). The successful ligation of vector was verified by digestion with BamHI and NotI and visualised on agarose gel.

2.2.18.3 Plasmid DNA purification

Positive clones were sequenced and the verified plasmid was amplified. Large scale plasmid DNA purification was performed using the Plasmid Maxi Kit (Qiagen) according to manufacturer's instructions. Briefly, 400ml transformed *E.coli* culture having been incubated for 15 hours at 37°C and 200rpm were pelleted, lysed and applied to a column. Plasmid DNA was resuspended in water and DNA concentration was quantified. The concentration was measured at 260 and 280nm absorbance using the NanoDrop (Fisher Scientific). Samples with the OD260=280 ratio above 1.8 were used.

2.2.19 Data representation and Statistical Analysis

The statistical significance of differences between the means of two groups was evaluated by unpaired Student's t test. All statistical tests were two-sided. Calculations were performed using Instat (GraphPad Software).

3. Monocyte effect on prostate and breast cancer cell proliferation

3.1 Introduction

Immune cells such as monocytes and macrophages have the capability to migrate to various parts of the body in response to specific stimuli. Histological data collected by several groups shows a strong infiltration of myeloid cells in malignant tumours which is associated with a poor clinical prognosis [227, 233, 239, 387]. In chronic inflammatory diseases, such as chronic inflammation or Crohn's disease, these cells are highly present and a correlation of these inflammatory diseases with cancer initiation and progression has been previously described. Conversely, the use of anti-inflammatory drugs has led to a reduction of cancer risk [226, 388]. Therefore, strong evidence exists to suggest that immune cells play an active role in tumour progression and promote tumour survival [389]. Within the tumour surroundings, the presence and recruitment of such cells creates a highly diverse microenvironment rich in cytokines and chemokines. These soluble factors act in a paracrine manner with positive effects on cancer progression and development [228, 390].

Genes that are involved in the regulation of these soluble factors are often candidates for the development of anticancer drugs. SPHK1 is one such gene that has been shown to be up-regulated in several types of cancer [391], [199, 204] and to be involved in cytokine regulation [270], [268]. This has led to the hypothesis that SPHK1 plays a role in inflammatory-mediated cell interactions; in this study I investigate whether SPHK1 is a key mediator of the interaction of monocytes/macrophage with cancer cells and a key regulator in the recruitment of these cells to the tumour site.

3.2 Co-culture with monocytes has differential effects on prostate cancer cell lines

To elucidate the role of SPHK1 in the interactions between prostate cancer cells and macrophages/monocytes during inflammation-related cancer progression, a prostate cancer cell line (PC-3) was co-cultured with a monocytic cell line (U937) in a transwell system. PC-3 cells were selected as a PCa cell line due to its highly metastatic phenotype and its androgen independency. This cell line is well characterised and has been widely used in several studies involving PCa [344]. U937 cells are also well characterised as a monocytic cell line and have been widely used in studies investigating the role of monocytes in cancer development [246, 247]. Similarly, THP-1 cells are another well characterised monocytic cell line widely used to evaluate the role of monocytes in several diseases including cancer [384, 392].

The transwell membrane pore size selected was 0.45 μm diameter so that only soluble molecules could be exchanged between the two cell types. For this purpose PC-3 cells were cultured in 6 well-plates for 24 hours before the addition of U937 cells to the upper chamber. In full media conditions PC-3 showed a mild increase in proliferation throughout the indicated time points which did not prove to be statistically significant (**Fig.3.1a**). Under starvation conditions (serum-free medium) PC-3 cells also showed an increase in cell proliferation when in co-culture with U937 cells. This effect was observed in a time-dependent manner, with a positive impact after 24 hours proving statistically significant from 48 hours onwards (**Fig 3.1b**).

To investigate the impact of SPHK1 signalling on PC-3 interactions with U937 cells, PC-3 cells were transfected with siRNA targeting SPHK1 for 48 hours prior to the addition of U937 cells. As expected, PC-3 cells transfected with siRNA for SPHK1 exhibited less cell proliferation in comparison with PC-3 cells transfected with the non-targeting siRNA (**Fig.3.1c**). As seen previously, PC-3 cells proliferated significantly more in the presence of U937 cells; interestingly SPHK1 knockdown in PC-3 cells completely abolished the effect of U937 cells on PC-3 cell proliferation. This effect was significant from 72 hours onwards, indicating a role for SPHK1 in communication between the cell types, likely in the regulation of the secretion of certain factors.

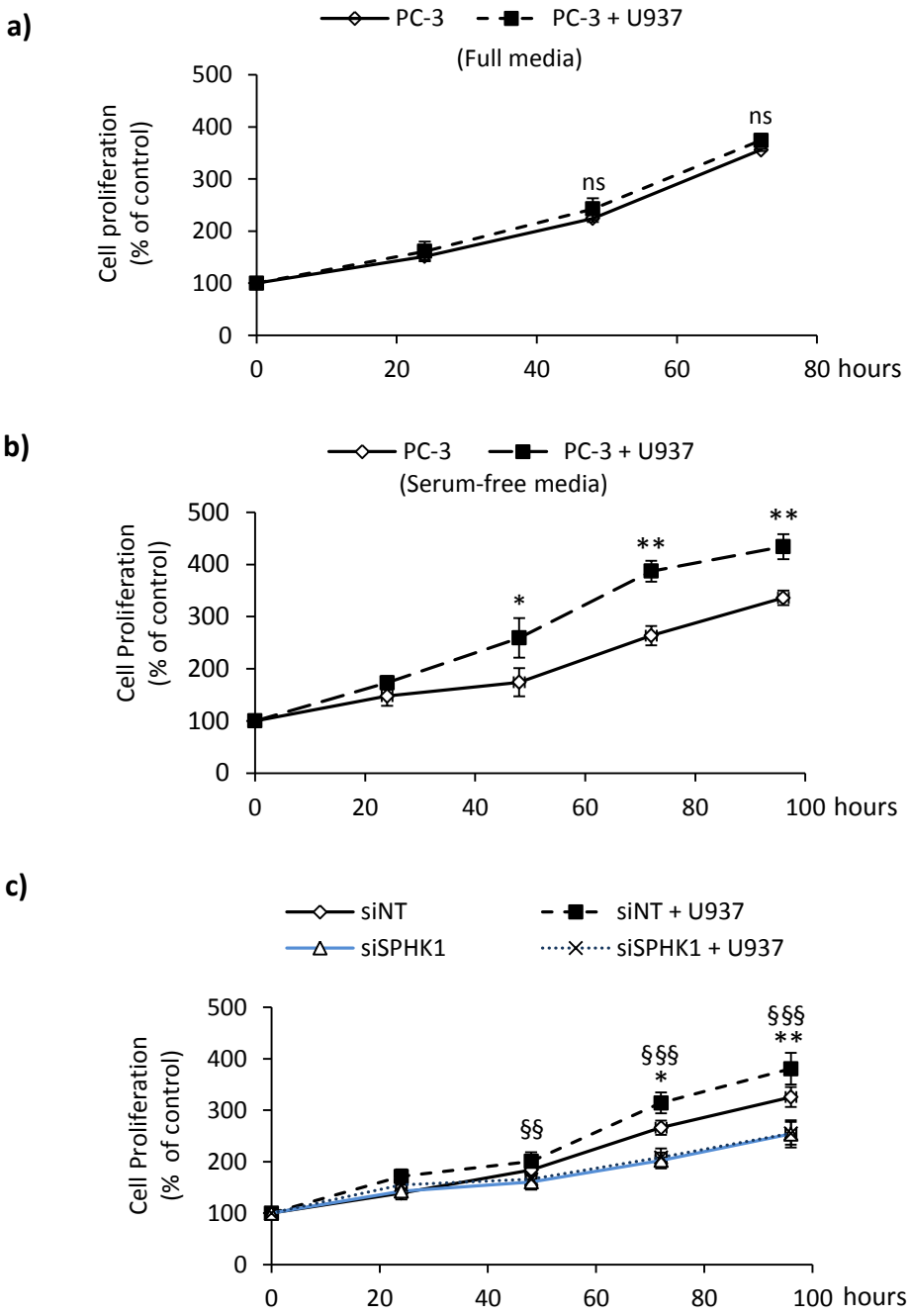


Figure 3.1: PC-3 cell proliferation in co-culture with U937 cells. PC-3 cells were cultured in **a)** full media or **b)** serum-free media for 24 hours before the addition of U937 cells which were added to the upper chamber and left incubating for up to 96 hours. **c)** PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1) or non-targeting control siRNA (siNT) for 24hours before cells were washed with PBS and cultured in serum-free media for another 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. SRB solution was added to the bottom chamber to determine PC-3 cell proliferation. Data shown is the mean ± SEM of triplicate measurements for each sample and is representative of three independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point. **a-b)** ns $p>0.05$, *$p<0.05$, **$p<0.01$, for PC-3 vs. PC-3+U937; **c)** *$p<0.05$, **$p<0.01$,for siNT vs. siNT+U937; §§$p<0.01$, §§§$p<0.001$ for siNT+U937 vs. siSPHK1+U937.

Following the previous assay, it was important to evaluate the effect of U937 cells on other prostate cancer cell lines. For this purpose DU-145 (**Fig.3.2a**) and c4-2 (**Fig.3.2b**) cells were co-cultured in the presence or absence of U937 cells for up to 72 hours. DU-145 showed a decrease in their proliferation when in the presence of U937 cells, an effect that was constant across all time points studied (**Fig.3.2a**). Similarly for c4-2 cell proliferation, the addition of U937 cells also proved to have an inhibitory effect on these prostate cancer cells, from 24 hours of co-culture there was a small decrease in c4-2 proliferation when in comparison with these cells cultured alone (**Fig.3.2b**).

3.3 Co-culture with monocytes has differential effects on breast cancer cell lines

To clarify whether the increase in cell proliferation was a universal event or a cell-tumour specific occurrence, BT549 and MDA-231 breast cancer cell lines were co-cultured with U937 cells. The metastatic characteristic of these cells and their triple negative genotype were taken into consideration to this study as they represent a proximal model to PC-3 cells which are androgen independent and highly metastatic prostate cancer cells.

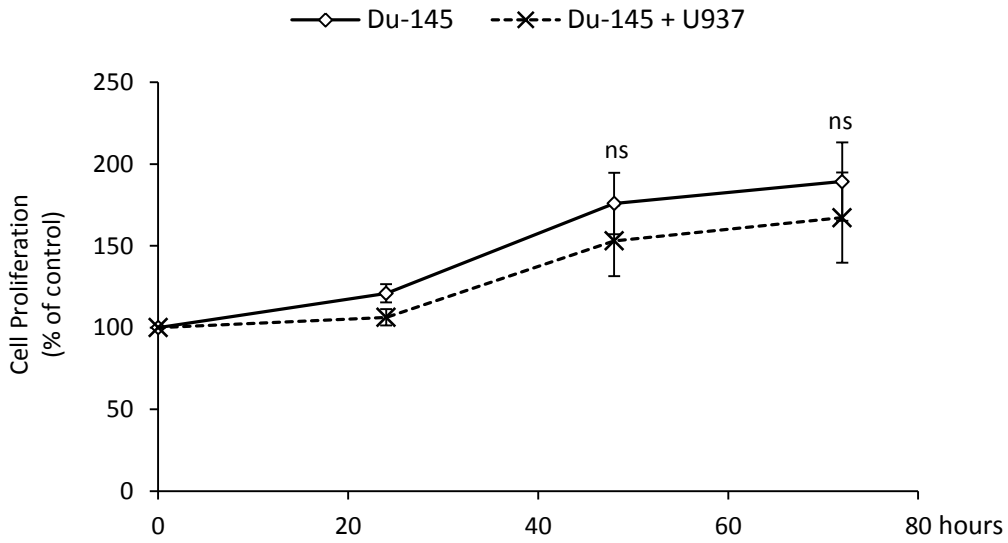
BT549 cells showed an increase in cell proliferation upon the addition of U937 cells, which was significant following 48 and 72 hours of co-culture (**Fig.3.3a**). Regarding MDA-231 cells, an increase in cell proliferation was also observed in the presence of U937 cells from 24 hours onwards; however although reproducible, this effect did not prove statistically significant (**Fig.3.3b**).

3.4 Effects of other monocytes or macrophages on PC-3 cells proliferation

To understand whether the U937-induced increase in PC-3 cell proliferation was monocyte-type specific, THP-1 cells were used as an alternative monocytic cell line for use in the co-culture model. THP-1 cells are a well characterised monocytic cell line as often used in to characterise the function and regulation of monocytes [392].

In full media conditions PC-3 cell proliferation was not affected by the presence of THP-1 cells (**Fig.3.4a**). Under starvation conditions, THP-1 cells increased PC-3 cell proliferation from 24 hours of co-culture onwards; however this increase did not prove statistically significant (**Fig.3.4b**). Similarly to that observed for U937 cells, this effect was not apparent when SPHK1 was targeted by siRNA in PC-3 cells as SPHK1 knockdown reduced cell proliferation and abolished the mild proliferative effects of THP-1 cells. (**Fig.3.4c**).

a)



b)

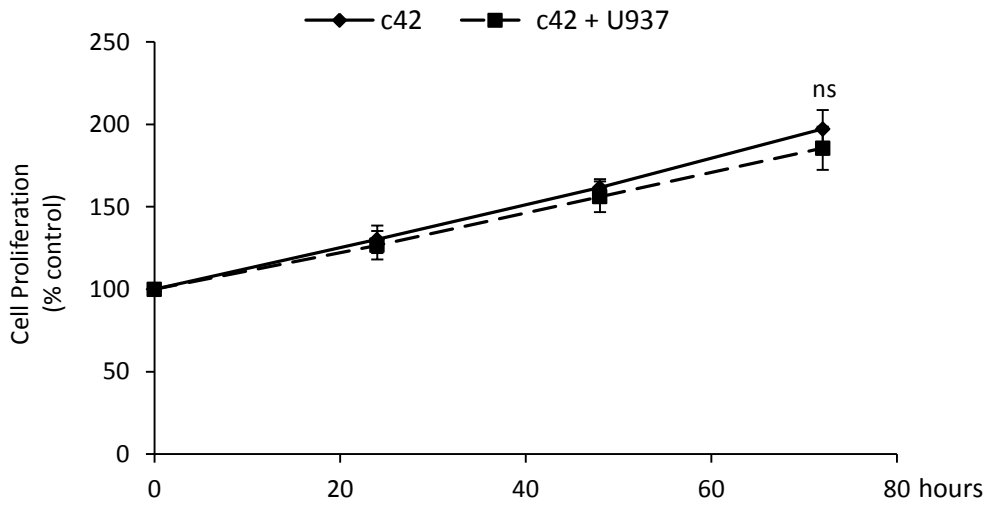


Figure 3.2: Prostate cancer cell proliferation in co-culture with U937 cells. **a)** DU-145 and **b)** C4-2 cells were cultured in serum-free media for 24 hours prior to the addition of U937 cells. U937 cells were added to the upper chamber and left incubating for up to 72 hours. SRB solution was added to the bottom chamber to determine PC-3 cell proliferation. Data shown is the mean ± SEM of three independent experiments performed in duplicate. Statistical analysis was performed using student's t-test relative to each individual time point. **a-b)** ns $p>0.05$ for DU-145 vs. DU-145+U937 or **c)** C4-2 vs. C4-2+U937.

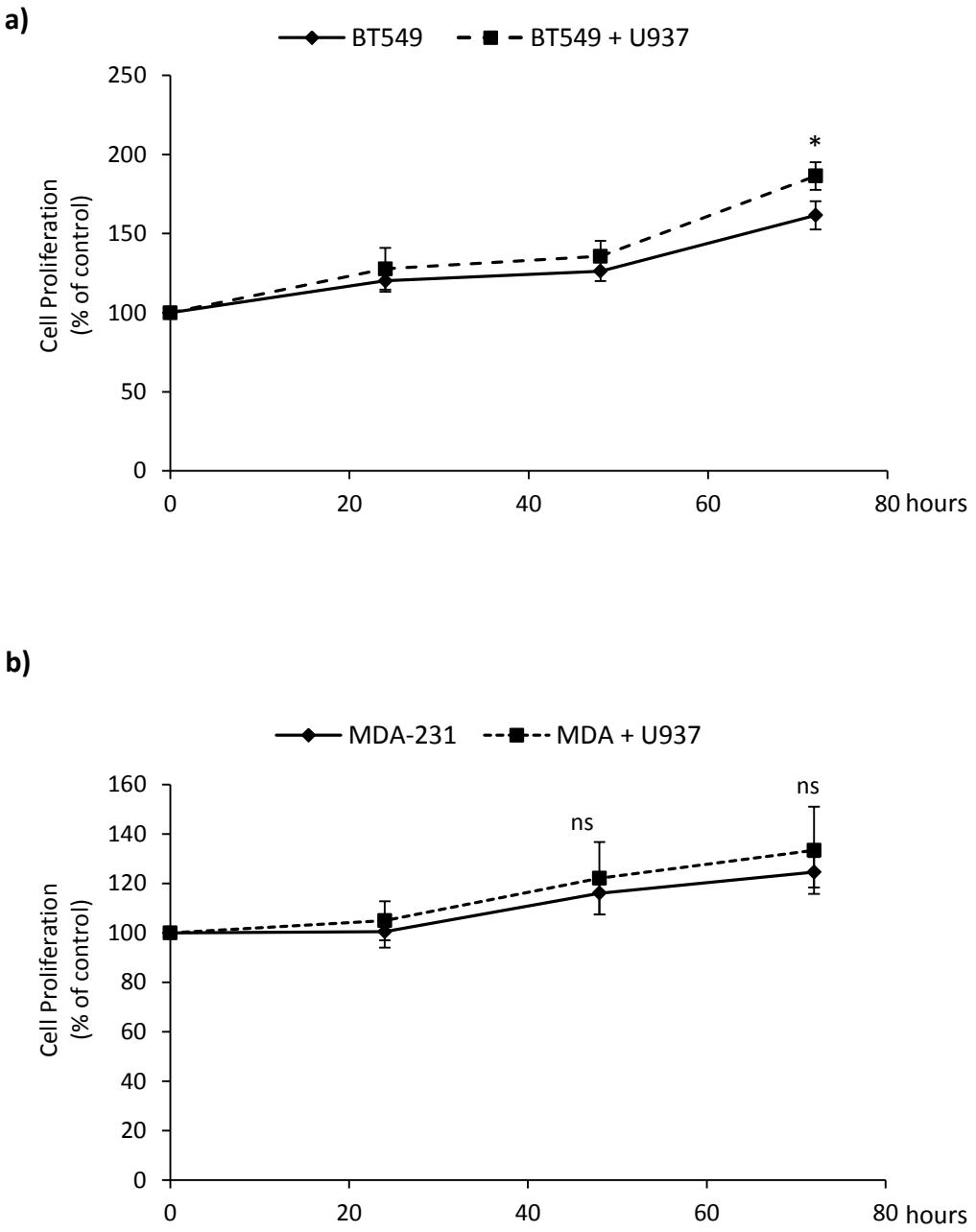


Figure 3.3: Breast cancer cells proliferation in co-culture with U937 cells. **a)** BT549 and **b)** MDA-231 cells were cultured in serum-free media for 24 hours prior to the addition of U937 cells. U937 cells were added to the upper chamber and left incubating for up to 72 hours. SRB solution was added to the bottom chamber to determined BT549 or MDA-231 cell proliferation. Data shown is the mean ± SEM of three independent experiments performed in duplicate. Statistical analysis was performed using student's t-test relative to each individual time point; ns $p>0.05$, *$p<0.05$, for BT549 vs. BT549+U937 or MDA-231 vs. MDA-231+U937.

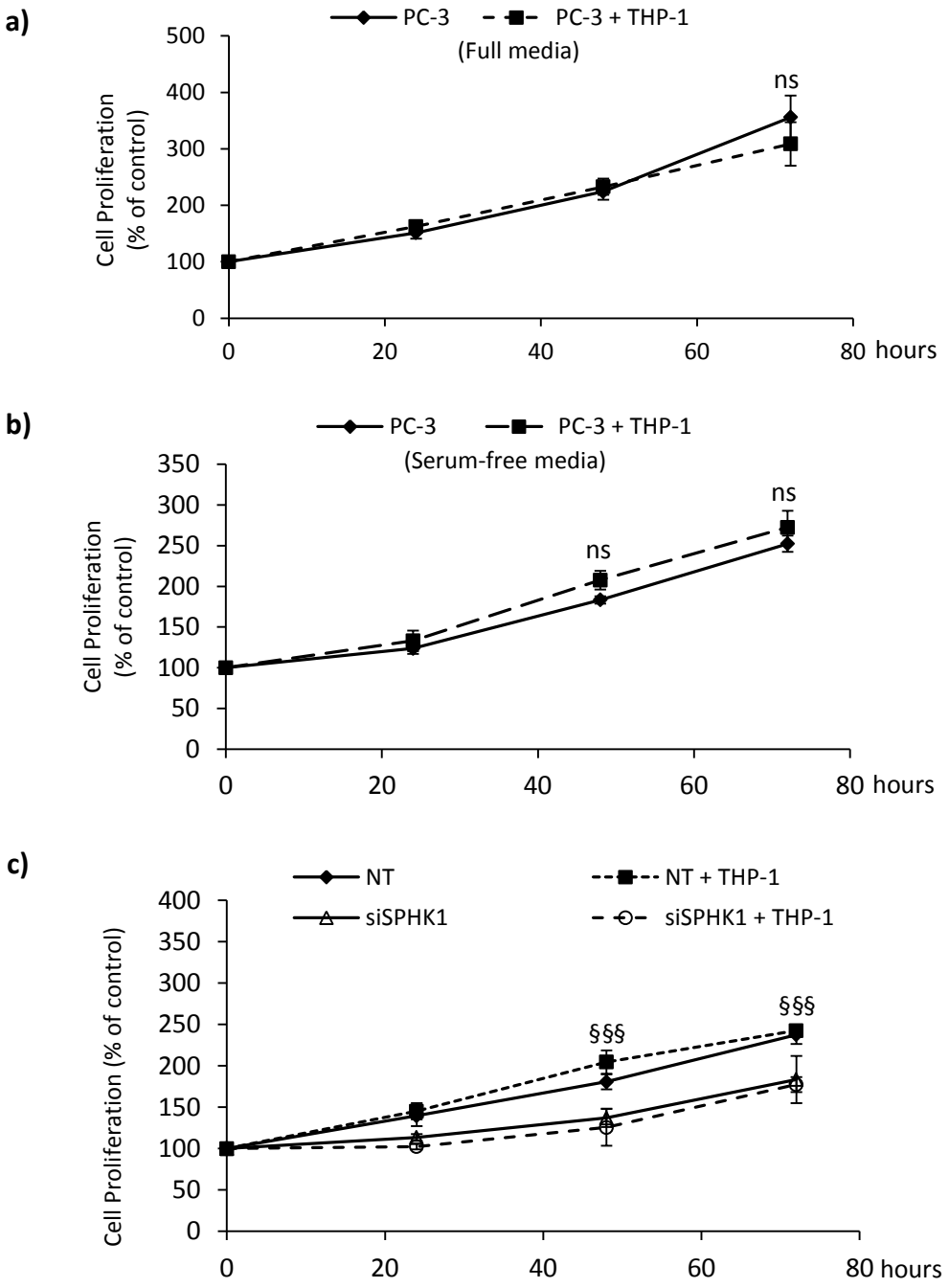


Figure 3.4: PC-3 cell proliferation in co-culture with THP-1 cells. PC-3 cells were cultured in **a)** full media or **b)** serum-free media for 24 hours before the addition of THP-1 cells. THP-1 cells were added to the upper chamber and left incubating for up to 72 hours. **c)** PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1) or non-targeting control siRNA (siNT) for 24hours before cells were washed with PBS and cultured in serum-free media for another 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. SRB solution was added to the bottom chamber to determine PC-3 cell proliferation. Data shown is the mean ± SEM of three independent experiments performed in triplicate. Statistical analysis was performed using student's t-test relative to each individual time point; **a-b)** ns $p<0.05$ for PC-3 vs PC-3 + THP-1; **c)** §§§$p<0.001$ for siNT+THP-1 vs. siSPHK1+THP-1.

Next, it was important to evaluate the effect of macrophages upon PC-3 cell proliferation. The use of a macrophage cell line (RAW 264.7) had no positive effect on PC-3 cell proliferation, in fact from 48 hours onwards there was a non-significant decrease in proliferation in either full media conditions (**Fig.3.5a**) or under starvation (**Fig.3.5b**). With the same purpose of testing macrophage cells in co-culture, THP-1 cells were differentiated into a macrophage-like cell type with PMA for 24 hours, after which non-adherent cells were removed and differentiated adherent THP-1 cells were added to the PC-3 cells**.** A modest, albeit transient, positive effect on PC-3 cell proliferation was observed after 24 hours which was followed by a reduction at 72 hours (**Fig.3.5c**).

3.5 Effect of increasing SPHK1 in PC-3 cell proliferation during co-culture

cDNA encoding human SPHK1 was cloned into the phmKO2-MNLinker expression vector as described in section (section **2.2.3**), with SPHK1-mKO2 constitutively expressed in PC-3 cells, as verified by fluorescent microscopy (**Fig.3.6a**). SPHK1 mRNA levels were quantified using qPCR. Figure **3.6b** shows a 4-fold increase in SPHK1 expression in PC-3 transfected with SPHK1-mKO2 (PC-3/OFP-SPHK1) when compared with PC-3 cells transfected with an empty vector (PC-3/OFP). The next step was to evaluate if an over-expression of SPHK1 would result in an increase in cell proliferation. As seen in figure **3.6c**, the over-expression of SPHK1 did not lead to a significant increase in PC-3 cell proliferation. However, the addition of U937 cells was shown to have very little effect on PC-3/OFP cell proliferation in this experiment (**Fig.3.6d**) as seen previously with parental PC-3 cells (**Fig.3.1b**).

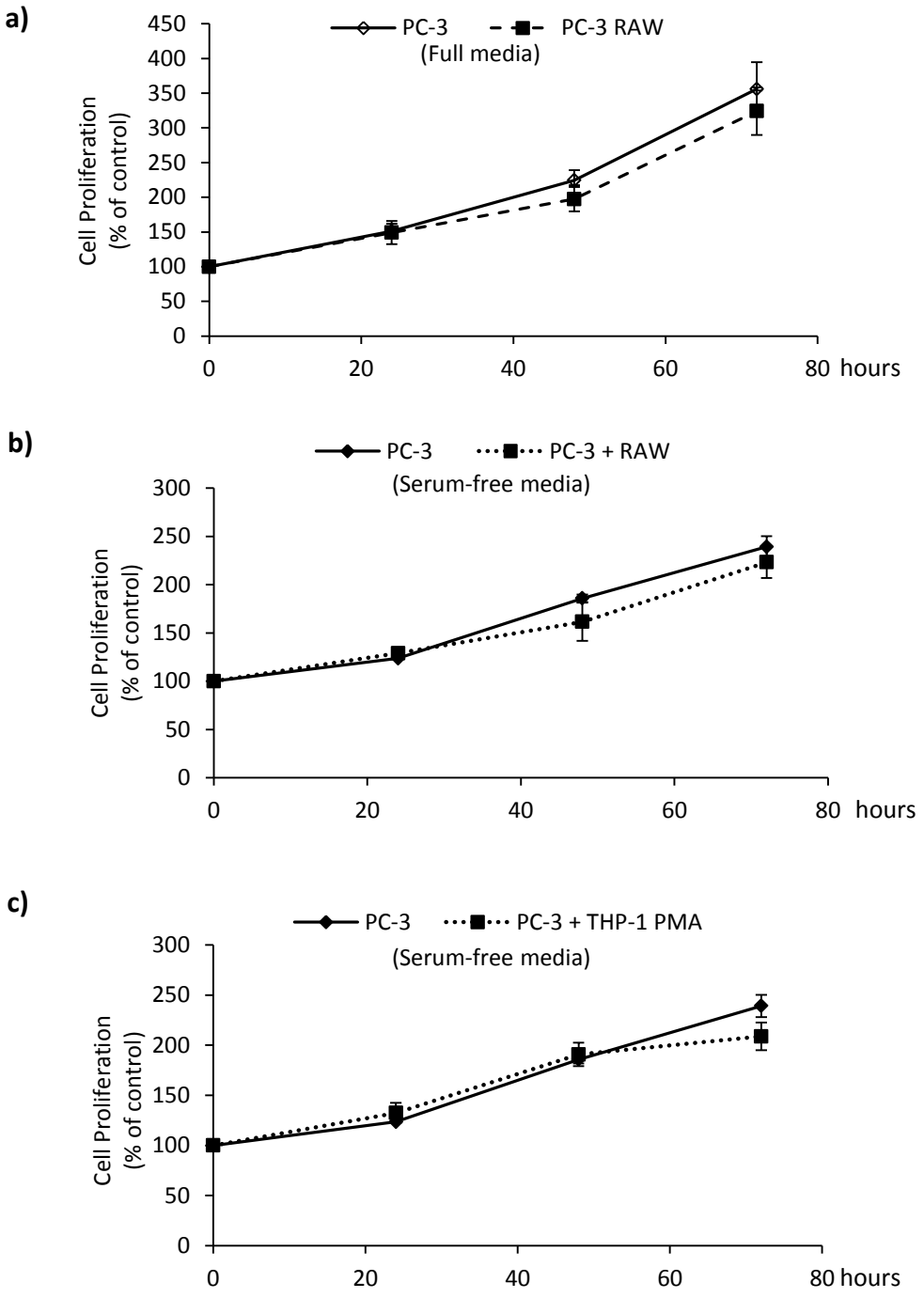


Figure 3.5: PC-3 cell proliferation upon co-culture with macrophages. PC-3 cells were cultured in **a)** full media or **b-c)** serum-free media for 24 hours before the addition of macrophages. **a-b)** RAW264.7 mouse macrophages were added to the upper chamber and left incubating for up to 72 hours **c)** THP-1 cells were pre-treated with PMA (100nM) for 24 hours before being added to PC-3 cells for up to 72 hours. SRB solution was added to the bottom chamber to determined PC-3 cell proliferation. Data shown is the mean ± SEM of two independent experiments performed in duplicate.

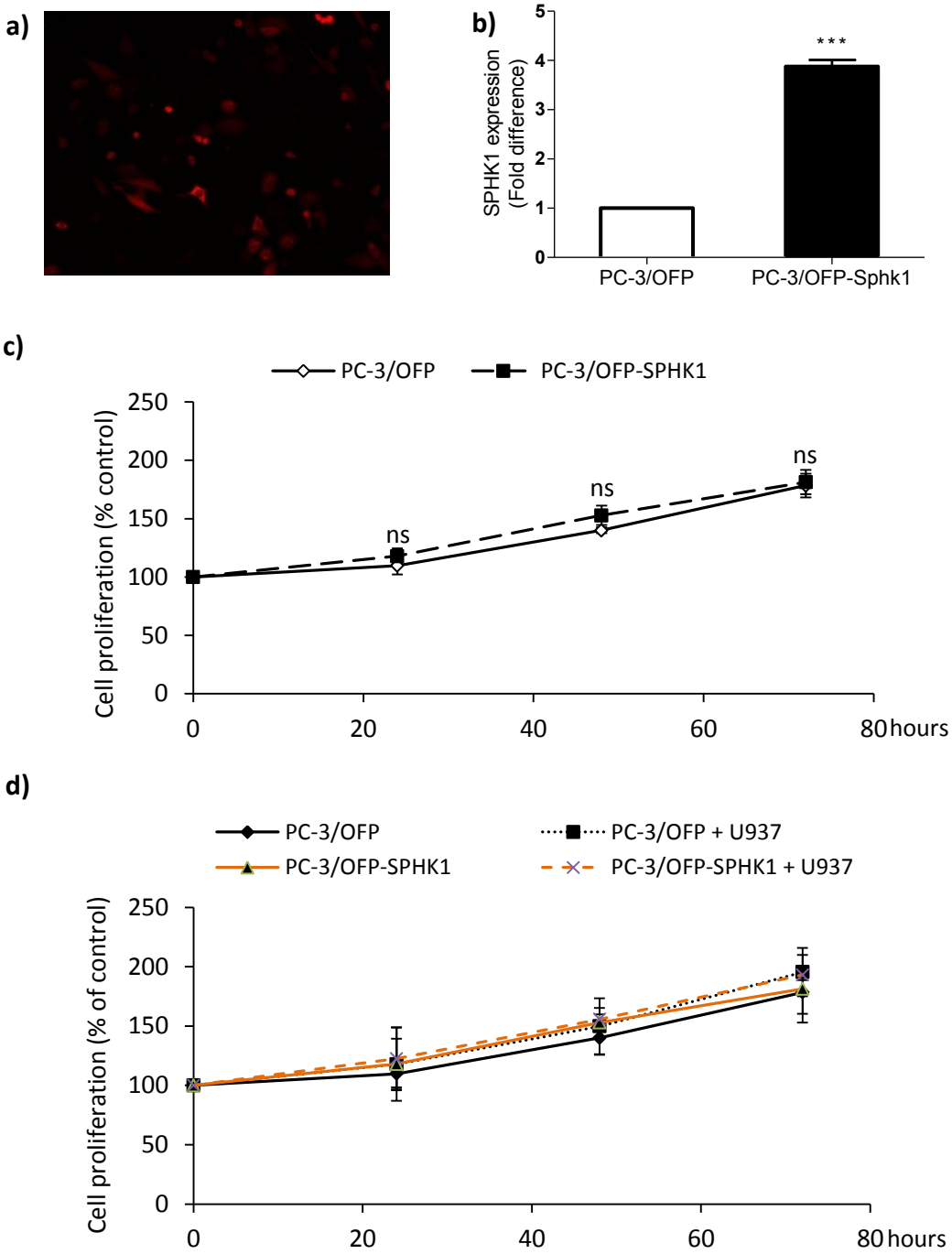


Figure 3.6: SPHK1 over-expression in PC-3 cells. PC-3 cells were transfected with a phmKO2 vector constitutively expressing SPHK1 or phmKO2 vector for control. Cell sorting was performed to isolate only OFP positive cells while G418 was used as a selective agent. **a)** Fluorescence microscopy was used to detect OFP positive cells (orange) and **b)** qRT-PCR was performed to verify SPHK1 over-expression. **c)** PC-3 tagged OFP- SPHK1 and OFP alone were cultured in the absence or **d)** presence of U937 cells for the indicated time points. SRB solution was added to the bottom chamber to determined PC-3 cell proliferation. Data shown is the mean ± SEM of two independent experiments performed in duplicate. Statistical analysis was performed using student's t-test relative to each individual time point; ns $p>0.05$, ***$p<0.001$ for PC-3 OFP vs. PC-3 OFP-SPHK1.

3.6 Effect of SPHK1 inhibition on PC-3 growth in an *in vivo* model

The effect of SPHK1 on tumour growth and recruitment of monocytes/macrophages was examined *in vivo* in collaboration with Dr. Alwyn Dart and Dr. Greg Brook. PC-3 cells over-expressing GFP were subcutaneously implanted with matrigel in the flanks of nude mice. Following tumour establishment, mice were divided into two groups and treated every two or three days by subcutaneous injection with 50µL SPHK1 inhibitor SKi-II (50mg/kg) or DMSO (control) diluted in PBS. The addition of SKi-II significantly reduced the tumour growth upon comparison with the control group (**Fig.3.7a**). Interestingly, after just one dose (day 2) of SKi-II, the tumour volume had decreased with respect to the control group, and by day 9 the relative tumour volume was reduced to approximately the size observed prior to treatment. The control group showed a constant increase in tumour volume, with an approximate 2.5-fold increase by day 9. After collection, tumours were divided into several parts and frozen for multiple analyses. Immunohistochemistry was used to measure cell proliferation in each tumour, with Ki67 staining used as a marker of cell proliferation. Ki67 is a nuclear protein present during all phases of cell cycle but is notable by its absence from resting cells which allows the specific detection of proliferative cells. Figure **3.7b-c** represents the differences in Ki67 staining between the two groups showing a reduction in proliferative cells in tumours from mice treated with SKi-II. H&E staining (Hematoxylin and eosin) was performed for visualisation of nuclear and cell structure (**Fig.3.7b**).

To evaluate whether SPHK1 was differentially expressed in SKi-II treated mice with respect to untreated control mice, immunohistochemistry analysis was performed on each tumour (**Fig.3.8a**). SPHK1 intensity was scored as high (2) or low (1) by blind scoring of each tumour section and showed no significant differences between control or treated tumours (**Fig.3.8b**). To further analyse SPHK1 levels, RNA was extracted from each tumour and SPHK1 mRNA expression levels were determined by qRT-PCR after 4 treatments (day 9). As seen in figure **3.8c**, the effect of SKi-II on tumour volume was paralleled with a significant reduction of SPHK1 expression in human PC-3 cells. Mouse cells of the tumour microenvironment were also evaluated for SPHK1 levels in order to determine the effect of the SKi-II inhibitor in the tissues surrounding the tumour; a similar reduction in SPHK1 expression was observed, although did not prove statistically significant (**Fig. 3.8d**).

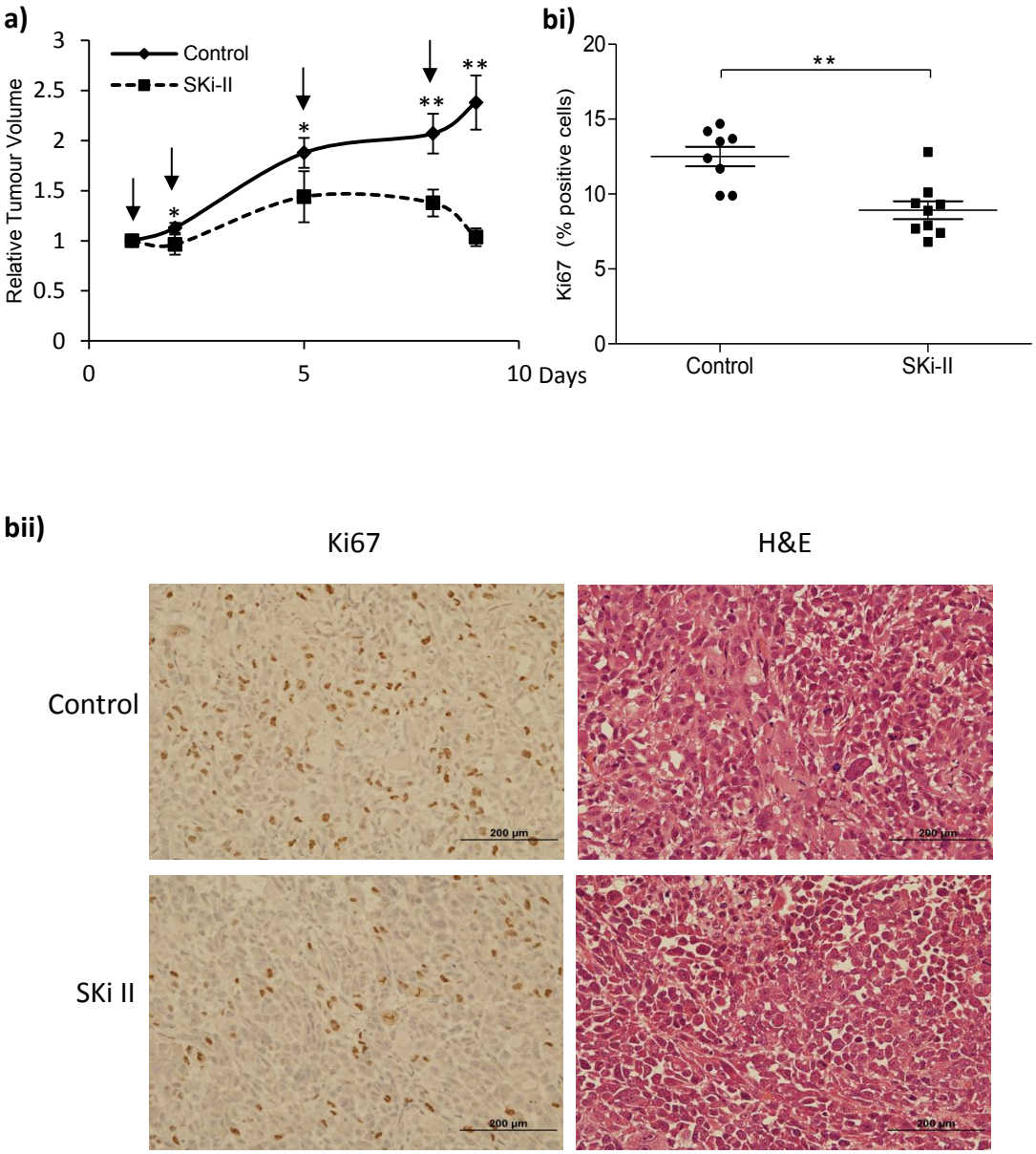


Figure 3.7: PC-3 Xenograft from nude mouse model. 1x10^6 PC-3 cells tagged with GFP were subcutaneously injected into nude mice, left to grow for ten days before the addition of SKi-II (50mg/ml) every three days for a total of four treatments (black arrows). DMSO was used as a control. **a)** Tumours were collected and volumes were measured using calipers (section 2.2.6). **b)** Immunohistochemistry was performed for the cell proliferation marker Ki67, **bi)** each data point represents the average three scorings of Ki67 positive cells per tumour section, **bii)** representative staining for each condition. Hematoxylin and Eosin (H&E) staining was used to verify cell structure. Student's t-test was used to determine statistical significance for SKi-II treated vs. control xenografts; ns $p>0.05$, *$p<0.05$, **$p<0.01$.

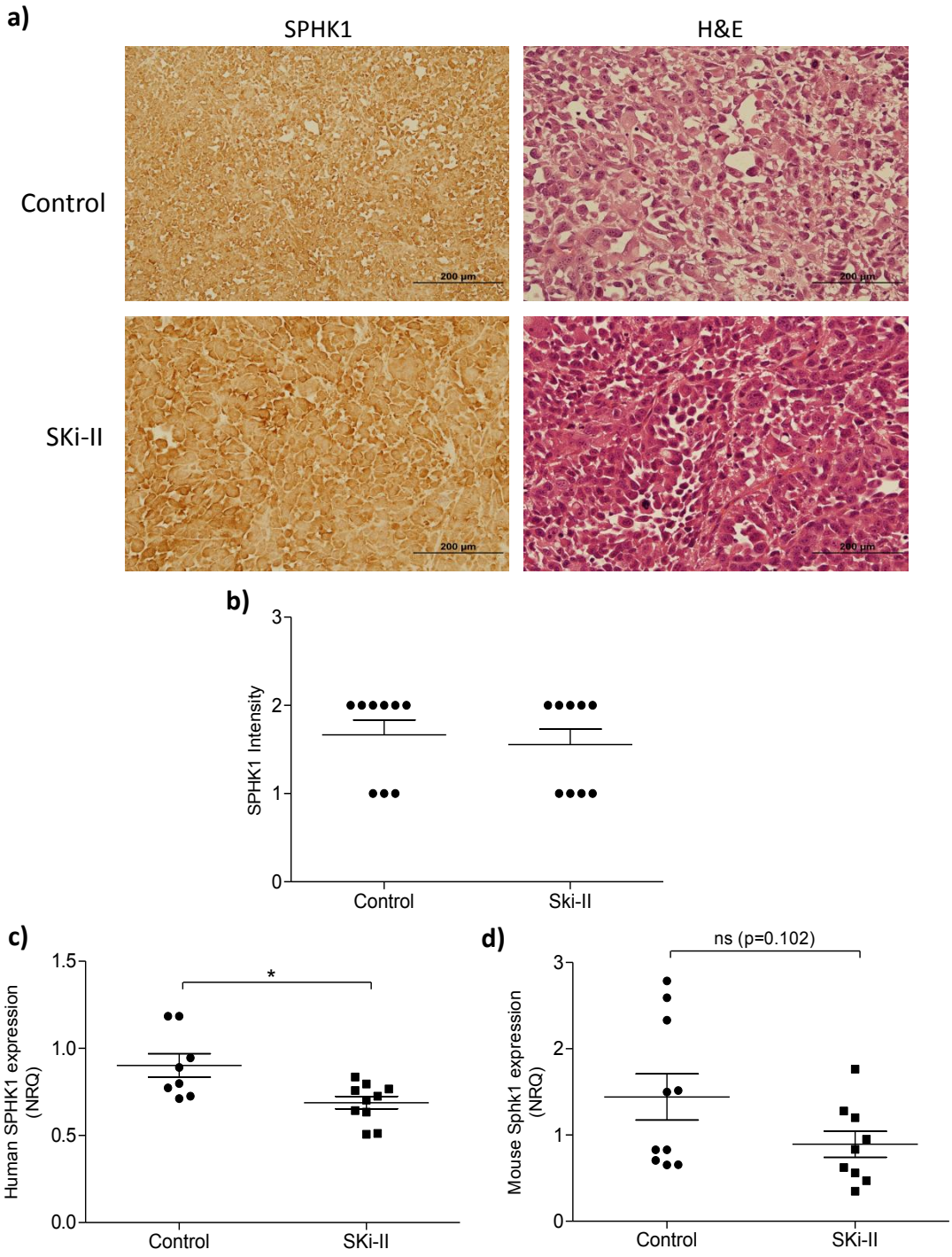


Figure 3.8: Immunohistochemistry analysis of PC-3 Xenograft tumours after SKi-II treatment. Immunohistochemistry was performed for SPHK1 and H&E. **a)** Representative staining of each condition. **b)** SPHK1 intensity was determined by blind scoring of each tissue sample. **c)** Human SPHK1 and **d)** mouse Sphk1 expression was determined by qRT-PCR of RNA extracted from each tumour sample, and expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. Data shown is the normalised relative quantification (NRQ) of each sample. Student's t-test was used to determine statistical significance for SKi-II treated vs. control xenografts; ns $p>0.05$, *$p<0.05$.

Next it was important to determine whether SKi-II treatment would affect the number of infiltrating monocytes and macrophages within the tumour. For this purpose MAC2 antibody was used as a monocyte/macrophage positive marker during immunohistochemistry analysis (**Fig.3.9a**). Each MAC2 stained section was blind scored and, as seen in figure **3.9b**, did not show a significant difference amongst treated versus untreated mice. It must be noted that the antibody showed a lack of specificity and in order to have a more accurate analysis staining with other monocyte/macrophage antibodies such as CD68, CD14 and F4/80 were also tested, with no success.

Nonetheless, the MAC2 antibody when used in spleens of PC-3 xenograft mice proved to be very specific and reliable in its staining profile. This analysis was performed to determine whether the use of SKi-II treatment would reduce the number of migratory monocyte/macrophages present. As seen in figure **3.10b** the use of SKi-II did not reduce the number of infiltrating monocytes/macrophages in the spleen upon comparison with untreated mice.

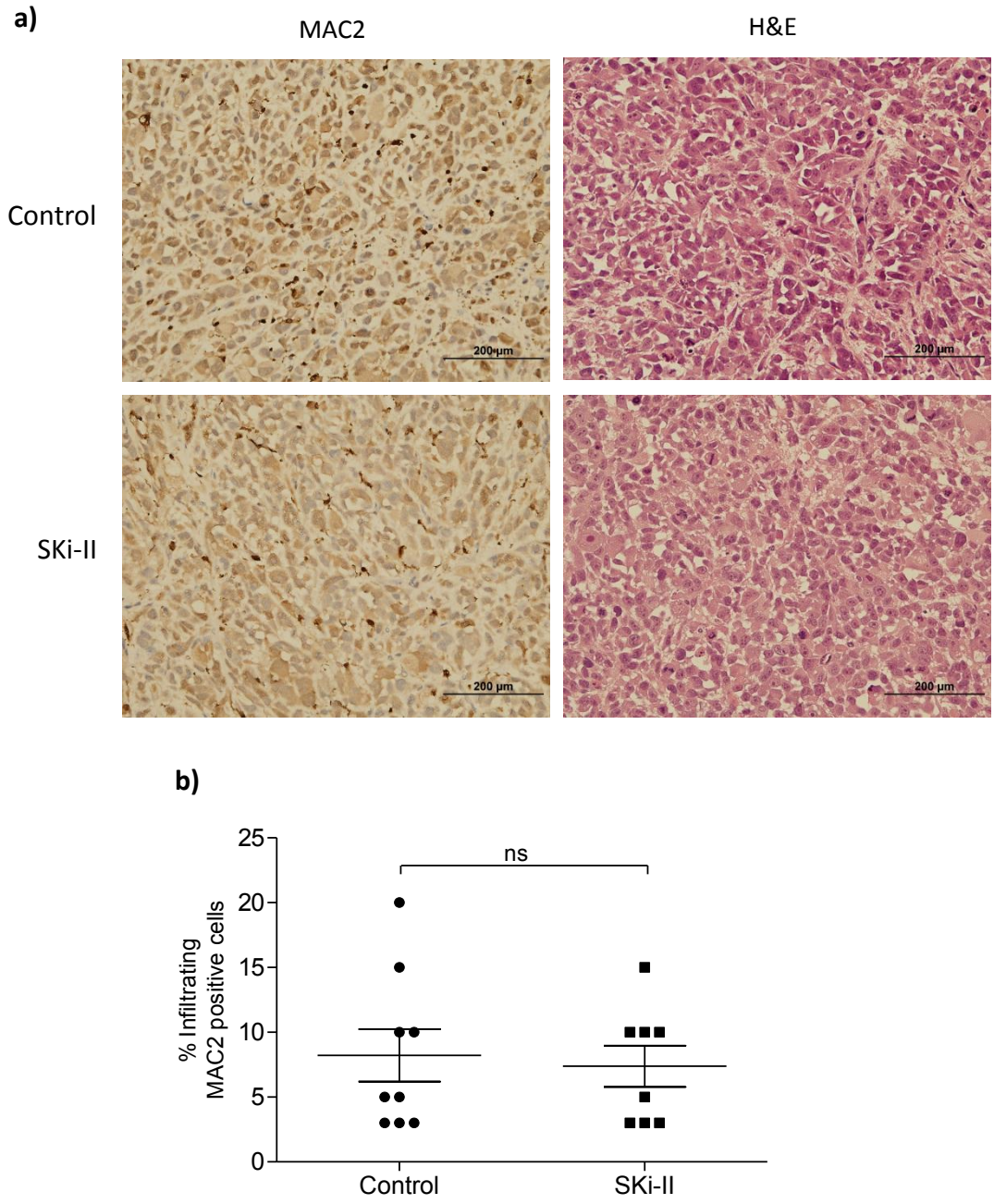


Figure 3.9: Immunohistochemistry analysis of PC-3 Xenograft tumours after SKi-II treatment. Immunohistochemistry was performed for MAC2 and H&E. **a)** Representative staining of each condition. **b)** Data shown is percentage of MAC2 positive staining as determined by blind scoring of each tissue sample within a field of approximately 100 cells. Student's t-test was used to determine statistical significance for SKi-II treated vs. control xenografts; ns $p>0.05$.

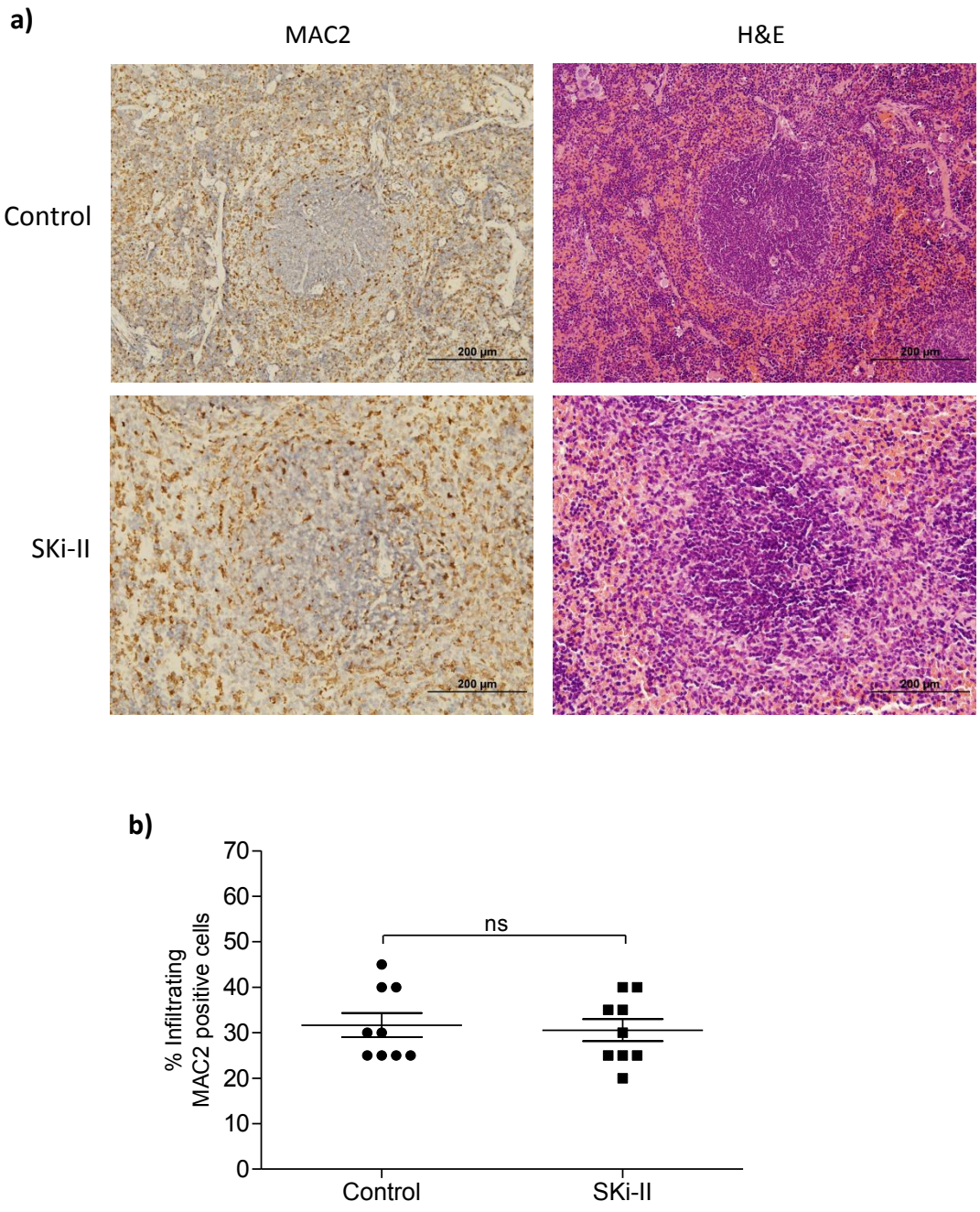


Figure 3.10: Immunohistochemistry analysis of PC-3 Xenograft mice spleens after SKi-II treatment. Immunohistochemistry was performed for MAC2 and H&E. **a)** Representative staining of each condition. **b)** Data shown is percentage of MAC2 positive staining as determined by blind scoring of each tissue sample within a field of approximately 100 cells. Student's t-test was used to determine statistical significance for SKi-II treated vs. control xenografts; ns $p>0.05$.

3.7 Discussion

The tumour microenvironment is one of the most rapidly growing areas of cancer cell biology research. It is readily accepted that tumour cells interact with their surroundings in order to create favourable conditions for their growth and progression. Tumour-stromal cell interactions are crucial for tumour development, with monocytes and macrophages being the main types of immune cells whose involvement has been described in tumour progression [233, 393]. In this context, it has been shown that co-inoculation of PC-3 and U937 cells in male athymic mice significantly increases tumour growth when compared with PC-3 cells injected alone [246], whilst another study indicates that THP-1 cells and blood derived monocytes were able to induce colon cancer cell proliferation and increase colony formation [248]. Here, in this study, it is shown that co-culturing cancer cells and monocytes can result in a beneficial effect on cancer cell proliferation; indeed PC-3 cells cultured with U937 (**Fig.3.1b**) and THP-1 cells (**Fig.3.4b**), to a lesser extent, provide an example of such an effect. Moreover, in BT549 (**Fig.3.3a**) and MDA-231 breast cancer cells (**Fig.3.3b**) a similar effect was observed, with the beneficial effect more accentuated and statistically significant in BT549 cells. It is to be noted that in breast cancer cell models the increase in proliferation was not as accentuated as that observed in PC-3 cells when in culture with U937; one plausible explanation could be the longer doubling time of these cells, MDA-231 at 25-35 hours and BT549 at 25-30 hours, in comparison to PC-3 cells doubling every 10-20 hours, which would result in a lower increase in the same period of time. Indeed, the characteristics of each cell line together with the culture conditions appear crucial in cancer cell development and may not always confer a beneficial effect on cancer cell proliferation. This is evident in the case of DU-145 (**Fig.3.2a**) and C4-2 (**Fig.3.2b**), other prostate cancer cell lines, models of CRPC, whereby the addition of U937 cells appeared to invoke a slight inhibitory effect on cancer cell proliferation. Likewise, PC-3 cell proliferation was also inhibited when co-cultured with RAW264.7 macrophages (**Fig.3.5a-b**) and THP-1-derived macrophages (**Fig.3.5c**). The influence of culture conditions is exemplified by the finding that in full media conditions U937 (**Fig.3.1a**) and THP-1 cells (**Fig.3.4a**) induced a lower level of proliferation in PC-3 cells when compared with the increase observed upon serum-free culture. Altogether these results indicate that tumour-monocyte or macrophage interactions vary from cell to cell with the outcome being determined by the signalling profile of each cell

type. A more extensive analysis of factors secreted by different cancer cell types would afford a better understanding and elucidation of the mediators of these interactions and could provide useful insights into the differential effects on cancer cells.

The involvement of SPHK1 in cell proliferation has been described previously; indeed SPHK1 silencing has been shown to reduce MCF-7 cell growth and glioblastoma cell proliferation by 2.5-fold [215, 353]. In support, our group has shown in a prostate cancer cell model (PC-3) that the knockdown of SPHK1 by siRNA can lead to a significant reduction in cell proliferation [78]. In this study I aimed to evaluate if monocytes were able to overcome the inhibitory effect on PC-3 cell proliferation induced by SPHK1 knockdown. As expected, the importance of SPHK1 on cell proliferation was confirmed as its knockdown reduced PC-3 cell proliferation; more importantly the inhibitory effects observed were not altered by the presence of U937 (**Fig.3.1c**) or THP-1 cells (**Fig.3.4c**). Several explanations can thus be inferred from these findings; a) SPHK1 plays a central role in PC-3 proliferation and it's absence renders ineffective any stimulus that could trigger proliferation (e.g U937 or THP-1 cell presence); b) the factors secreted by monocytes are mediated by SPHK1 in PC-3 cells (discussed in chapter 4 and 5); or c) soluble factors secreted by PC-3 cells in the absence of SPHK1 are different from those secreted by parental PC-3, which could modulate the monocytic response towards PC-3 cells. While these data support an interpretation that monocytes are unable to increase cell proliferation in SPHK1 siRNA targeted PC-3 cells, a question remains whether soluble factors involved in the interaction between monocytes and cancer cells may be regulated by SPHK1. This aspect will be further discussed in chapters 4 and 5.

Over-expression of SPHK1 is credited in the literature for increasing proliferation in intestinal epithelial cells [394] and colon cancer cells [395]; the effect of SPHK1 over-expression upon PC-3 cell proliferation together with its effect on U937-induced cancer cell proliferation was therefore evaluated in this study. The constitutive over-expression of SPHK1 in PC-3 cells did not result in a significant increase in PC-3 cell proliferation (**Fig.3.5c**) and no further increase in proliferation was observed upon co-culture with U937 cells (**Fig.3.5d**). The hypothesis that increased SPHK1 would lead to an increase in SPHK1-mediated signalling molecules, in turn activating U937 cells to promote PC-3 cell proliferation, is not supported by these findings. Nonetheless this result should be analysed

carefully as the proliferation of PC-3 cells transfected with an empty vector (PC-3/OFP) (**Fig.3.6c**) was markedly lower than in parental PC-3 cells (**Fig.3.1.a-b**) and in the presence of U937 cells (**Fig.3.1b**) indicating a possible negative effect of the transfected plasmid DNA upon cell proliferation. Such an effect could mask not only the positive influence of SPHK1 on PC-3 proliferation but also the SPHK1-induced proliferative effects of U937s on PC-3 cells.

The effect of SPHK1 modulation on tumour growth and monocyte/macrophage interaction was next examined *in vivo*. SKi-II inhibition of tumour growth supported my previous findings that identified SPHK1 as a key element for tumour growth and progression. Indeed, the 2.5-fold decrease in tumour volume caused by the SPHK1 inhibitor (**Fig.3.7a**) together with a significant reduction in cell proliferation (**Fig.3.7b-c**) indicates its clinical and potential therapeutic value. These findings are supported by other *in vivo* studies showing SPHK1 involvement in tumour growth; SPHK1 inhibition by FTY720 led to a decrease of PC-3 cell growth, metastasis and increase in apoptosis in a time and concentration dependent manner [144]. Moreover, Ski-I induced reduction of tumour size in a NSCLC xenograft model [349], whilst in breast cancer the tumour reduction was accompanied by a reduction in the number of metastasis to lymph nodes and lungs [396].

An important observation is that SPHK1 expression for both human and mouse tissue was reduced by SKi-II treatment (**Fig.4.7b-c**). This effect could be attributed to the ability of SKi-II to regulate SPHK1 though inhibition of SPHK1 enzymatic activity or by rendering it ineffective via the induction of proteosomal degradation as described in previous studies [397-399]. The reduction in SPHK1 levels is also believed to cause inhibition of SPHK1-activated signalling-related proteins, affecting not only intracellular pathways that are involved in SPHK1 transcription, but also the secretion of extracellular proteins that in turn will affect the tumour microenvironment [270, 326]. In this study, the impact of SPHK1 inhibition in an *in vivo* mouse model did not correlate with a decrease in macrophage infiltration (**Fig.3.9**). These results should however be interpreted with caution as MAC2 staining did not meet an acceptable quality to be considered a viable and specific marker for macrophage and monocyte staining. A strong background in tumour sections associated with non-specific cell staining is the most likely reason as to why these results may not be accurate. Nonetheless, MAC2 staining of the spleen showed good levels of cell specificity and was deemed reliable in its staining profile. This indicated that preparation of the tumour

sections, rather than poor specificity of the antibody itself, was the most likely cause of the unreliable staining profile. This could also explain the apparent lack of reduction of SPHK1 as observed by immunohistological analysis of SKi-II treated tumour preparations as well as the unreliable performance of other monocyte/macrophage markers tested such as CD68, F4/80 and CD14. It is therefore important to optimize and clarify the quality of the tumour sections in order to obtain more assured conclusions regarding the role of SPHK1 in monocyte infiltration, and subsequently be able to determine if, within the tumour sections, the presence of monocytes is localised with high SPHK1 expression. Possible future approaches to resolve the high background staining observed could entail the fixing of new tumour sections onto new slides or the use of frozen sections to increase antibody specificity and binding. With respect to macrophage staining the use of antibodies such as cd11b or cd11c could offer an alternative to the antibodies explored to date and may provide an increased specificity to the cell type. The use of two different antibodies in mirror sections could also further validate the presence of infiltrating macrophages in the tumour.

Altogether this data supports the role of SPHK1 as a key mediator in cancer cell proliferation, with both *in vitro* and *in vivo* models showing an anti-proliferative effect on cancer cells when SPHK1 was inhibited. The ability of monocytes to induce cancer cell proliferation appears to vary amongst different cell types; however SPHK1 inhibition was able to suppress this positive effect, identifying SPHK1-targeted therapy as a potential approach for prostate cancer associated with a high inflammatory condition.

4. The role of tumour cell-derived SPHK1 in intercellular interactions

4.1 Introduction

The inflammatory tumour microenvironment contains an abundance of cytokines, chemokines and growth factors that are secreted by both tumour cells and stromal cells such as macrophages [226, 244]. Recent studies reveal that direct communication between macrophages and tumour cells is critical to the invasion and egress of tumour cells into blood vessels, and to subsequent metastasis [387, 389]. Indeed, tumour cells have the ability to recruit myeloid derived cells, such as monocytes and macrophages, through the production and secretion of various stimulatory molecules such as growth factors, cytokines and chemokines [232, 304]. The presence of infiltrating cells within the tumour is thought to induce the activation of important signalling pathways such as PI3K/AKT or MAPK/ERK1/2, known promoters of cancer development [328, 400, 401]. In this context, it is becoming clear that a more effective approach to new cancer drugs should not only target molecules involved in pro-tumorigenic events within the cancer cells but also molecules that are able to modulate the tumour cell microenvironment. As such, the role of the SPHK1/S1P pathway in immune cell trafficking and inflammatory events such as cytokine release, together with regulation of and its ability to be regulated by important intracellular signalling mediators such as AKT and ERK1/2, has led to the hypothesis that SPHK1/S1P could be an important mediator of tumour-monocyte interactions. Therefore I aimed to study the role of SPHK1/S1P in tumour-monocyte interactions, understand the molecular mechanisms by which tumour cells benefit from this interaction, and identify the soluble mediators involved.

4.2 The role of SPHK1 in PC-3 prostate cancer cells during co-culture with U937 cells

SPHK1 regulation was monitored in PC-3 cells following their co-culture with U937 cells (**Fig.4.1**). SPHK1 activity in cell lysates was assessed though its incubation with Sphingosine and radiolabeled ATP-P32. The formation of S1P with incorporated P32 was measured as an indication of SPHK1 activity.

SPHK1 activity was increased by 30% in PC-3 cells following one and three hours of co-culture with U937 cells when compared with PC-3 cells cultured alone (**Fig.4.1a**). A smaller non-significant increase in SPHK1 activity was observed following five and eight hours of co-culture, whilst 24 hours of co-culture led to a 70% increase in SPHK1 activity in PC-3 cells.

Alongside an increase in SPHK1 activity, SPHK1 transcript levels were also observed to be altered in PC-3 cells upon co-culture with U937 cells. As shown in figure **4.1b**, there were initial (one and three hour) and later (24 hour) increases in SPHK1 expression levels observed in PC-3 cells following their co-culture with U937 cells when compared with PC-3 cells cultured alone. After one and three hours of co-culture there were respective 1.8- and 2-fold increases observed in SPHK1 expression in PC-3 cells; this was followed by a reduction in expression in PC-3 cells after five and eight hours of co-culture in comparison with one and three hours (**Fig.4.1b**). In concomitance with SPHK1 activity, at 24 hours there was a 2-fold increase of SPHK1 mRNA in PC-3 cells.

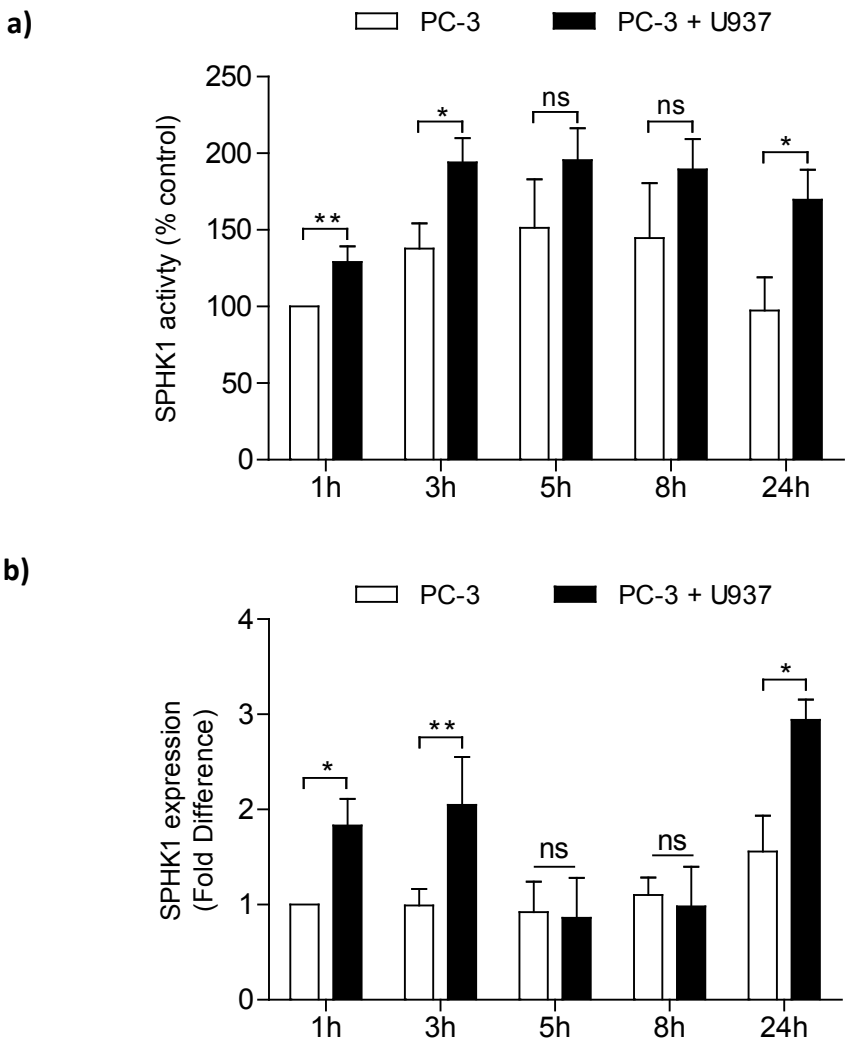


Figure 4.1: SPHK1 activity and expression levels in human PC-3 cells following their co-culture with U937 cells. PC-3 cells were seeded in the bottom chamber under serum-free media 24 hours before the addition of U937 cells for the indicated duration. **a)** SPHK1 activity and **b)** SPHK1 expression were determined in PC-3 cell lysates containing equal amounts of protein or mRNA using radiolabelling and qRT-PCR respectively. For qRT-PCR, SPHK1 mRNA expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. Data shown is the mean ± SEM of three independent experiments performed in duplicate. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$ for PC-3 cells vs. PC-3 + U937 cells.

To study the possible mechanisms of SPHK1 regulation in the co-culture system, I investigated the signal transduction pathways that have previously been shown to be involved in both the tumour microenvironment and in SPHK1 regulation. In this context, Akt is a well documented regulator of several pro-survival pathways involved in tumour development and inflammatory processes and importantly is regulated by SPHK1. To evaluate its levels in PC-3 cells during co-culture with U937 cells, a western blotting assay was performed at the indicated time points (**Fig.4.2a**).

My data show that upon one hour of co-culture a highly significant increase in Akt phosphorylation of approximately 3-fold was evident. Furthermore, following three hours of co-culture, U937 cells were still able to induce Akt phosphorylation in PC-3 cells when compared with PC-3 cells cultured alone. This increase is lower than that observed at one hour; however the 2-fold increase in Akt phosphorylation observed at three hours proved to be statistically significant (**Fig.4.2a-b**). In a later stage of co-culture (24 hours), Akt phosphorylation was not affected by the presence of U937 cells (**Fig.4.2a**)

To further investigate Akt regulation by SPHK1, siRNA was used to target SPHK1 at a transcriptional level in PC-3 cells. To assess the efficacy of the RNAi, qRT-PCR was performed using cDNA derived from mRNA extracted from PC-3 cells. SPHK1 expression was shown to be down-regulated by approximately 70%, independently of the presence of U937 cells (**Fig.4.2e**). The use of a non-targeting siRNA (siNT) showed a similar pattern of Akt phosphorylation as shown in figure **4.2a**, with an increase in Akt phosphorylation at one and three hours followed by no effect at 24 hours (**Fig.4.2c** and **d**). SPHK1 knockdown induced a significant down-regulation in Akt phosphorylation upon one, three and 24 hours of co-culture and abolished the effect of U937 cells (**Fig.4.2c**). Notably, SPHK1 knockdown didn't affect the total Akt levels, indicating a possible role for SPHK1 in Akt phosphorylation. Another known regulator of SPHK1 is ERK1/2; in this study I attempted to measure ERK1/2 phosphorylation levels in PC-3 cells during co-culture. However it was not possible to detect any level of ERK1/2 phosphorylation in the absence or presence of monocytes, which could be explained by the fact that PC-3 cells have almost undetectable levels of phosphorylated ERK1/2. It was therefore not possible to assess whether the increase in SPHK1 activity and expression was correlated with ERK1/2 phosphorylation.

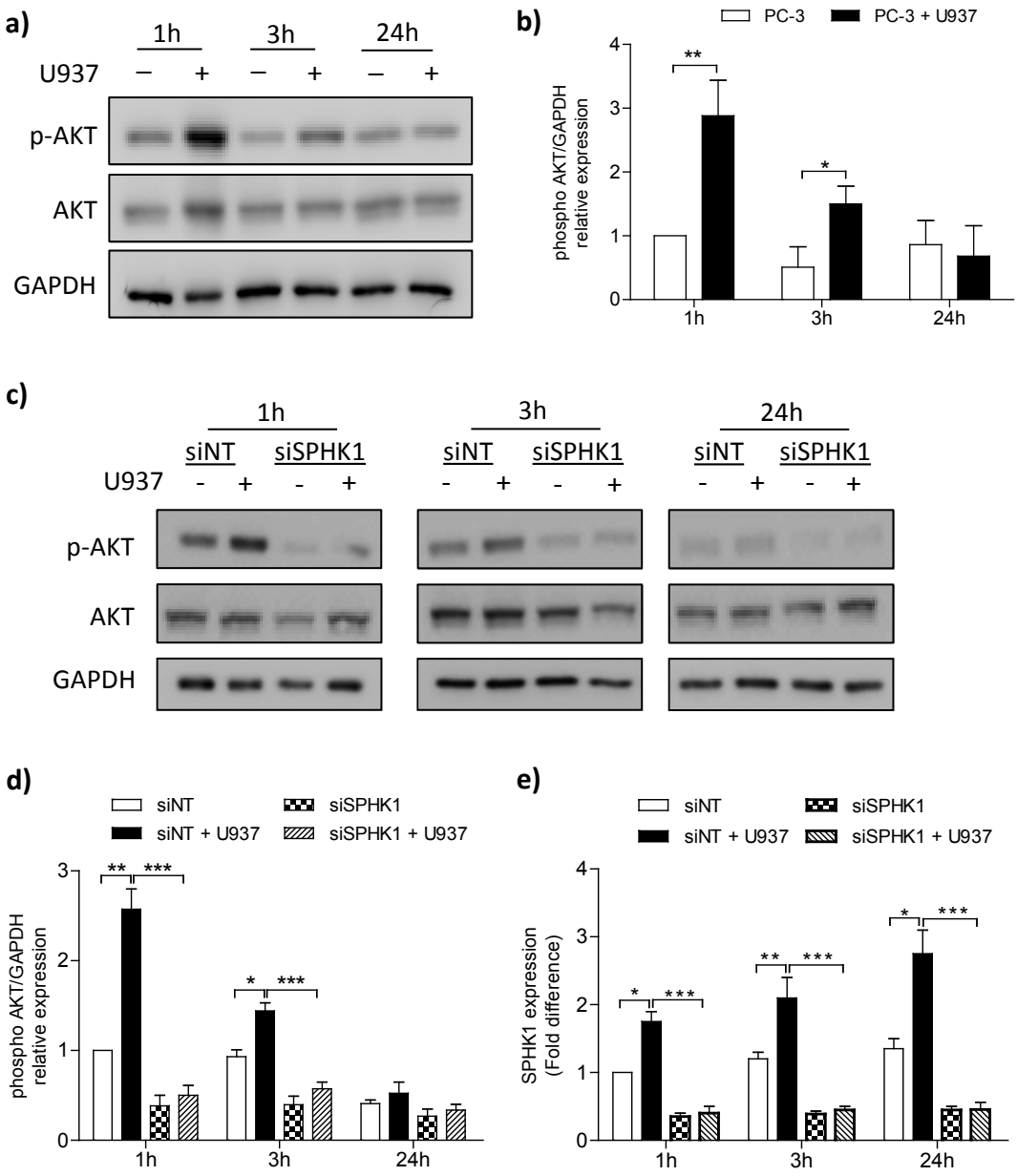


Figure 4.2: AKT regulation in PC-3 cells during co-culture with U937 cells. PC-3 cells were seeded in the bottom chamber under serum-free media 24 hours before the addition of U937 cells for the indicated duration. **a)** AKT phosphorylation and total levels were determined by western blotting **b)** quantified by Image J software and normalised to GAPDH. **c-d)** PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1) or non-targeting control siRNA (siNT) for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. AKT phosphorylation and total levels were determined as described above (**a-b**). **e)** SPHK1 expression in U937 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used for normalisation Data shown is representative of **a)**, **c)** three independent experiments and **b)**, **d-e)** the mean ± SEM of three independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$ for **b)** PC-3 vs. PC-3+U937 or **d-e)** siNT PC-3 vs. siNT PC-3 + U937 or siNT PC-3 + U937 vs. siSPHK1 + U937.

4.3 Impact of Akt and ERK1/2 regulation in PC-3 prostate cancer cells during co-culture with U937 cells

Following on from initial findings, the next step was to evaluate the role of Akt and ERK1/2 signalling pathways in SPHK1 regulation during co-culture. In a similar manner to that described for SPHK1, PC-3 cells were transfected with siRNAs targeting Akt or ERK1/2. As seen in figure **4.3a**, Akt knockdown led to an increase in SPHK1 expression after one hour of co-culture, this effect was further increased by the presence of U937 cells (**Fig.4.3ai**). For the remaining two time points the presence of U937 did not induce an increase in SPHK1 expression in Akt siRNA-treated PC-3 cells (**Fig.4.3aii** and **iii)**.

With regards to the effect on SPHK1 mRNA levels, ERK1/2 knockdown proved to have no effect on SPHK1 basal expression in PC-3 cells cultured alone for the indicated time points (**Fig.4.3ai-iii**). However, following just one hour of co-culture, in PC-3 cells where ERK1/2 mRNA was targeted, the previously observed U937-dependent increase in SPHK1 expression was abolished in these cells (**Fig.4.3ai)**. ERK1/2 knockdown was confirmed by total forms of the protein and not specifically for its phosphorylated variants, as ERK1/2 phosphorylation levels in PC-3 cells proved very difficult to determine (**Fig.4.3b**).

As for ERK1/2 and Akt cross-regulation, it was apparent that ERK1/2 knockdown reduced Akt phosphorylation in non U937-stimulated PC-3 cells at all time points (**Fig.4.3b**). Moreover, in PC-3 cells with ERK1/2 knockdown the presence of U937 cells led to an increase in Akt phosphorylation after one hour of co-culture; however this U937-induced effect on Akt phosphorylation was not observed after three and 24 hours of co-culture (**Fig.4.3b**). As for Akt siRNA-mediated PC-3 cells there was no effect in Akt phosphorylation levels as shown for one and three hours of co-culture. Nonetheless, Akt knockdown inhibits U937-induced increase in Akt phosphorylation in PC-3 cells, importantly this inhibitory effect was observed across all time points. At the 24 hour time point, i.e. 72 hours post-siRNA transfection, phosphorylated Akt basal levels were reduced when compared alongside PC-3 cells transfected with a non-targeting siRNA (**Fig.4.3b**).

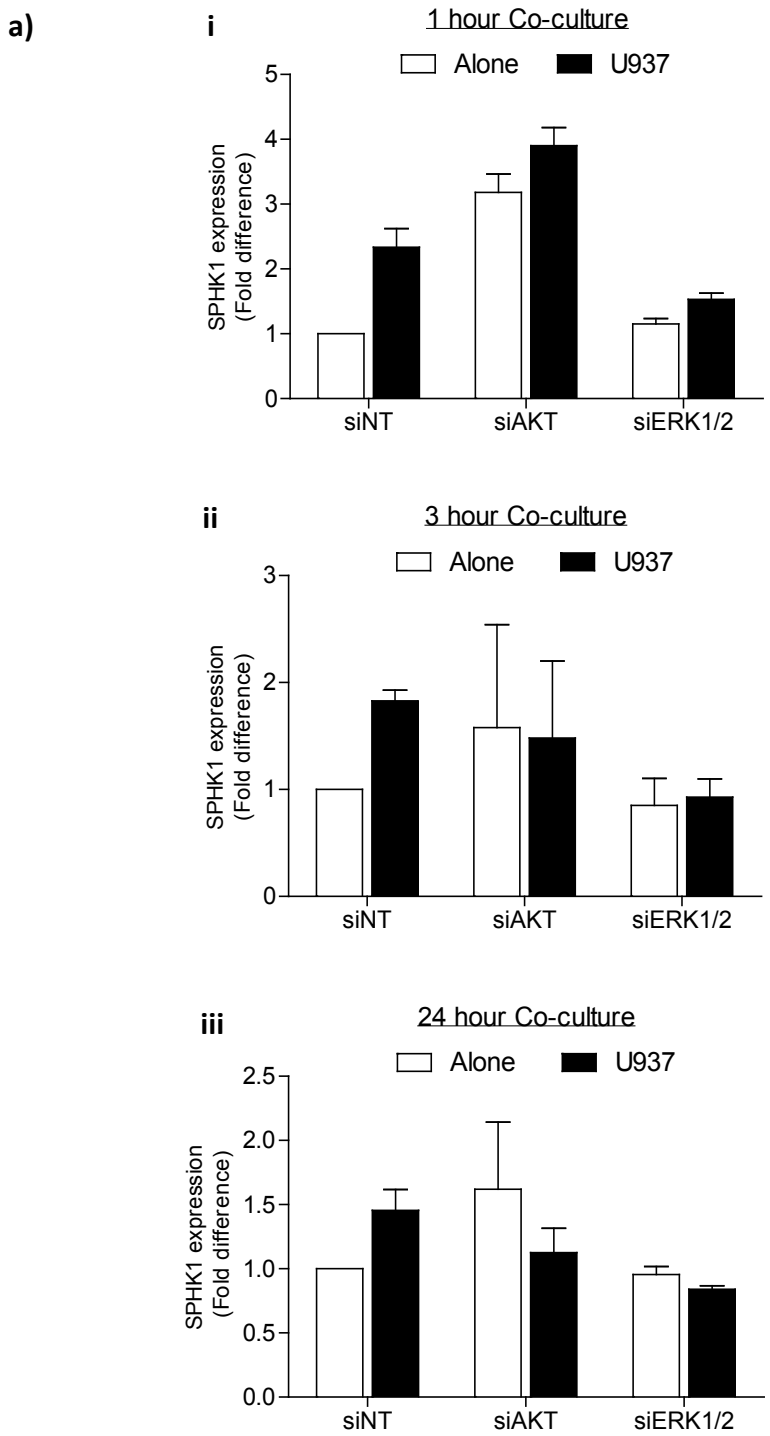


Figure 4.3a: Effect of AKT and ERK1/2 knockdown on SPHK1 expression in PC-3 cells during co-culture with U937 cells. PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1), AKT (siAKT), ERK1/2 (siERK1/2), or non-targeting control siRNA (siNT) for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. SPHK1 expression in U937 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. Data shown for is the mean ± SEM of two independent experiments.

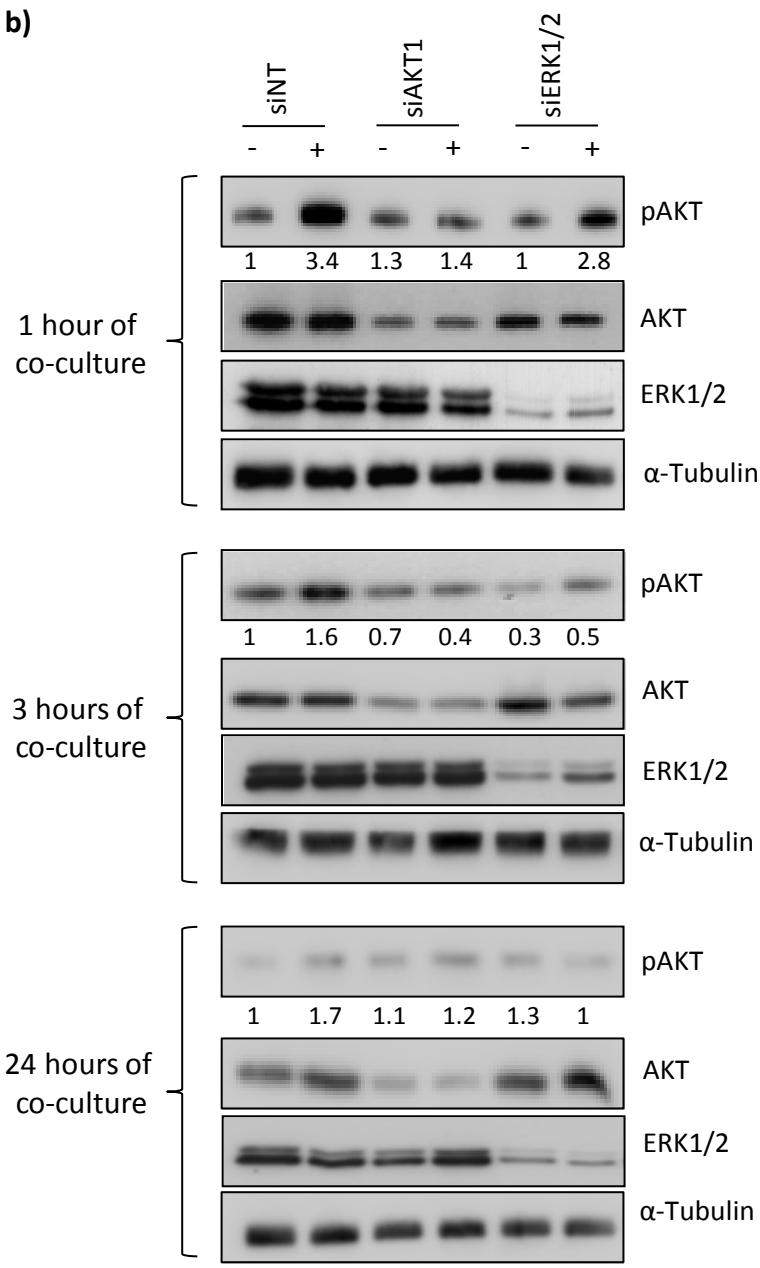


Figure 4.3b: Effect of AKT and ERK1/2 knockdown in PC-3 cells during co-culture with U937 cells. PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1), AKT (siAKT), ERK1/2 (siERK1/2) or non-targeting control siRNA (siNT) for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. AKT phosphorylation and total levels, and ERK1/2 total levels were determined by western blotting, quantified by Image J software and normalised to alpha-tubulin. Data shown is representative of one independent experiment.

4.4 Effect of THP-1 monocytic cell line on SPHK1 levels in PC-3 cells

The ability of THP-1 cells to confer a positive effect on PC-3 cell proliferation (chapter 3, **Fig.3.3b**), akin to that observed for U937 cells, prompted a preliminary investigation of THP-1 involvement in SPHK1 regulation in PC-3 cells. As seen in figure **4.4a**, after one hour of co-culture SPHK1 activity in PC-3 cells increased by approximately 40% upon THP-1 stimulation. This effect is transient as for the remaining studied timed points there was no THP-1 induction on SPHK1 activity in PC-3 cells (**Fig.4.4a**). As for SPHK1 mRNA expression levels, no effect was observed after only one hour of co-culture. However, for the remaining time points, THP-1 cells were able to induce an increase in SPHK1 expression, being more accentuated after 24 hours of co-culture (**Fig.4.4b**). In addition, it was important to understand if the increase in SPHK1 levels was accompanied by an alteration in Akt phosphorylation. Indeed, in PC-3 cells the presence of THP-1 led to an increase in Akt phosphorylation at all time points, this positive effect being more accentuated after three hours of co-culture with a 3.7-fold increase (**Fig.4.4c**).

4.5 Effect of U937 monocytic cell line on SPHK1 levels in MDA0-231 breast cancer cells

In order to evaluate the ability of U937 cells to regulate SPHK1 in another cancer cell line, MDA-231 cells were co-cultured with the monocytes. SPHK1 activation in MDA-231 was triggered by the presence of U937 cells upon three hours of co-culture, however this effect was transient as for the remaining six and 24 hour time points, the U937-induced increase in SPHK1 activity was not observed (**Fig.4.5a)**. SPHK1 expression was transiently elevated by the presence of U937 cells; this effect was only observed after six hours of co-culture (**Fig.4.5b**). In terms of the molecular mechanism underpinning this activation, ERK1/2 phosphorylation levels were measured across the same time points described previously. As seen in figure **4.5c**, the addition of U937 cells to MDA-231 led to a clear up-regulation of ERK1/2 phosphorylation at all studied time points. It is Important to note that the Akt phosphorylation levels were measured without success, as the basal levels and/or the monocyte-induced phosphorylation levels were too low to be detected by western blotting (data not shown).

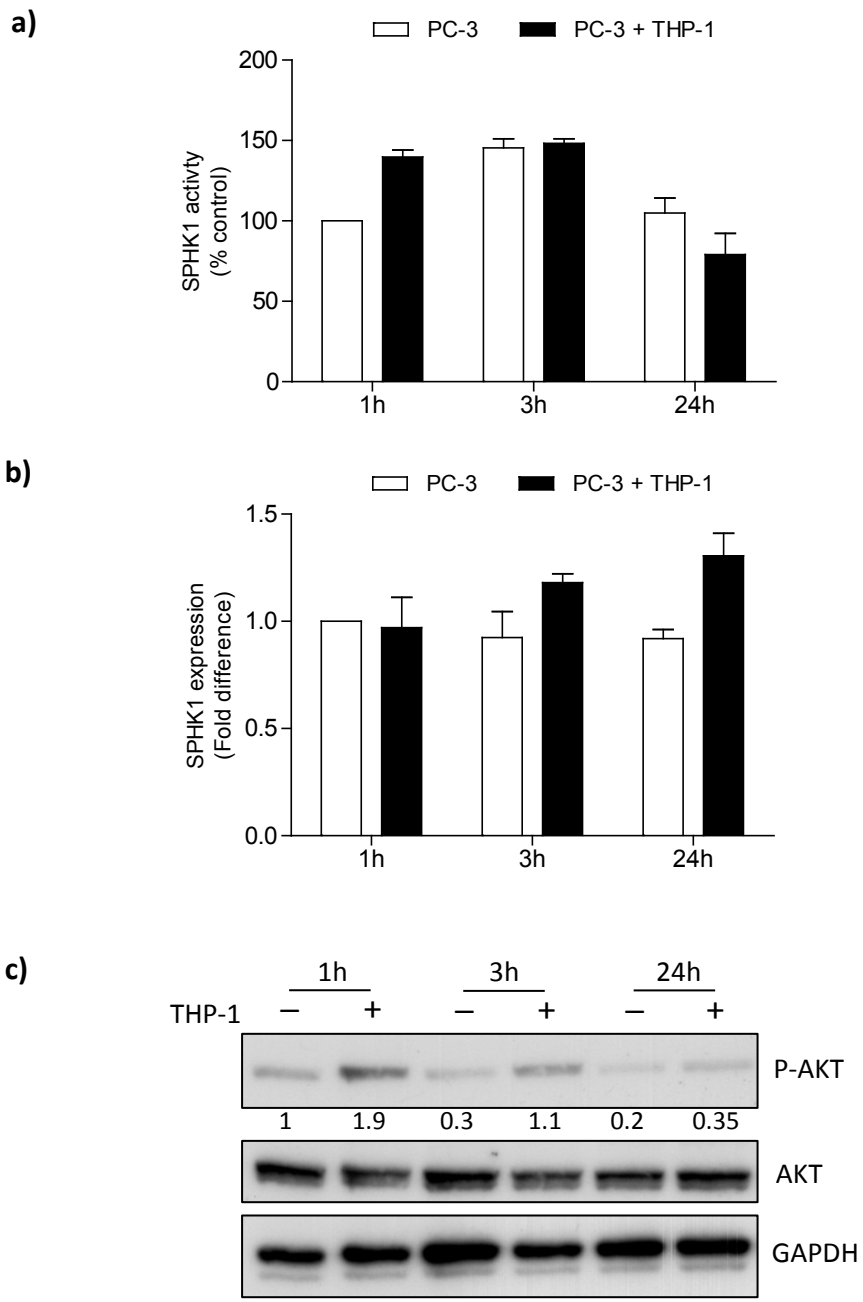


Figure 4.4: SPHK1 activity and expression levels in human PC-3 cells following their co-culture with THP-1 cells. PC-3 cells were seeded in the bottom chamber under serum-free media 24 hours before the addition of THP-1 cells to the upper chamber for the indicated duration. **a)** SPHK1 activity and **b)** SPHK1 expression was determined in PC-3 cell lysates containing equal amounts of protein or mRNA respectively using radiolabelling and qRT-PCR respectively. For qRT-PCR, SPHK1 mRNA expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. **c)** AKT phosphorylation and total levels were determined by western blotting, quantified by Image J software and normalised to GAPDH Data shown is **a-b)** mean ± SEM of two independent experiments and **c)** representative of one independent experiment.

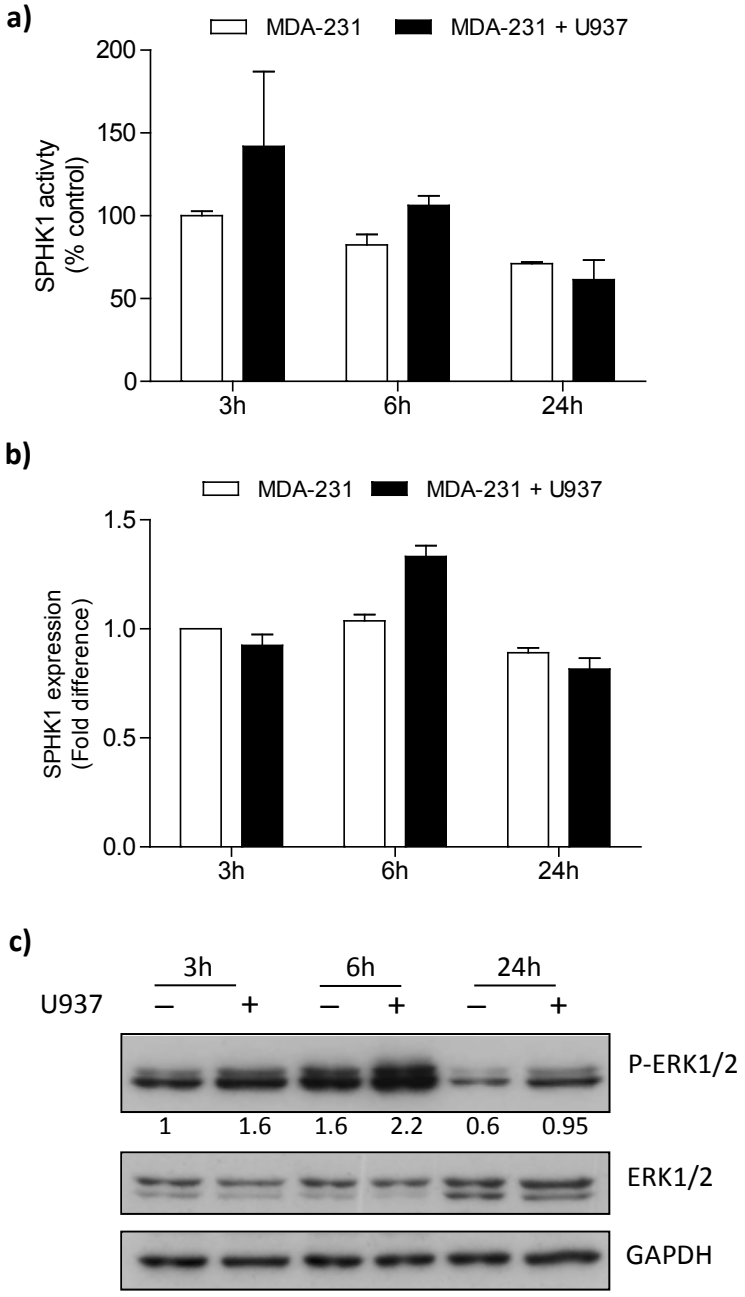


Figure 4.5: SPHK1 activity and expression levels in human MDA-231 cells following their co-culture with U937 cells. MDA-231 cells were seeded in the bottom chamber under serum-free media 24 hours before the addition of U937 cells to the upper chamber for the indicated duration. **a)** SPHK1 activity and **b)** expression were determined in MDA-231 cell lysates containing equal amounts of protein or mRNA respectively using radiolabelling and qRT-PCR respectively. For qRT-PCR, SPHK1 mRNA expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. **c)** ERK1/2 phosphorylation and total levels were determined by western blotting, quantified by Image J software and normalised to GAPDH. Data shown is **a-b)** mean ± SEM of two independent experiments and **c)** representative of one independent experiment.

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4.6 Signalling mediators involved in PC-3/U937 cell interactions during co-culture

The ability of U937 to regulate SPHK1 in PC-3 cells by increasing its activity and mRNA levels led to the hypothesis that S1P, produced by SPHK1 activation, could be acting in an autocrine manner. To investigate this, PC-3 cells were treated with S1P for several time points up to a maximum of 24 hours. S1P treatment provoked a significant increase in SPHK1 expression after one hour of treatment. At three hours the S1P-induced increase in SPHK1 expression was not significant, whilst at 24 hours there was no effect on mRNA levels when compared with untreated PC-3 cells (**Fig.4.6a**). Akt phosphorylation was also increased by S1P treatment; this effect was observed at three hours and more accentuated at 24 hours (**Fig.4.6b**) with a 1.5- and 2.5-fold increase respectively.

4.6.1 The role of MCP-1 in PC-3/U937 co-culture

The cytokine MCP-1 has been reported to be up-regulated in tumours [320, 402], is identified as a key chemo-attractant for monocytes and macrophages [259, 403] and can be regulated by SPHK1 [324]. To investigate whether MCP-1 could be involved in PC-3-monocyte interactions the conditioned media of PC-3 co-cultured with U937 cells was analysed by ELISA. MCP-1 secretion was increased by the presence of U937 cells after three hours of co-culture, with the highest levels observed after 24 hours (**Fig.4.7a**). Interestingly, the siRNA-mediated inhibition of SPHK1 expression in PC-3 cells led to a significant decrease in MCP-1 secretion at three and 24 hours with a 3- and 2-fold drop respectively. Basal levels of MCP-1 in the media of PC-3 cells cultured alone have shown no difference upon SPHK1 inhibition, suggesting SPHK1 independence (**Fig.4.7b**). In this context, I wanted to ascertain whether MCP-1 secreted levels were accompanied by an increase in MCP-1 at the transcriptional level. It was of note that MCP-1 mRNA levels were increased at three hours and more significantly at 24 hours upon PC-3 co-culture with U937 cells (**Fig.4.7c**). Furthermore, MCP-1 basal expression was not affected by SPHK1 knockdown; however an effect was observed in PC-3 cells that were co-cultured with U937 cells. By comparing PC-3 transfected with non-targeting or SPHK1-targeting siRNA in the presence of U937 cells, it is clear that MCP-1 expression is reduced in the siSPHK1 PC-3 cells, with the result being more significant following three hours of co-culture (**Fig.4.7d**).

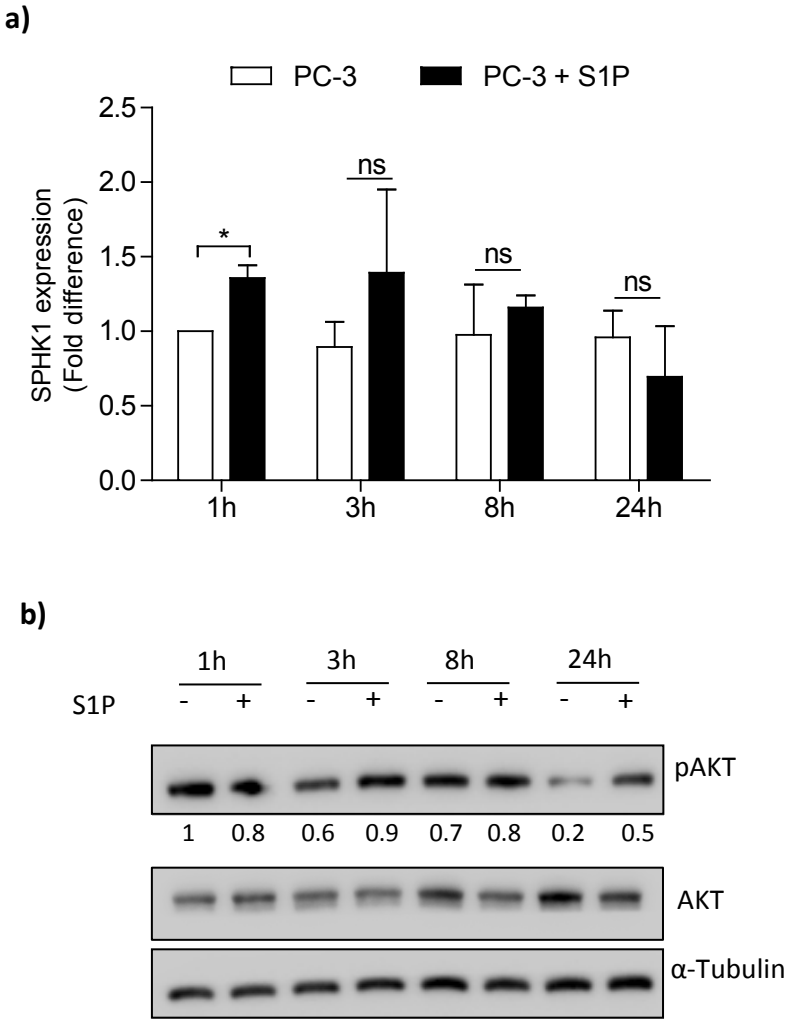


Figure 4.6: Effect of S1P in PC-3 cells signalling pathways and cytokine production. a) PC-3 cells were seeded in a 6 well plate for 24 hours before the addition of S1P at a final concentration of 1μM. **a)** SPHK1 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. **b)** AKT phosphorylation and total levels were determined by western blotting, quantified by Image J software and normalised to alpha tubulin. Data shown **a) i**s the mean ± SEM of three independent experiments and **b)** representative of two independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point, **a**) ns $p>0.05$, *$p<0.05$ for PC-3 vs. PC-3+S1P.

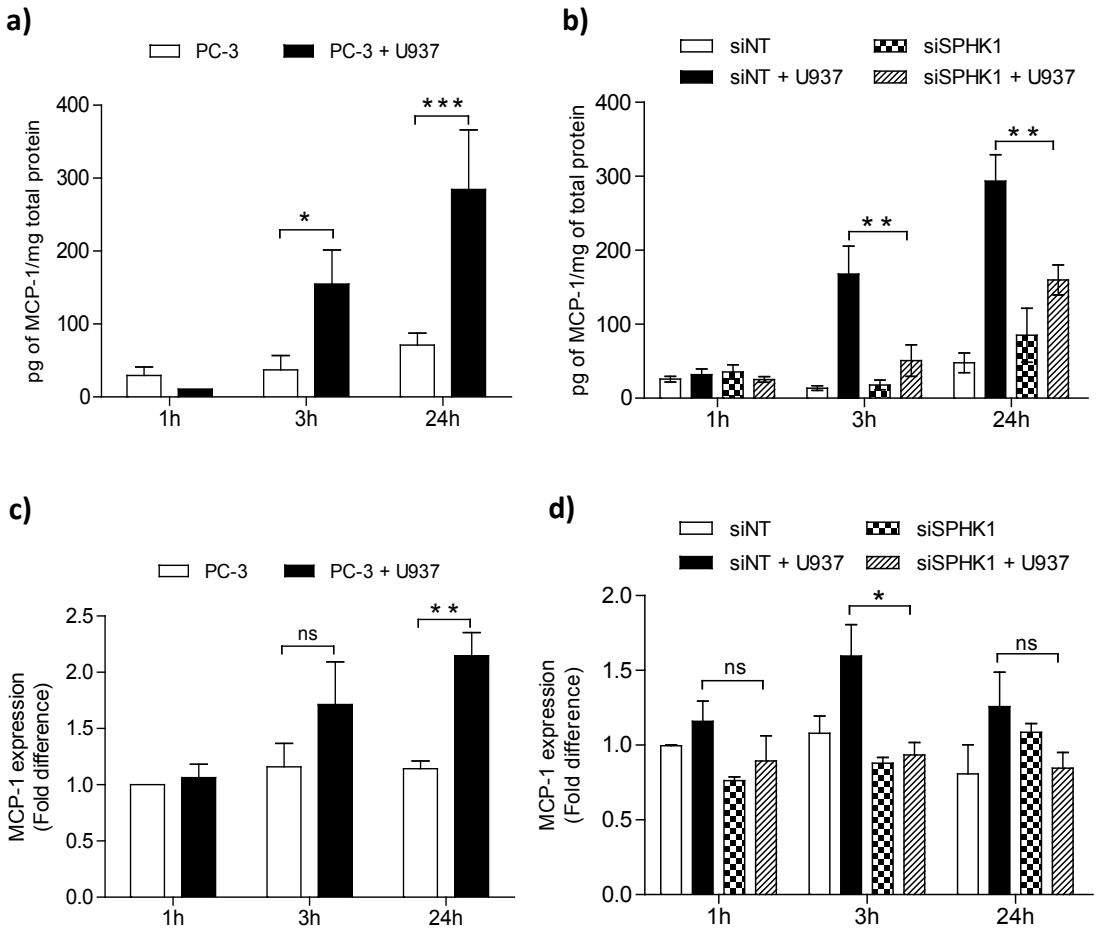


Figure 4.7: MCP-1 regulation in PC-3 cells during co-culture. a,c) PC-3 cells were seeded in the bottom chamber under serum-free media 24 hours before the addition of U937 cells for the indicated duration. **b,d)** PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1), or non-targeting control siRNA (siNT) for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. **a-b)** MCP-1 concentration in the media of the lower compartment where PC-3 cells were present was determined by ELISA. Media was incubated with MCP-1 specific antibodies and a standard curve was used to quantify the relative amount of MCP-1 in each sample. Protein concentration of each cell lysate was used for normalisation. **c-d)** MCP-1 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. Data shown is mean ± SEM of three independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point, ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$ for **a,c)** PC-3 vs. PC-3+U937 or **b,d)** siNT+U937 vs. siSPHK1+U937.

As a consequence of my initial findings, it was important to clarify the role, if any, of SPHK1 in MCP-1 regulation. For this purpose, I generated a stable PC-3 cell line that constitutively over-expresses human SPHK1. Using qRT-PCR, an 8-fold increase in MCP-1 mRNA levels was observed in PC-3 cells over-expressing SPHK1 (PC-3/OFP-SPHK1) in comparison with PC-3 cells transfected with an empty vector (**Fig.4.8ai**). In concomitance, secreted MCP-1 levels in the supernatant were also increased by approximately 4-fold in PC-3/OPF-SPHK1 cells (**Fig.4.8aii**).

To assess the impact of SPHK1 over-expression on MCP-1 during co-culture, PC-3 cells over-expressing SPHK1 were co-cultured with U937 cells. ELISA of the supernatant showed that MCP-1 secretion was significantly up-regulated in the presence of U937 cells at one, three and 24 hours (**Fig.4.8aiii**). Surprisingly, there was no increase in MCP-1 mRNA levels upon co-culture with U937 cells at any of the studied time points (**Fig.4.8aiv**) suggesting that the increase in MCP-1 is mainly due to its secretion from the monocytes (revised on **chapter 5**). SPHK1 expression was also measured in this co-culture and surprisingly no increase was observed when U937 cells were present (**Fig.4.8av**). Interestingly, the increase in MCP-1 secretion was partially reduced by the use of siRNA targeting SPHK1 in these stable PC-3 cells (**Fig.4.8b**).

To elucidate whether MCP-1 has a role in SPHK1 regulation and Akt phosphorylation, human recombinant MCP-1 was added to PC-3 cells for the indicated time points. My results showed that MCP-1 addition had no effect on SPHK1 mRNA levels (F**ig.4.9a**) or Akt phosphorylation (**Fig.4.9b**) at any of the indicated time points.

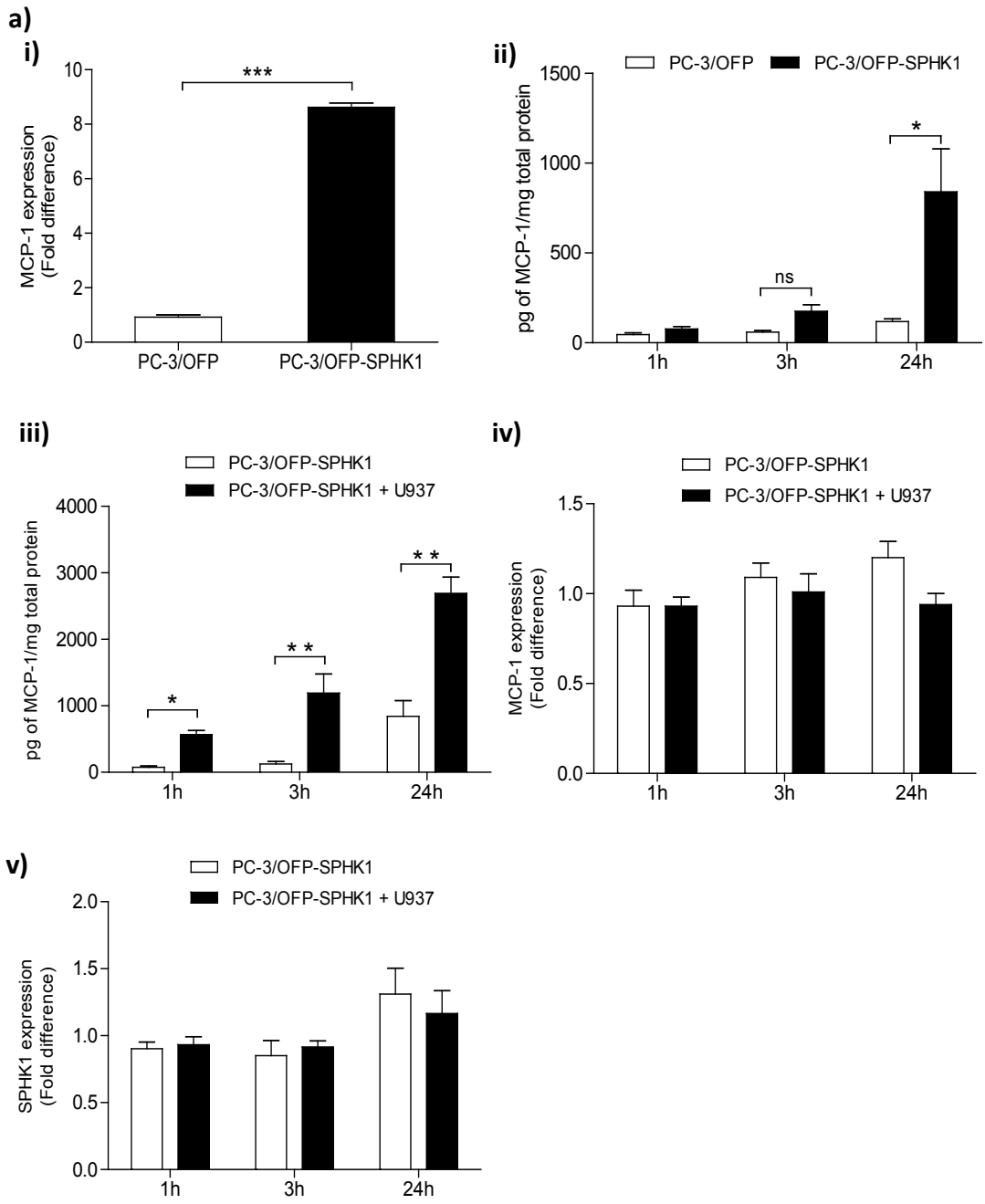


Figure 4.8: MCP-1 regulation and secretion in PC-3 cells over-expressing SPHK1. a) PC-3 cells over-expressing SPHK1 (PC-3/OFP-SPHK1) or empty phmKO2 vector (PC-3/OFP) were cultured for 24 hours. **i)** MCP-1 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. **ii)** MCP-1 concentration in the media was determined by ELISA. Media was incubated with MCP-1 specific antibodies and a standard curve was used to quantify the relative amount of MCP-1 in each sample. Protein concentration of each cell lysate was used for normalisation. **iii-v**) PC-3/OFP and PC-3/OFP-SPHK1 cells were seeded in the bottom chamber under serum-free media 24 hours before the addition of U937 cells for the indicated duration. **c)** MCP-1 concentration in the media was determined by ELISA as described in **ii)**, **iv)** MCP-1 and **v)** SPHK1 expression was determined by qRT-PCR as described in **i)**. Data shown is mean ± SEM of three independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for **i-ii)** PC-3/OFP vs. PC-3/OFP-SPHK1 or **c)** PC-3/OPF-SPHK1 vs. PC-3/OFP-SPHK1 + U937 cells.

b)

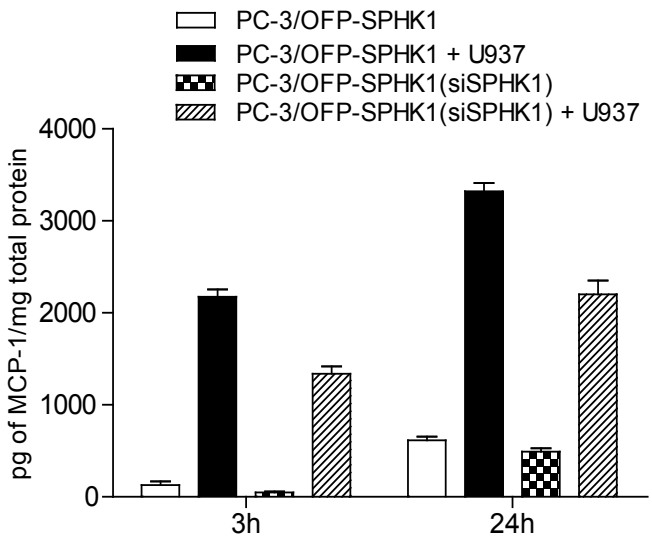


Figure 4.8: MCP-1 secretion in PC-3 cells over-expressing SPHK1. PC-3 cells over-expressing SPHK1 (PC-3/OFP-SPHK1) or empty phmKO2 vector (PC-3/OFP) were transfected with siRNA targeting SPHK1 (siSPHK1), or non-targeting control siRNA (siNT) for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. MCP-1 concentration in the media was determined by ELISA. Media was incubated with MCP-1 specific antibodies and a standard curve was used to quantify the relative amount of MCP-1 in each sample. Protein concentration of each cell lysate was used for normalisation. Data shown is representative of one independent experiment performed in duplicate.

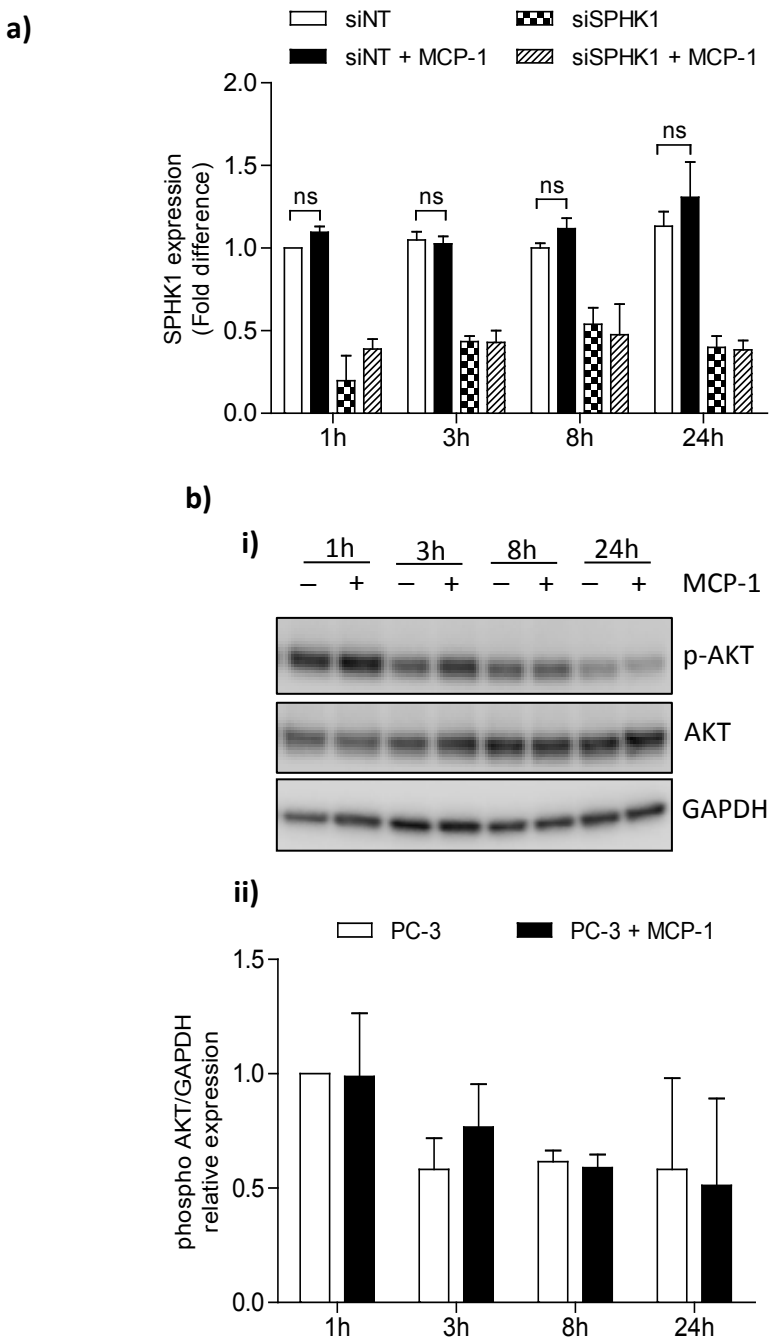


Figure 4.9: Effect of human recombinant MCP-1 on SPHK1 and AKT activation. a) PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1) or non-targeting control siRNA (siNT) 48 hours before the addition of human recombinant MCP-1 (rhMCP-1) at a final concentration of 100ng/µl. **a)** SPHK1 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. **b)** PC-3 cells were seeded in a 6-well plate for 24 hours before the addition of MCP-1 as described above **a)**, **i)** AKT phosphorylation and total levels were determined by western blotting, **ii)** quantified by Image J software and normalised to GAPDH. Data shown is mean ± SEM of **a)** three independent experiments and **b)** two independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point. **a**) ns $p>0.05$ for siNT vs. siNT+MCP-1

4.6.2 The role of IL-6 in PC-3/U937 co-culture

IL-6 has been shown to modulate prostate cancer survival and proliferation [404] and is also under the regulatory control of SPHK1 [270, 294]. Consequently, I decided to explore any possible involvement of IL-6 in either SPHK1 up-regulation or Akt phosphorylation. As previously described for MCP-1, an ELISA was performed on PC-3 cell supernatant as a first step, in order to determine whether IL-6 secretion was affected by the presence of U937 cells. Figure **4.10a** indicates that the presence of U937 cells does not lead to a significant increase in IL-6 secretion during co-culture with PC-3 cells. A similar effect was observed at the transcriptional level in PC-3 cells, where IL-6 expression did not change with the presence of U937 cells. Also, SPHK1 down-regulation by siRNA showed no effect on IL-6 expression (**Fig.4.10b**).

Next I wished to determine whether SPHK1 could directly regulate IL-6. For this purpose a quantitative RT-PCR was performed on cDNA derived from cell lysates of PC-3 over-expressing SPHK1. The analysis showed an increase in IL-6 transcripts of approximately 2.5-fold in comparison with PC-3 containing an empty vector (**Fig.4.11a**). In accordance with these findings, IL-6 levels in the supernatant were also increased when SPHK1 was over-expressed, with a more accentuated effect observed after 24 hours of culture (**Fig.4.11b**). Furthermore, PC-3/OFP-SPHK1 cells were co-cultured with U937 cells, in order to assess any effect on secreted IL-6 protein levels and mRNA expression in PC-3 cells upon the presence of U937. As anticipated, with the presence of U937 cells increased levels of IL-6 were detected in the supernatant of PC-3. Interestingly, this increase was observed at a very early stage of co-culture (one hour) and increased to its highest level at 24 hours (**Fig.4.11c**). Surprisingly this increase in secretion was not accompanied by an increase in IL-6 mRNA expression (**Fig.4.11d**) suggesting once more that IL-6 increase is mainly due to presence of the monocytes (revised on **Chapter 5**).

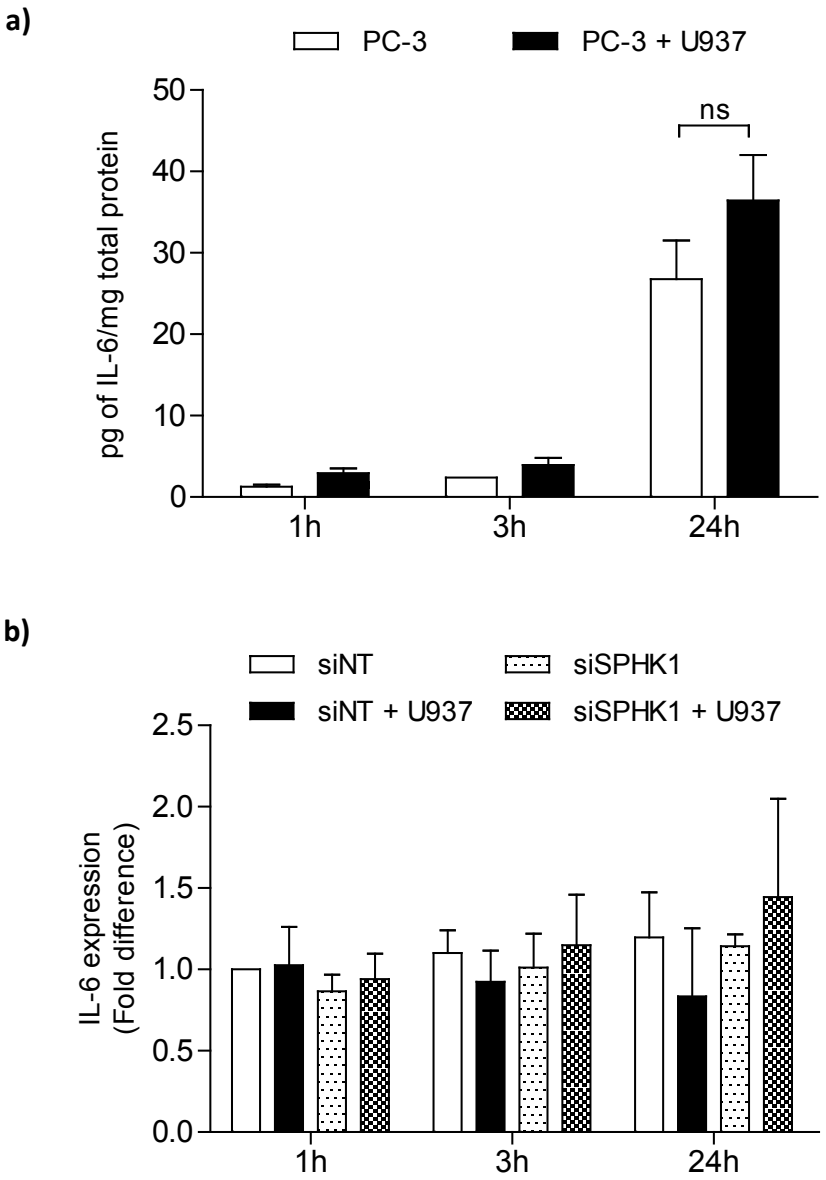


Figure 4.10: IL-6 regulation and secretion in PC-3 cells over-expressing SPHK1. a) PC-3 cells were seeded in the bottom chamber under serum-free media 24 hours before the addition of U937 cells for the indicated duration. IL-6 concentration in the media was determined by ELISA. Media was incubated with IL-6 specific antibodies and a standard curve was used to quantify the relative amount of IL-6 in each sample. Protein concentration of each cell lysate was used for normalisation. **b)** PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1) or non-targeting control siRNA (siNT) for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. IL-6 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. Data shown is mean ± SEM of **a)** three independent experiments and **b)** two independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point. **a**) ns $p>0.05$ for siNT vs. siNT+U937**.**

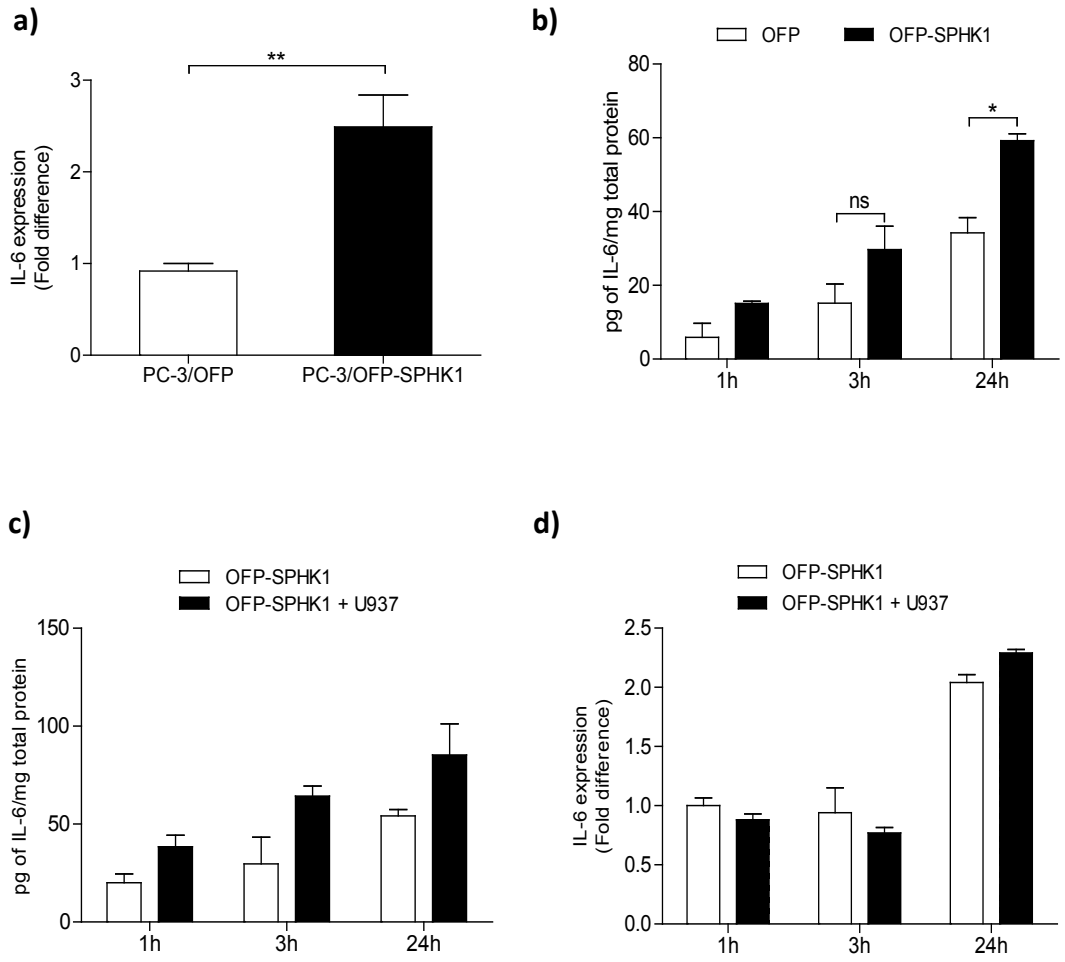


Figure 4.11: IL-6 regulation and secretion in PC-3 cells over-expressing SPHK1. PC-3 cells over-expressing SPHK1 (PC-3/OFP-SPHK1) or empty phmKO2 vector (PC-3/OFP) were cultured for 24 hours. **a)** IL-6 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise **b)** IL-6 concentration in the media was determined by ELISA. Media was incubated with IL-6 specific antibodies and a standard curve was used to quantify the relative amount of IL-6 in each sample. Protein concentration of each cell lysate was used for normalisation. **c-d**) PC-3/OFP and PC-3/OFP-SPHK1 cells were seeded in the bottom chamber under serum-free media 24 hours before the addition of U937 cells for the indicated duration. **c)** IL-6 concentration in the media was determined by ELISA as described in **b)**, **d)** IL-6 expression was determined by qRT-PCR as described in **a)**. Data shown is mean ± SEM of **a-b)** three and **c-d)** two independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$, **$p<0.01$, for **a-b)** PC-3/OFP vs. PC-3/OFP-SPHK1 or **c)** PC-3/OPF-SPHK1 vs. PC-3/OFP-SPHK1 + U937 cells.

It was also important to understand whether IL-6 could affect SPHK1 and/or Akt regulation. For this purpose human recombinant IL-6 was added to the PC-3 cells at a final concentration of 100ng/ml, arrived at by preliminary titration to determine an effective working concentration (data not shown). The addition of IL-6 to PC-3 cells led to a significant early increase in SPHK1 mRNA at three hours, albeit by only 20% (**Fig4.12a).** As for the remaining time points, IL-6 did not induce SPHK1 expression. The early effect on SPHK1 mRNA levels following the addition of IL-6 was further explored by investigating whether IL-6 showed any effect on SPHK1 activation at the protein level. Using a radiolabeling assay, SPHK1 activity was measured in PC-3 cells following the addition of recombinant IL-6. The cytokine rapidly induced SPHK1 activation; 15 minutes after treatment SPHK1 activity increased by approximately 50%. However it appeared to be a transient process as the positive effect was lost for the remaining time points (**Fig.4.12b**). Furthermore, IL-6 treatment did not induce Akt phosphorylation in PC-3 cells at any studied time point (**Fig.4.12c**).

As described previously in chapter **3**, section **3.7**, PC-3 xenografts showed a decrease in SPHK1 expression after SKi-II treatment and this was observed in both human and host mouse cells. To further extend this study and to understand if in an *in vivo* setting MCP-1 and IL-6 expression in PC-3 cells were affected by SPHK1 modulation, an analysis of mRNA expression levels of these two molecules in the same xenografts was performed using human specific primers. With regards to MCP-1, a significant decrease in mRNA expression was observed in mice treated with SKi-II upon comparison with respective controls (**Fig.4.13a**). No effect was apparent on IL-6 expression following treatment with SKi-II (**Fig.4.13b**).

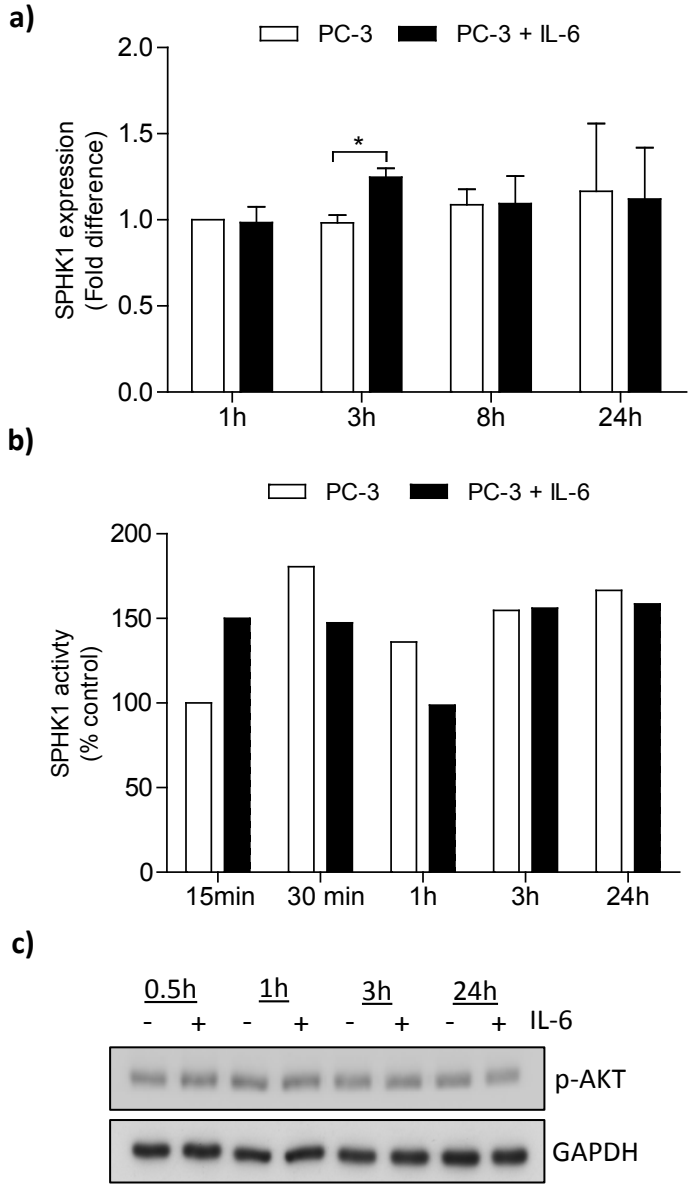


Figure 4.12: Effect of human recombinant IL-6 on SPHK1 and AKT in PC-3 cells. PC-3 cells were seeded in a 6-well plate under serum-free media 24 hours before the addition of IL-6 at a final concentration of 100ng/µl for the indicated duration. **a)** SPHK1 expression and **b)** SPHK1 activity were determined in PC-3 cell lysates containing equal amounts of mRNA or protein using qRT-PCR and radiolabelling respectively. For qRT-PCR, SPHK1 mRNA expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. **c)** AKT phosphorylation and total levels were determined by western blotting. Data shown is mean ± SEM of **a)** three **b)** one and **c)** two independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point, **a**) ns $p>0.05$, *$p<0.05$ for PC-3 vs. PC-3+IL-6.

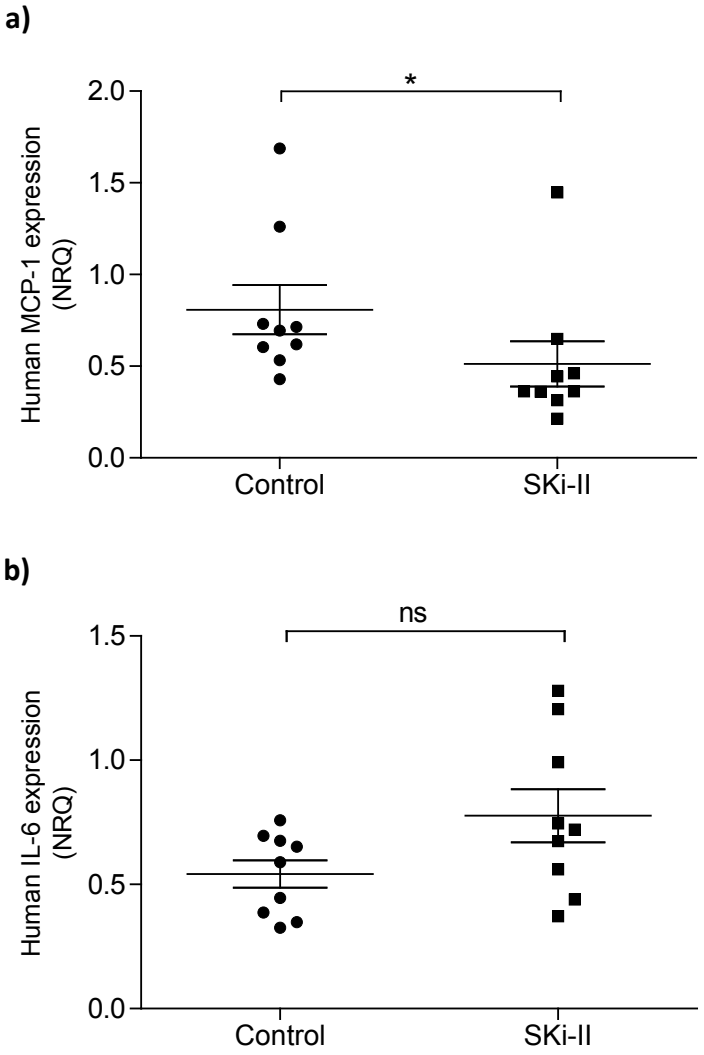


Figure 4.13: Effect of SKi-II on MCP-1 and IL-6 expression in PC-3 xenograft model. $1x10^6$ PC-3 cells tagged with GFP were subcutaneously injected into nude mice, and left to grow for ten days before the addition of SKi-II (50mg/ml), every three days for a total of four treatments (chapter 3, fig. 3.7). DMSO was used as a control. Tumours were collected and volumes were measured using calipers. **a)** MCP-1 expression and **b)** IL-6 expression were determined by qRT-PCR of RNA extracted from each tumour sample, and expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. Data shown is the normalised relative quantification (NRQ) of each sample. Student's t-test was used to determine statistical significance for SKi-II treated vs. control xenografts; ns $p>0.05$, *$p<0.05$.

As both MCP-1 and IL-6 levels in the media were up-regulated during the co-culture of PC-3 and U937 cells, it was of interest to evaluate their ability to regulate one another. As seen in figure **4.14a**, the addition of rhMCP-1 to PC-3 led to an approximate 1.5- and 1.8-fold increase in IL-6 mRNA expression levels after three and eight hours respectively (**Fig.4.14a**). This effect is not mediated by SPHK1 as siRNA-mediated knockdown did not affect the MCP-1-induced increase in IL-6mRNA, at any of the studied time points (**Fig.4.14a**). The addition of rhIL-6 to PC-3 proved to have a positive impact on MCP-1 expression, as characterised by an approximate 1.8-fold increase after three hours of treatment (**Fig.4.14b**). This effect was deemed transient as after eight and 24 hours there was no significant increase in IL-6 mRNA. As previously observed in SPHK1 siRNA transfected PC-3 cells, the IL-6-induced effect on MCP-1 expression was not mediated by SPHK1 as no changes were observed in MCP-1 expression after SPHK1 knockdown (**Fig.4.14b**).

Initial results indicated that S1P is involved in SPHK1 regulation (**Fig.4.6a**), it was therefore important to evaluate its effect on MCP-1 and IL-6 expression. For this purpose PC-3 cells were treated with S1P for different time points up to 24 hours. MCP-1 expression was increased by approximately 2-fold in S1P-treated PC-3 cells after one hour, whereas at three and 24 hours the increase observed was not significant (**Fig.4.15a**). In a similar manner to that observed for MCP-1 expression, S1P treatment of PC-3 cells led to an increase in IL-6 expression after one and three hours. Indeed, there was an approximate 4-fold increase after one hour whilst at three hours the S1P-induced effect on IL-6 expression resulted in an approximate 2-fold increase (**Fig.4.15b**).

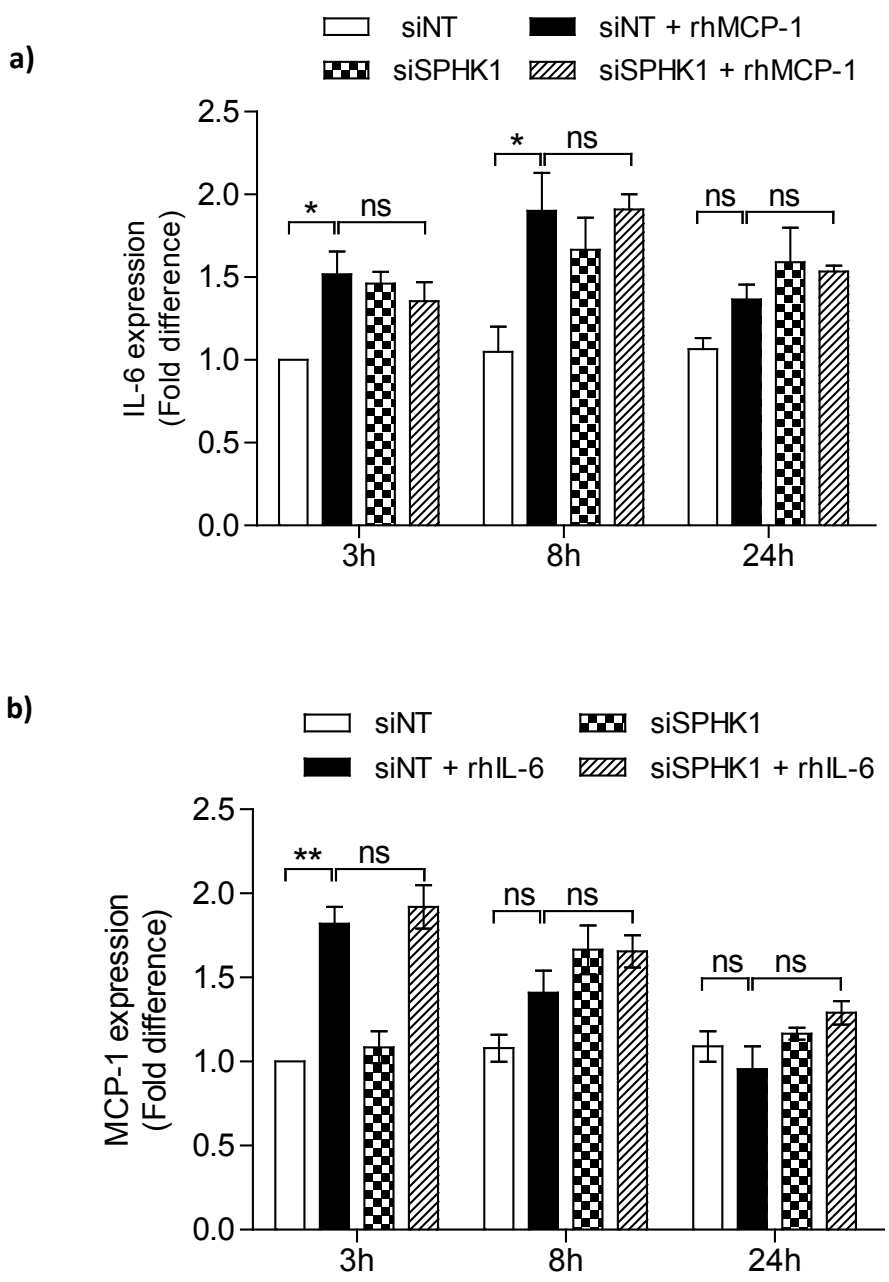


Figure 4.14: Effect of SPHK1 in the IL-6/MCP-1 axis in PC-3 cells. PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1) or non-targeting control siRNA (siNT) for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. **a)** MCP-1 (rhMCP-1) or **b)** IL-6 were then added at final concentration of 100ng/µl for the indicated times. **a)** IL-6 expression and **b)** MCP-1 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. Data shown is mean ± SEM of three independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point, ns $p>0.05$, *$p<0.05$, **$p<0.01$ for **a**) siNT vs. siNT+MCP-1. siNT+MCP-1 vs. siSPHK1 +MCP-1 or **b)** siNT vs. siNT+IL-6 or siNT+IL-6 vs. siSPHK1 +IL-6.

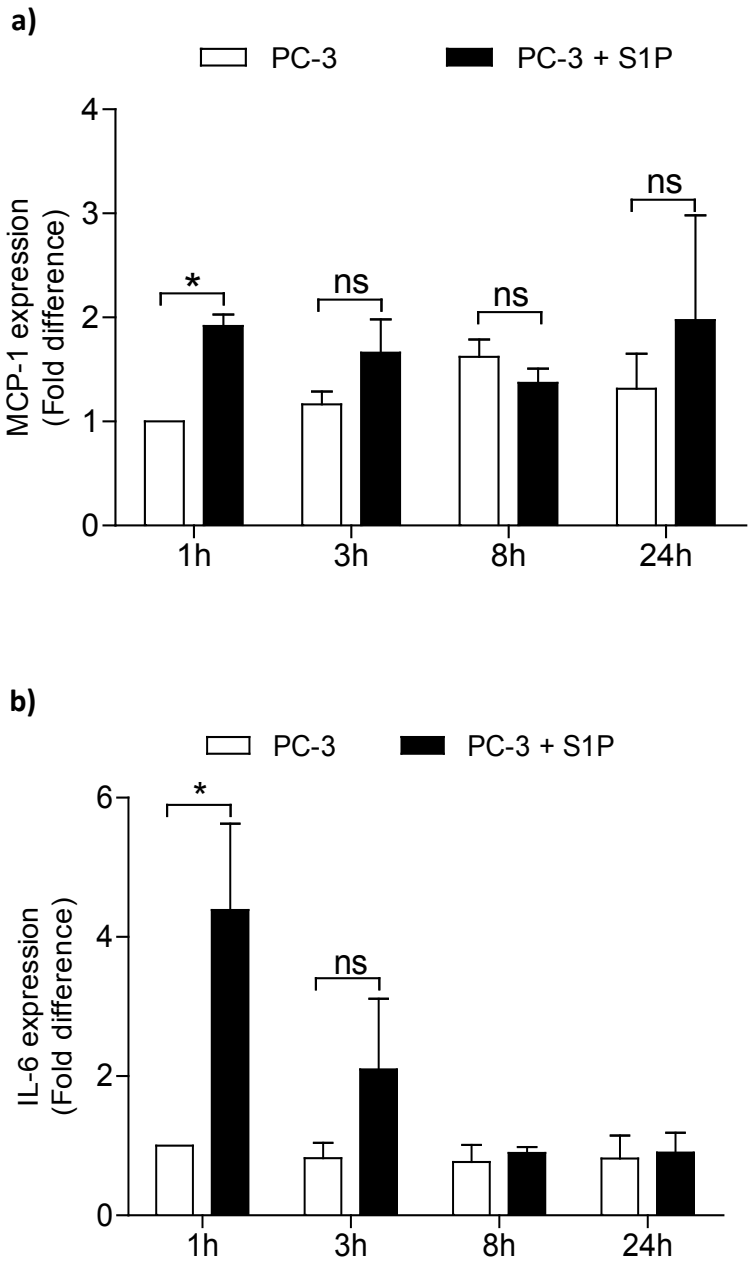


Figure 4.15: Effect of S1P on MCP-1 and IL-6 expression in PC-3 cells. PC-3 cells were seeded in a 6-well plate under serum-free media 24 hours before the addition of S1P at a final concentration of 1μM for the indicated duration. **a)** MCP-1 expression and **b)** IL-6 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used for normalisation. Data shown is mean ± SEM of three independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point, ns $p>0.05$, *$p<0.05$ for PC-3 vs. PC-3 + S1P.

According to my results it is clear that IL-6 and MCP-1 are involved in PC-3–U937 cell interactions, also it appears that their regulation is affected by SPHK1 levels, however my data indicates that other factors may be involved in SPHK1 and Akt phosphorylation. Therefore, to study a broader range of signalling molecules that 1) could be involved in PC-3-U937 interactions; or 2) are under SPHK1, and ERK1/2 regulation and therefore affecting the monocytic response, a proteome profiler assay was performed using media from PC-3 cells transfected with a non-targeting siRNA (siNT). SPHK1 down-regulation was validated by qRT-PCR and a 70% decrease in SPHK1 mRNA levels was observed when compared with PC-3 cells transfected with a non-targeting control siRNA (**Fig.4.16ai**). For ERK1/2 a clear decrease in ERK1/2 protein levels upon knockdown was observed by western blotting (**Fig.4.16aii**). The supernatant from each condition was then individually incubated with a pre-probed membrane containing 36 different targets for soluble molecules such as cytokines, chemokines and growth factors. Each membrane contains three reference spots (**Fig.4.16aiii**) in order to normalise each spot intensity to its own membrane. As the reference spot only indicates Streptavidin-HRP intensity for each membrane a further normalisation was made with the total protein levels of each transfected PC-3 cells. Therefore the quantification showed in figure **4.16aiv** is corrected for the variations in cell number caused by the effects of the different siRNA on PC-3 cells. The assay indicated that growth regulatory hormone alpha (GROα) and granulocyte macrophage colony-stimulating factor (GM-CSF) secretion were affected by SPHK1, as its knockdown decreased secretion of both of these molecules (**Fig.4.16iii** and **iv**). As this assay was only performed once, further analysis is necessary to validate these results and confirm their role in tumour-monocyte interactions. Other target molecules were detected in this assay; however their secretion patterns were not affected by any of the siRNAs, indicating that they were not regulated by SPHK1 or ERK1 (**Fig.4.17ai-ii**) Nonetheless, their possible roles in myeloid and tumour cell regulation should not be disregarded.

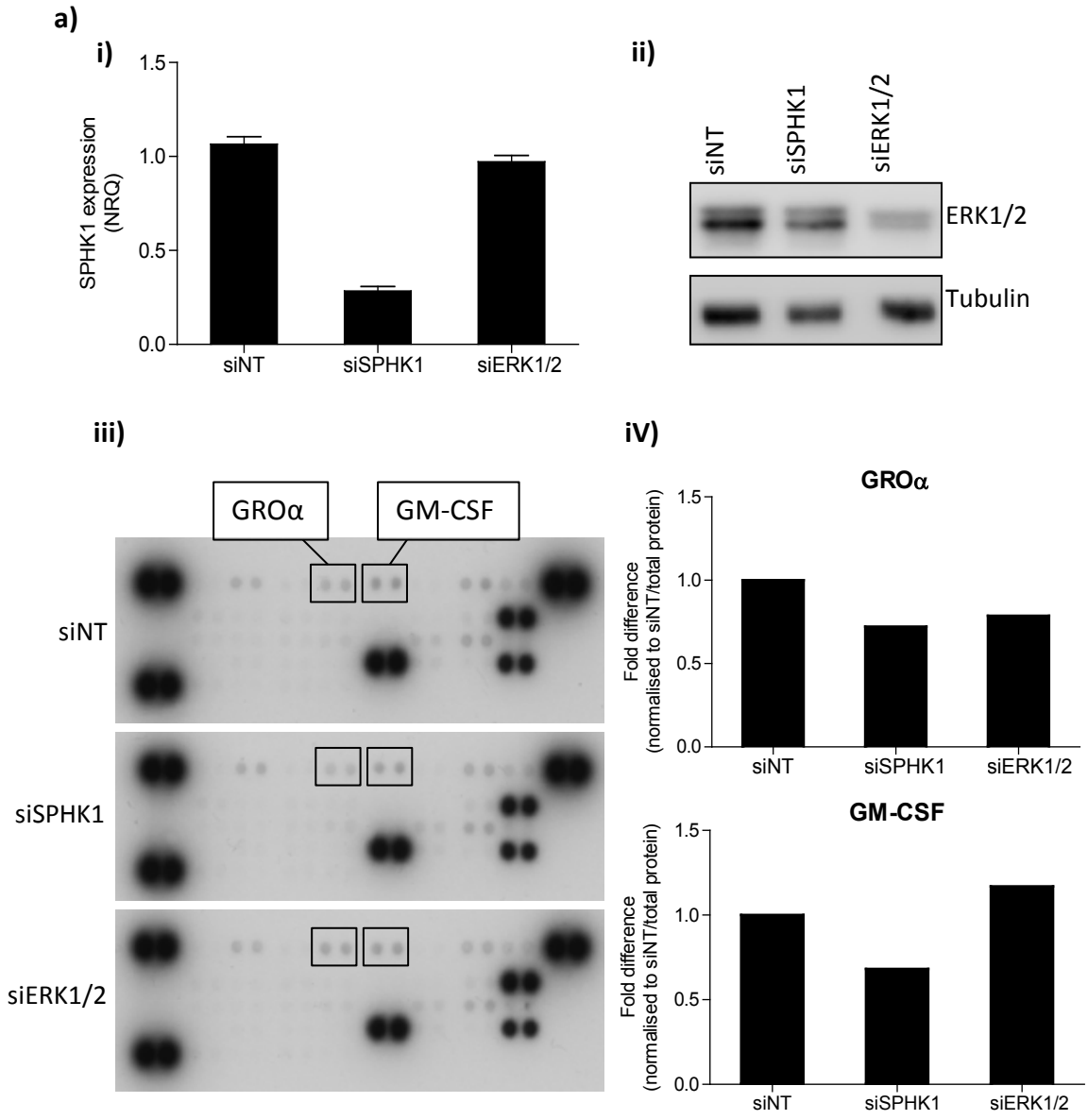
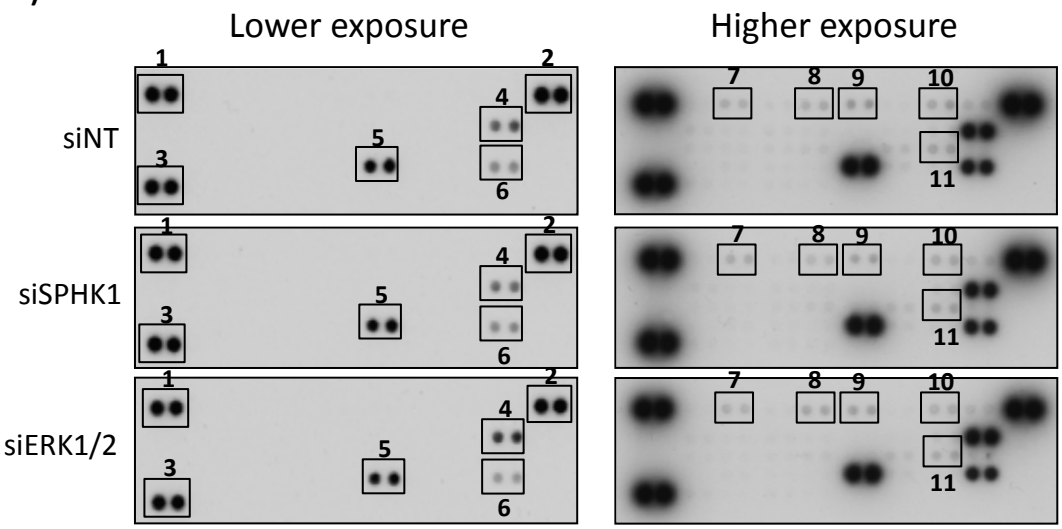


Figure 4.16: Proteome Profiler Assay: targets regulated by SPHK1 and ERK1/2. PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1), ERK1/2 (siERK1/2) or non-targeting control siRNA (siNT) for 48 hours. PC-3 cell lysates were collected for **a)** mRNA, **b)** western blotting and **c)** proteome profiler assay. **a)** SPHK1 expression was determined by qRT-PCR where three housekeeping genes, GAPDH, YWHAZ and UBC were used for normalisation. **b)** ERK1/2 total levels were determined by western blotting, with tubulin used as a loading control. **c)** Media was collected and centrifuged in order to remove non-adherent cells. Each media was incubated individually with a pre-probed membrane. Signal intensity for each of the 36 molecules on the membrane was measured using Image J software and the average intensity from the duplicate samples calculated. The results obtained for selected molecules are presented graphically and were normalised to the protein content; data is representative of a single experiment.

i)



ii)

Position	Target	Abbreviation/ Alternative nomenclature
1, 2, 3	Reference Spot	
4	Interleukin-8	IL-8
5	Macrophage migratory inhibitory factor	MIF-1/GIF/DER6
6	Plasminogen activator inhibitor-1	PAI-1/SERPIN E1
7	CD40 Ligand	CD154
8	Growth regulated oncogene-alpha	GROα/CXCL1
9	Granulocyte-macrophage colony-stimulating factor	GM-CSF/ CSFa/CSF-2
10	Soluble Intracellular adhesion molecule 1	siCAM-1/CD54
11	Interleukin-23	IL-23

Figure 4.17a: Proteome Profiler Assay: identified targets. a) PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1), ERK1/2 (siERK1/2) or non-targeting control siRNA (siNT) for 48 hours. Media was collected and centrifuged in order to remove non-adherent cells. Each media was incubated individually with a pre-probed membrane. This figure represents different exposures with respect to figure 4.16a. **b)** The table shows the identified targets, and corresponding alternative nomenclature.

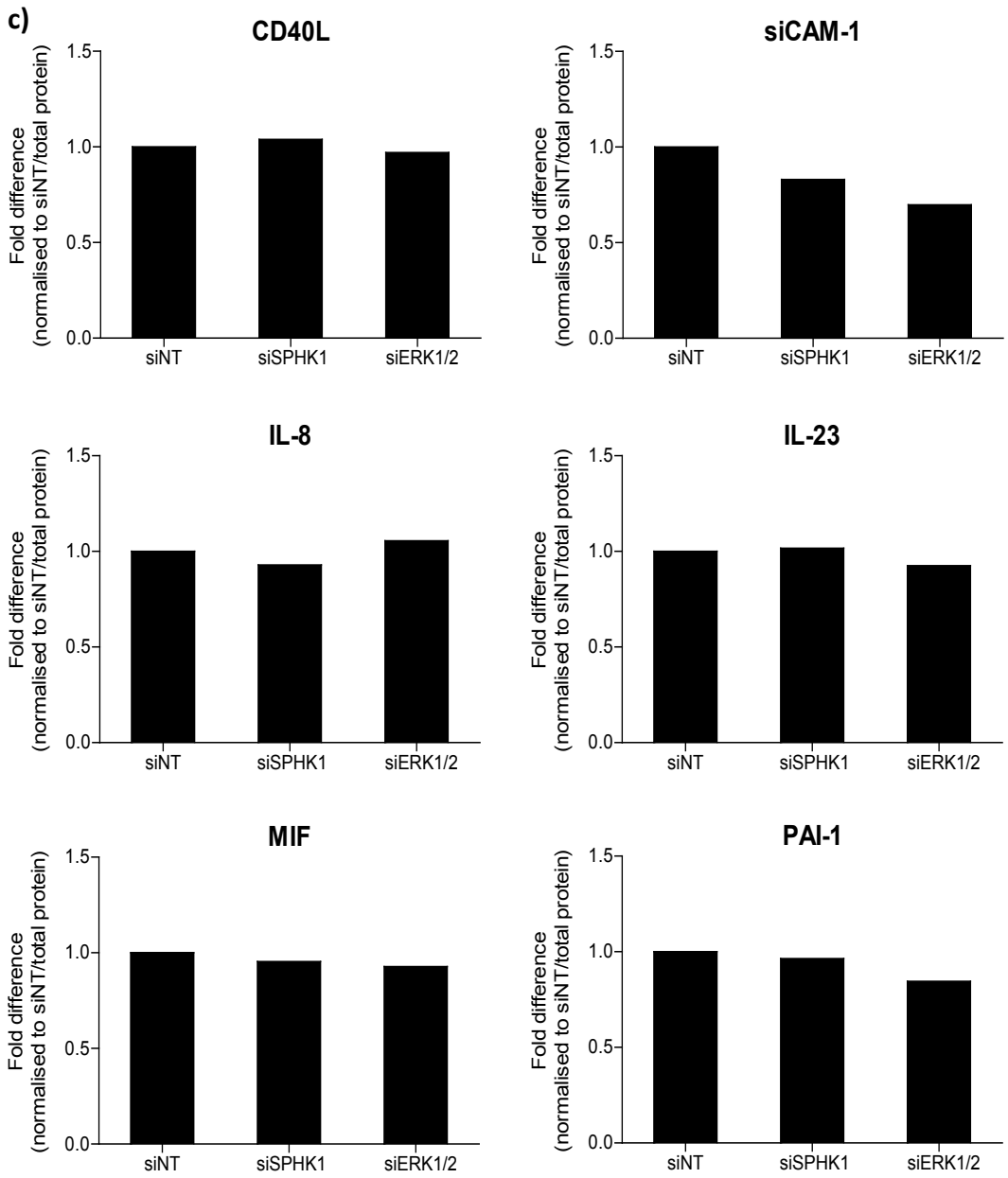


Figure 4.17b: Proteome Profiler Assay: Graphical analysis of unmodified targets. The results obtained in figure 4.17 are presented graphically and were normalised to the protein content. Data is representative of a single experiment.

4.7 Discussion

The increase in SPHK1 enzymatic activity and expression in PC-3 cells (**Fig.4.1a** and **b**) when co-cultured with U937s in a transwell model, supports the concept of tumour-immune cell interactions through signalling molecules that lead to an alteration of gene regulation. Indeed, SPHK1 activity seems to be continuously induced by the presence of U937 cells (**Fig.4.1a**). Rather than a consequence of an increase in SPHK1 mRNA, it is proposed that the initial increase in activity could also result from the presence of a strong activator, possibly secreted by monocytes, that is capable of inducing SPHK1 phosphorylation and activation. It is known that SPHK1 has intrinsic activity which can be further increased by several membrane receptors or signalling molecules. Billich *et al* showed that TNF-alpha and IL-1beta induced SPHK1 activation after only ten minutes in A549 epithelial lung carcinoma cells. However, no corresponding increase was found in mRNA levels even after 2.5 hours, suggesting that the observed increase in activity most likely resulted from a post-translational or post-transcriptional modification [326]. Conversely, in the transwell model described in this study, an increase in SPHK1 mRNA levels is evident at the earliest timepoint tested (one hour), suggesting that transcriptional activation could also be related to increased SPHK1 activity. In accordance, PMA was shown to rapidly induce SPHK1 activation and later increase corresponding mRNA levels in a leukemic cell line [184]. A biphasic activation of SPHK1 is documented in the literature [190, 405] and could explain the later 24 hour increase in SPHK1 mRNA observed using the transwell model (**Fig.4.1b**). The same interpretation could be inferred from the findings that in both PC-3 cells co-cultured with THP-1 (**Fig.4.4)** and MDA-231 co-cultured with U937 cells (**Fig.4.5**) there is an early activation in SPHK1 activity followed by a later increase in mRNA expression levels. Nonetheless, it is important to note that SPHK1 activation and expression may not be regulated by the same soluble signalling molecules during co-culture; with this being a dynamic interaction between the two cell types it is reasonable to assume that the media content changes over time. The significance of the inferences from co-culture experiments using THP-1 (**Fig.4.4**) and MDA-231 (**Fig.4.5**) cells could be established by increasing the number of experimental repeats.

Mechanistically, both Akt and ERK1/2 appear to play a role in this interaction. AKT phosphorylation in PC-3 cells was highly increased by the presence of U937 (**Fig.4.2a**) and THP-1 cells (**Fig.4.4c**); this activation in PC-3 cells correlated with increased SPHK1 activity and expression (**Fig.4.1 and Fig.4.4**), suggesting a possible cross-regulation between Akt and SPHK1. Indeed, this was further supported by the finding that U937-induced Akt phosphorylation was abrogated in PC-3 transfected with SPHK1 siRNA (**Fig.4.2c**), indicating that Akt is under the regulatory control of SPHK1 independent of the presence of U937 cells. This regulation appears most likely to be post-translational, as the total Akt levels were not affected by SPHK1 siRNA. This specific effect on Akt phosphorylation was also reported by another group using either chemical inhibition or siRNA mediated knock-down of SPHK1 in PC-3 cells (Cho, Lee et al. 2011). Likewise, in glioblastoma and ovarian cancer cells SPHK1 inhibition reduced Akt phosphorylation whilst concurrently inhibiting tumour growth [352, 353], reinforcing the concept that Akt is a downstream target of SPHK1 and its inhibition is strongly correlated with anti-proliferative effects in cancer cells. In this study I showed for the first time that U937 cells-induced Akt phosphorylation in PC-3 cells is dependent upon SPHK1 activation in the latter cells, implying that SPHK1 inhibition is required to block monocyte-induced activation of this important signalling pathway. The precise mechanisms underlying this phenomenon remain unclear; indeed, a direct interaction between SPHK1 and Akt, leading to AKT phosphorylation, or a regulation of Akt by molecules that are under SPHK1 regulatory control are both plausible and require further elucidation. In this regard, a recent study proposed a model whereby activation of Akt2, but not Akt1 or Akt3, was shown to induce S1P formation via SPHK1 transcriptional regulation; S1P extracellular binding to its receptor would then create a positive feed-back loop through PI3K/Akt2/mTOR [406]. However, the authors failed to prove that S1P levels were being elevated by SPHK1 activation or that S1P was indeed activating Akt2; nonetheless this report comes in support of another report identifying Akt2 as a positive regulator of SPHK1 [187]. In fact the regulatory effect of Akt2, but not Akt1, on SPHK1 could explain why in this study Akt1 siRNA transfected PC-3 cells did not show any significant reduction in SPHK1 expression (**Fig.4.3a**).

In addition, my results show that the addition of S1P to PC-3 cells leads to SPHK1 transcriptional activation as well as Akt phosphorylation (**Fig.4.6**). Interestingly, Akt phosphorylation is preceded by an increase in SPHK1 mRNA, suggesting that this event may

be a prerequisite for Akt phosphorylation and raising the hypothesis that S1P could be involved in Akt activation. Unfortunately, it was not possible to verify S1P levels during co-culture and therefore assert with a higher degree of confidence that S1P was indeed required for Akt activation upon monocyte stimulation. S1P levels are usually increased in response to an increase in SPHK1 expression, and whilst I could not verify this in my system, this hypothesis provides a possible explanation for the simultaneous up-regulation of SPHK1 (mRNA and activity) and AKT phosphorylation during co-culture.

As mentioned initially, the MAPK pathway, more specifically ERK1/2, was also hypothesised as a possible regulator for SPHK1 due to its well documented ability to phosphorylate and activate this kinase [182]. ERK1/2 knockdown by siRNA abrogated the U937-induced increase of SPHK1 expression (**Fig.4.3ai-iii**) suggesting an important role for ERK1/2 in SPHK1 activation. In PC-3 cells ERK1/2 phosphorylation could not be detected and it was therefore not possible to assess if ERK1/2 phosphorylation was differentially regulated in PC-3 cells during co-culture. However in a breast cancer cell line, MDA-231, ERK1/2 phosphorylation was clearly increased by the presence of U937 cells across all studied time points (**Fig.4.5c**). This effect was accompanied by an increase in SPHK1 activity (**Fig.4.5a**) at three hours and a later increase in SPHK1 mRNA expression levels after six hours of co-culture (**Fig.4.5b**). It is therefore reasonable to hypothesise that in PC-3 cells ERK1/2 phosphorylation could be involved in SPHK1 activation and could indeed act as an important regulator of molecules under SPHK1 regulatory control. In fact, an interesting observation arises from the finding that ERK1/2 knockdown regulates U937-induced Akt phosphorylation in PC-3 cells; this effect was observed after three and 24 hours of co-culture (**Fig.4.3b**). It is plausible that SPHK1 inhibition by siERK1/2 would block SPHK1 signalling and consequently Akt phosphorylation, as seen previously in this study (**Fig.4.2c**). It is also important to note that SPHK1 and ERK1/2 knockdown had no effect on U937-induced Akt phosphorylation in PC-3 cells after one hour of co-culture (**Fig.4.3b**), indicating an ability of U937 cells to regulate Akt independently of SPHK1 and ERK1/2 levels in PC-3 cells. To note that the significance these experiment (**Fig.4.3b**) could be established by increasing the number of experimental repeats as it was only performed once.

4.7.1 Candidate signalling molecules involved in PC-3/U937 co-culture: Effect on SPHK1 and Akt phosphorylation

In order to explore and interrogate any molecules that could be involved in the aforementioned pathways my first approach was to evaluate the cytokine levels of MCP-1, IL-6, TNF-α, IFN-γ and IL-10. These 4 cytokines and one chemokine were initially chosen to evaluate the pro-inflammatory and anti-inflammatory microenvironment present during co-culture due to their role in modulating monocytes and macrophages into either pro- or anti-inflammatory phenotypes. Surprisingly, TNF-α, IFN-γ and IL-10 were not detected in co-culture assays presumably due to their low levels (data not shown). MCP-1 and IL-6 are cytokines well documented to be highly expressed in tumour and tumour stromal cells such as monocytes and macrophages [286, 291, 322]. My data supports the theory that MCP-1 (**Fig.4.7**) and, to a lesser extent, IL-6 levels (**Fig.4.10**) are increased in tumour-monocyte interactions. In terms of MCP-1 this increase was observed at a transcriptional level (**Fig.4.7c**) and extracellular protein (**Fig.4.7a**), whilst SPHK1 RNA-interference reduced U937-mediated up-regulation of MCP-1 expression (**Fig.4.7d**) and extracellular secretion (**Fig.4.7b**). In contrast, SPHK1 over-expression significantly increased MCP-1 expression (**Fig.4.8ai**) and secretion (**Fig4.8aii**); an effect that was accentuated by the presence of U937 cells (**Fig.5.8aiii**), and partially reduced by SPHK1 siRNA (**Fig.4.8b**). This data not only supports other studies indicating that MCP-1 was under the regulatory effect of SPHK1 but also indicates that the presence of monocytes can further stimulate its production. Indeed Sica *et al.* showed that macrophages can be recruited to the tumour site by MCP-1, and once resident within the tumour site they increase the secretion of MCP-1 [407]. Particularly in prostate cancer studies, MCP-1 was found to be four-fold higher in the tumour-bone microenvironment when compared to non-neoplastic bone [320] whilst MCP-1 can act as a direct mediator of prostate cancer cell growth, invasion and migration [323]. A more recent study has shown that MCP-1 production was inhibited in a SPHK1 deficient mouse model [293] whlist TNF-alpha induced MCP-1 expression was dependent upon SPHK1 activation ([326]. In this study I show for the first time that in a PC-3/U937 co-culture model, MCP-1 stimulation in PC-3 by U937 cells is partially dependent on SPHK1. In support, SKi-II treated mice carrying PC-3 xenografts showed lower levels of human MCP-1 mRNA when compared

with the control group (**Fig.4.13a**), an effect that was accompanied by a reduction in SPHK1 expression in both mouse and human cells (**Chapter 3**, **figure 3.7d-e**).

It is Important to note that MCP-1 failed to induce SPHK1 transcriptional activation (**Fig.4.9a**) as well as Akt phosphorylation in PC-3 cells (**Fig.4.9b**); indeed this latter event was unexpected as several other studies using the same cell line (PC-3) have indicated MCP-1 as a strong activator of Akt phosphorylation in a time and concentration-dependent manner [323, 408, 409].

Interestingly, in my study S1P was found to induce MCP-1 expression in PC-3 cells (**Fig.4.15a**) which, together with an S1P-induced increase in SPHK1 expression and Akt phosphorylation (**Fig.4.6**) supports the concept that MCP-1 is being positively regulated by the activation of a signalling loop between SPHK1/S1P/Akt. In support of this theory, in human aortic endothelial cells and mast cells S1P was shown to increase MCP-1 mRNA and protein secretion levels via an SPHK1-dependent mechanism [324, 325]. It is reasonable to propose that increased MCP-1 levels during co-culture could result from an initial SPHK1 activation triggered by other monocytic secreted molecules, or that MCP-1 could be produced directly by monocytes in response to tumour cells (Reviewed and discussed in Chapter 5, **Figure 5.5**). Indeed this latter explanation could also be implied from the fact that in PC-3 cells over-expressing SPHK1 no increase in either MCP-1 (**Fig.4.8aiv**) or SPHK1 mRNA (**Fig.4.8av**) levels was detected upon co-culture with U937 cells, despite the increase in MCP-1 secreted protein (**Fig.4.8aiii**).

With regards to IL-6, the increased levels in PC-3 media (**Fig.4.10a**) did not correlate with an increase in mRNA in these cells (**Fig.4.10b**). However, over-expression of SPHK1 in PC-3 cells increased levels of IL-6 mRNA and protein secretion (**Fig.4.11a** and **b**); an effect that was further increased (secreted protein only) by co-culture with U937 cells (**Fig.4.11c** and **d**). The lack of increase in IL-6 expression could be interpreted as SPHK1 having little effect on IL-6 expression, although I did see a 2.5-fold increase in PC-3/OFP-SPHK1 cells. The increase in SPHK1 during co-culture may not be significant enough to induce IL-6 expression, indicating that the observed increase in IL-6 in the media could result from U937 cells (described in chapter 5). Furthermore, it must be considered that IL-6 could be regulating SPHK1, as is supported by my findings whereby recombinant IL-6 induced SPHK1 activation

and increased its mRNA levels (**Fig.4.12a-b**). This finding is supported by a report stating that in multiple myeloma cells IL-6 was shown to induce SPHK1 activation and cell proliferation [294]. Further, Kun-Yun *et al* showed that IL-6 initially secreted from macrophages induces a later surge in IL-6 secretion in human colon cancer cells [402]. Due to its paracrine action, it is possible that in my co-culture system monocytes initially secrete IL-6 in response to PC-3 cells during the early stages of co-culture and this leads to a subsequent later IL-6 secretion from PC-3 cells. Furthermore, IL-6 expression was also transiently induced by the addition of exogenous MCP-1 (**Fig.4.14a**) or S1P (**Fig.4.15b**) raising the hypothesis that during co-culture of PC-3 with U937 cells the dynamic balance of these factors, coupled with variations in their levels throughout time, could explain the activation of important signalling pathways such as Akt or SPHK1.

The proteome screening assay was performed with the purpose of studying a wider range of cytokines or chemokines potentially secreted by PC-3 cells. I also sought to understand if these cytokines were regulated by SPHK1, ERK1/2 or Akt, and finally to determine whether they act in a paracrine and/or autocrine manner. siRNA-mediated SPHK1 knockdown in PC-3 cells showed that granulocyte colony stimulating factor (GM-CSF) and growth regulated oncogene-alpha (GROα) were reduced in comparison with PC-3 transfected with a non-targeting siRNA. To date, there are no reports associating SPHK1 with GM-CSF. GM-CSF is known to stimulate the production, survival and proliferation of myeloid cells such as macrophages [410]. As such, it emerges as an important regulator of myeloproliferative disorders and myeloid leukaemias, more specifically, in a mouse model GM-CSF was identified as being crucial for the maintenance of juvenile myelomonocytic leukemia [411]. An interesting report shows that GM-CSF not only led to an increase in cell growth, but also activated Akt phosphorylation in several lung cancer cells [412]. Furthermore, it showed that Akt up-regulation led to an increase in GM-CSF, indicating a possible autocrine function of GM-CSF through Akt. An autocrine function of GM-CSF has also been reported in human glioma cells [413] and malignant B-lymphocytes [414]. Other reports indicate that in the prostate cancer cell lines DU-145 and PC-3, GM-CSF increases cell proliferation [415, 416]. At present there are no reports associating SPHK1 with GM-CSF, however in correlation with my data, GM-CSF could play a role in cell proliferation, with SPHK1 being an important mediator. It could also be involved in Akt

phosphorylation in PC-3 and in the modulation of the monocytic response towards PC-3 cells.

With respect to GROα, its regulation by SPHK1 is not currently described in the literature. However a role in cancer has been reported; Reiland J. *et al* showed that PC-3 cells increased their invasiveness by two-fold in response to GROα [417]. In support, more recent work has shown that GROα-induced increased in PC-3 and DU-145 cell proliferation was mediated by Akt [418]. An immunohistochemistry study on primary colorectal specimens showed a strong correlation between GROα expression and tumour size, lymph node metastasis and patient survival [419]. GROα is expressed by endothelial, epithelial, fibroblast and myeloid cells such as monocytes and macrophages and it is reported that in inflammatory sites its expression is significantly raised. A potential autocrine and paracrine role for GROα in my system remains to be elucidated; however, evidence suggests that its role in the tumour-monocyte interaction may be a worthwhile avenue of investigation. Other target molecules were detected in this assay (**Fig.5.17**), however their patterns of secretion were not affected by any of the siRNAs employed, indicating that they were not regulated by SPHK1 or ERK1. Nonetheless their potential roles in myeloid and tumour cell regulation should not be excluded and should be further validated by increasing the number of experimental repeats (proteome profiler assay).

In summary, I show for the first time that in both prostate cancer cells (PC-3) and breast cancer cells (MDA-231) SPHK1 expression and activity is regulated by monocytes. This appears to be part of a highly dynamic process, whereby the constant exchange of signals dictates a molecule's importance and regulation over time. I have shown that SPHK1 plays a central role in MCP-1, but not in IL-6 regulation. In fact conversely, IL-6 was proven to regulate SPHK1 at the protein and mRNA level, a phenomenon that was not observed for MCP-1. Evidence indicates that MCP-1 and IL-6 play an important role in these interactions as well as cross-regulation of one another, however the specifics of their roles and the co-ordination of the timing of their actions is not yet fully understood. Akt has been shown to be positively affected by U937 cell stimulus; however this effect was blocked by SPHK1 inhibition. The increase in Akt phosphorylation could be due to S1P, as both MCP-1 and IL-6 fail to induce its phosphorylation. As a consequence GROα and GM-CSF, both identified as

being under SPHK1 regulatory control, could be potential candidates as their ability to activate Akt has been previously documented. A schematic representation of the above findings, highlighting the dynamic interactions between PC-3 and U937 cells as well as the role of SPHK1/S1P in this interaction is shown in figure **4.18a-b**.

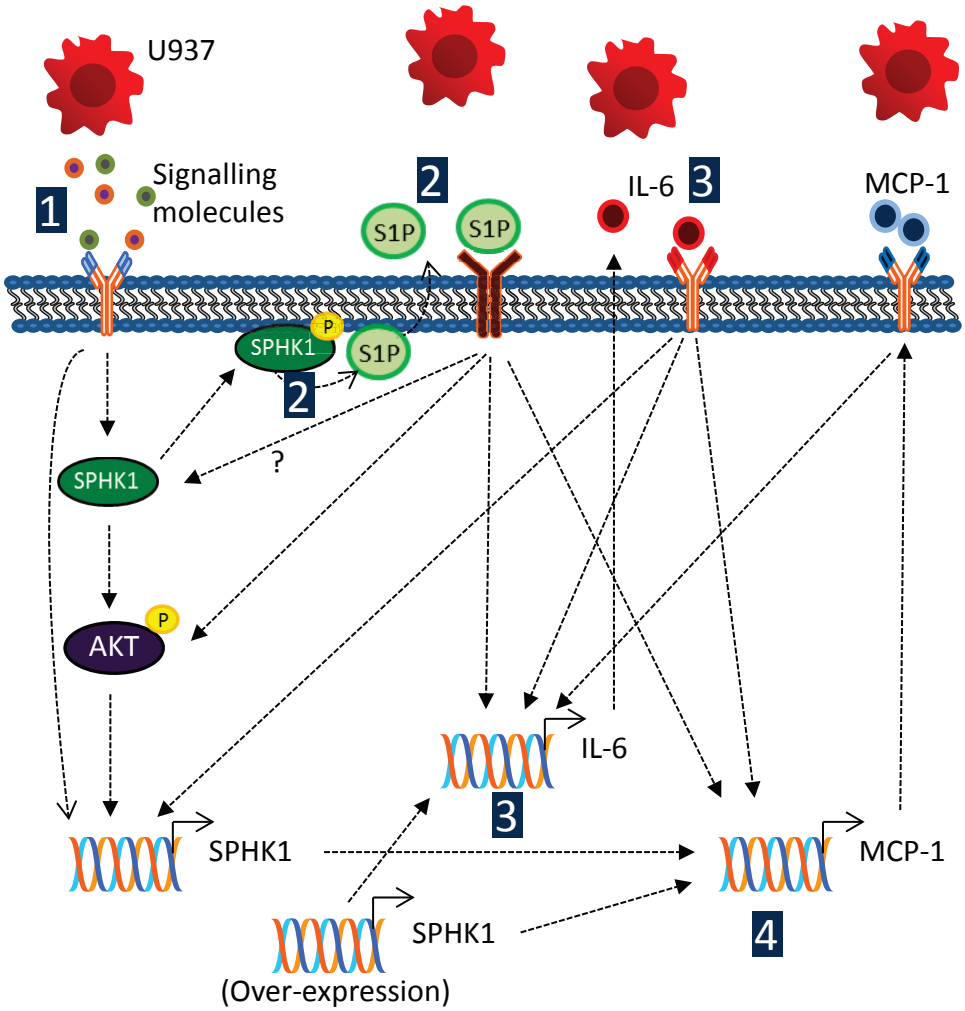


Figure 4.18a: Proposed model for SPHK1 activation in PC-3 during their co-culture with U937 cells. 1) The secretion of unknown molecules by U937 cells leads to the activation of SPHK1 at mRNA levels and enzymatic activity and AKT phosphorylation. **2)** SPHK1 is most likely to produce S1P that can itself lead to the increase of AKT phosphorylation as well as transcriptional activation of several genes, including SPHK1, IL-6 and MCP-1. **3)** Monocytes induce IL-6 secretion whilst mRNA expression levels are not affected. Extracellular IL-6 can induce an increase in SPHK1 and MCP-1 mRNA expression levels as well as acting in an autocrine manner to promote its own expression. No effect on AKT phosphorylation. SPHK1 over-expression induces IL-6 mRNA. **4)** MCP-1 mRNA and secreted levels are up-regulated during co-culture with U937 cells, where secreted MCP-1 can induce IL-6 transcriptional activation but has no effect on SPHK1 or AKT phosphorylation. SPHK1 over-expression leads to an increase in MCP-1 mRNA expression. The sequence of events may not reproduce the real dynamics existing between PC-3 and U937 cells as some of the represented events may occur simultaneously.

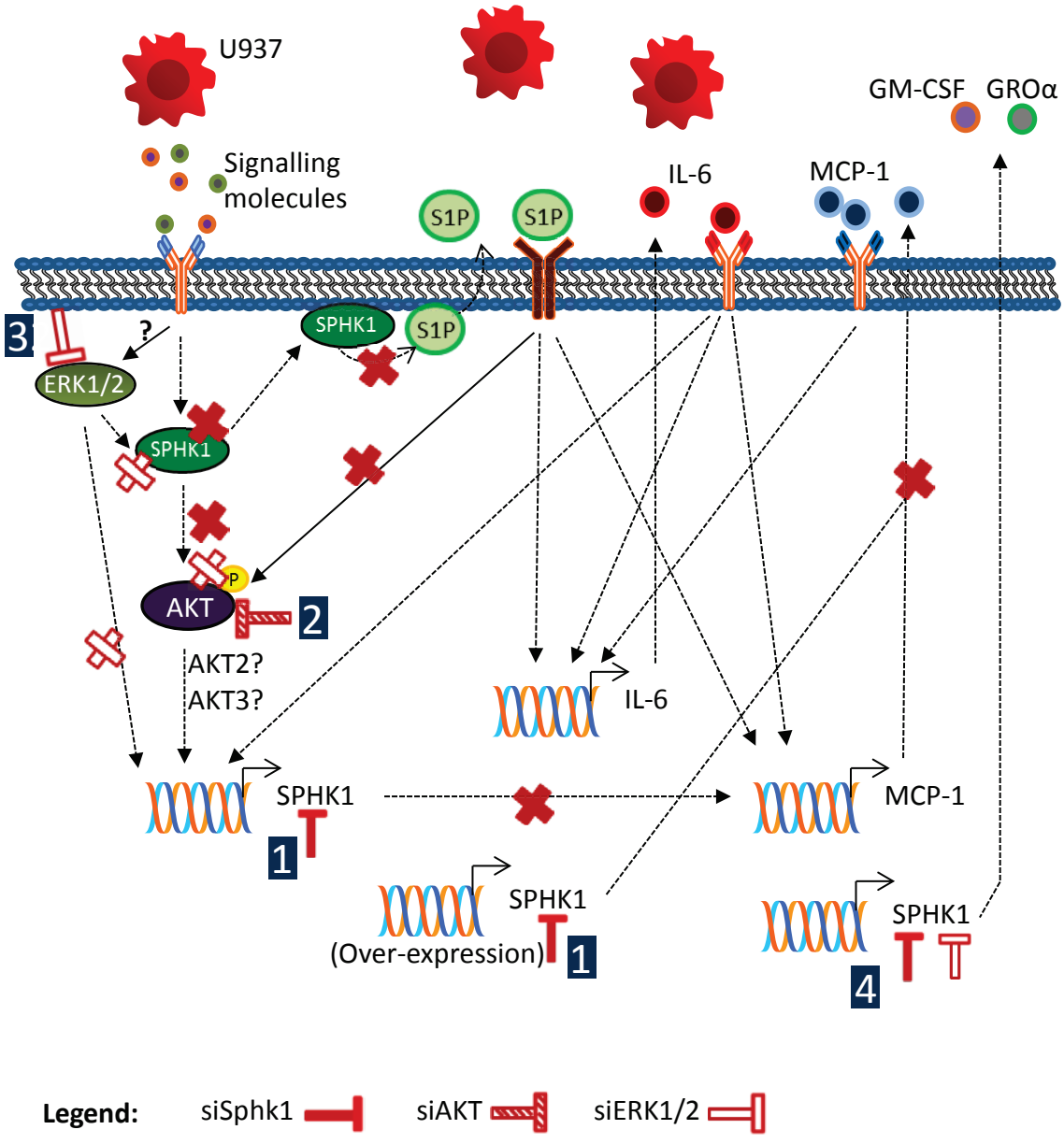


Figure 4.18b: Proposed model for intracellular pathways involved in SPHK1 regulation in PC-3 cells during their co-culture with U937 cells. 1) SPHK1 knockdown by siRNA inhibits U937-induced AKT phosphorylation. A decrease in SPHK1 levels would lead to a decrease in S1P production, which in turn affect AKT phosphorylation. MCP-1 expression and secreted levels are also reduced by SPHK1 down-regulation. Similarly, in PC-3 cells over-expressing SPHK1, the use of siRNA targeting SPHK1 reduces MCP-1 secreted levels. IL-6 mRNA or secreted protein was not affected by SPHK1 knockdown. **2**) The use of siRNA targeting AKT1 did not have an effect on SPHK1 expression; a possible regulatory mechanism could involve AKT2 or AKT3 instead of AKT1. **3)** ERK1/2 knockdown abrogated the positive effect of U937 in SPHK1 mRNA expression, it also reduced AKT phosphorylation suggesting a possible regulatory mechanism of AKT via ERK1/2 and SPHK1. **4)** SPHK1 or ERK1/2 knockdown decreases the extracellular levels of GRO and GM-CSF, two possible mediators involved in the PC-3/U937 interaction.

5. SPHK1 regulation in U937 cells upon co-culture with PC-3 cells: Role of AKT, ERK1/2 and cytokines/chemokines.

5.1 Introduction

The capacity of tumours to influence their surroundings has been widely reported [244, 420, 421]. Their ability to attract different cell types to their "neighbourhood" and alter the transcriptional response in these cells is identified as a very important event in tumour development. Monocytes and macrophages are well described in literature as one of the main components of tumour stoma and their presence is associated with tumour progression and aggressiveness [247, 389, 407]. Their interaction with cancer cells is thought to be a bidirectional dynamic process, in which signalling molecules such as cytokines and chemokines play an important role [390, 422]. In this context, the involvement of SPHK1 in cytokine production has been described. In microglia cells (brain resident macrophages) SPHK1 was found to regulate the expression of pro-inflammatory cytokines such as TNF-α and IL-1β. Interestingly, S1P added to these cells also induced the expression of these cytokines indicating an active role of SPHK1 and S1P in cytokine regulation [270]. Another study indicated that S1P secreted from cancer cells provoked the induction of Akt and ERK1/2 in macrophages [254], suggesting an important role of the SPHK1/S1P pathway in the macrophage response and in the activation of important associated signal transduction pathways. Here I aim to investigate whether SPHK1 regulation in monocytes is affected by PC-3 cells, and if by modulating SPHK1 in PC-3 cells this response is altered. Finally, I aim to uncover the molecular mechanisms and possible mediators behind SPHK1 activation in monocytes during co-culture with PC-3 cells.

5.2 SPHK1 regulation in U937 during co-culture with PC-3 cells

Using the same co-culture model as described previously (Section **2.2.3**), PC-3 prostate cancer cells and the U937 monocytic cell line were co-cultured for up to 24 hours, followed by determination of SPHK1 levels in U937 cells. The presence of PC-3 cells induced a consistent increase in SPHK1 enzymatic activity in U937 cells, being significant at one, three and 24 hours (**Fig.5.1a**). SPHK1 activity was increased by 50% and 100% at one and three hours respectively, and by approximately 100% at 24 hours when U937 cells were co-cultured with PC-3 cells. As for SPHK1 transcriptional regulation (**Fig.5.1b**), there was an initial increase in expression of approximately 1.2- and 2-fold at one and three hours respectively, and a later 1.5-fold increase at 24 hours when in co-culture with PC-3 cells. Despite a non-significant increase in SPHK1 enzymatic activity at five and eight hours induced by PC-3 cells (**Fig.5.1a**), this effect was not correlated with an alteration of SPHK1 mRNA at the respective time points (**Fig.5.1b**).

Following on from the previous experiment, it was important to evaluate whether the SPHK1 knockdown in PC-3 cells had any influence on SPHK1 regulation in U937 cells. The U937 cells co-cultured with PC-3 cells transiently transfected with SPHK1 siRNA (siSPHK1) showed a lower SPHK1 enzymatic activity in comparison to those co-cultured with a non-targeting siRNA (siNT) (**Fig.5.2**). This effect was higher at one and three hours with a decrease of approximately 60%, followed by an approximate 30% decrease after 24 hours of co-culture (**Fig.5.2**).

In terms of SPHK1 transcriptional activation in U937 cells, it was observed that the PC-3-induced increase in SPHK1 mRNA expression in U937 cells was reduced when SPHK1 was knocked down in PC-3 cells (**Fig.5.2b**). In comparison with U937 cells co-cultured with siNT PC-3 cells, there was a reduction of 30%, 35% and 37% in SPHK1 expression at one, three and 24 hours respectively. In summary, the positive effect of PC-3 cells co-culture upon SPHK1 in U937 cells was entirely abolished when SPHK1 was knocked down in PC-3 cells.

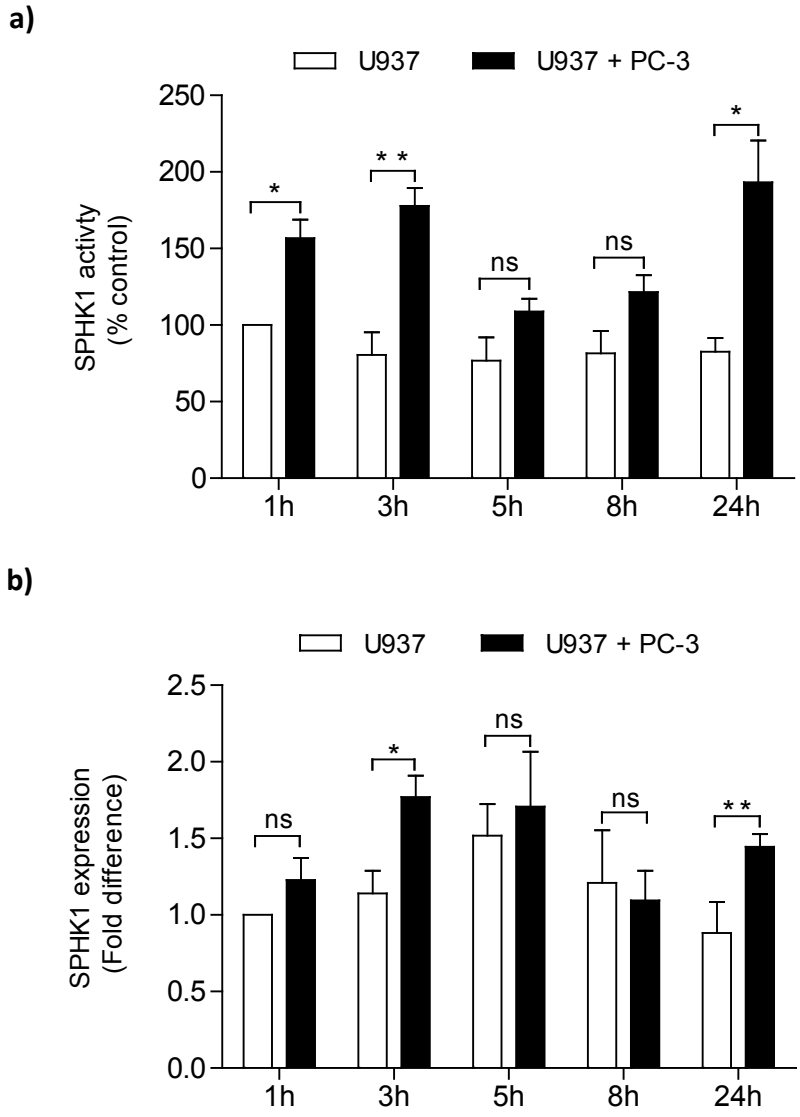


Figure 5.1: SPHK1 activity and expression levels in human U937 cells following their co-culture with PC-3 cells. PC-3 cells were seeded in the bottom chamber under serum-free media 24 hours before the addition of U937 cells to the upper chamber for the indicated duration. **a)** SPHK1 activity and **b)** expression were determined in U937 cell lysates containing equal amounts of protein or mRNA using radiolabelling and qRT-PCR respectively. For qRT-PCR, SPHK1 mRNA expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. Data shown is the mean ± SEM of three independent experiments performed in duplicate. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$, **$p<0.01$, for U937 vs. U937+PC-3.

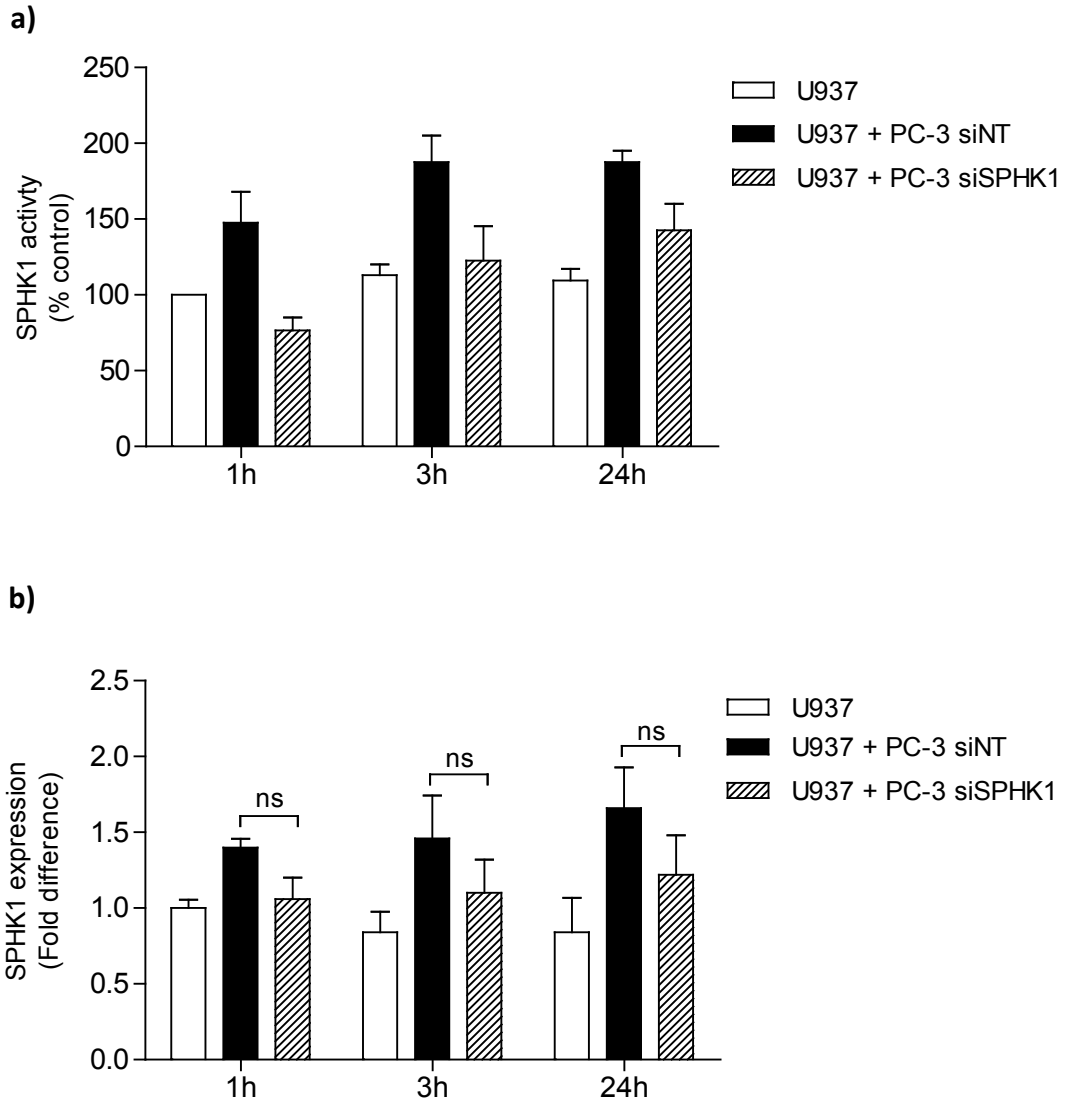


Figure 5.2: SPHK1 activity and expression levels in human U937 cells following co-culture with PC-3 cells treated with RNAi. PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1) or non-targeting control siRNA (siNT) for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. **a)** SPHK1 activity and **b)** expression were determined in U937 cell lysates containing equal amounts of protein or mRNA respectively using radiolabelling and qRT-PCR respectively. For qRT-PCR, SPHK1 mRNA expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. Data shown is the mean ± SEM of **a)** two or **b)** three independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$ for U937+ PC-3siNT vs. U937 + PC-3 siSPHK1

5.3 Signal transduction pathways in U937 cells during co-culture with PC-3 cells

In order to elucidate the molecular mechanisms by which SPHK1 is regulated in U937 cells during the presence of PC-3 cells, a western blotting assay was performed in U937 cells following co-culture with PC-3 cells, as described previously. As shown in figure **5.3a**, there is an increase in Akt, ERK1/2 and NF-KB phosphorylation upon stimulation with PC-3 cells across all of the indicated time points. The 3-fold increase in Akt phosphorylation is significant at three and 24 hours (**Fig.5.3bi**). ERK1/2 phosphorylation levels significantly increase by 7- and 3-fold at three and 24 hours respectively, when compared with U937 cells cultured in the absence of PC-3 cells (**Fig.5.3bii**). The presence of PC-3 cells also had a positive impact on p65 phosphorylation (a subunit of NF-KB) in U937 cells (**Fig.5.3biii**); however the significance of this finding proved difficult to determine due to technical constraints.

To further investigate the impact of SPHK1 absence on PC-3 cells, and the subsequent influence on the identified regulators of important signalling pathways in U937 cells, PC-3 cells were transiently transfected with siRNA for SPHK1 and a non-targeting siRNA as a control, prior to co-culture with U937 cells for three or 24 hours (**Fig.5.4a**). Interestingly, Akt and NF-KB phosphorylation in U937 cells was affected in a similar way to that observed following SPHK1 knockdown in PC-3 cells, with a small reduction observed at three hours and a more accentuated effect seen at 24 hours (**Fig.5.4ai-ii** and **bi**). ERK1/2 phosphorylation was also affected by SPHK1 down-regulation in PC-3 cells (**Fig.5.4ai**); in this case the decrease in ERK1/2 phosphorylation at three hours was more accentuated than with Akt or NF-KB phosphorylation levels (**Fig.5.4ai** and **b**). Furthermore, this decrease in EKR1/2 phosphorylation was also observed at 24 hours, with a 4-fold decrease in comparison with U937 co-cultured with a non-targeting siRNA (**Fig.5.4ii**).

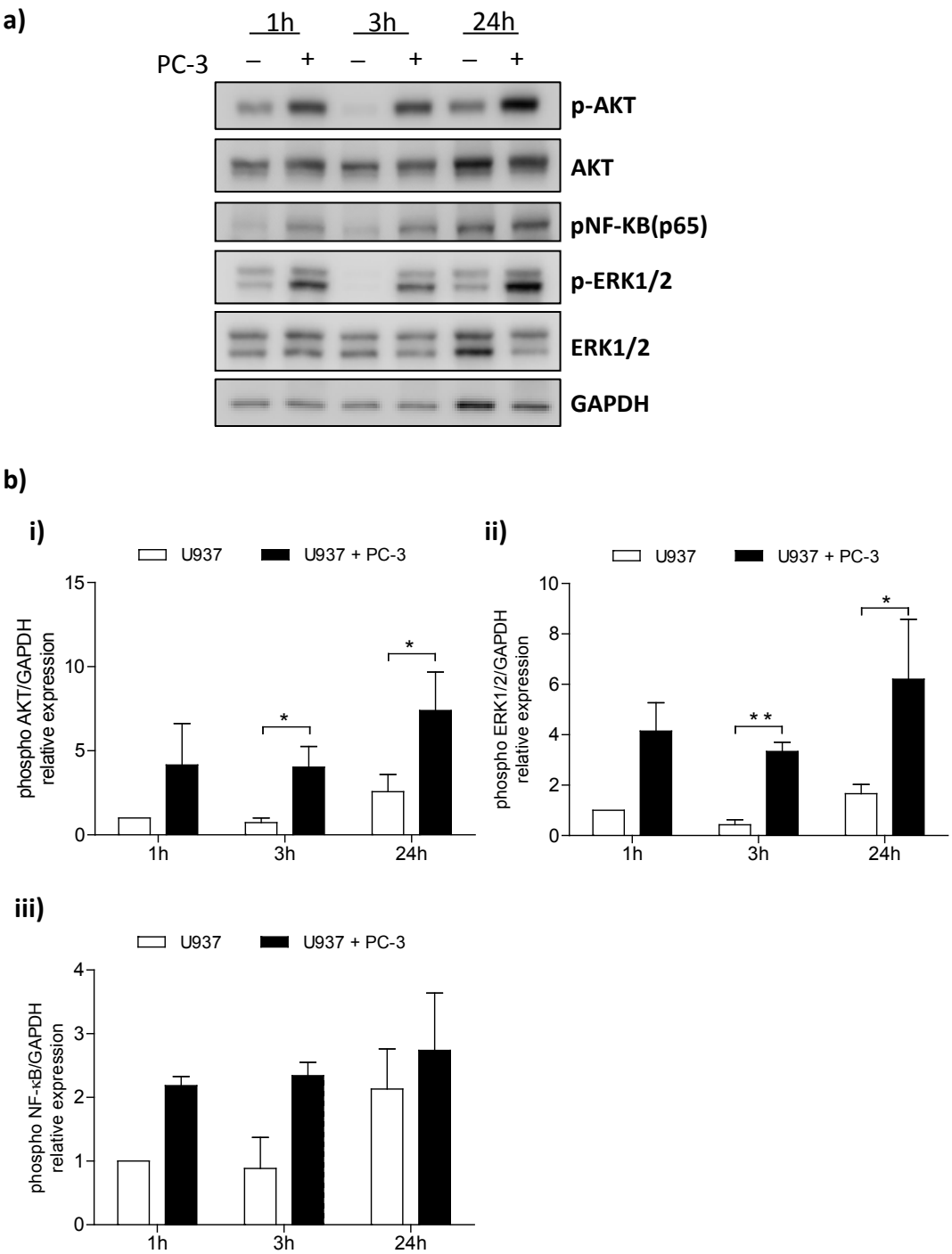


Figure 5.3: Changes in U937 cells signal transduction pathways during co-culture with PC-3 cells. PC-3 cells were seeded in the bottom chamber under starvation 24 hours before the addition of U937 cells to the upper chamber and co-cultured for the indicated duration. **a)** AKT, ERK1/2 and NF-KB phosphorylation and total levels were determined by western blotting, **b)** quantified by Image J software and normalised to GAPDH. Data shown is the mean ± SEM of three independent experiments with the exception of NF-kB phosphorylation levels that were only detected in two independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$, **$p<0.01$, for U937 vs. U937+PC-3.

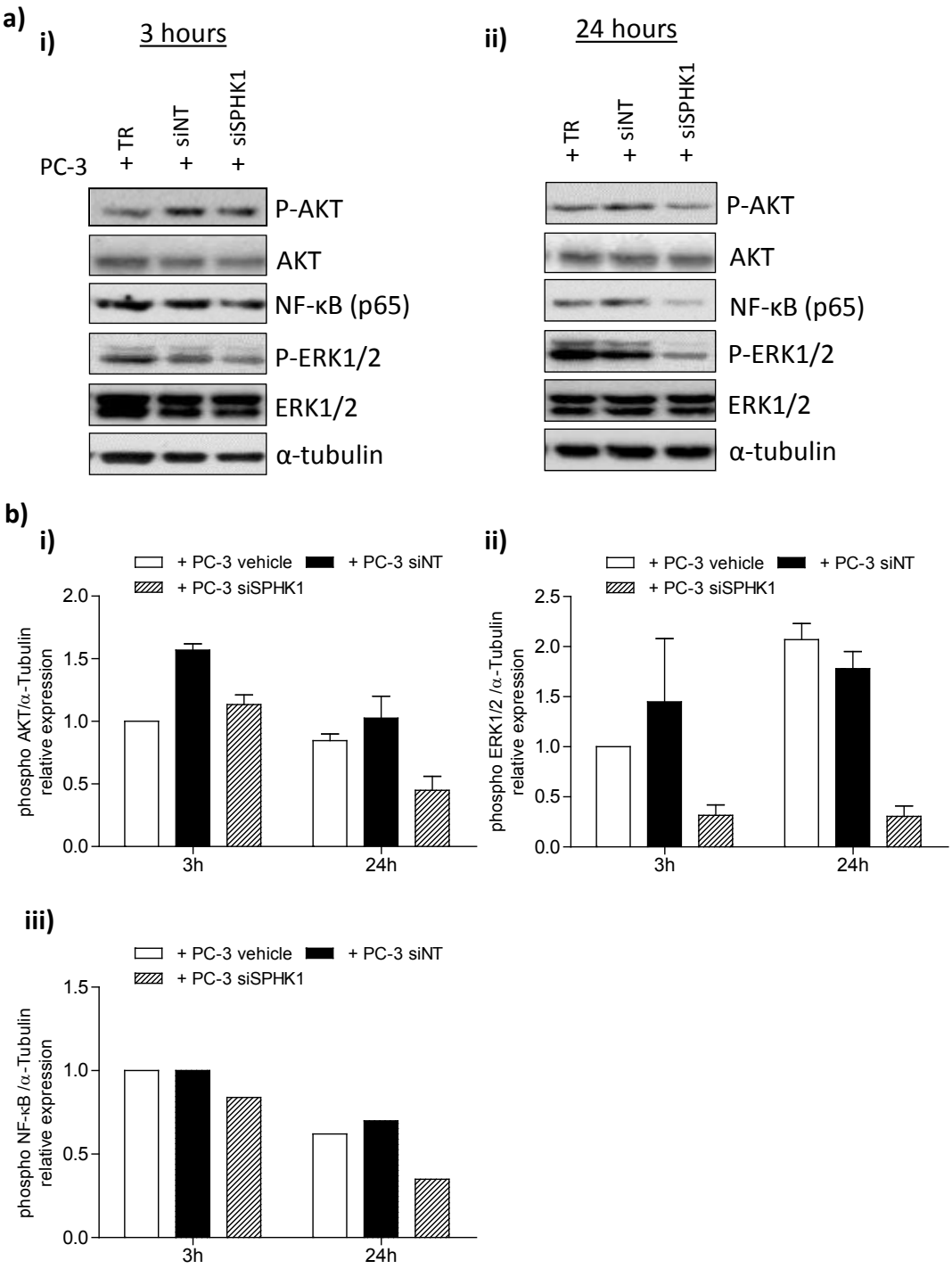


Figure 5.4: Changes in U937 cells signal transduction pathways during co-culture with siRNA-SPHK1 treated PC-3 cells. PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1), non-targeting control siRNA (siNT) or transfection reagent (TR) for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. **a)** AKT, ERK1/2 and NF-KB phosphorylation and total levels were determined by western blotting, **b)** quantified by Image J software and normalised to alpha-tubulin. Data shown is the representative of two independent experiments, with the exception of NF-kB phosphorylation levels that were only detected in one independent experiment.

Findings from these initial experiments led me to conclude that not only was it important to evaluate the role of SPHK1 in this interaction, but just as important was to determine how Akt and ERK1/2 activation in PC-3 cells are important for SPHK1 activation in U937 cells. For this purpose PC-3 cells were transiently transfected with Akt and ERK1/2 siRNA separately (chapter 4, **Fig.4.3b**), before being added to U937 cells for the indicated time points. This transient transfection in PC-3 cells proved to impact on SPHK1 mRNA, with Akt and ERK1/2 knockdown inducing a similar response upon SPHK1 transcriptional regulation in U937 cells (**Fig.5.5a**). Following one hour of co-culture SPHK1 expression was increased by the presence of PC-3 cells and was not affected by the absence of Akt or ERK1/2 in PC-3 cells. However, after three and 24 hours of co-culture, SPHK1 expression in U937 cells was reduced when PC-3 Akt and ERK1/2 levels were decreased by siRNA-mediated knockdown in comparison with U937 cells co-cultured with PC-3 treated with non-targeting siRNA (**Fig.5.5a**).

Using the previous experimental design as a basis, I sought to investigate if ERK1/2 or Akt knockdown in PC-3 cells would affect the phosphorylation levels of these molecules in monocytes. Western blotting was performed on U937 cell lysates following three hours of co-culture with PC-3 cells transfected with siRNA targeting SPHK1, Akt or ERK1/2. As shown in figure **5.5b**, the initial increase in Akt phosphorylation induced by co-culture with PC-3 cells transfected with a non-targeting siRNA, was reduced when Akt or ERK1/2 were targeted in PC-3 cells. A similar effect was observed when ERK1/2 was targeted in PC-3 cells; the expected positive effect of these cells on ERK1/2 phosphorylation, with a 3.2-fold increase compared to U937 cells alone, was reduced to 1.6- and 0.7-fold when PC-3 cells were transfected with siRNA targeting Akt and ERK1/2 respectively (**Fig.5.5b**).

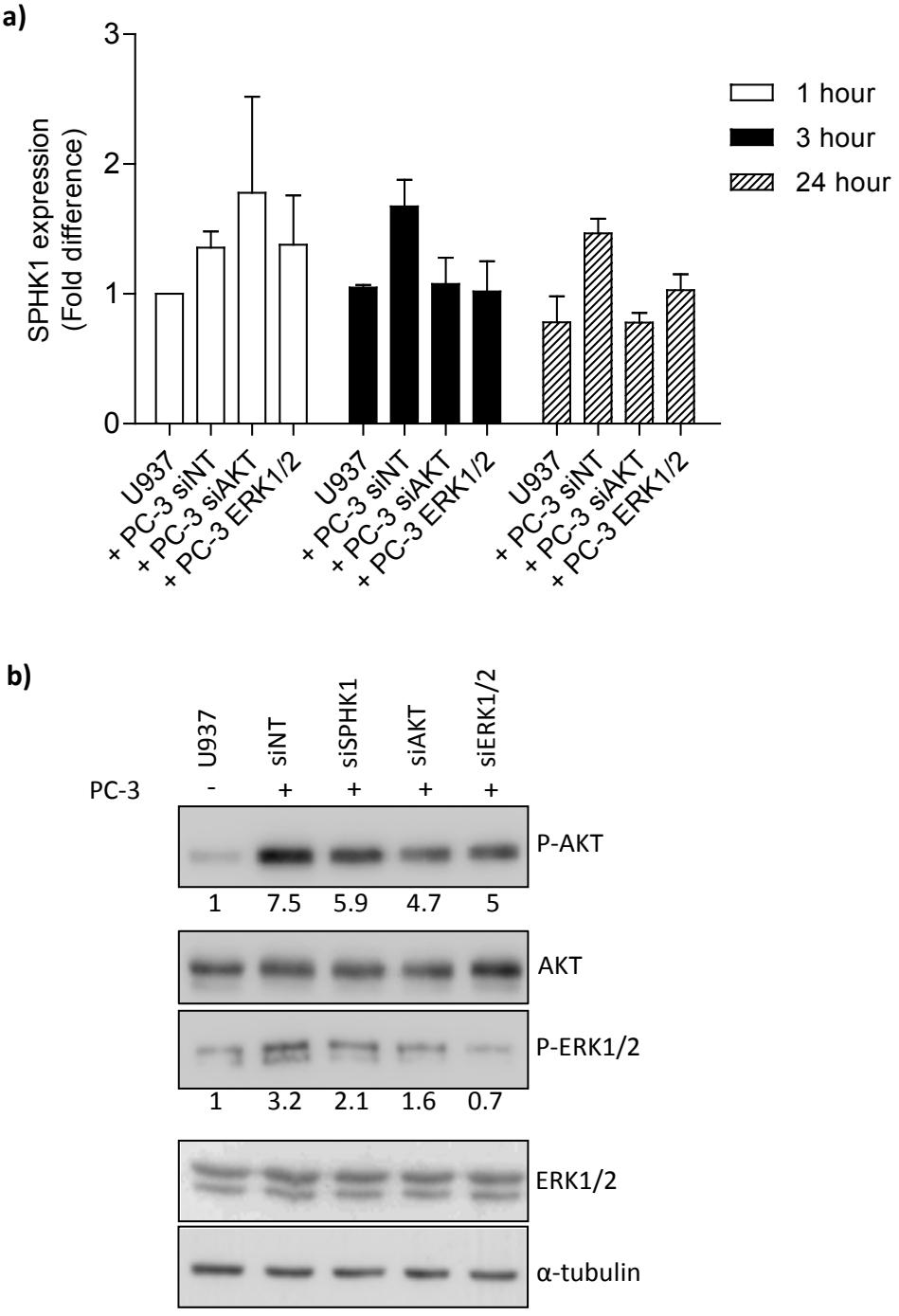


Figure 5.5: Changes in SPHK1 and signal transduction pathways in U937 cells following co-culture with PC-3 cells targeted with several siRNAs. PC-3 cells were transfected with siRNA targeting SPHK1(siSPHK1), AKT (siAKT), ERK1/2 (siERK1/2) or non-targeting control siRNA (siNT) for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. **a)** SPHK1 expression in U937 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise SPHK1 expression. **b)** AKT and ERK1/2 phosphorylation and total levels were determined by western blotting, quantified by Image J software and normalised to alpha-tubulin, values under the immunoblot represent the normalised fold difference to U937 cells cultured alone. Data shown is representative of **a)** two and **b)** one independent experiment.

5.4 Effect of PC-3 cells on SPHK1 levels in THP-1 monocytic cell line

During co-culture the presence of THP-1 cells was shown to induce a modest increase in proliferation of neighbouring PC-3 cells (chapter 3, **Fig.3.4**). Based upon this observation, and with the supporting knowledge that SPHK1 activation in cancer cells is associated with cell proliferation, I decided to investigate whether SPHK1 was being activated in THP-1 cells upon co-culture with PC-3. For this purpose THP-1 cells were co-cultured with PC-3 cells in similar conditions as described previously for U937 cells. SPHK1 enzymatic activity was found to be increased in THP-1 cells after one hour of PC-3 cell co-culture (**Fig.5.6a**). This positive effect on SPHK1 activity was deemed transient as after three hours of co-culture no variation in activity was observed when compared to THP-1 cells cultured alone. Nonetheless, SPHK1 biphasic activation appears to occur; this is shown by the later increase in SPHK1 activity when THP-1 were in the presence of PC-3 cells, an effect that is not observed when THP-1 cells were cultured alone (**Fig.5.6a**).

SPHK1 mRNA expression levels were also measured in THP-1 cells following their co-culture with PC-3 cells (**Fig.5.6b**). After one and three hours of co-culture there were no observed differences in SPHK1 expression when THP-1 were cultured alone or co-cultured with PC-3 cells. However, after 24 hours of co-culture, the presence of PC-3 cells induced an approximate 1.5-fold increase in SPHK1 mRNA in THP-1 cells.

To evaluate whether the molecular mechanisms underpinning the THP-1-induced effects in PC-3 cells were similar to those identified for U937 cells, it was important to determine if intracellular signalling pathways such as NF-KB, Akt or ERK1/2 were also being activated in THP-1 upon co-culture with PC-3 cells. Contrary to U937 cells (**Fig.5.3a**), co-culture of THP-1 with PC-3 did not result in an increase of NF-KB, Akt or ERK1/2 phosphorylation (**Fig.5.6c**). In fact, the presence of PC-3 cells induced a decrease in the phosphorylation levels of these molecules across all analysed time points (**Fig.5.6c**)

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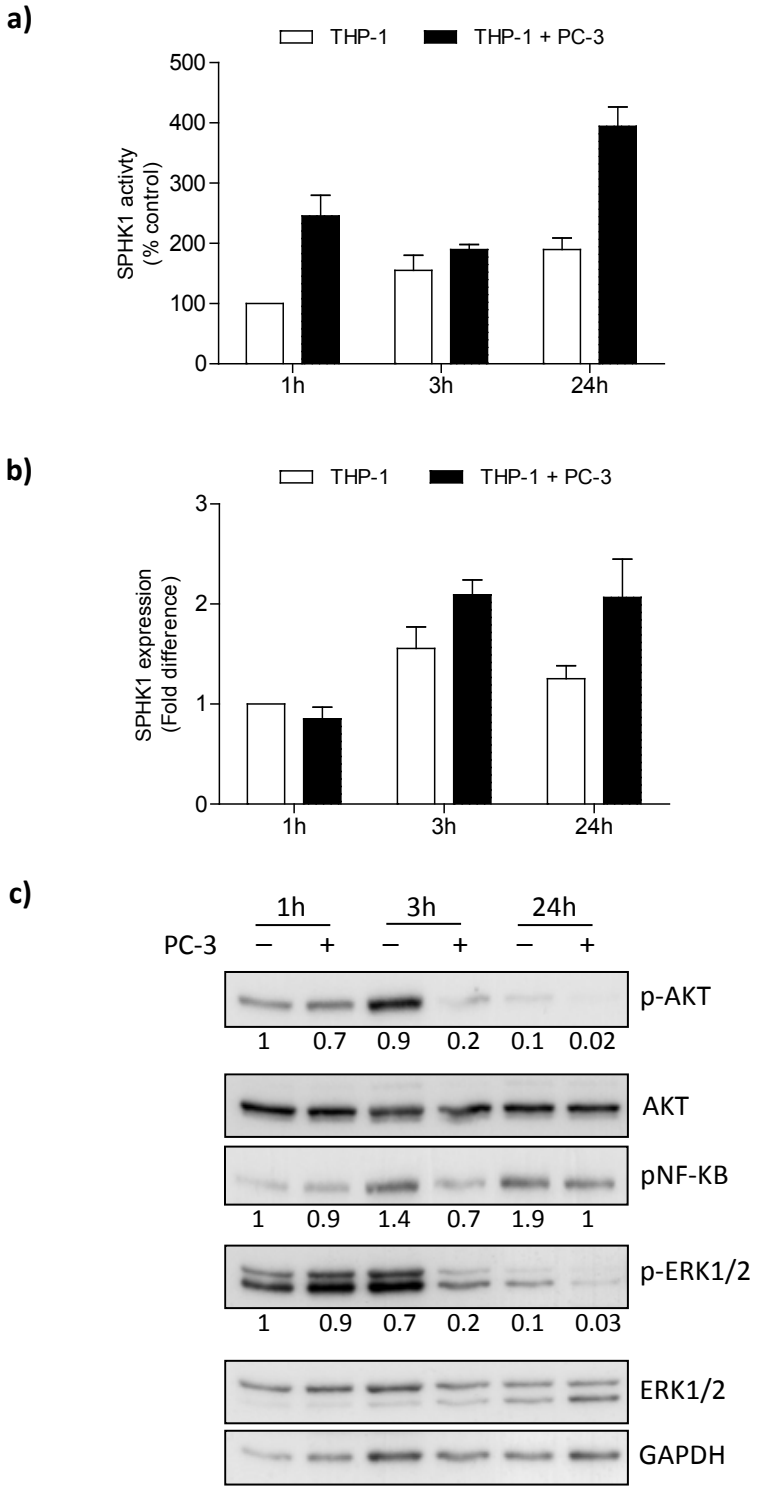


Figure 5.6: SPHK1 activity and expression levels in human THP-1 cells following co-culture with PC-3 cells. PC-3 cells were seeded in the bottom chamber under serum-free media 24 hours before the addition of THP-1 cells to the upper chamber for the indicated duration. **a)** SPHK1 activity and **b)** expression were determined in THP-1 cell lysates containing equal amounts of protein and mRNA respectively using radiolabelling and qRT-PCR respectively. For qRT-PCR, SPHK1 mRNA expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. **c)** NF-kB, AKT and ERK1/2 phosphorylation and total levels were determined by western blotting, quantified by Image J software and normalised to GAPDH. Data shown is **a-b)** mean ± SEM of two independent experiments and **c)** representative of one independent experiment.

5.5 Effect of MDA-231 breast cancer cells on SPHK1 levels in U937 monocytic cell line

As observed with THP-1 cells inducing an increase in PC-3 cell proliferation, MDA-231 cells were also more proliferative when in co-culture with U937. To evaluate whether SPHK1 levels in U937 cells were affected in a similar way as to when in co-culture with PC-3 cells, a co-culture assay was performed with U937 cells and MDA-231 cells. As shown in figure **5.7a**, the co-culture with MDA-231 induced a decrease in SPHK1 enzymatic activity in U937 cells after three hours. A further three hours later no difference in SPHK1 activity between U937 cells cultured alone or co-cultured with MDA-231 was observed, whereas following 24 hours of co-culture MDA-231 were able to induce a modest increase in SPHK1 activity in U937 cells (**Fig.5.7a**).

As with previous co-culture experiments, SPHK1 mRNA levels in U937 cells were also measured after being cultured alone or in the presence of MDA-231 cells. As shown in figure **5.7b**, MDA-231 induced a marginal decrease in SPHK1 mRNA levels of U937 cells across all studied time points, suggesting that the U937 cells-induced increase in MDA-231 proliferation is not regulated by SPHK1 in U937 cells (**Fig.5.7b**).

Next, it was important to analyse NF-KB, Akt and ERK1/2 phosphorylation levels in order to understand if phosphorylation events in these molecules were of a similar pattern to that observed for SPHK1. A western blotting analysis on U937 cell lysates revealed a MDA-231-mediated increase of 1.4- and 1.5-fold in NF-KB and Akt phosphorylation levels respectively after three hours of co-culture (**Fig.5.7c**). This effect proved to be transient as following six and 24 hours of co-culture no differences in NF-KB and Akt phosphorylation levels were observed (**Fig.5.7c**). ERK1/2 phosphorylation was not altered at any of the studied time points.

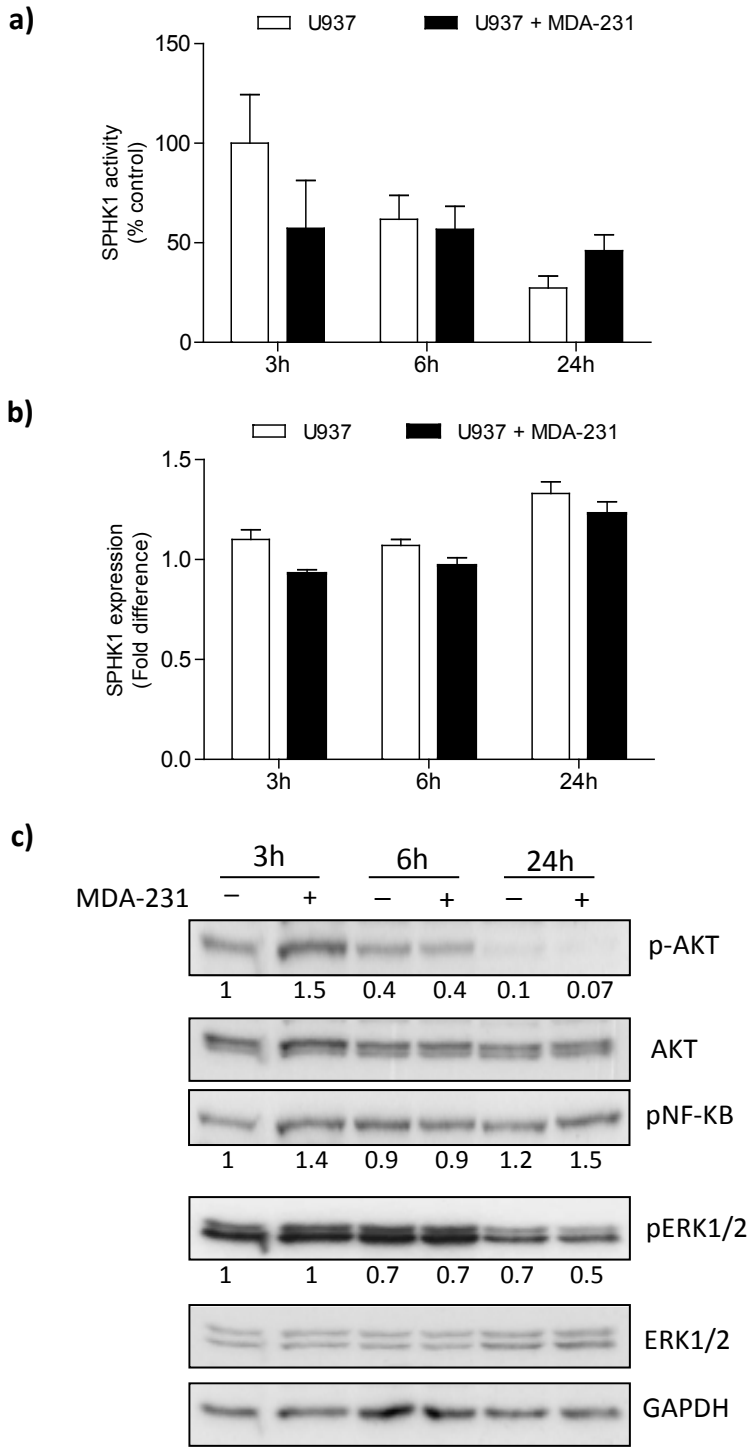


Figure 5.7: SPHK1 activity and expression levels in human U937 cells following co-culture with MDA-231 cells. MDA-231 cells were seeded in the bottom chamber under serum-free media 24 hours before the addition of U937 cells to the upper chamber for the indicated duration. **a)** SPHK1 activity and **b)** expression were determined in U937 cell lysates containing equal amounts of protein and mRNA respectively using radiolabelling and qRT-PCR respectively. For qRT-PCR, SPHK1 mRNA expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. c) NF-kB, AKT and ERK1/2 phosphorylation and total levels were determined by western blotting, quantified by Image J software and normalised to GAPDH. Data shown is representative of one independent experiment performed in duplicate (**a-b**).

5.6 Effect of conditioned media on U937 cells

As shown previously, U937 and PC-3 cells are able to communicate though signalling molecules resulting in an activation of several intracellular pathways. To date, it remains unclear whether cancer cells act as the trigger to initiate the activation of these pathways or if the first stimulus is derived from a monocyte secreted molecule. In an attempt to further elucidate the roles of each cell type in the orchestration of the cross-talk, conditioned media from PC-3 cells cultured for 24 hours in starvation conditions was added to the bottom chamber of a transwell to mimic the presence of PC-3 cells. U937 cells were then added to the top chamber and allowed to incubate for the indicated time points (**Fig.5.8a-b**). As seen in figure **5.8a**, SPHK1 mRNA expression levels in U937 cells were not altered by the presence of conditioned media when compared with serum-free co-culture alone. The presence of conditioned media negatively regulated Akt and ERK1/2 phosphorylation as in both cases a decrease in the phosphorylation levels of each in comparison with U937 cells cultured without conditioned media was observed (**Fig.5.8b**). These results indicate that the initial increase in SPHK1, Akt and ERK1/2 levels could result by an initial activation of PC-3 cells caused by U937 cells that, in turn, are activated by PC-3 cells in a secondary activation loop.

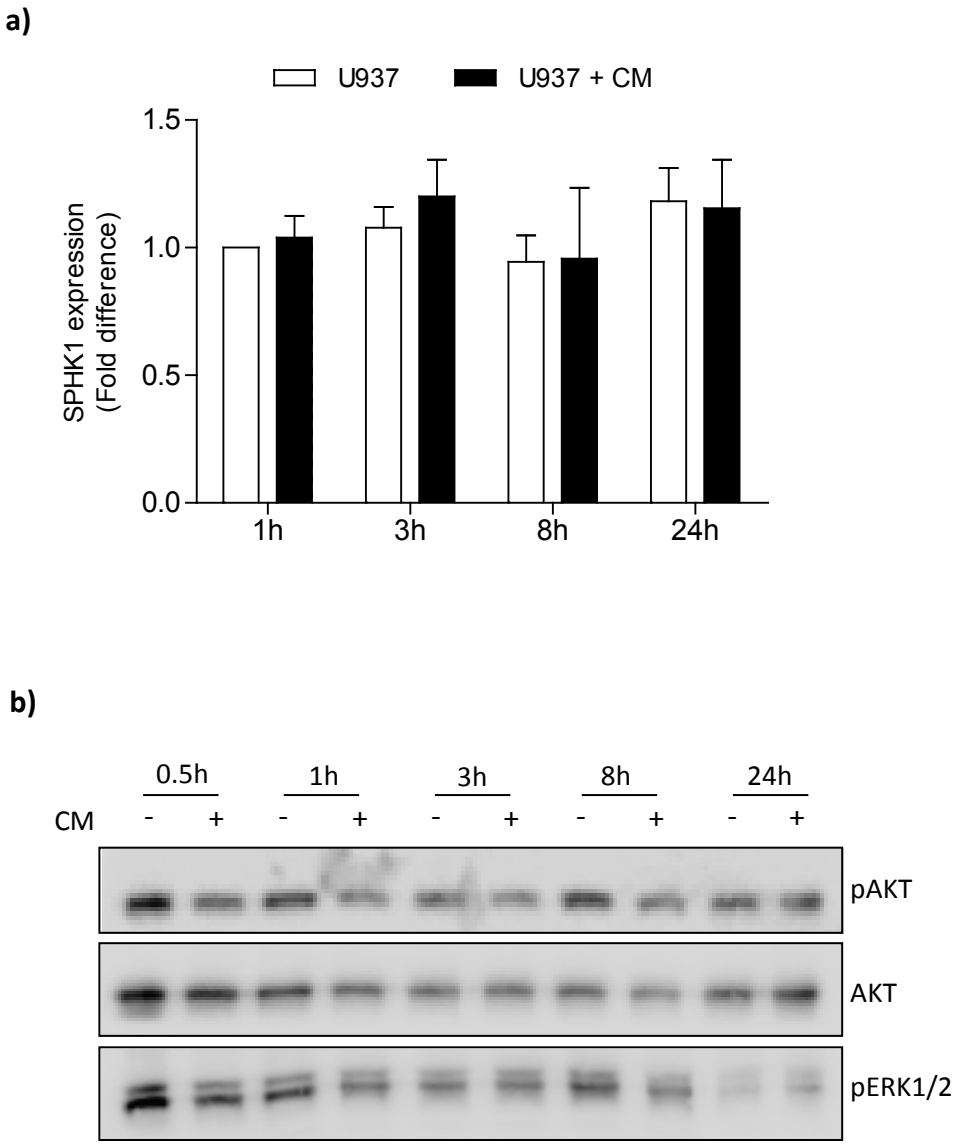


Figure 5.8: Effect of conditioned media on U937 cells. PC-3 cells were cultured for 24 hours under serum-free media. The conditioned media (CM) was collected and centrifuged to remove any PC-3 cells debris. CM was then added to the bottom chamber of the transwell and U937 cells were added to the top chamber and left incubating for the indicating time. **a)** SPHK1 expression in U937 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise SPHK1 expression. **b)** AKT and ERK1/2 phosphorylation and total levels were determined by western blotting. Data shown is the **a)** mean of two and **b)** representative of one independent experiment.

5.7 The effect of S1P on U937 cells

Previously, I hypothesised that an increase in SPHK1 would lead to the production and secretion of S1P that could act in a paracrine manner during co-culture. To understand if S1P could indeed be responsible for the activation of not only SPHK1 but also other important mediators such as Akt and ERK1/2, U937 cells were treated with exogenous S1P for the indicated times (**Fig5.9**). As shown in figure **5.9a**, the addition of S1P led to an initial increase in SPHK1 enzymatic activity after three and six hours of treatment, followed by a decrease at 24 hours (**Fig.5.9a**). SPHK1 mRNA levels were also increased by S1P treatment; this effect was observed after 24 hours with an approximate 1.8-fold increase in comparison with non-treated U937 cells (**Fig.5.9b**).

The addition of S1P to U937 cells also led to an increase in Akt phosphorylation with a constant activation throughout all the time points observed; this effect was most pronounced following one and 24 hours of S1P treatment (**Fig.5.9c**). In terms of ERK1/2 phosphorylation, a similar constant activation was observed with the most accentuated differences observed at one and eight hours with a 1.4- and 2-fold increase respectively (**Fig.5.9c**).

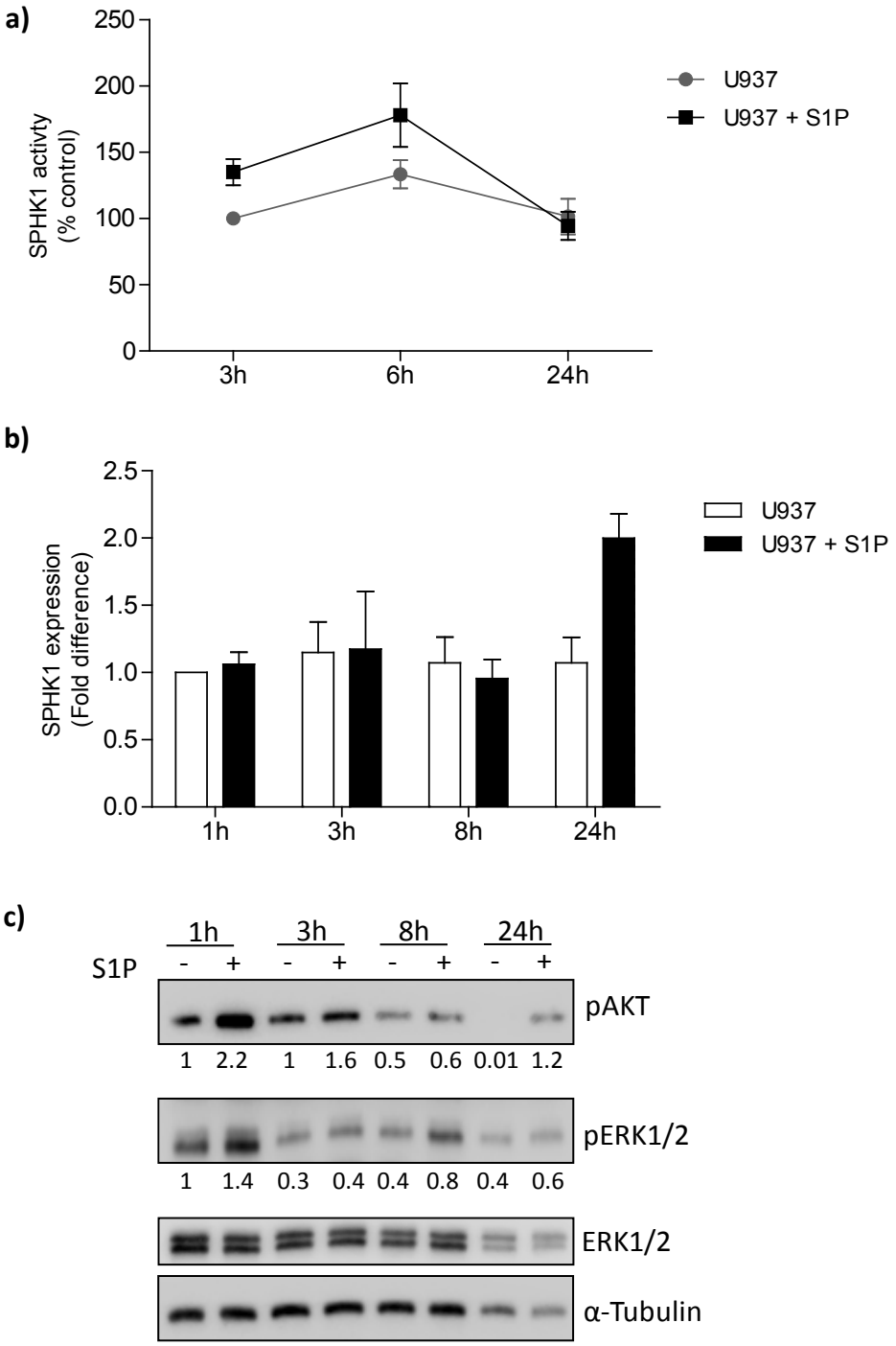


Figure 5.9: Changes in SPHK1 and signal transduction pathways in U937 cells following S1P treamtent. a) U937 cells were seeded in a 6-well plate before the addition of S1P (1μM) for the indicated times. **a)** SPHK1 activity and **b)** SPHK1 expression were determined in U937 cell lysates containing equal amounts of protein or mRNA respectively using radiolabelling and qRT-PCR respectively. For qRT-PCR, SPHK1 mRNA expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. **c)** AKT and ERK1/2 phosphorylation and total levels were determined by western blotting in U937 cells, quantified by Image J software and normalised to GAPDH. Data shown is the representative of **a), c)** one and **b)** the ± SEM of two independent experiment performed in duplicate .

5.8 MCP-1 regulation in U937 cells during co-culture with PC-3cells

With MCP-1 and IL-6 levels being increased in PC-3 cell media when co-cultured with U937 cells, it was important to evaluate their role in SPHK1 regulation and the activation of Akt and ERK1/2 in U937 cells. Interestingly, U937 MCP-1 expression is increased by the presence of PC-3 cells at three and 24 hours (**Fig.5.10a**). This increase is correlated to an increase in secreted MCP-1 levels in the media in which U937 cells were present (**Fig.5.10b**). ELISA analysis of U937 cell conditioned media showed a reduction in MCP-1 extracellular levels in comparison with U937 co-cultured with PC-3 transfected with a non-targeting siRNA, and abolished the effect of PC-3 cells when compared to U937 cells alone. (**Fig.5.11a**). However, this reduction was not accompanied by a reduction in MCP-1 expression in U937 cells at any of the studied time points (**Fig.5.11b**). The effect of Akt and ERK1/2 knockdown in PC-3 also had an impact upon MCP-1 mRNA levels in U937 cells. Surprisingly, at one hour, MCP-1 expression was increased when these two molecules were subject to knockdown in PC-3 cells; however this effect was reversed at three hours where there is no increase in U937 cells cultured with PC-3 siAkt and a significant reduction in U937 cultured with PC-3 siERK1/2 in comparison with U937 cultured with PC-3 siNT cells (**Fig.5.11c**).

Previous results had shown that over-expression of SPHK1 led to an increase in MCP-1 expression and secretion in PC-3 cells (chapter 4, **Fig.4.7**); therefore it was important to understand if this over-expression in PC-3 cells could have an impact on MCP-1 and SPHK1 regulation in U937 cells during their co-culture. As described previously (chapter 4, **Fig.4.8**) PC-3 constitutively over-expressing SPHK1 (PC-3/OFP-SPHK1) and control vector (PC-3/OFP) were co-cultured with U937 cells for the indicated time points. U937 SPHK1 enzymatic activity showed an increase at 24 hours in response to both PC-3/OFP and PC-3/OFP-SPHK1 (**Fig5.12a**). A similar effect was observed for SPHK1 mRNA, where no effect of altering the SPHK1 levels in PC-3 cells was detected (**Fig.5.12b**). Similarly for MCP-1 expression, no difference was observed between PC-3/OFP and OFP-SPHK1 (**Fig.5.13a**). However, MCP-1 levels in the media proximal to U937 cells were higher when these cells were co-cultured with PC-3/OFP-SPHK1 at three and 24 hours compared to PC-3/OFP. This effect was reduced when PC-3/OFP-SPHK1 were transfected with SPHK1 siRNA following three and 24 hours of co-culture, although the reduction did not prove to be statistically significant (**Fig.5.13b**).

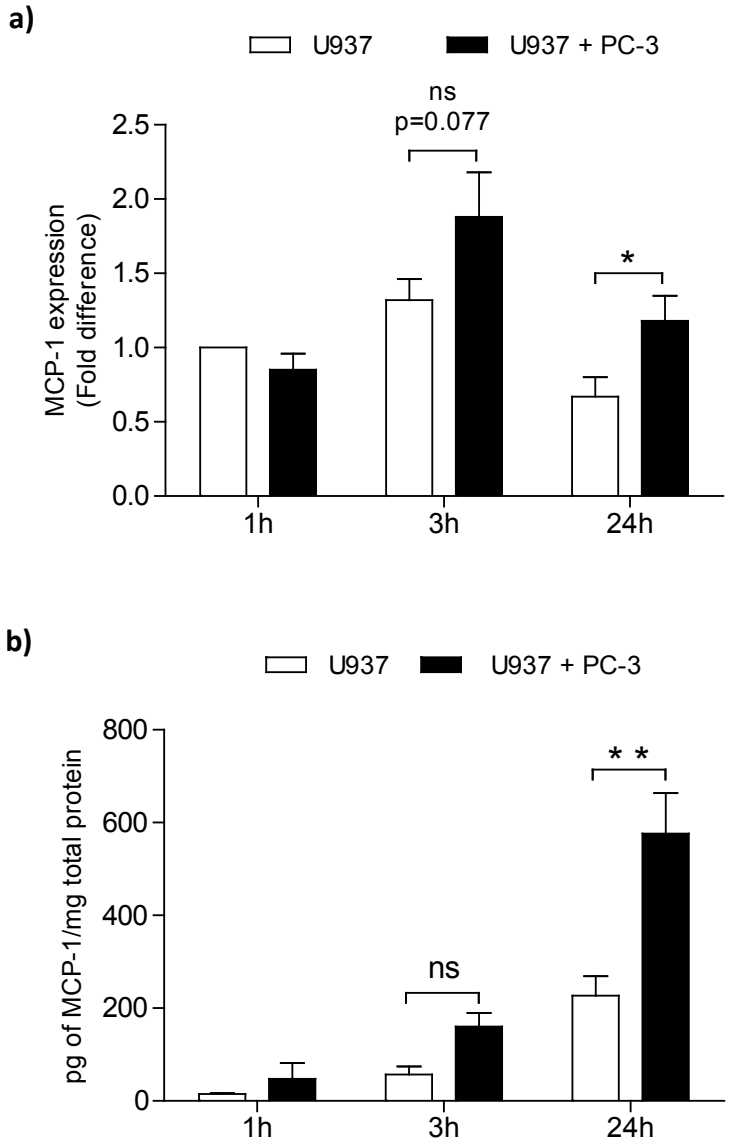


Figure 5.10: MCP-1 regulation in U937 cells during co-culture. PC-3 cells were seeded in the bottom chamber for 24 hours under starvation before the addition of U937 cells to the upper chamber and co-cultured for the indicated duration. **a)** MCP-1 expression in U937 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise MCP-1 expression. **b)** MCP-1 concentration in the media of the top compartment where U937 cells were present was determined by ELISA. Media were incubated with MCP-1 specific antibodies and a standard curve was used to quantify the relative amount of MCP-1 in each sample. Protein concentration of each cell lysate was used for normalisation. Data shown is the mean ± SEM of three independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, * $p<0.05$, ** $p<0.01$, for U937 vs. U937+PC-3

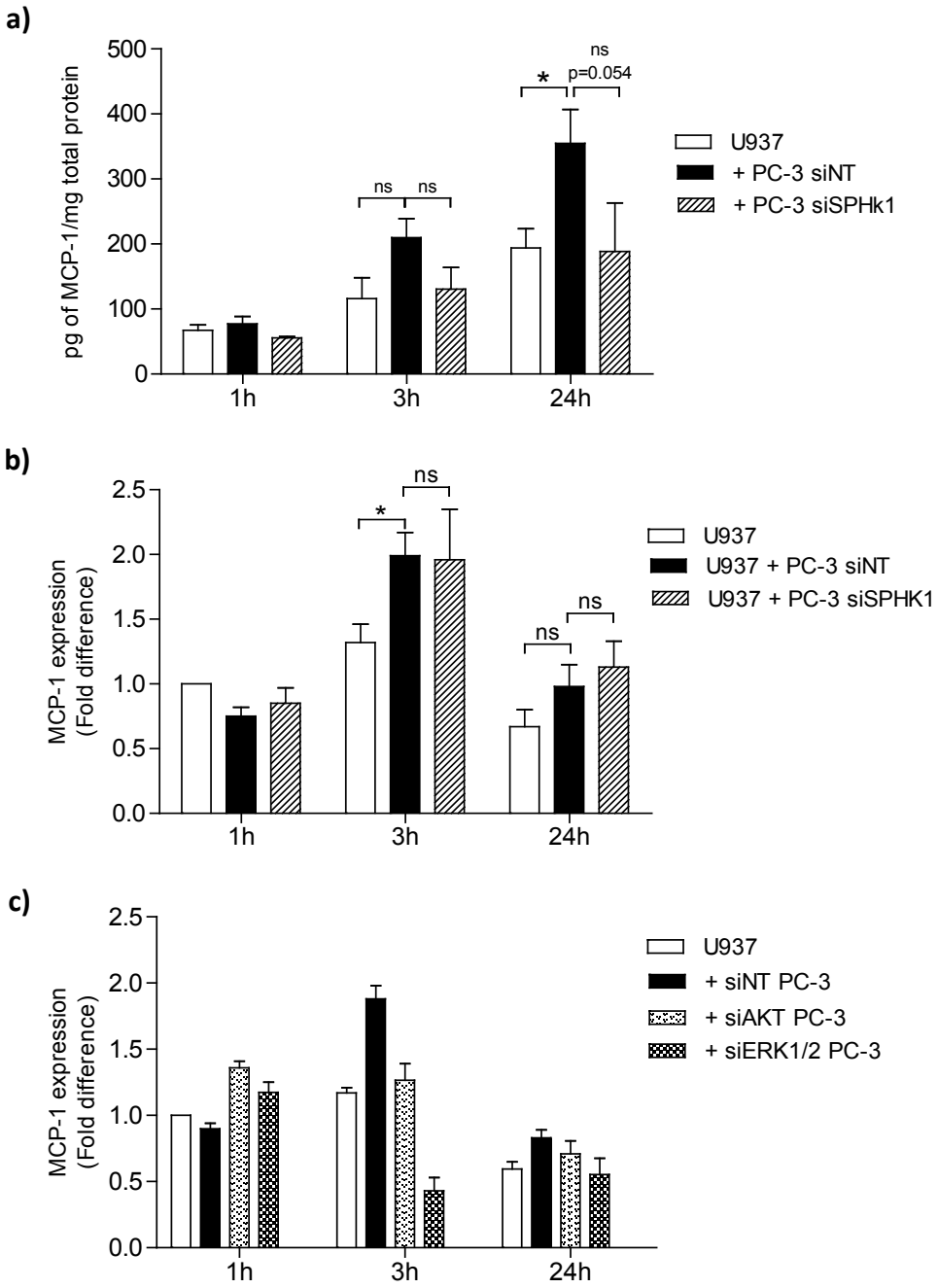


Figure 5.11: MCP-1 regulation in U937 cells during co-culture with PC-3 treated with several targeting siRNAs. PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1), AKT (siAKT), ERK1/2(siERK1/2) or non-targeting siRNA (siNT) for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. **a)** MCP-1 concentration in the media of the top compartment where U937 cells were present was determined by ELISA. Media were incubated with MCP-1 specific antibodies and a standard curve was used to quantify the relative amount of MCP-1 in each sample. Protein concentration of each cell lysate was used for normalisation. **b-c)** MCP-1 expression in U937 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise MCP-1 expression. Data shown is the mean ± SEM of **a-b)** three and **c)** one independent experiments performed in duplicate. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$, **$p<0.01$, for U937 vs. U937+siNTPC-3 or U937 + siCP-3 vs. U937 + siSPHK1 PC-3.

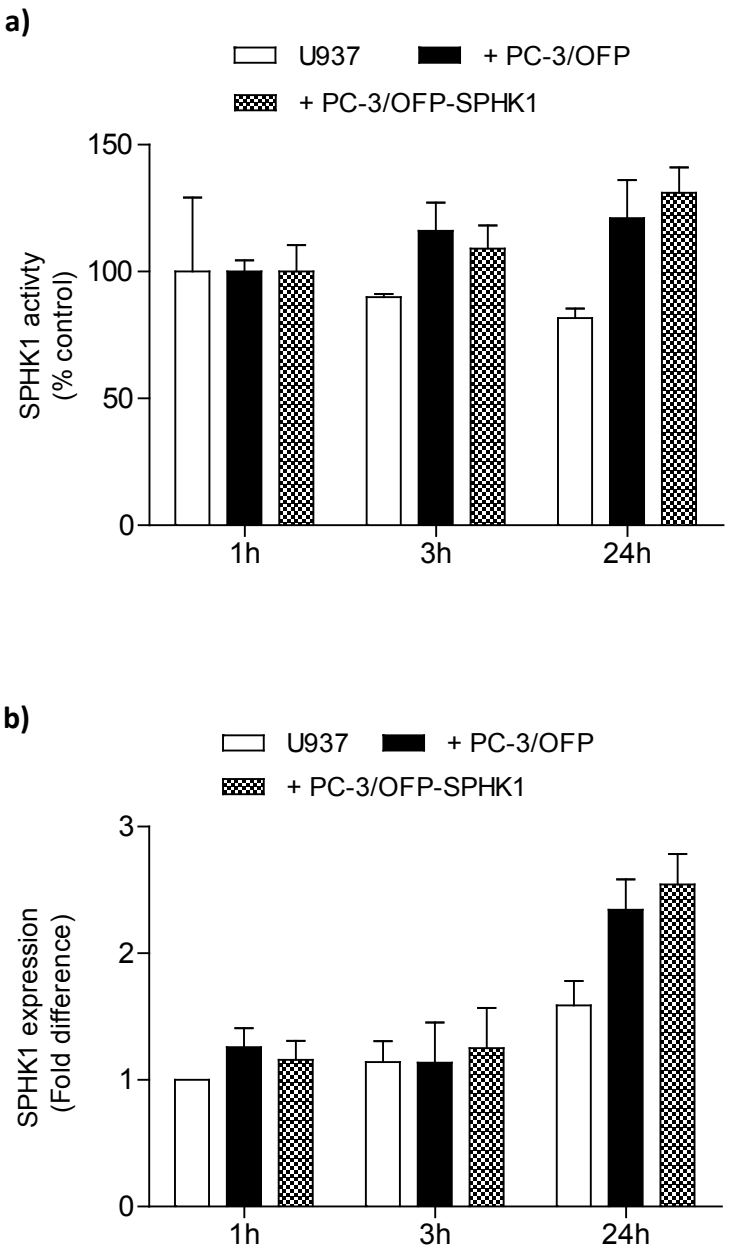


Figure 5.12: SPHK1 activity and expression levels in human U937 cells following co-culture with PC-3 cells over-expressing SPHK1. PC-3 cells over-expressing SPHK1 (PC-3/OFP-SPHK1) or empty phmKO2 vector (PC-3/OFP) were seeded in the bottom chamber for 24 hours under serum-free media before the addition of U937 cells to the upper chamber for the indicated duration. **a)** SPHK1 activity and **b)** expression were determined in U937 cell lysates containing equal amounts of protein or mRNA respectively using radiolabelling and qRT-PCR respectively. For qRT-PCR, SPHK1 mRNA expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. Data shown is the mean ± SEM of two independent experiments performed in duplicate.

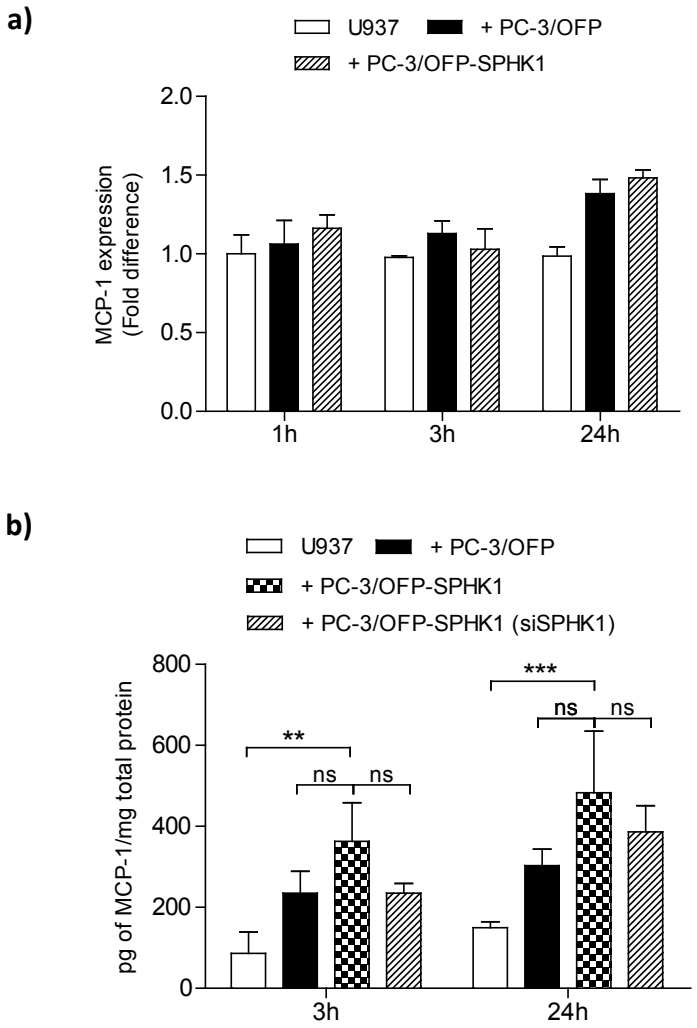


Figure 5.13: MCP-1 expression and secretion in human U937 cells following co-culture with PC-3 cells over-expressing SPHK1. **a)** PC-3 cells over-expressiong SPHK1 (PC-3/OFP-SPHK1) or empty phmKO2 vector (PC-3/OFP) were seeded in the bottom chamber under serum-free media 24 hours before the addition of U937 cells to the upper chamber for the indicated duration. **a)** MCP-1 expression in U937 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. **b)** PC-3/OFP-SPHK1 were transfected with siRNA targeting SPHK1 for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. In the other indicated co-culture (U937 + PC-3/OFP), cells were culture as described in (a) with the exception of the seeding that occurred 48 hours before the addition of U937 cells. MCP-1 concentration in the media of the top compartment where U937 cells were present was determined by ELISA. Media were incubated with MCP-1 specific antibodies and a standard curve was used to quantify the relative amount of MCP-1 in each sample. Protein concentration of each cell lysate was used for normalisation. Data shown is the mean $\pm$ SEM of **a)** two and **b)** three independent experiments performed in one single replicate. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$ for U937 vs. U937+PC/OFP-3 or U937+PC-3/OFP vs. U937+PC-3/OFP-SPHK1 or U937+PC-3/OFP-SPHK1 vs. U937+PC-3/OFP-SPHK1 (siSPHK1) .

With MCP-1 levels being increased during co-culture of U937 and PC-3 cells, it was natural to investigate the role of MCP-1 in SPHK1 regulation and activation of other signalling pathways, such as Akt and ERK1/2, in U937 cells. Human recombinant MCP-1 (rhMCP-1) was added to U937 cells at a concentration of 100ng/ml, and resultant SPHK1 enzymatic activity (**Fig.5.14a**) and transcriptional activation (**Fig.5.14b**) were measured at the indicated time points. rhMCP-1 induced a rapid increase in SPHK1 enzymatic activity until one hour; however this increase proved transient as no increase was observed at the later time points (**Fig.5.14a**). SPHK1 mRNA was increased by the addition of rhMCP-1 only after 24 hours (**Fig.5.14b**), suggesting a possible involvement of MCP-1 cascade signalling in SPHK1 transcriptional regulation. Furthermore, it was decided to investigate whether MCP-1 was a key molecule in Akt and ERK1/2 phosphorylation in U937 cells. As seen in figure **5.14c**, rhMCP-1 did not increase either Akt or ERK1/2 phosphorylation; in fact, there was a decrease at one and three hours, which was an unexpected finding. However as this experiment was performed only once, firm conclusions cannot be drawn from this preliminary data.

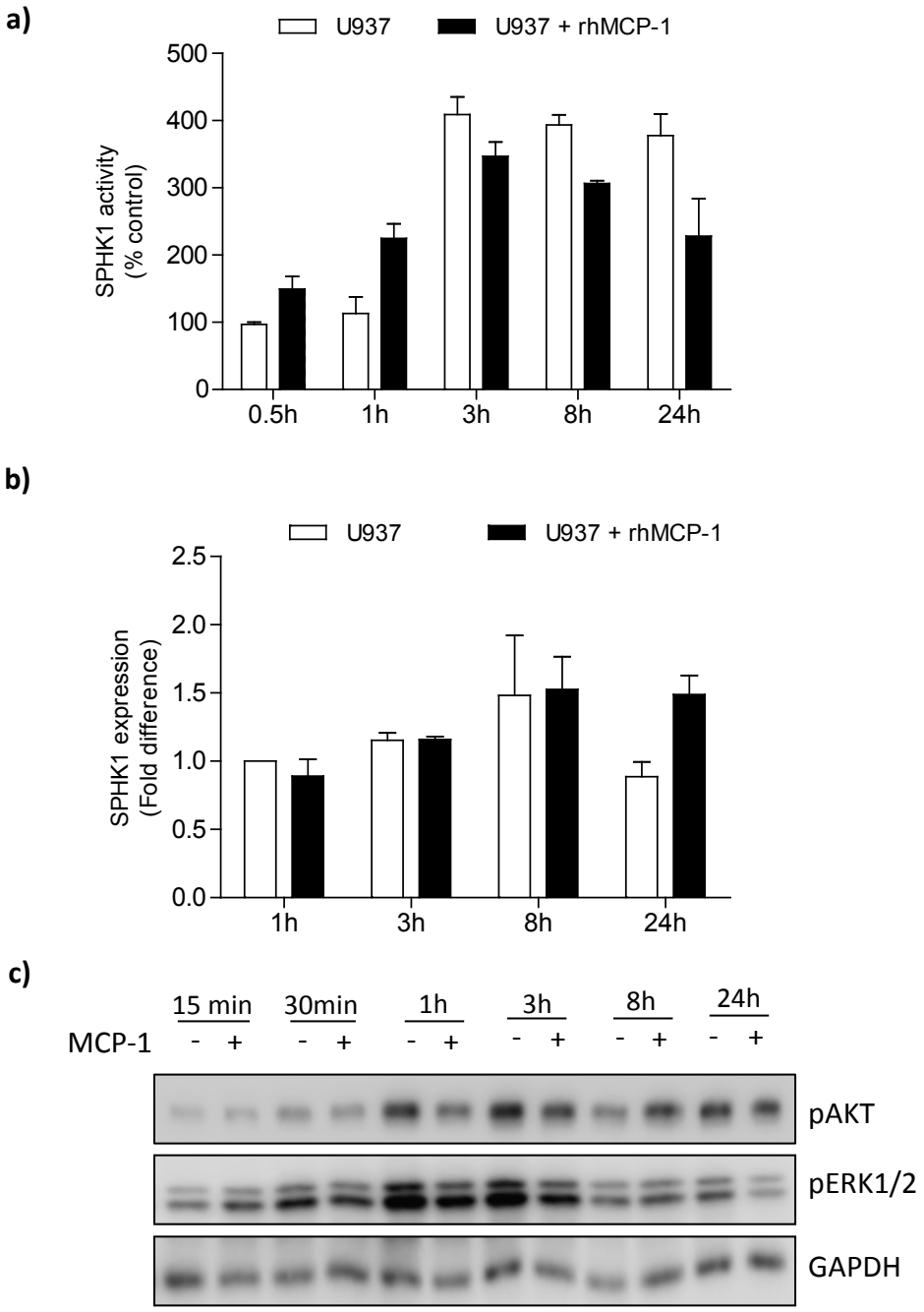


Figure 5.14: Effect of human recombinant MCP-1 on signal transduction pathways in U937 cells. U937 cells were seeded in a 6-well plate before the addition of MCP-1 (100ng/µl) for the indicated times. **a)** SPHK1 activity and **b)** SPHK1 expression were determined in U937 cell lysates containing equal amounts of protein or mRNA respectively using radiolabelling and qRT-PCR respectively. For qRT-PCR, SPHK1 mRNA expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. **c)** AKT and ERK1/2 phosphorylation and total levels were determined by western blotting in U937 cells, quantified by Image J software and normalised to GAPDH. Data shown is the mean ± SEM of **a-b)** two and **c)** one independent experiment performed in duplicate .

5.9 IL-6 regulation in U937 cells during co-culture with PC-3 cells

In order to understand the significance of the role of IL-6 in U937 cells during co-culture with PC-3 cells a similar study to that designed for MCP-1 was performed. Firstly, IL-6 expression and secretion levels were measured in U937 cells upon co-culture with PC-3 cells. As seen in figure **5.15a**, IL-6 mRNA was increased in U937 cells when stimulated with PC-3 cells; this effect was highest at one hour of co-culture. Analysis of IL-6 secretion in U937 cell supernatant showed an increase in IL-6 extracellular levels over time, with the highest levels of secretion observed upon 24 hours of co-culture (**Fig.5.15b**). The use of SPHK1 siRNA in PC-3 cells, and subsequent co-culture with U937 cells, led to a modest reduction in IL-6 expression in U937 cells at one and 24 hours, whilst at three hours a modest increase was observed. However, and despite no statistical analysis, this variation in IL-6 mRNA does not appear to be significant (**Fig.5.16a**). However, Akt or ERK1/2 siRNA transfected PC-3 cells induced different IL-6 mRNA responses in U937 cells. Akt knockdown in PC-3 cells had no effect on IL-6 expression at one hour, but was shown to decrease IL-6 transcriptional activation at three and 24 hours respectively (**Fig.5.16b**). The knockdown of ERK1/2 in PC-3 cells reduced the previously observed increase in IL-6 mRNA induced by PC-3 cells transfected with non-targeting siRNA at one hour. This effect was also observed at three hours, albeit to a lesser extent (**Fig.5.16b**). Unfortunately IL-6 extracellular levels for the different siRNA co-cultures (**Fig.5.16a and b**) were not possible to obtain due to technical difficulties during the IL-6 ELISA.

To further investigate the role of IL-6 in SPHK1, Akt and ERK1/2 regulation in U937 cells, human recombinant IL-6 (rhIL-6) was added to U937 cells at a final concentration of 100ng/ml. It was interesting to note that rhIL-6 induced a rapid SPHK1 transcriptional activation causing statistically significant 1.5- and 1.4-fold increases at one and three hours respectively (**Fig.5.17a**). In terms of Akt phosphorylation, IL-6 induced a modest phosphorylation after both 30 minutes and one hour of treatment (**Fig.5.17b**). ERK1/2 phosphorylation was also increased with S1P treatment; this effect was apparent after 30 minutes and greater after one hour. STAT3 phosphorylation was used as a positive control for IL-6 treatment (**Fig.5.17a**).

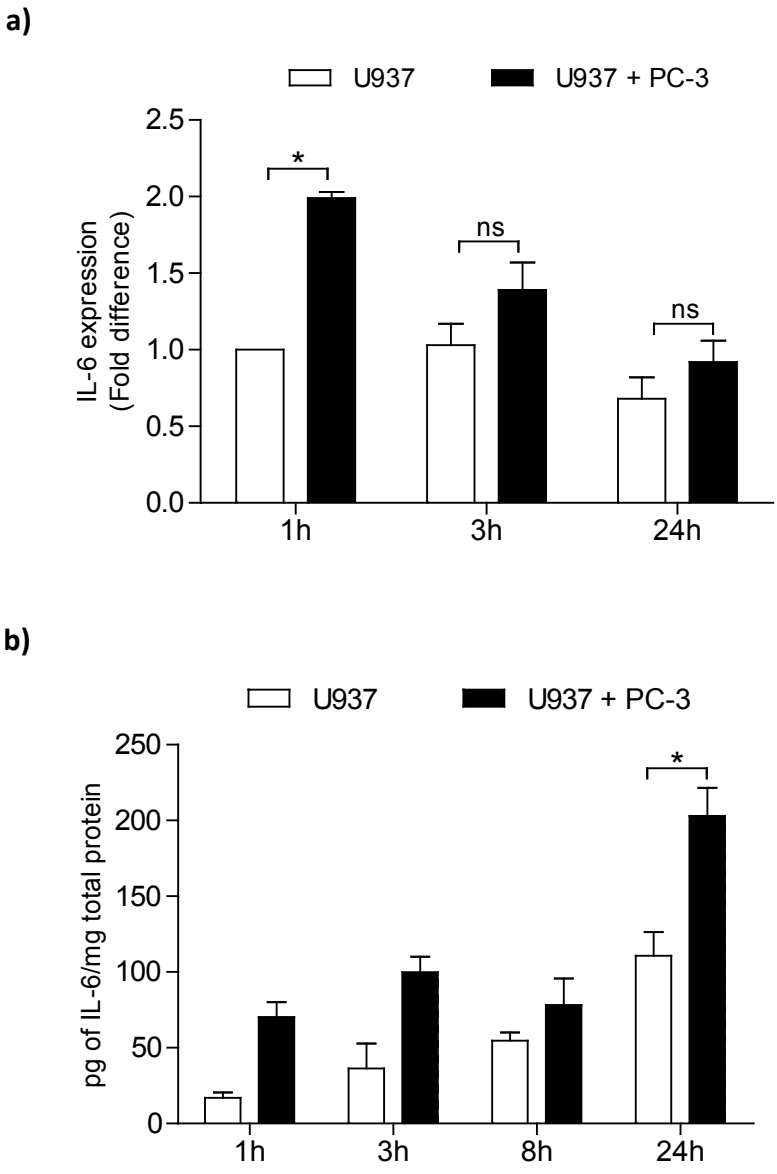


Figure 5.15: IL-6 regulation in U937 cells during co-culture. PC-3 cells were seeded in the bottom chamber under starvation 24 hours before the addition of U937 cells to the upper chamber and co-cultured for the indicated duration. **a)** IL-6 expression in U937 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. **b)** IL-6 concentration in the media of the top compartment where U937 cells were present was determined by ELISA. Media were incubated with IL-6 specific antibodies and a standard curve was used to quantify the relative amount of IL-6 in each sample. Protein concentration of each cell lysate was used for normalisation. Data shown is the mean ± SEM of three independent experiments performed in duplicate. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$, **$p<0.01$, for U937 vs. U937+PC-3

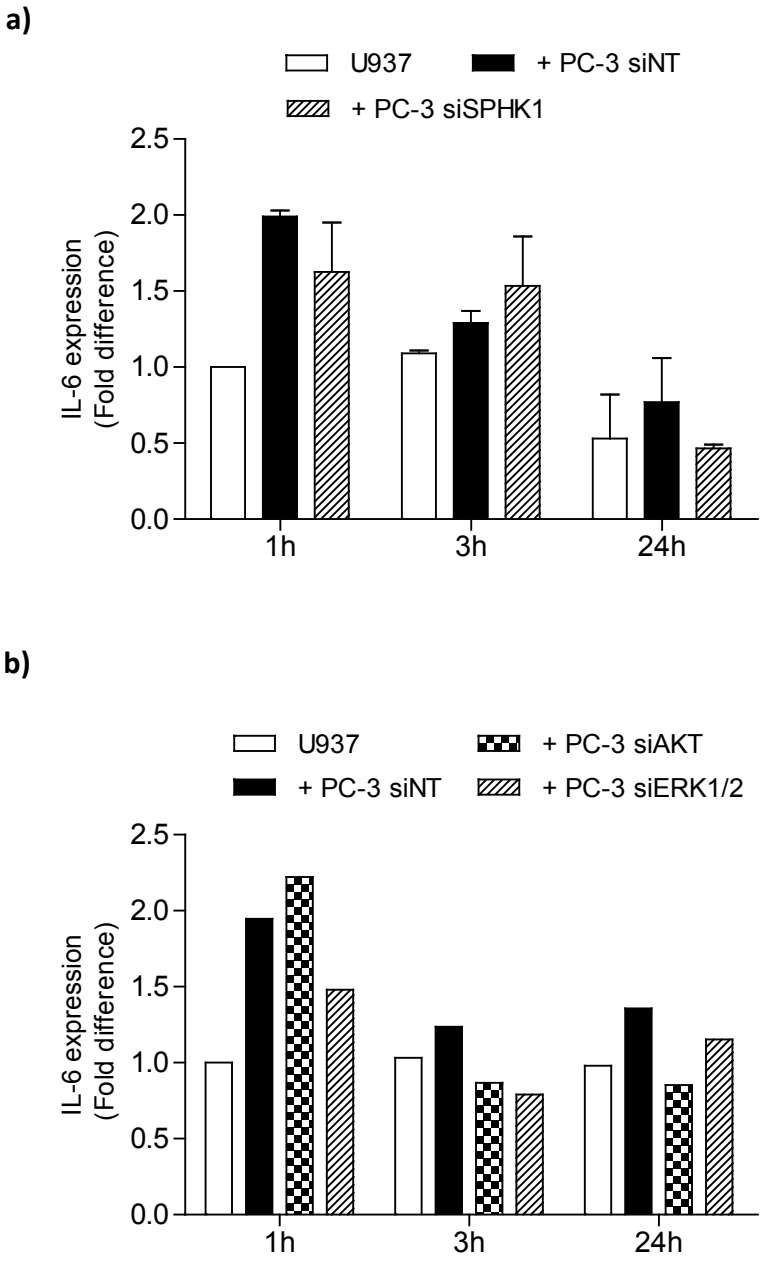


Figure 5.16: IL-6 regulation in U937 cells during co-culture with PC-3 treated with several targeting siRNAs. a) PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1), or non-targeting siRNA (siNT) for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. IL-6 expression in U937 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. **b)** PC-3 cells were transfected with siRNA targeting AKT (siAKT), ERK1/2 (siERK1/2) or non-targeting siRNA (siNT) were cultured as described above (a). IL-6 expression was determined by qRT-PCR as described above **(a)**. Data shown is the **a)** mean ± SEM of two independent experiments performed in duplicate or **b)** fold-difference to control (U937 cell cultured alone) of a single biological replicate.

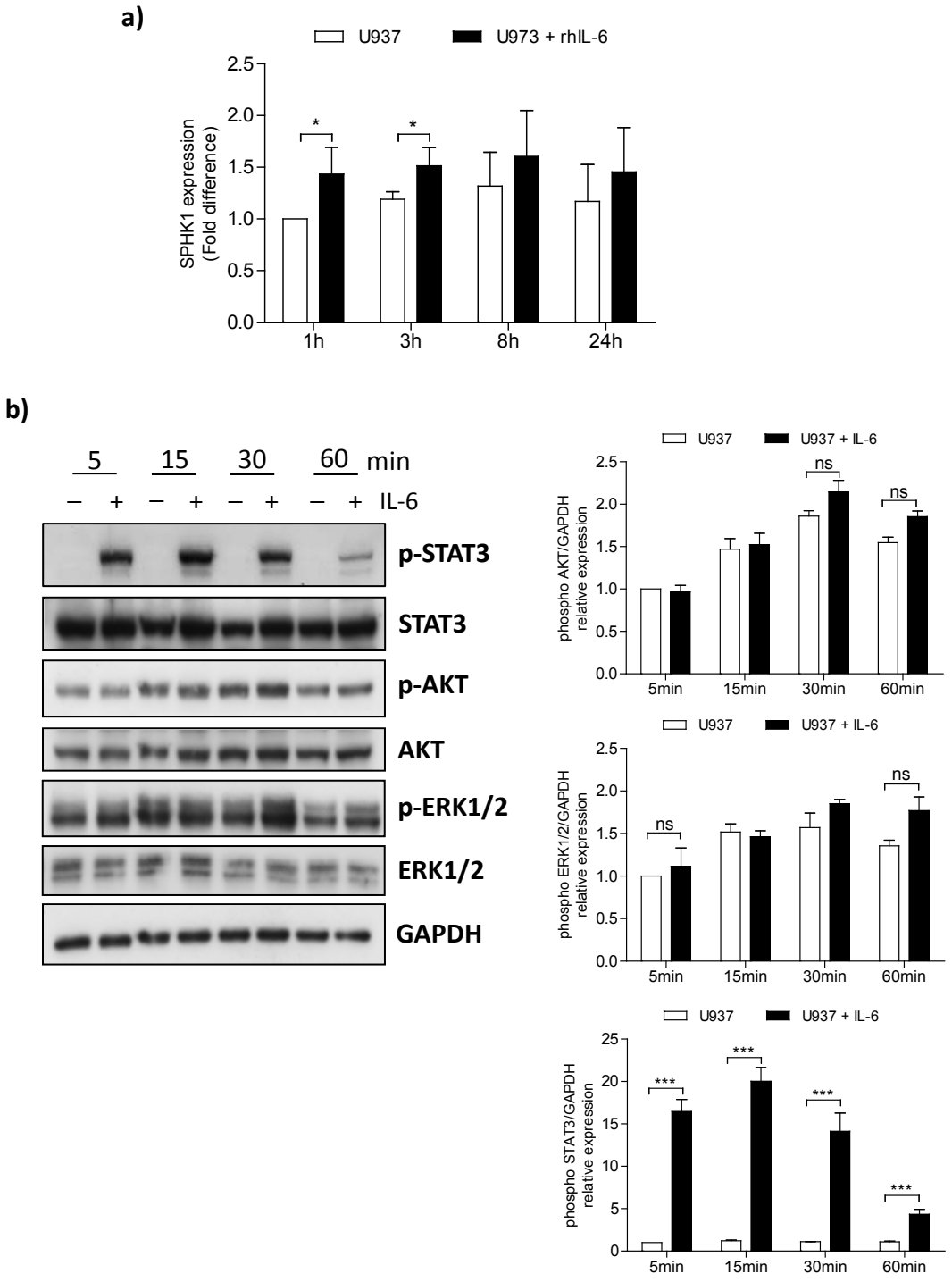


Figure 5.17: Effect of human recombinant IL-6 on signal transduction pathways in U937 cells. U937 cells were seeded in a 6-well plate before the addition of IL-6 (100ng/µl) for the indicated times. **a)** SPHK1 expression in U937 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. **b)** AKT, ERK1/2 and STAT3 phosphorylation and total levels were determined by western blotting in U937 cells, quantified by Image J software and normalised to GAPDH. Data shown is the mean ± SEM of three independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$, **$p<0.01$ or ***$p<0.001$ for U937 vs. U937+IL-6.

SPHK1 constitutive expression in PC-3 cells was previously shown to increase IL-6 expression and secretion. In this regard it was important to evaluate if this over-expression in PC-3 cells would increase IL-6 levels in U937 cells during co-culture. After one hour, IL-6 expression in U937 cells cultured with PC-3 over-expressing SPHK1 (PC-3/OFP-SPHK1) was up-regulated by approximately 1.8-fold in comparison with U937 cells cultured alone; however, no significant difference were observed when compared to U937 cells cultured with PC-3 cells containing the control vector (PC-3/OFP) (**Fig.5.18a**). A similar effect was observed on IL-6 extracellular levels where no differences were observed between PC-3 over-expressing SPHK1 and the control vector (**Fig.5.18b**). The use of siRNA targeting SPHK1 in PC-3 over-expressing SPHK1 did not make any difference to IL-6 extracellular levels in U937 cell proximity media (**Fig.5.18b**).

5.10 Cross-regulation between MCP-1 and IL-6 in U937 cells

With both IL-6 and MCP-1 being involved in the U937/PC-3 interaction, it was important to understand if there was any cross-regulation between these two proteins. Using cDNA from rhMCP-1-treated U937 cells, IL-6 expression was measured at the indicated time points (**Fig.5.19**). It was of great interest to note that MCP-1 can induce IL-6 transcriptional activation in U937 cells, with the highest effect apparent after 24 hours (**Fig.5.19a**). In U937 cells, rhIL-6 led to a consistent increase of IL-6 transcriptional levels across all studied time points, with the highest increase being observed after three hours (**Fig.5.19b**). Furthermore, MCP-1 expression was induced in rhIL-6-treated U937 cells; an effect that was observed after only three hours and reaching its highest at 24 hours where a 2-fold increase was observed (**Fig.5.19c**). This increase was accompanied by an increase in MCP-1 secretion, where IL-6 treatment led to a significant increase in MCP-1 secreted levels at eight and 24 hours (**Fig.5.19d**). These results not only indicate an autocrine role for IL-6 in U937 cells but also suggest that MCP-1 and IL-6 can regulate each other, most likely through the activation of their respective signalling cascades, resulting in an increase of their respective mRNA expression levels.

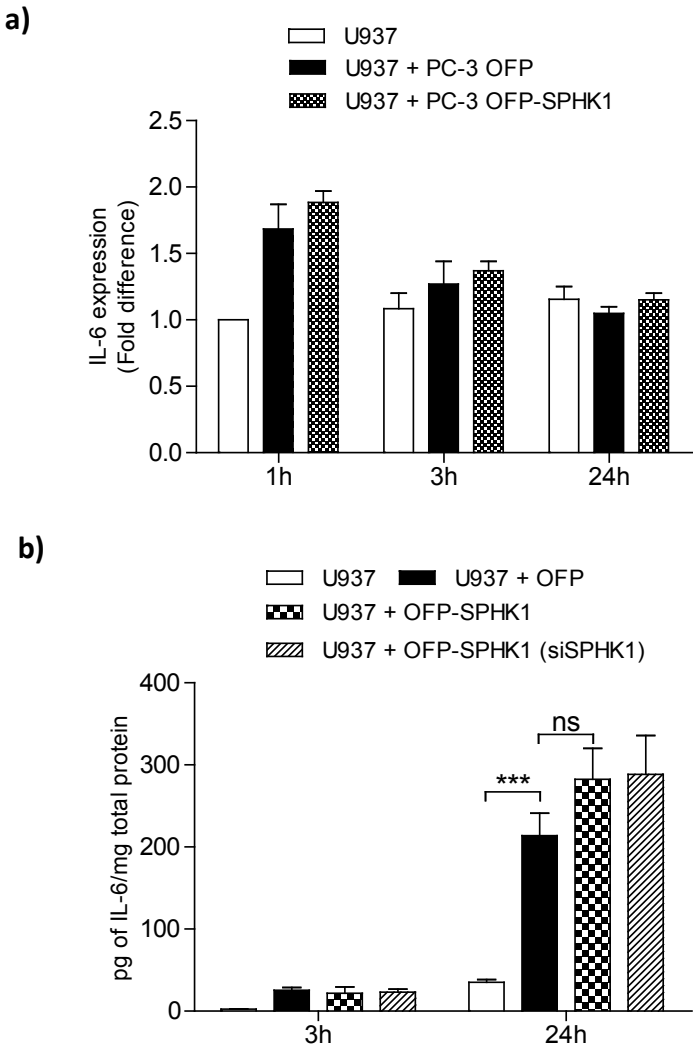


Figure 5.18: IL-6 expression and secretion in human U937 cells following co-culture with PC-3 cells over-expressing SPHK1. **a)** PC-3 cells over-expressing SPHK1 (PC-3/OFP-SPHK1) or empty phmKO2 vector (PC-3/OFP) were seeded in the bottom chamber for 24 hours under serum-free media before the addition of U937 cells to the upper chamber for the indicated duration. **a)** IL-6 expression in U937 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. **b)** PC-3/OFP-SPHK1 were transfected with siRNA targeting SPHK1 for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. In the other indicated co-culture (U937 + PC-3/OFP) cells were culture as described in **(a)** with the exception of the seeding that occurred 48 hours before the addition of U937 cells. IL-6 concentration in the media of the top compartment where U937 cells were present was determined by ELISA. Media were incubated with IL-6 specific antibodies and a standard curve was used to quantify the relative amount of IL-6 in each sample. Protein concentration of each cell lysate was used for normalisation. Data shown is the mean ± SEM of **a)** two and **b)** three independent experiments performed in 1 single replicate. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$ for U937 vs. U937+PC/OFP-3 or U937+PC-3/OFP vs. U937+PC-3/OFP-SPHK1 or U937+PC-3/OFP-SPHK1 vs. U937+PC-3/OFP-SPHK1 (siSPHK1) .

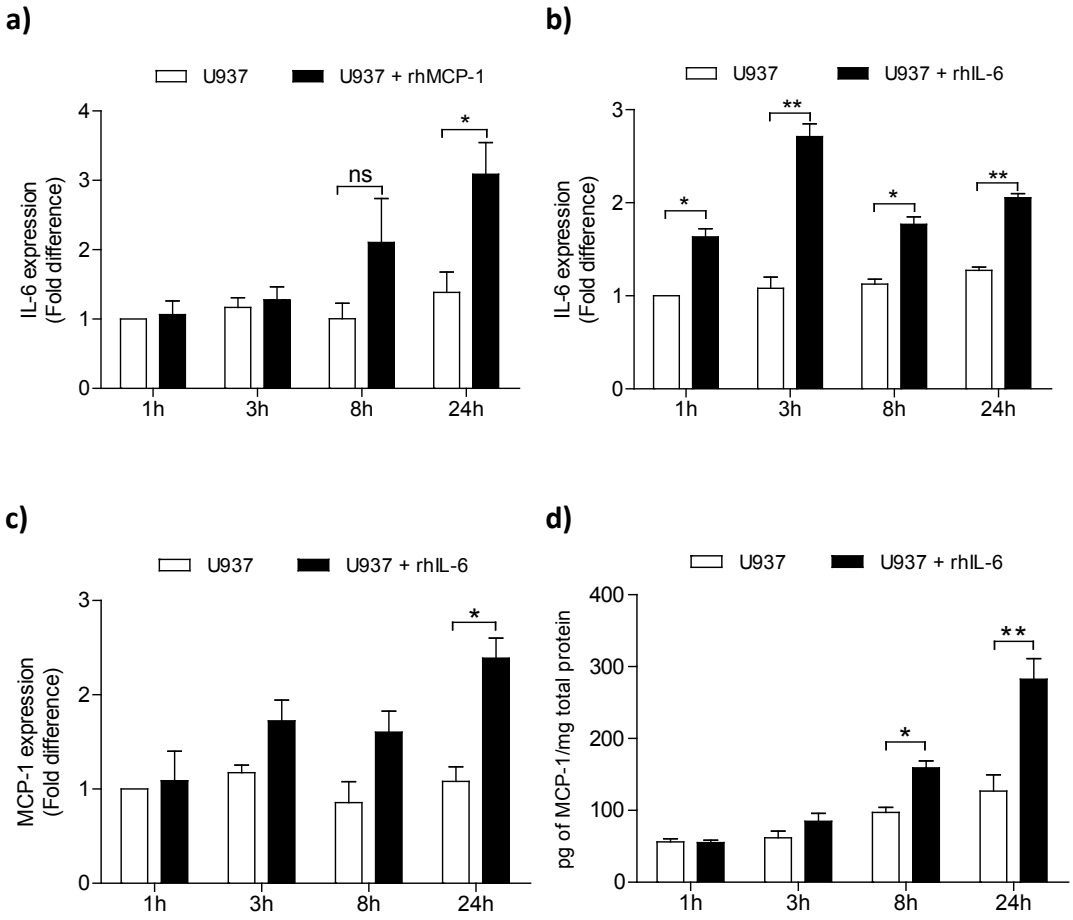


Figure 5.19: Effect of the IL-6/MCP-1 axis in U937 cells. U937 cells were seeded in a 6-well plate before the addition of **a)** MCP-1 (100ng/µl) or **b-d)** IL-6 (100ng/µ) for the indicated times. **a-b)** IL-6 expression and **c)** MCP-1 expression in U937 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used for normalisation. **d)** MCP-1 concentration in the media was determined by ELISA. Media were incubated with MCP-1 specific antibodies and a standard curve was used to quantify the relative amount of MCP-1 in each sample. Protein concentration of each cell lysate was used for normalisation. Data shown is the mean ± SEM of three independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$, **$p<0.01$, for U937 vs. U937+IL-6 or U937 vs. U937 + MCP-1.

Previously, I have shown that the addition of S1P to U937 cells resulted in an increase in SPHK1 activity and mRNA levels. Here, I sought to investigate whether S1P could also be involved in MCP-1 and IL-6 regulation and therefore contribute to the signalling loop involved in the regulation of these two inflammatory mediators. Figure **5**.**20a** shows that S1P addition to U937 cells led to an increase in MCP-1 expression; this effect was only visible 24 hours after S1P treatment, where a 1.2-fold increase was observed when compared to MCP-1 expression in untreated U937 cells. IL-6 expression in S1P-treated U937 cells was also measured; figure **5**.**20b** shows initial increases in IL-6 expression of 3- and 1.4-fold after one and three hours respectively. No differences in IL-6 expression were observed for the remaining time points.

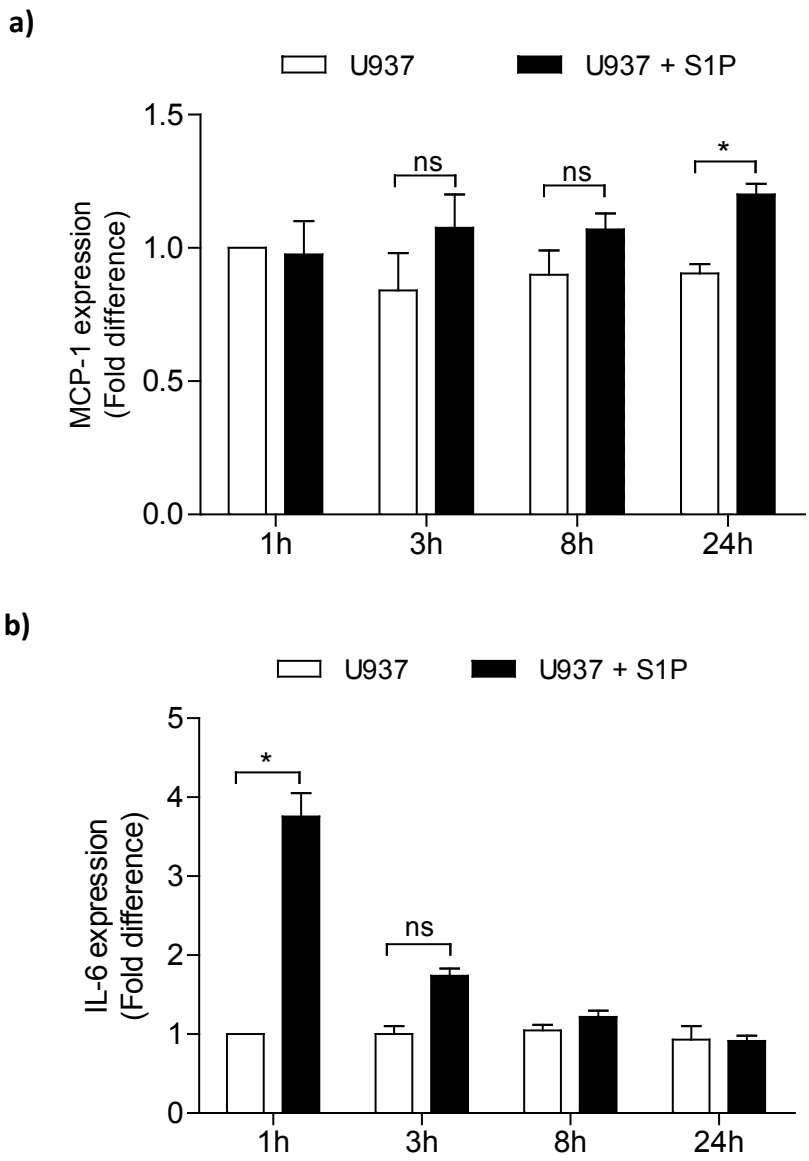
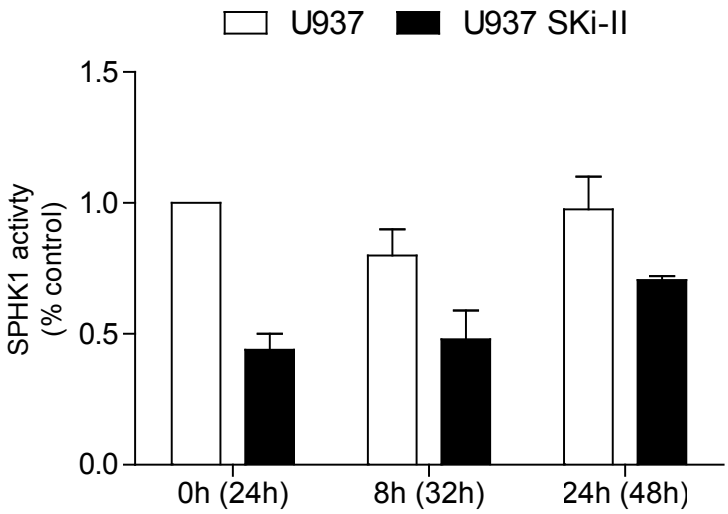


Figure 5.20: Effect of S1P on IL-6 and MCP-1 mRNA expression in U937 cells U937 cells were seeded in a 6-well plate before the addition of S1P (1μM) for the indicated times. **a)** MCP-1 expression and **b)** IL-6 expression in U937 cell lysates were determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used for normalisation. Data shown is the mean ± SEM of three independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$ for U937 vs. U937+S1P.

5.11 Effect of SPHK1 inhibition in U937 cells on PC-3 cells during co-culture

Finally, in order to understand if SPHK1 activation in U937 cells had an impact on SPHK1 levels in PC-3 cells, U937 cells were treated with SKi-II at a final concentration of 20μM. U937 cells were treated with the inhibitor for 24 hours before media was removed and cells washed with serum-free medium to remove any traces of the inhibitor. Cells were then cultured up to a further 24 hours in order to evaluate the levels of SPHK1 repression caused by the inhibitor, during which time-point samples were captured. SPHK1 activity in U937 cells was markedly reduced at recovery start point after 24 hours in the presence of SKi-II (0 hours post-inhibitor removal, 24 hours total) (**Fig.5.21a**); this inhibitory effect was maintained for a further eight hours (32 hours total), with an approximate 50% reduction in SPHK1 activity. A 40% reduction in SPHK1 activity was still present 24 hours after SKi-II removal (48 hours total) (**Fig.5.21a**); the persistence of this reduction is of significance as it corresponds to the 24 hour duration of the co-culture model used in these studies. In terms of SPHK1 mRNA, no significant differences were observed upon SKi-II treatment (**Fig.5.21b**).

a)



b)

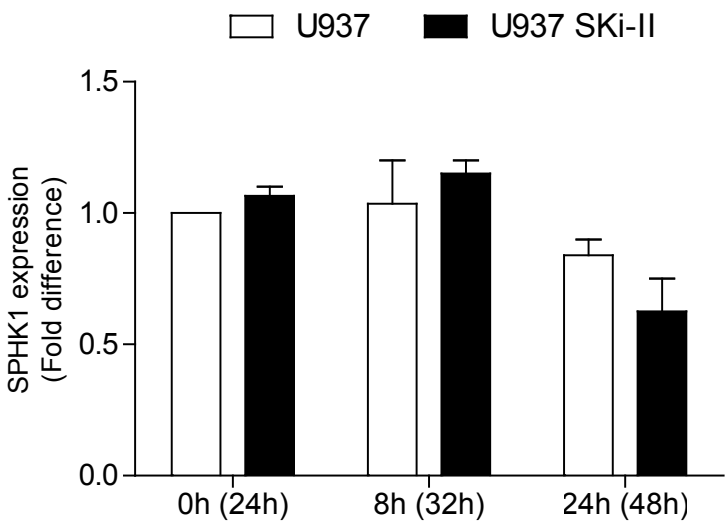


Figure 5.21: Effect of SKi-II on SPHK1 activity and expression in U937 cells. U937 cells were treated with SKi-II (20μM) for 24 hours in complete media. Following this incubation, cells were centrifuged and washed two times with PBS to remove any traces of SKi-II inhibitor. U937 cells were then re-suspended in serum –free media (0h(24h)) prior to culture into 6-well plates for a further 24 hours (24h(48h)). **a)** SPHK1 activity and **b)** expression were determined in U937 cell lysates containing equal amounts of protein or mRNA respectively using radiolabelling and qRT-PCR respectively. For qRT-PCR, SPHK1 mRNA expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. Data shown is the mean ± SEM of two independent experiments performed in duplicate.

Following the assessment of SPHK1 levels after treatment with SPHK1 inhibitor, U937 cells were treated for 24 hours prior to co-culture with PC-3 cells. As shown in figure **5.22ai**, SPHK1 inhibition in U937 cells induced an increase in SPHK1 mRNA in PC-3 cells after one hour of co-culture; at three and 24 hours the effect was reversed and there was a reduction in SPHK1 mRNA of approximately 30% and 35% respectively in PC-3 cells when in comparison to those cultured with non-treated U937 cells. MCP-1 expression was also determined in the same PC-3 cells lysates as described above. Following one hour of co-culture, U937 cells treated with the inhibitor induced a 50% increase in MCP-1 mRNA (**Fig.5.22aii**). This effect was reduced after three hours of co-culture, where no difference was observed for MCP-1 mRNA in PC-3 cells that were co-cultured with treated or non-treated U937 cells. At a later stage of co-culture (24 hours) PC-3 cells co-cultured with SKi-II showed reduced levels of MCP-1 expression of approximately 20% in comparison with PC-3 culture with non-treated U937 cells (**Fig.5.22aii**).

As shown previously (chapter 4 , **Fig.4.10**), U937 cells induced an early increase in IL-6 mRNA in PC-3 cells. Here I have shown that this effect was not affected by SPHK1 inhibition in U937 cells, as no reduction in IL-6 mRNA was identified in PC-3 cells after one hour (**Fig.5.22iii**). The remaining time points confirmed that SPHK1 inhibition in U937 cells did not affect IL-6 expression levels in PC-3 cells during co-culture (**Fig.5.22iii**).

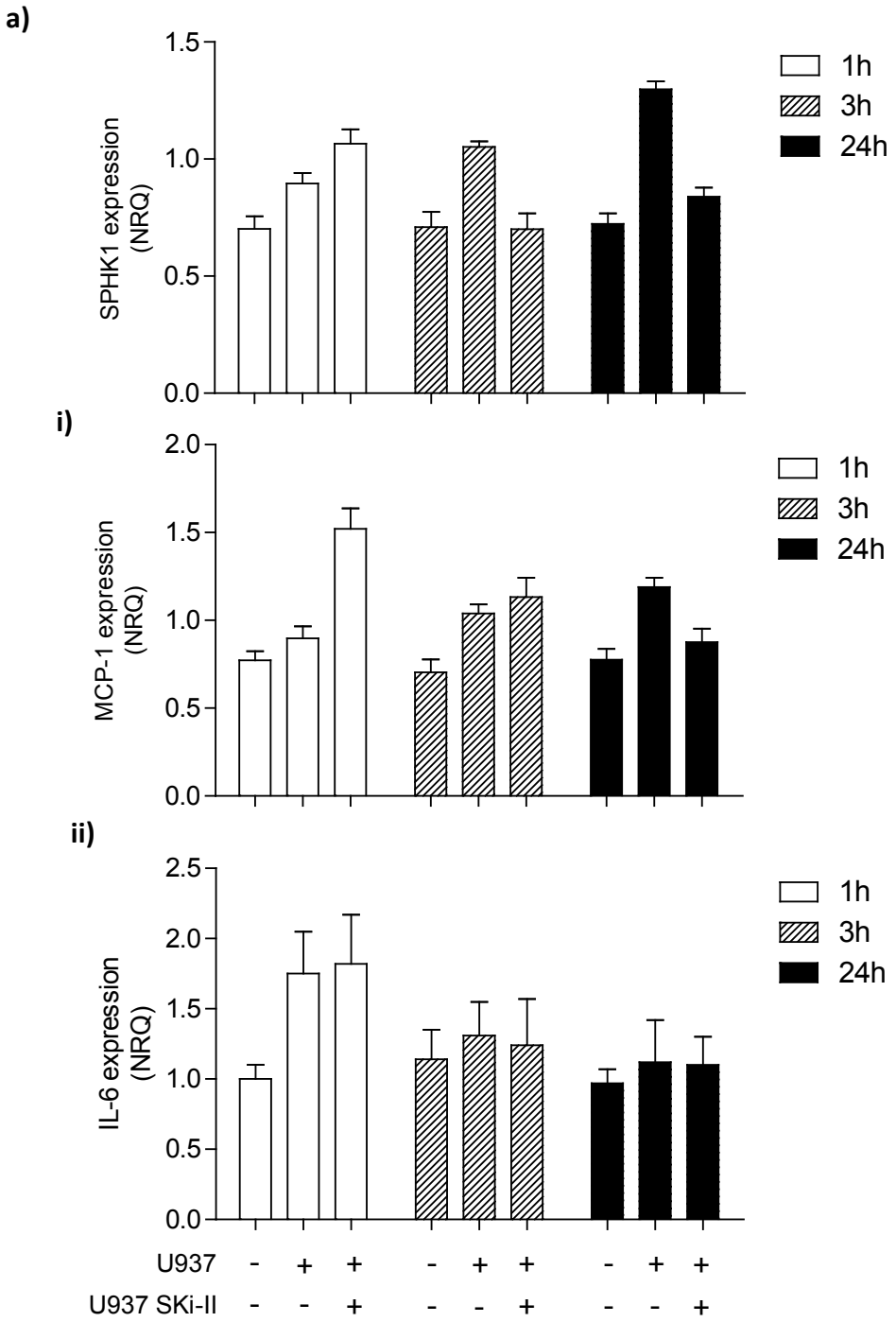


Figure 5.22: Effect of SKi-II on SPHK1, MCP-1 and IL-6 expression in PC-3 cells during co-culture with U937 cells. PC-3 cells were seeded into the bottom chamber for 24 hours under starvation before the addition of U937 cells. Simultaneously, U937 cells were treated with and without SKi-II (20μM) for 24 hours in complete media. Following this incubation, cells were centrifuged and washed two times with PBS to remove any traces of SKi-II inhibitor. For control U937 cells the same procedure was followed as to SKi-II treated U937 cells. Cells were then re-suspended in serum–free media before being added to the top chamber of the transwell with PC-3 cells cultured in the bottom chamber. **a)** SPHK1, **b)** MCP-1 and **c)** IL-6 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. Data shown is the mean ± SEM of one independent experiments performed in one single replicate.

5.12 Discussion

Inflammatory cells are known to play a well-recognised role in cancer progression and their interactions with tumour cells are important determinants of tumour biology. Paracrine loops between tumour and immune cells have emphasised the potential role of some molecules in tumour development. In this study SPHK1 enzymatic activity (**Fig.5.1a**) and expression (**Fig.5.1b**) in U937 cells and THP-1 cells (**Fig.5.6a-b**) are increased during their co-culture with PC-3 cells. This indicates that soluble signalling molecules secreted from PC-3 cells are inducing SPHK1 activation in monocytes. Surprisingly this activation only occurs when cells are in co-culture as the use of conditioned medium from PC-3 cells did not induce SPHK1 activation in U937 cells (**Fig.5.8a**). The activation of SPHK1 and other important signalling mediators (discussed later on) in U937 cells is therefore dependent on a bidirectional, rather than unidirectional, signalling loop whereby an initial "cross-talk" is crucial to trigger cell responses towards one another.

The increase in SPHK1 enzymatic activity may result from a post-translational modification rather than as a consequence of mRNA increase. SPHK1 has been described to have intrinsic catalytic activity independent of post-translational modifications; however, upon phosphorylation induced by different growth factors its enzymatic activity can be further increased [175]. It is therefore possible that during co-culture the increase in SPHK1 enzymatic activity in U937 cells is a result of a phosphorylation event caused by signalling molecules secreted from PC-3 cells.

For U937 cells the increase in SPHK1 activity is accompanied by an increase in SPHK1 mRNA levels at one and more significantly at three hours of co-culture (**Fig.5.1a-b**). In this case, the increase in mRNA could lead to a later increase in SPHK1 activity; however, there is a possibility of these events occurring in an independent manner as the transcriptional activation and the kinase enzymatic increase may result from different signalling mediators.

In THP-1 cells there was no increase in mRNA levels at an early stage of co-culture (**Fig.5.6b**), suggesting that the increase in SPHK1 activity (**Fig.5.6a**) was caused by signalling molecules secreted by PC-3 cells. The later increase, upon 24 hours of co-culture, in SPHK1 activity could be due to an increase in SPHK1 mRNA. Indeed, SPHK1 biphasic activation has

been described, where an initial increase in SPHK1 enzymatic activity is followed by a later increase in mRNA expression levels [190, 405].

The SPHK1 response to stimuli has been reported previously [179, 423]; however in a co-culture system I show for the first time that SPHK1 is not only activated in tumour cells, but also in monocytes. Curiously, this double activation appears to be extremely coordinated as there is a "synchronised" increase in SPHK1 for both tumour (chapter 4, **Fig.4.1a**-**b**) and immune cells (**Fig.5.1a**-**b**). This suggests that SPHK1 is involved in the secretion of signalling molecules that are able to regulate its own expression in the "partner cells", either through a common molecule (such as S1P, as discussed later on) or by several molecules, whose effect are time-dependent (as discussed in chapter 4). Interestingly, SKi-II treatment of U937 cells before co-culture with PC-3 cells provoked a reduction in SPHK1 mRNA in the latter cells (**Fig.5.22**); this finding reinforces the importance of SPHK1, with its activation in U937 cells seemingly a necessary factor for its activation in PC-3 cells. An interesting observation is made when siRNA is used to knockdown SPHK1 in PC-3 cells; this results in a lower SPHK1 enzymatic activation and lower mRNA levels in U937 cells (**Fig.5.2a-b**). This data also show for the first time that SPHK1 is involved in the regulation of one or more soluble molecule/s messaging from PC-3 to U937 cells and is responsible for SPHK1 activation in monocytes.

A possible candidate for such a soluble molecule (apart from those identified using the proteome profiler assay, chapter 4, **Fig.4.16**) would be S1P as it is directly regulated by SPHK1 through sphingosine phosphorylation. The use of siRNA against SPHK1 in PC-3 cells would lead to a reduction of available S1P for U937 cells, leading to a lower activation of SPHK1 in U937 cells. The increase in SPHK1 activity and mRNA (**Fig.5.9a-b**), together with an induction of Akt and ERK1/2 phosphorylation (**Fig.5.9c**), by S1P in U937 cells strongly indicates S1P as a possible mediator of the U937/PC-3 cell interaction. A more recent study shows that in RAW264.7 macrophages treated with exogenous S1P, SPHK1 mRNA levels were increased in a concentration-dependent manner. This effect was also accompanied by an increase in S1P secretion, indicating the existence of a self-regulatory effect through a positive feed-back loop [424]. In this current study, the reduction in Akt and ERK1/2 phosphorylation in U937 cells, caused by the use of SPHK1 siRNA in PC-3 cells (**Fig.5.4**), could also be credited to a reduction in S1P available in the media. The fact that S1P is able to activate both Akt and ERK1/2 phosphorylation levels in U937 cells supports the role of S1P as

a key mediator in PC-3/U937 cell interactions. Since both Akt and ERK1/2 are up-regulated during co-culture (**Fig.5.3**), a possible positive effect of Akt or ERK1/2 on S1P levels in U937 cells (dependent or independent of SPHK1) should also not be excluded. Other regulatory soluble molecules, aside from S1P, could also have an effect on the regulation of Akt (including VGEF,cAMP and GM-CSF), ERK1/2 (including IL-6, CD40 and VGEF) or NF-KB (IL-1 and TNF-α) in monocytes during cancer-monocyte interactions, including [226, 389].

According to my results it is unlikely that S1P is being initially secreted from PC-3 cells in the absence of U937 cells as conditioned media from PC-3 cells did not increase ERK1/2 phosphorylation or SPHK1 mRNA levels in U937 cells (**Fig.5.8a-b**). However, the presence of monocytes could lead to an increase in S1P secretion from PC-3 cells, triggering the U937 cells response. A study by Weigert *et al.*, using primary human macrophages, showed that S1P secreted from dying cells led to an increase in ERK1/2 and phosphoinositide 3-kinase (PIK3), a known upstream regulator of Akt, in comparison with normal Jurkat cells [254]. This re-enforces the idea that S1P may not be constantly secreted from cells but its induction can trigger differential cell type specific responses.

The use of Akt and ERK1/2 siRNA in PC-3 cells indicated that SPHK1 regulation in U937 cells is dependent on these pathways (**Fig.5.5a**); however, their importance appears to vary during co-culture. At the very initial phase of co-culture the SPHK1/S1P pathway is the main driver for its own activation in U937 cells and is independent of Akt or ERK1/2 activation in PC-3 cells. However at later stages, Akt and ERK1/2 also play an important role in this activation as their absence proved to be crucial for SPHK1-induced expression (**Fig.5.5a**). In order to draw more assertive conclusions from this preliminary data, further repetitions need to be performed. Also, the analysis of SPHK1 activity levels in U937 cells could be an important indicator as to when SPHK1 is involved in the regulation of these signalling transduction pathways.

To further determine if these effects were cell specific, and to understand a possible correlation between SPHK1, Akt and ERK1/2, I have assessed these pathways in other cell lines. The analysis of SPHK1 regulation in THP-1 cells during co-culture with PC-3 showed biphasic enzymatic activation (**Fig.5.6a**); however, in this case there was no increase in Akt or ERK1/2 phosphorylation, which suggests that SPHK1 is not being mediated by any of these

molecules in this system (**Fig.5.6b**). Furthermore, MDA-231 cells induced a transient increase in Akt and ERK1/2 phosphorylation in U937 cells but not in SPHK1 activity or mRNA (**Fig.5.7a-c**) suggesting activation of these pathways in MDA-231 does not require SPHK1 activation during their interaction with monocytes.

Altogether these findings suggest that cancer cell SPHK1 may be important for the secretion of molecules that may potentially activate SPHK1 in neighbouring monocytes, which in turn mediates its activation in PC-3 cells. Also, Akt and ERK1/2 regulation during cancer cell-monocyte interactions are also affected by SPHK1 regulation; however its importance as to when its role is essential is not clear and seems to vary according to time and cell type. A schematic representation of a possible mechanism for tumour and monocyte interactions is shown below (**Fig.5.23a**).

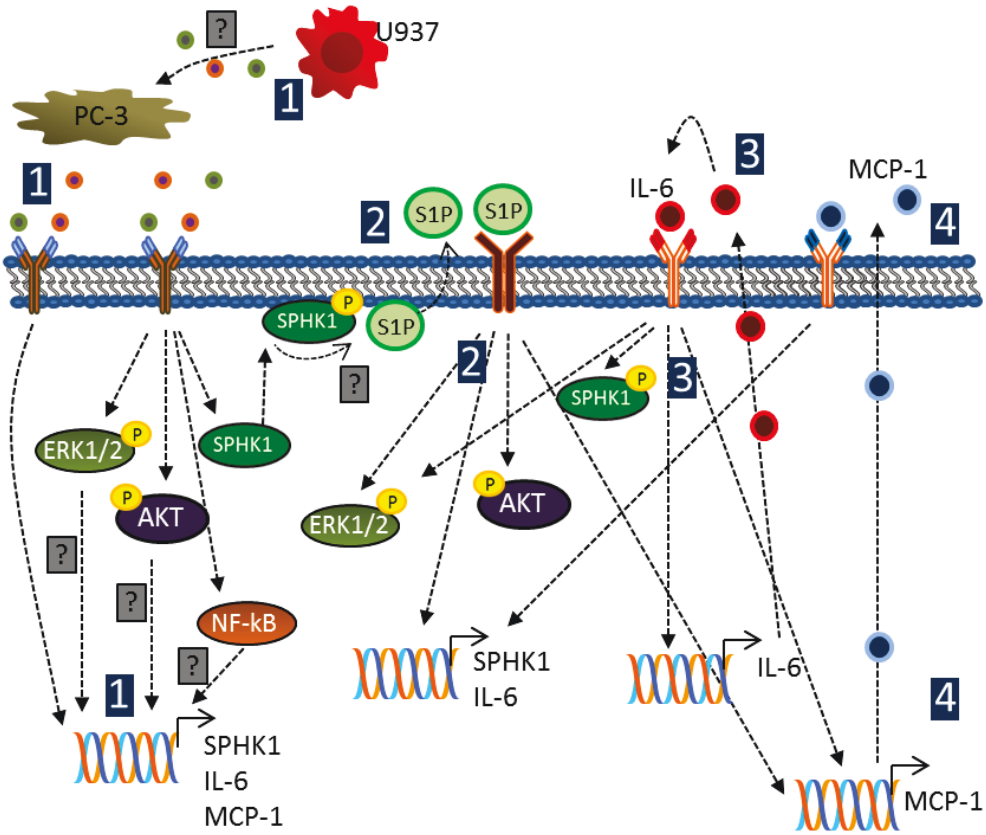


Figure 5.23a: Proposed model for SPHK1 activation and important signal transduction pathways in U937 during co-culture with PC-3 cells. 1) The secretion of so far unknown molecules by U937 cells leads to the secretion of also unidentified molecules by PC-3 cells. This initial cross-talk results in the activation of SPHK1 at mRNA levels and enzymatic activity, IL-6 and MCP-1 expression, as well as AKT, NF-KB and ERK1/2 phosphorylation in U937 cells. The involvement of these important signal transduction pathways in SPHK1 regulation remains unclear. **2)** SPHK1 is likely to produce S1P that can itself lead to the increase of AKT and ERK1/2 phosphorylation as well as transcriptional activation of several genes, including SPHK1, IL-6 and MCP-1. **3)** Extracellular IL-6 can induce an increase in SPHK1 and MCP-1 mRNA expression levels as well as acting in an autocrine manner to promote its own expression. IL-6 can induce a very modest increase in AKT phosphorylation, for ERK1/2 phosphorylation this effect appears to be stronger. **4)** MCP-1 can induce SPHK1 mRNA levels and enzymatic activity as well as IL-6 expression. The sequence of events may not reproduce the real dynamics existing between U937 and PC-3 cells as some of the represented events may occur simultaneously.

5.12.1 Signalling molecules involved in monocyte/cancer cell interactions

It is known that MCP-1 expression and macrophage infiltration correlates with a poor clinical prognosis. Moreover, the increase in MCP-1 expression from monocytes and tumour cells during their interaction was also described in a recent *in vivo* study. Human inflammatory monocytes were recruited to the site where pulmonary metastases of human breast cancer were formed; it was shown that MCP-1 expression was increased in both cell lines. Interestingly the use of an MCP-1 blocking antibody resulted in a decrease in the number of metastasis [313]. Together with previous findings in PC-3 cells (chapter 4, **Fig.4.7-9**), it was important to evaluate MCP-1 expression in U937 cells and to determine how MCP-1 can affect SPHK1 regulation in these cells during co-culture with PC-3 cells. The finding that MCP-1 expression and secretion levels are increased in U937 cells by the presence of PC-3 cells (**Fig.5.10a-b**) indicates the involvement of extracellular signalling molecules capable of inducing transcriptional activation of this chemokine in U937 cells. In this context, S1P could be a potential candidate; however, SPHK1 knockdown in PC-3 cells showed no effect on MCP-1 expression in U937 cells. Also, the over-expression of SPHK1 in PC-3 cells did not increase MCP-1 expression in U937 cells (**Fig.5.13a**) in comparison with control PC-3 cells (PC-3/OFP), indicating that S1P is not likely to be the key regulator in MCP-1 expression. It is important to note that the results obtained from PC-3 cells transfected with empty vector (PC-3/OFP) and PC-3 transfected with vector carrying SPHK1 (PC-3/OFP-SPHK1) may not be the best model to analyse the effect of SPHK1 over-expression in U937 cells. This is based on the observations that PC-3/OFP cells were not able to increase SPHK1 enzymatic activity or expression in U937 cells; an effect that was previously observed with parental PC-3 cells (**Fig.5.1a**-**b**). Therefore it is not possible to draw firm conclusions of the PC-3/OFP-SPHK1 effect on U937 cells as the expression profile and normal homeostasis of these cells may be affected by the vector used in this study, and therefore masking the effect of SPHK1 regulated signalling molecules on U937 cells.

SKi-II treatment in U937 cells did not alter the induced effect of PC-3 on MCP-1 expression in the monocytes at early stages of co-culture; in fact, a repressive effect was only observed after 24 hours in co-culture (**Fig.5.22a**), indicating a possible regulation, but most likely through secondary activated loops that, in turn, could be mediated by SPHK1 in

the first instance. This preliminary data should be analysed with caution; further repetitions need to be performed in order to justifiably place greater emphasis on the conclusions drawn.

There is evidence in the literature to support the regulation of MCP-1 by SPHK1/S1P; a study by Oskeritzian *et al.* have shown that S1P induced MCP-1 secretion in mast cells after 30 minutes [324]. Unfortunately the authors did not show the MCP-1 mRNA expression levels and therefore a comparison with my study (**Fig.5.20a**) cannot be established; however, these findings are indicative of the ability of S1P to induce a regulatory effect on MCP-1. Furthermore, SPHK1 has been described to regulate the TNF-α-induced increase of MCP-1 in endothelial cells [325], whilst DMS, a SPHK1 inhibitor, significantly reduced the TNF-α-induced increase in MCP-1 protein levels in Jurkat-U937 cells and PBMCs [292]. Despite these literary evidences of SPHK1-mediated regulation of MCP-1 in other cell systems, my study suggests that this regulation does not play a significant role in cancer-cell/monocyte interactions in an early stage of co-culture, but may have an impact at later stages. Also, there could be other important factors that have a stronger prevalence in MCP-1 regulation than SPHK1 during different stages of co-culture.

Despite the reduced effect of SPHK1 inhibition on MCP-1 in U937 cells, it is clear that MCP-1 plays an important role in our system. In support, rhMCP-1 was shown to have a role in SPHK1 regulation by inducing a transient increase in SPHK1 enzymatic activity and a later mRNA increase (**Fig.5.13a-b**). This later increase in SPHK1 mRNA could indicate a second activation loop caused by either the MCP-1 signalling cascade itself or by other MCP-1 activated genes such as IL-6 (**Fig.5.19**). In fact, recombinant MCP-1 led to an increase in IL-6 mRNA (**Fig.5.19a**), which in turn can also act in an autocrine manner by regulating its own expression in U937 cells (**Fig.5.19b**). This direct transcriptional regulation has been described previously; for example, a 3D co-culture system showed that IL-6 was important for macrophage infiltration in Spheroid tumour derived fibroblasts by regulating MCP-1 levels, whilst an anti-IL-6 antibody was shown to decrease MCP-1 levels and subsequent macrophage infiltration [425]. In support, an interesting study investigating the cross-talk between glioma and microglia cells through the MCP-1/IL-6 axis, showed that, initially, glioma cells secrete MCP-1 to microglia cells resulting in an increase in IL-6 secretion from microglia cells during co-culture. More interestingly, IL-6 was shown to be essential for the

increase in glioma invasiveness, as the neutralising antibody reversed this effect [426]. The ability of IL-6 to promote SPHK1 mRNA up-regulation (**Fig.5.17a**) and possibly Akt and ERK1/2 phosphorylation (**Fig.5.17b**), supports the role of this cytokine as a driver for the activation of important genes involved in oncogenic processes. Interestingly IL-6 expression levels in U937 cells appear to be independent of SPHK1 levels in PC-3 cells (**Fig.5.16a**), but partially dependent on Akt and ERK1/2 levels in PC-3 cells. Further experimental repeats are needed in order to draw more definite conclusions (**Fig.5.16b**), however it is interesting to find that SPHK1 regulated molecules such as Akt and ERK1/2 are able to reduce IL-6 expression despite no effect of SPHK1 itself.

A further clarification of IL-6 autocrine signalling in U937 cells during co-culture could have come from the analysis of STAT3 phosphorylation levels; however, for as yet determined reasons it was not possible to detect any levels of STAT3 phosphorylation.

In summary, this study shows that targeting SPHK1 in tumour cells not only has an effect on the tumour cells themselves but also affects the surrounding cells, in this case monocytes. The reduction of SPHK1, Akt and ERK1/2 phosphorylation in these cells indicate for the first time that targeting SPHK1 in tumour cells also has an indirect effect on SPHK1 itself and other important signalling molecules in monocytes. As for soluble molecules, IL-6 and MCP-1 will most certainly play an important role in cancer cell-monocyte interactions, however, their dependency on SPHK1 levels still remains unclear, although are likely to be time-dependent. To simplify the current findings a suggested model is shown the schematic representation below.

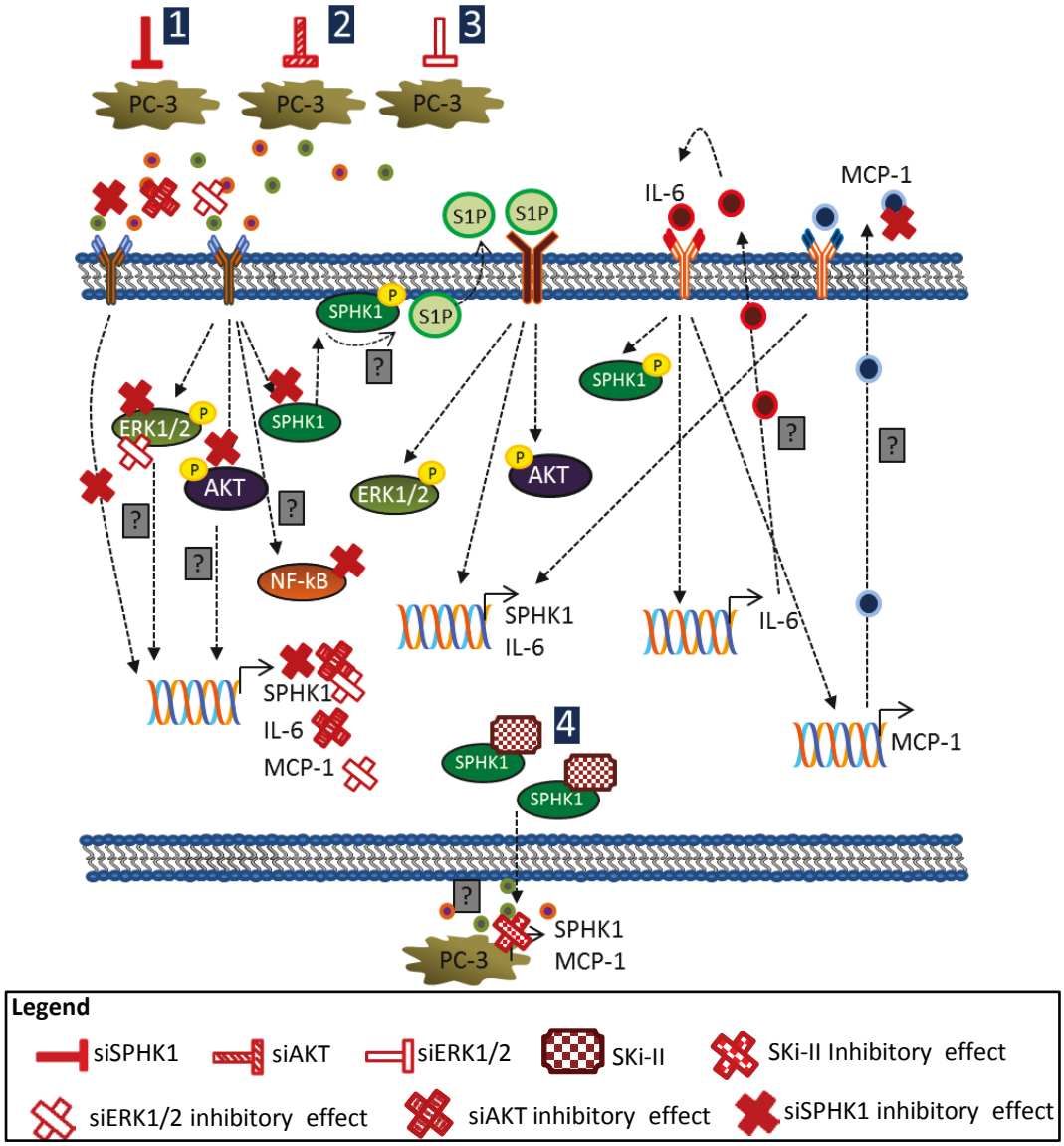


Figure 5.23b: Proposed model for the role of intracellular pathways involved in SPHK1 regulation in U937 cells during their co-culture with PC-3 cells. 1) SPHK1 knockdown by siRNA in PC-3 cells inhibits SPHK1 activity and expression in U937 cells; most likely by reducing the levels of a secreted molecule that is able to activate SPHK1 in monocytes, this molecule may well be S1P. AKT, NF-kB and ERK1/2 phosphorylation are also reduced by SPHK1 down-regulation. IL-6 and MCP-1 expression were not affected by this in PC-3 cells, nonetheless, MCP-1 secreted levels were reduced. **2**) AKT targeted knockdown in PC-3 cells decrease the PC-3 cells-induced increase of SPHK1 expression in U937 cells. In addition, AKT and ERK1/2 phosphorylation levels were also reduced when AKT was down-regulated in PC-3 cells. Similarly, IL-6 and MCP-1 expression were also reduced in U937 cells during this co-culture. **3)** ERK1/2 knockdown by siRNA in PC-3 cells markedly reduced the ability of these cells to increase SPHK1 expression in U937 cells; a similar effect was also observed for MCP-1 and IL-6 expression. The consequence of AKT and ERK1/2 knockdown on the secretion of MCP-1 and IL-6 by U937 was not studied; it therefore remains unclear as to what extent these knockdowns affect the regulation of these two inflammatory mediators. **4)** SPHK1 inhibition by SKi-II in U937 cells provoked a reduction in SPHK1 and MCP-1 expressio,n indicating the importance of SPHK1 activation in monocytes as critical for the regulation of molecules capable of activating SPHK1 in cancer cells.

6. STAT1 involvement in SPHK1 transcriptional regulation in PC-3 cells during co-culture with U937 cells

6.1 STAT1 transcriptional binding to SPHK1 encoding gene

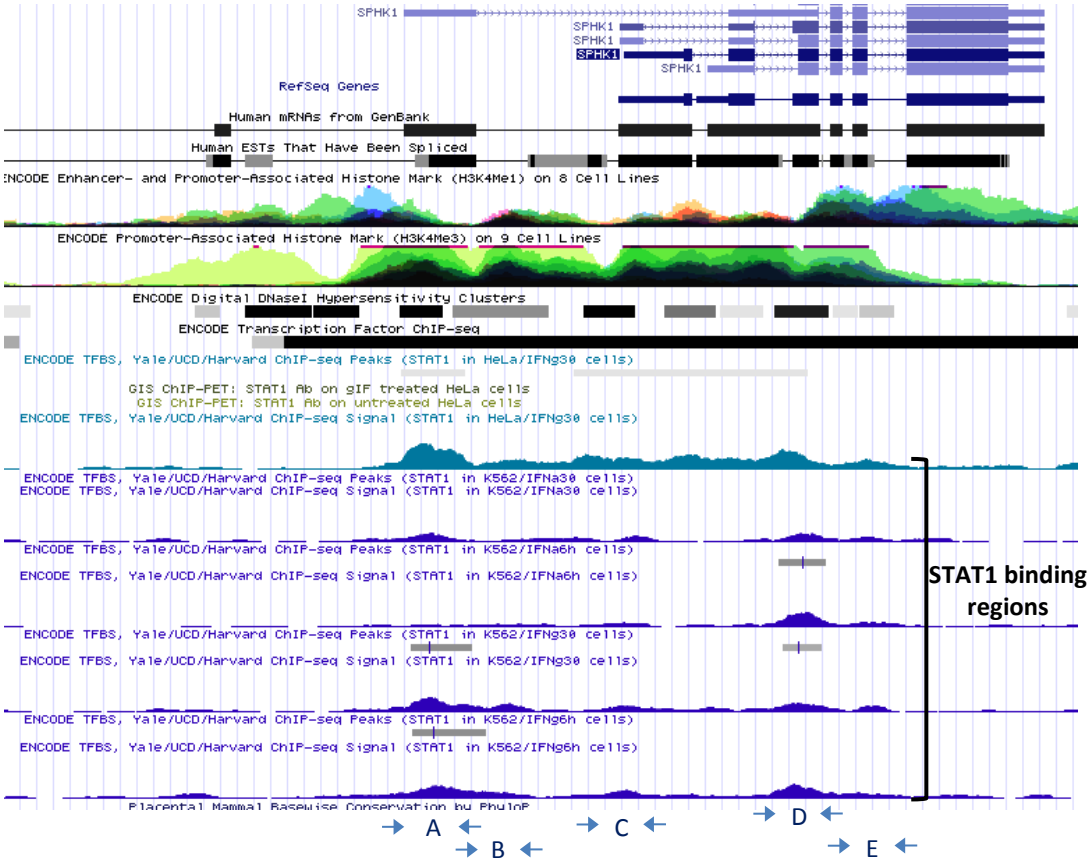
Some cytokines [325, 326] and growth factors [130, 176] are known to activate SPHK1 transcriptional expression; however the transcriptional factors or mediators involved in this activation are currently poorly described and remain unclear. So far, activator protein 2 (AP-2) and specificity protein 1 (SP1) binding to SPHK1 promoter regions have been identified as critical for SPHK1 transcriptional activation upon PMA [184], NGF (nerve growth factor) [427] and GDNF (glial cell line-derived neurotropic factor) stimulation [428]. In this context, signal transducers and activators of transcription (STATs) are known to be regulated through the activation of cytokine and hormone receptors. Activation of STATs has been correlated with cell survival, apoptosis, proliferation [355, 429] and are known to modulate inflammatory responses by maintaining an inflammatory microenvironment with pro- and anti-survival benefits for cancer development [362, 430]. STAT3 and STAT1 have similar structures, with both having activated tyrosine residues, and despite their binding to very specific elements their sets of activated downstream genes can sometimes be overlaid. Both can form dimers or heterodimers with each other upon given specific stimuli (e.g. cytokines or growth factors). The very low endogenous levels of STAT3 in the PC3 cell line [431, 432] prompts the hypothesis that STAT1 is being activated in the “absence” of STAT3 and therefore regulating and activating the STAT3 set of target genes. Here, I propose to investigate the role of STAT1 as a transcription factor responsible for SPHK1 regulation in PC-3 cells during co-culture with U937 cells.

CHIP-Seq data in the public domain (UCSC Genome Bioinformatics, genome.ucsc.edu) indicated several SPHK1 genomic regions where STAT1 was shown to bind in HeLa-S3 and the leukaemia K562 cell lines after IFN-gamma treatment for 30 minutes and six hours (**Fig.6.1a**). In accordance with these available data, several sets of primers were

designed to recognise five different sequences within the vicinity of the SPHK1 encoding gene where STAT1 was shown to bind (**Fig.6.1b**). Each primer pair was tested in PC-3 cell lysates after a chromatin immunoprecipitation assay (ChIP), designed to evaluate if U937 cells were inducing STAT1 binding to SPHK1 genomic DNA. For this purpose PC-3 and U937 cells were co-cultured for two hours before the PC-3 cell pellet was collected, with lysates from PC-3 cells cultured with or without U937 cells compared. Indeed, U937 cells were able to induce STAT1 binding within the vicinity of the SPHK1 encoding gene in PC-3 cells when compared with cells cultured alone. Of the five tested primer pairs, STAT1 enrichment was observed in two different regions, A and D (**Fig.6.2a-b**). Region **A**, situated up-stream of the SPHK1 coding region, had a 4-fold increase in STAT1 binding upon U937 cell stimulation after normalisation with the respective IgG control (**Fig.6.2a**). Region **D**, situated inside the coding region, showed a 2-fold increase after co-culture (**Fig.6.2b**).

6.2 SPHK1 modulation of STAT1 phosphorylation

Following on from these initial findings it was important to understand whether STAT1 binding to putative SPHK1 regulatory regions was accompanied by an increase in its phosphorylation. A co-culture assay was performed where PC-3 cells were cultured in the presence or absence of U937 cells for one, three or 24 hours. Western blotting analysis of PC-3 cell lysates showed an increase in STAT1 phosphorylation levels when cultured with U937 cells for one and three hours (**Fig.6.3a**), whereas the use of siRNA targeting SPHK1 abrogated U937 induced-increase in STAT1 phosphorylation in PC-3 cells. Figure 6.3b is a representation of figure 4.3e (chapter 4) to show SPHK1 knockdown in PC-3 cells.



Region/Primer nomenclature	Position on SPHK1 encoding gene (Range)	Product Length
A	**chr17:74378958-74379370**	**91bp**
B	**chr17:74379401-74379628**	**95 bp**
C	**chr17:74379671-74379970**	**107 bp**
D	**chr17:74381110-74381582**	**107 bp**
E	**chr17:74381806-74382343**	**115bp**

Figure 6.1: Schematic representation of putative STAT1 binding sites to SPHK1 encoding gene. a) Representation of CHIP-Seq data on SPHK1 DNA for possible STAT1 binding sites (UCSC Genome Bioinformatics; genome.ucsc.edu). Blue peaks indicate STAT1 binding after IFN- treatment, with the different lines representative of the different cell lines and time of treatment. **b)** Table shows the regions in SPHK1 genomic DNA where primers were designed to detect STAT1 binding (represented by blue arrows in **(a)**).

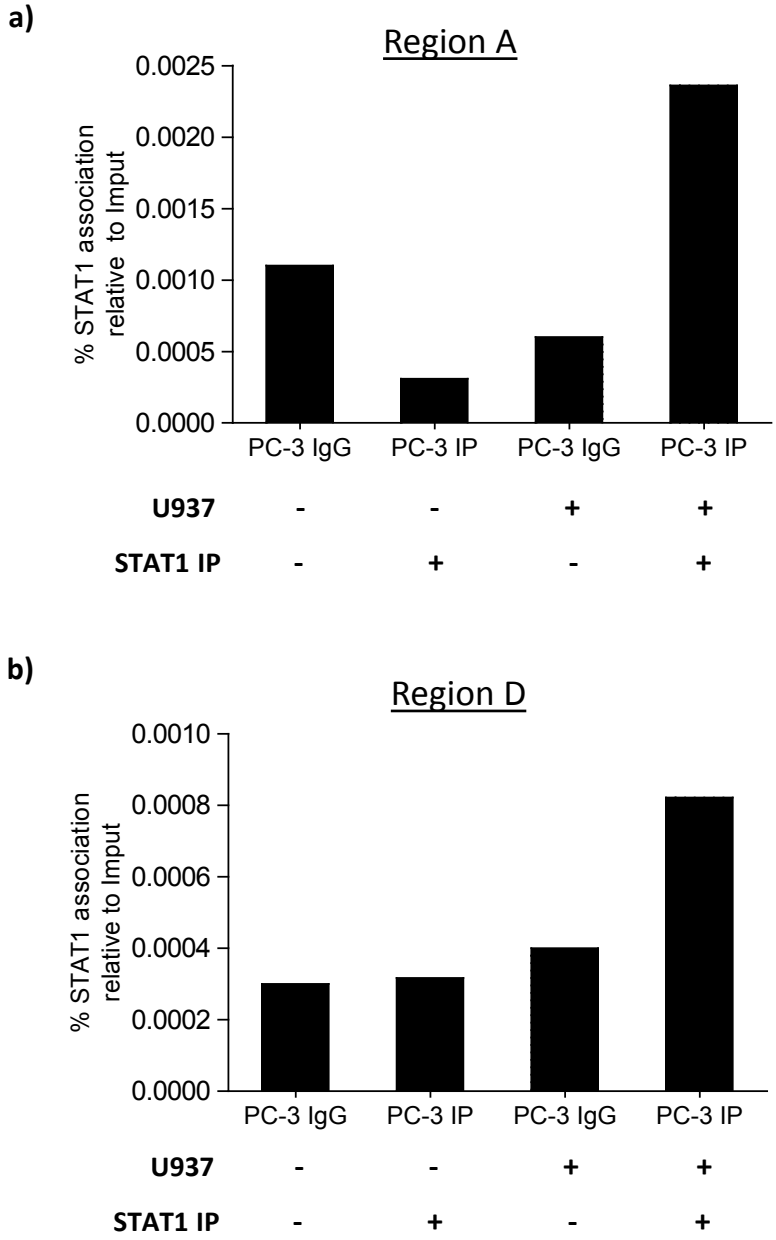


Figure 6.2: ChIP assay on PC-3 cells during co-culture. PC-3 cells were seeded in the bottom chamber of a transwell for 24 hours in serum-free media prior to the addition of U937 cells to the upper chamber for a further two hours. After removal of the top chamber, PC-3 cells were cross-linked by the addition of 1% formaldehyde for 10 minutes, at which point glycine was added. PC-3 cells were then lysed and sonicated followed by immunoprecipitation with STAT1 antibody. Purified DNA was quantified by real time PCR where two primer pairs were used for **a)** region A and **b)** region D. The values were normalised for the initial amount of chromatin used in each condition (input) and compared with each antibody isotype (IgG) for background signalling. Data shown is from one experiment.

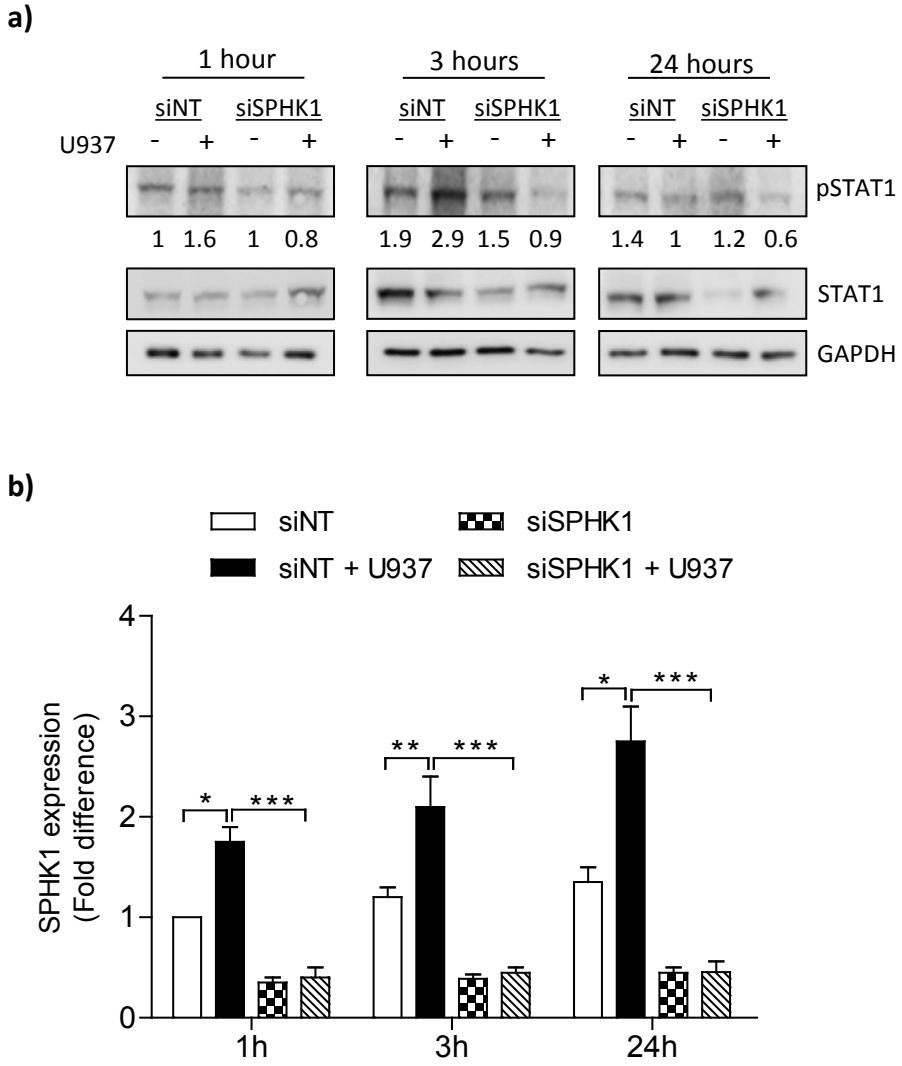


Figure 6.3: STAT1 regulation in PC-3 cells during co-culture with U937 cells. PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1) or non-targeting control siRNA (siNT) for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. **a)** STAT1 phosphorylation and total levels were determined by western blotting quantified by Image J software and normalised to GAPDH. **b)** SPHK1 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used for normalisation. **a)** Data shown is for one experiment, **b)** Data shown is the mean ± SEM of three independent experiments. **b)** Statistical analysis was performed using student's t-test relative to each individual time point. *p<0.05, **p<0.01, ***p<0.001 for PC-3 siNT vs. PC-3 siTN + U937 or PC-3 siSPHK1 vs PC-3 siSPHK1 + U937.

To further investigate the role of STAT1 in SPHK1 transcriptional regulation, PC-3 cells were transfected with a pool of four siRNAs targeting STAT1 for 48 hours prior to collection of total RNA (**Fig.6.4a**). Surprisingly, STAT1 knockdown increased SPHK1 mRNA levels by 2-fold relative to PC-3 cells transfected with a non-targeting siRNA. To rule out off-target effects of individual STAT1 siRNAs, a deconvolution was performed in each of the individual siRNAs composing the pool. As seen in figure **6.4b**, all four individual siRNA targeting STAT1 led to an increase in SPHK1 expression in PC-3 cells.

Next, to assess if the absence of STAT1 in PC-3 cells would affect the interaction with U937 cells, a co-culture assay was performed for one, three and 24 hours. As expected, STAT1 knockdown in PC-3 cells induced SPHK1 expression, an effect that proved to be independent of monocyte presence as there was no further increase or reduction in SPHK1 mRNA when U937 cells were added to the co-culture (**Fig.6.5a**). Since I have previously shown a level of "cross-talk" between SPHK1 and Akt (chapter 3 and 4), it was important to understand whether an inhibition of STAT1, and consequently activation of SPHK1, would lead to an increase in Akt phosphorylation in PC-3 cells when in the presence of U937 cells. Western blotting analysis showed that Akt phosphorylation was down-regulated by STAT1 knockdown; indeed, basal levels of Akt phosphorylation were reduced across all time points when in comparison with PC-3 cells transfected with a non-targeting siRNA (**Fig.6.5d**). However, the presence of U937 cells had a positive effect as it restored Akt phosphorylation levels in STAT1 siRNA transfected PC-3 cells at one and three hours. After 24 hours of co-culture this increase was not observed as AKT levels were very low (**fig.6.5b**). It should be noted that STAT1 knockdown was not directly assessed during this assay, however, this siRNA proved to be efficient, as depicted in figure **6.6**.

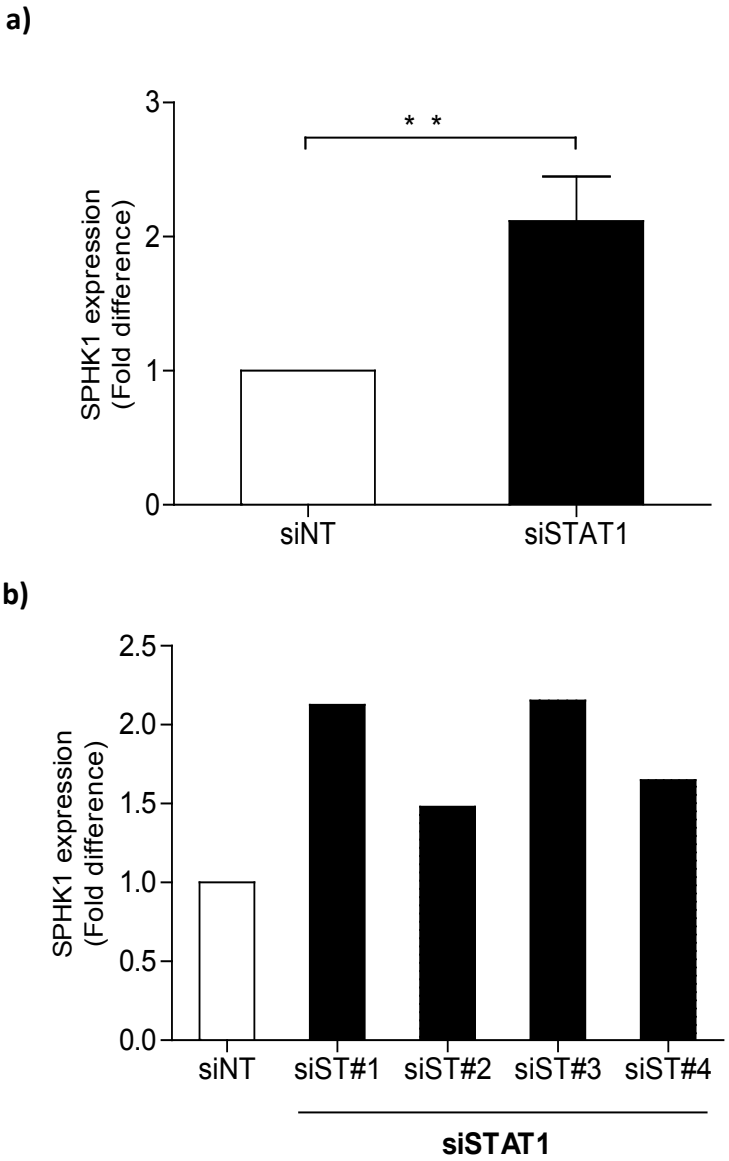


Figure 6.4: Effect of STAT1 knockdown on PC-3 cells. PC-3 cells were transiently transfected with **a)** siRNA smart pool targeting STAT1 or **b)** four independent siRNA also targeting STAT1, for 48 hours. A non-targeting siRNA (siNT) was used as a control. SPHK1 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used for normalisation. **a)** Data shown is the mean ± SEM of three independent experiments, **b)** data shows the fold difference of each siSTAT1 relative siNT for one single experiment.

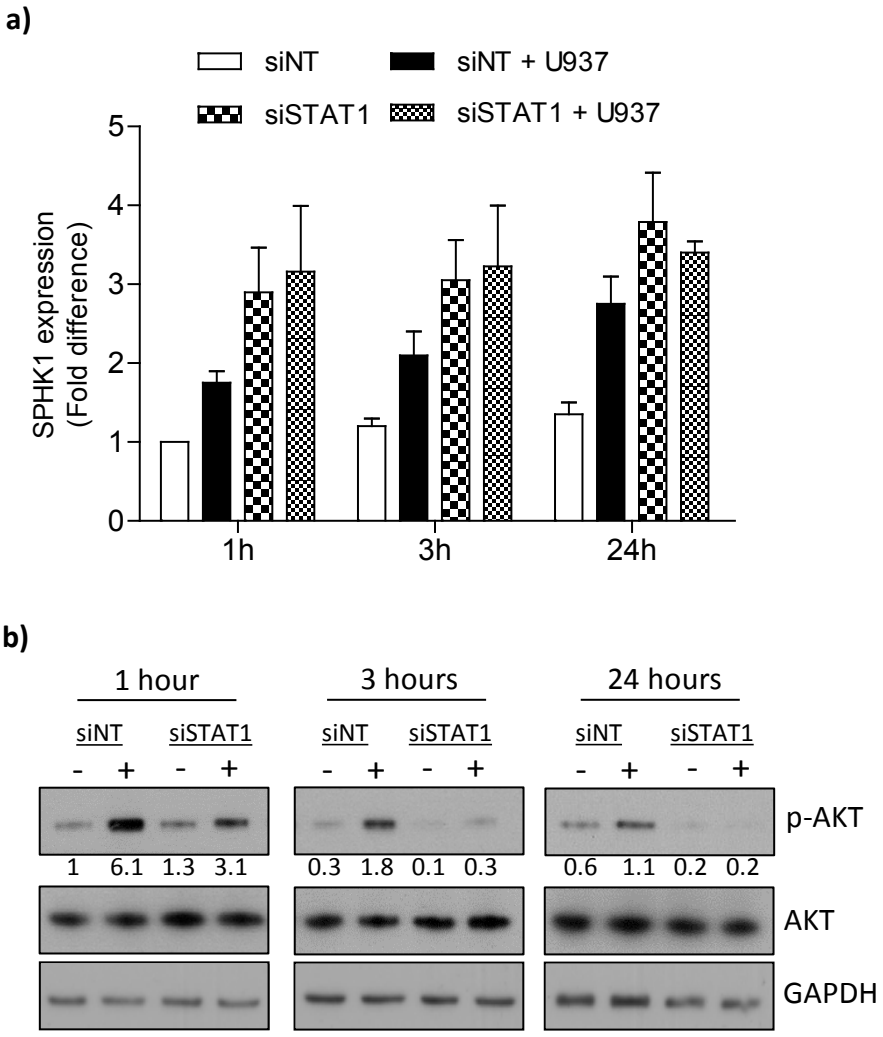


Figure 6.5: Effect of STAT1 knockdown on PC-3 cells during co-culture with U937 cells. PC-3 cells were transfected with siRNA smart pool targeting STAT1 (siSTAT1) or non-targeting control siRNA (siNT) for 24 hours before cells were washed with PBS and cultured in serum-free media for 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated duration. **a)** SPHK1 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used for normalisation. **b)** AKT phosphorylation and total levels were determined by western blotting, quantified by Image J software and normalised to GAPDH. Data shown is the mean ± SEM of **a)** two independent experiments, **b)** data shown represents one experiment.

6.3 Phosphorylation of STAT1 and STAT1-regulated genes by IL-6 and MCP-1: the role of SPHK1

I have previously shown that IL-6 and MCP-1 were regulated during co-culture of PC-3 cells with U937 cells. To further assess their effect on STAT1 phosphorylation in PC-3 cells, human recombinant IL-6 and MCP-1 were added at a final concentration of 100ng/ml to PC-3 cells for the indicated time points before collection of lysates for western blotting analysis. STAT1 phosphorylation was strongly induced by IL-6 across all of the indicated time points, peaking at 30 minutes (**Fig.6.6a**). This effect was partially inhibited by the use of siRNA targeting SPHK1, when the IL-6 induction of STAT1 phosphorylation was reduced by approximately 2-fold (**Fig.6.6b**). The effect of rhMCP-1 on STAT1 phosphorylation (**Fig.6.6c**) was not as accentuated as that observed for IL-6, nonetheless there was still a 1.5-fold increase in comparison with non-treated PC-3 cells. SPHK1 knockdown completely blocked MCP-1 induced STAT1 activation (**Fig.6.6c**); this could indicate that MCP-1 and IL-6 (to a greater extent) can regulate STAT1 phosphorylation through a SPHK1 dependent mechanism.

To determine whether STAT1 activation regulates SPHK1, PC-3 cells were treated with Interferon-gamma (IFN-γ). IFN-γ is a well-known activator of STAT1 [433], and several of its downstream genes, such as interferon regulatory factor 1 (IRF-1), are dependent on STAT1. IFN-γ was added to PC-3 cells at a final concentration of 500 IU/ml for 24 hours. After cell lysates were collected SPHK1 expression was measured by qRT-PCR. A STAT1 activated gene, IRF-1, was also measured as a control for STAT1 activation. As expected, IRF-1 expression showed a 16-fold increase upon IFN-γ stimulation (**Fig.6.7a**), whilst SPHK1 expression was decreased by 2-fold after treatment (**Fig.6.7b**). In terms of IL-6 and MCP-1 mRNA expression, IFN-γ treatment did not affect IL-6 expression (**Fig.6.7c**) but markedly reduced MCP-1 expression with a 5-fold decrease when compared to untreated PC-3 cells (**Fig.6.7d**). Furthermore, to assess whether IL-6 or MCP-1 were inducing an IFN-γ/STAT1 response in PC-3 cells, human recombinant IL-6 and MCP-1 were added to the cells for 24 hours at a final concentration of 100ng/ml. IRF-1 expression in these cells showed a 1.3-fold increase after IL-6 treatment and a 2-fold decrease after addition of MCP-1 (**Fig.6.7e**).

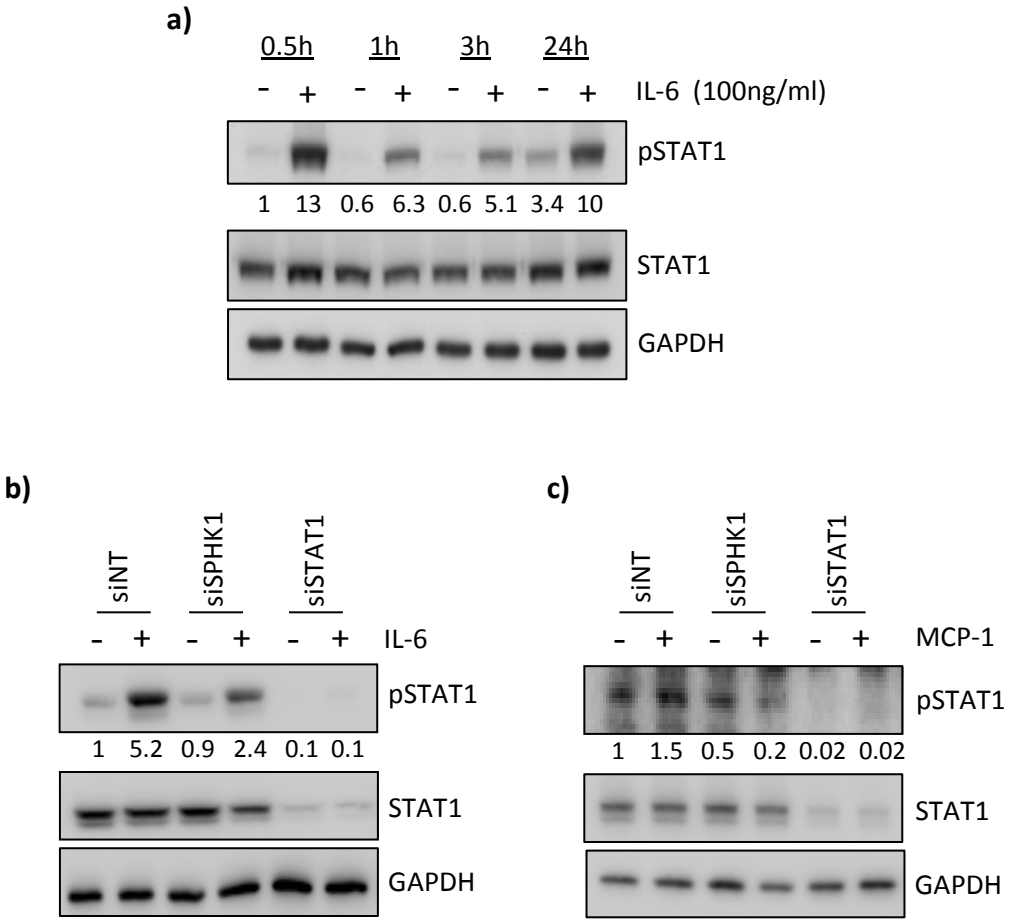


Figure 6.6: IL-6 and MCP-1 effects on STAT1 phosphorylation in PC-3 cells. a) PC-3 cells were seeded in a 6-well plate in serum-free medium for 24 hours before the addition of IL-6 (100ng/ml) for the indicated time points. STAT1 phosphorylation and total levels were determined by western blotting, quantified by image J and normalised to GAPDH. **b)** PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1), siRNA smart pool targeting STAT1 (siSTAT1) or a non-targeting control siRNA (siNT) for 24 hours before cells were washed with PBS and cultured in serum-free medium for 24 hours. **b)** IL-6 or **c)** MCP-1 were added at a final concentration of 100ng/ml for 30 minutes. STAT1 phosphorylation and total levels were determined by western blotting as described above. Data shown is representative of two independent experiments.

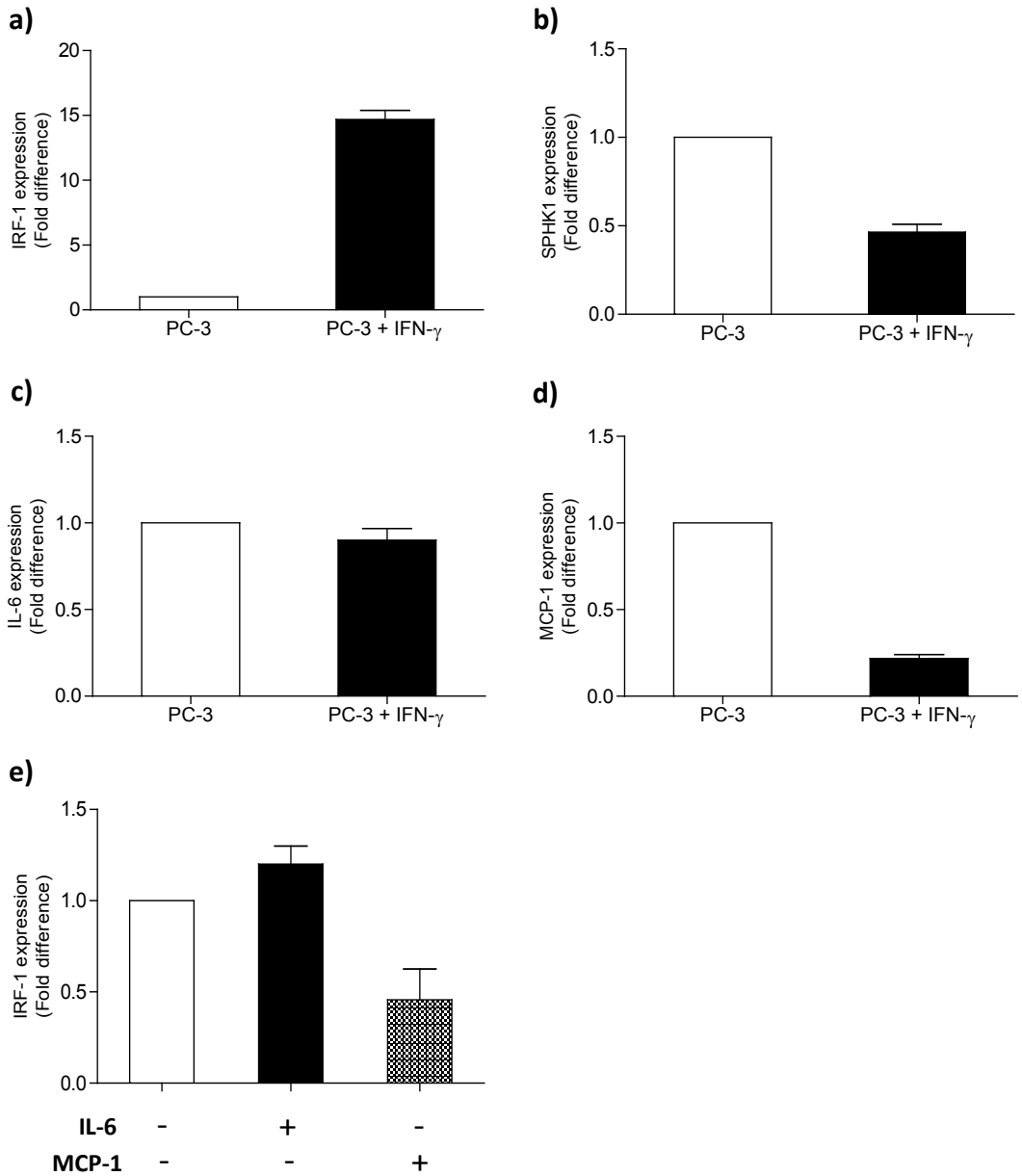


Figure 6.7: IFN-γ effect on PC-3 cells gene expression. PC-3 cells were seeded in a 6-well plate in serum-free media for 24 hours before the addition of IFN-γ (500 IU/ml) for 24 hours. **a)** IRF-1, **b)** SPHK1, **c)** IL-6 and **d)** MCP-1 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise each gene expression. **e)** PC-3 cells were seeded as described above, IL-6 (100ng/ml) and MCP-1 (100ng/ml) were added for a further 24 hours. IRF-1 expression was measured as indicated above **(a-d)**. Data shown is the SD of two replicates from one experiment normalised to untreated PC-3 cells.

To further confirm that IL-6 and MCP-1 were not inducing IFN-γ type responses in PC-3 cells, HLA-Class I protein levels were measure by flow cytometry. HLA class I molecules are cell surface proteins constitutively expressed in most cells, and IFN-γ can further induce their expression through a secondary loop involving the activation of IRF-1 and STAT1. For this purpose PC-3 cells were treated with IL-6 and MCP-1 for 24 (**Fig.6.8a**) and 48 hours (**Fig.6.8b**) before HLA-Class I protein levels were determined by flow cytometry. IFN-γ was also added as a positive control for the induction of HLA-Class I molecules. As seen in figure **6.8** IFN-γ induces a clear increase in HLA-Class I at both time points (panel **3** and **8**). MCP-1 treatment did not induce any increase in HLA-Class I expression levels when compared with untreated PC-3 cells stained with the control fluorescent fluorochrome FITC antibody (panel **4** and **9**). At 24 hours, IL-6 addition provoked a mild increase in HLA-Class I relative to untreated PC-3 cells (panel **5**), however the increase proved transient as at 48 hours there was a reduction of HLA-Class I upon IL-6 treatment (panel **10**).

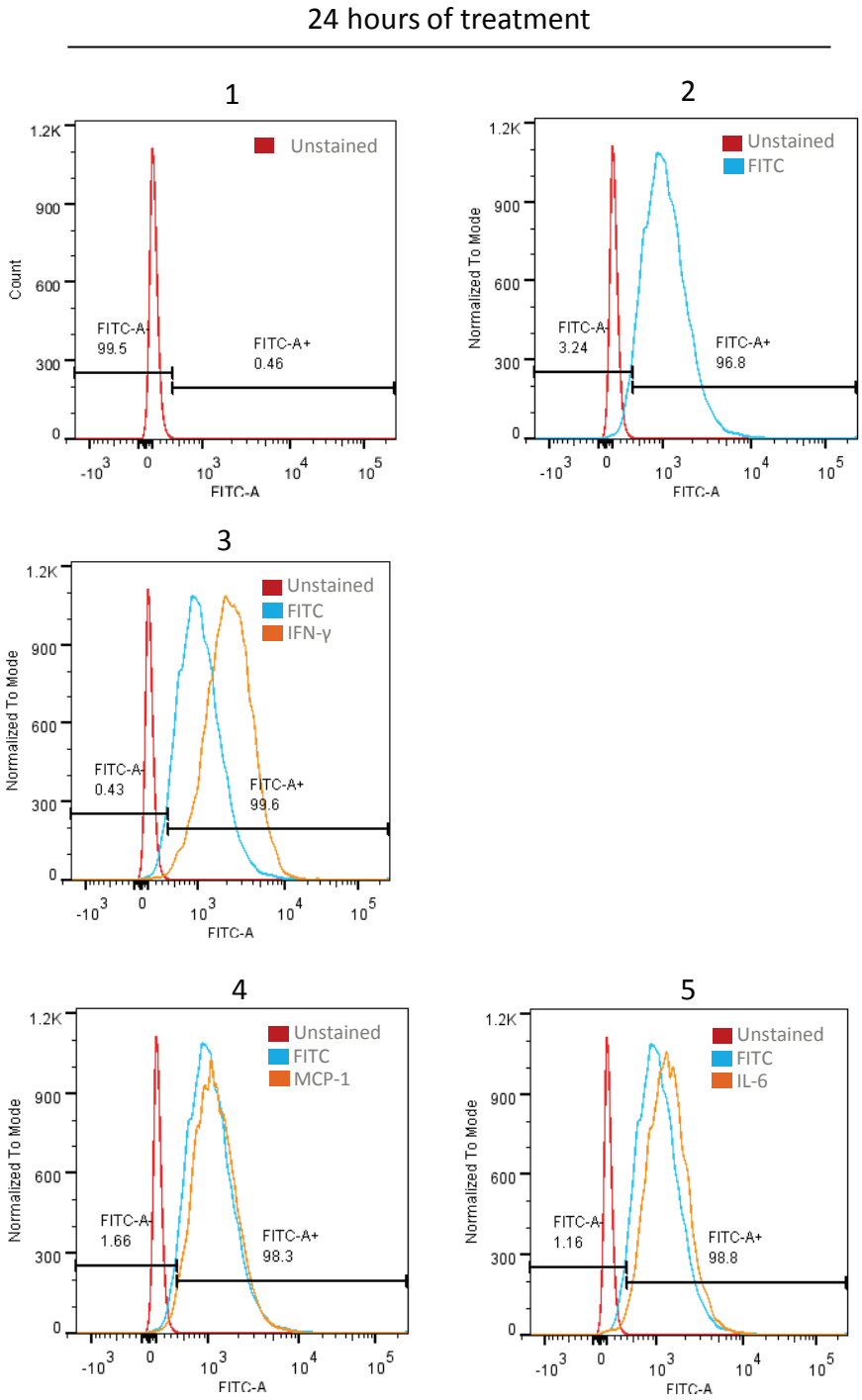


Figure 6.8: IL-6 and MCP-1 effect on IFN-γ response gene HLA-Class I in PC-3 cells. a) PC-3 cells were seeded for 24 hours before the addition of IFN-γ (500 IU/ml), IL-6 (100ng/ml) and MCP-1 (100ng/ml) for 24 hours. At each time point PC-3 cells were fixed with 4% PFA before the addition of FITC-HLA-Class I a,b,c antibody. Fluorescence was detected using a BD FACSCanto and results were analysed using FlowJo software. Panel **1** and **2** show staining controls for **(1)** unstained background signalling and **(2)** FITC background signalling of untreated PC-3 cells. Panels **3, 4** and **5** show staining profiles of IFN-γ, MCP-1 and IL-6 respectively. Results are presented as the geometric mean values of stained samples, and data shown is representative of two independent replicates of one independent experiment.

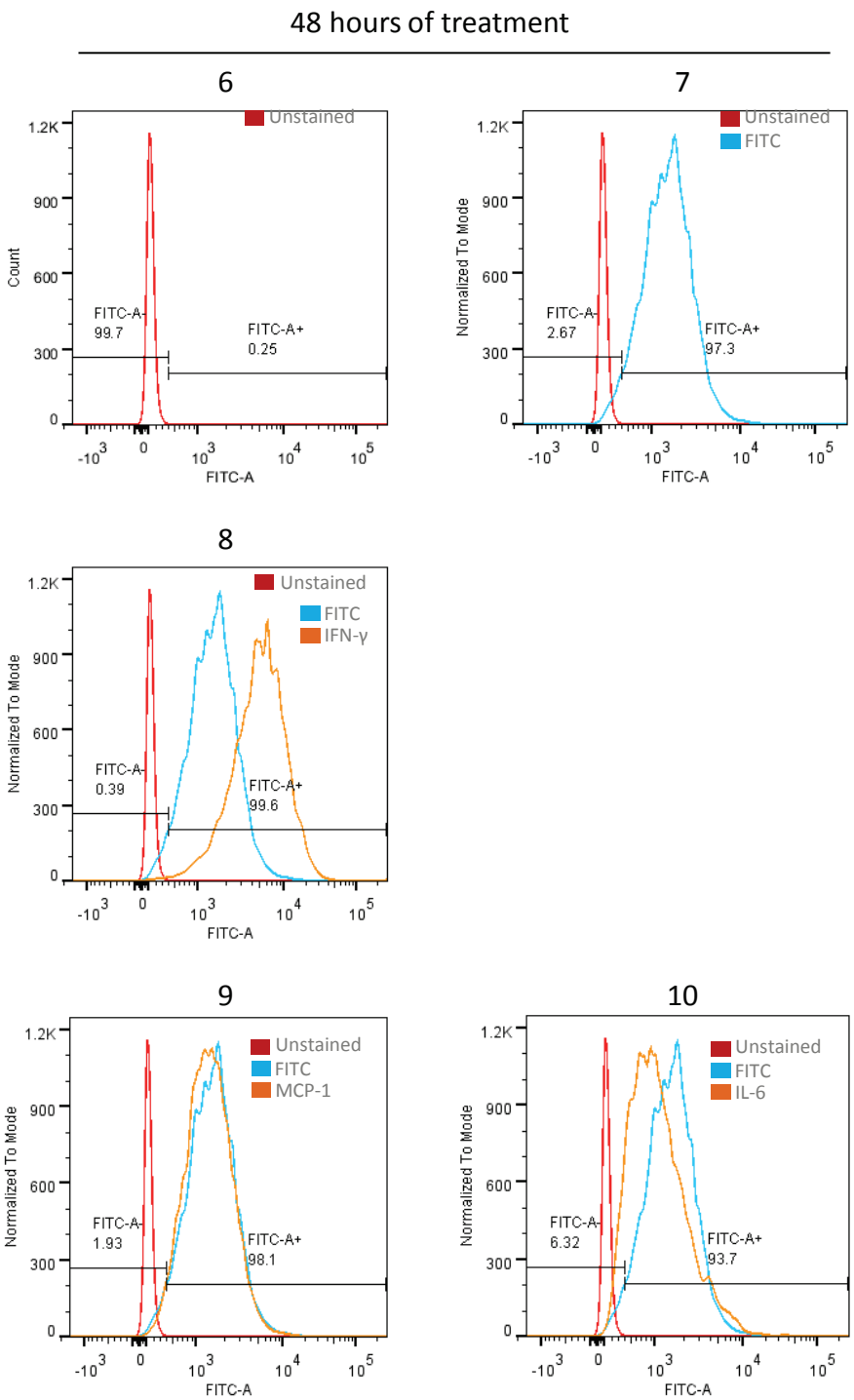


Figure 6.8b: IL-6 and MCP-1 effect on IFN-γ response gene HLA-Class I in PC-3 cells. a) PC-3 cells were seeded for 24 hours before the addition of IFN-γ (500 IU/ml), IL-6 (100ng/ml) and MCP-1 (100ng/ml) for 48 hours. At each time point PC-3 cells were fixed with 4% PFA before the addition of FITC-HLA-Class I a,b,c antibody. Fluorescence was detected using a BD FACSCanto and results were analysed using FlowJo software. Panels **1** and **2** show staining controls for **(1)** unstained background signalling and **(2)** FITC background signalling of untreated PC-3 cells. Panels **3, 4** and **5** show staining profiles of IFN-γ, MCP-1 and IL-6 respectively. Results are presented as the geometric mean values of stained samples, and data shown is representative of two independent replicates of one independent experiment.

6.4 Discussion

Signal transducer and activator of transcription 1 (STAT1) is an important mediator of the Janus Activated Kinase signalling pathway. STAT1 plays a crucial role in mediating immune and pro-inflammatory responses [434, 435] and antitumor responses [433, 436]. STAT1 is generally believed to be an antitumor molecule, however some reports describe the up-regulation of STAT1 in cancer and therapy resistant cancer cells in comparison with normal cells [437, 438]. In support of a tumour-promoting role, STAT1 regulated genes have been shown to have anti-apoptotic [439], proliferative [440], and metastatic properties [441].

STAT1 activation is triggered by cytokines, growth factors or hormone binding to cell receptors. In this context, interferons (IFN) are the most potent and well known regulators of STAT1 activation; upon stimulation STAT1 is phosphorylated forming homo or heterodimers that translocate to the nucleus and activate a specific set of genes known as IFN-stimulated genes (ISGs). In this regard, SPHK1 has been shown to be regulated by STAT1 upon IFN-γ stimulation, with CHIP-Seq data indicating several regions where STAT1 was binding to SPHK1 DNA either up-stream of the coding region, mostly likely within the SPHK1 promoter region, or inside the coding region (**Fig.6.1)**; this could indicate SPHK1 as a putative ISG. Our CHIP data analysis on PC-3 cells after co-culture indicated an increase in STAT1 binding to SPHK1 promoter after U937 cell stimulation (**Fig.6.2**). Therefore I hypothesise that STAT1 was regulating SPHK1 transcriptional expression during co-culture.

The U937-induced increase in STAT1 phosphorylation in PC-3 cells after one and three hours (**Fig.6.3a**) came in support of the notion that this phosphorylation may precede STAT1 translocation to the nucleus to regulate SPHK1. Interestingly, this effect was abrogated by SPHK1 knockdown, indicating that STAT1 could also be regulated by SPHK1 activated genes. With respect to my previous results it came as a surprise that the absence of STAT1 in PC-3 cells, by siRNA targeting, increased SPHK1 expression (**Fig.6.4a-b**), suggesting that STAT1 was having an inhibitory effect on SPHK1 transcription. The effect of STAT1 siRNA on SPHK1 expression was not altered by the presence of U937 cells (**Fig.6.5a**),

suggesting that increased SPHK1 expression does not necessarily result in a further increase in its expression through the monocyte feedback loop.

A very recent and interesting work by the Spiegel laboratory, showed that an SPHK1 increase leads to S1P production and activation of STAT3 through Sphingosine 1-phosphate receptor 1 (S1PR1). STAT3 activation then induces a positive feed-back loop involving IL-6, resulting in an increase of S1PR1 [299]. Prior to this, Lee *et al.* indicated that STAT3 is a transcription factor for S1PR1 and its activation leads to a persistent activation of STAT3 resulting in an increase in tumour growth and metastasis [298]. These published works indicate that STAT3, and not STAT1, acts as a modulator of this positive feedback loop involving S1P, however as STAT3 levels are very low in PC-3 cells, I hypothesised that STAT1 could be "replacing" STAT3 as an S1P down-stream activator molecule. Indeed, Costa-Pereira *et al.*, showed that in STAT3-/- mouse embryo fibroblasts IL-6 induced IFN-γ like responses suggesting that IL-6 dependent activation of STAT3 was dependent on STAT1 [442]. In my experiments, STAT1 phosphorylation decreases in the absence of SPHK1 (upon U937 cell stimulation), suggesting an S1P regulation of STAT1 in a SPHK1 secondary loop-dependent mechanism. However without stimulation, STAT1 represses SPHK1 transcription indicating that STAT1 can act as an antitumor modulator in the absence of "specific stimuli".

Indeed, STAT1 responses appear to be affected by different soluble factors. I have shown that IL-6 strongly induces STAT1 phosphorylation in PC-3 cells (**Fig.6.6a**) and it was partially dependent on SPHK1 (**Fig.6.6b**). To a lesser extent, MCP-1 showed a similar effect on STAT1 phosphorylation and its dependency on SPHK1 (**Fig.6.6c**). Interestingly, the effect of these cytokines on IRF-1, an interferon gamma- and STAT1-dependent gene was minimal. The finding that both IL-6 and MCP-1 did not result in a great increase in IRF-1 expression, indeed MCP-1 induced a clear reduction (**Fig.6.7e**), indicates that STAT1 activation does not necessarily lead to the response of an anti-tumour related gene such as IRF-1 [443]. Whether IL-6 induces the activation of a pro-survival set of genes in a STAT1-dependent manner remains unclear and should be addressed to clarify the importance of STAT1 in an IL-6 induced response. The study by Lesinski *et al.* in melanoma indicated that STAT1 levels were not affected by IFN-γ treatment, and in recurrent tumours STAT1 expression proved to be higher than in primary tumours [444]. Other groups have reported that STAT1 regulates a sub-set of genes involved in radio [438] and chemoresistance [445] upon interferon

treatment. It is possible that in the absence or very low levels of STAT3, STAT1 is being regulated by cytokines or other soluble molecules that would normally activate STAT3. As a consequence, a STAT3 activated set of genes could then be regulated by STAT1, provoking a more tumorigenic response in PC-3 cells and consequently a more productive interaction with U937 cells. Conversely, IFN-γ strongly induced IRF-1 expression (**Fig.6.7a**); in concomitance HLA class I molecule levels were also increased by IFN-γ (**Fig.6.8a-b**). HLA class I molecules are constitutively expressed in most human cells and can be used as an indicator of a secondary phase of IFN-γ transcription activation. Their involvement in antigen presenting cell interaction with T lymphocytes leads to an antitumor response [446]. A more recent study showed that reduced levels of HLA class I correlate with a poor prognosis in patients with non-small cell lung cancer [447]. In my study IL-6 and MCP-1 did not induce HLA-Class I molecules despite inducing STAT1 activation (**Fig.6.8a-b**), and no significant increase in IRF-1 expression was observed after either IL-6 or MCP-1 treatment.

Altogether I have shown that STAT1 is involved in SPHK1 regulation, however the repression or activation mechanisms involved still remain unclear. It appears to be dependent upon or regulated by different soluble molecules, prompting the notion that the tumour microenvironment could dictate a pro or antitumor STAT1 response. Finally I propose that the SPHK1/S1P pathway and STAT1 regulation occur through a feedback loop, whereby S1P may act as secondary messenger in activating STAT1. A proposed model of this possible mechanism is shown below (**Fig.6.9**).

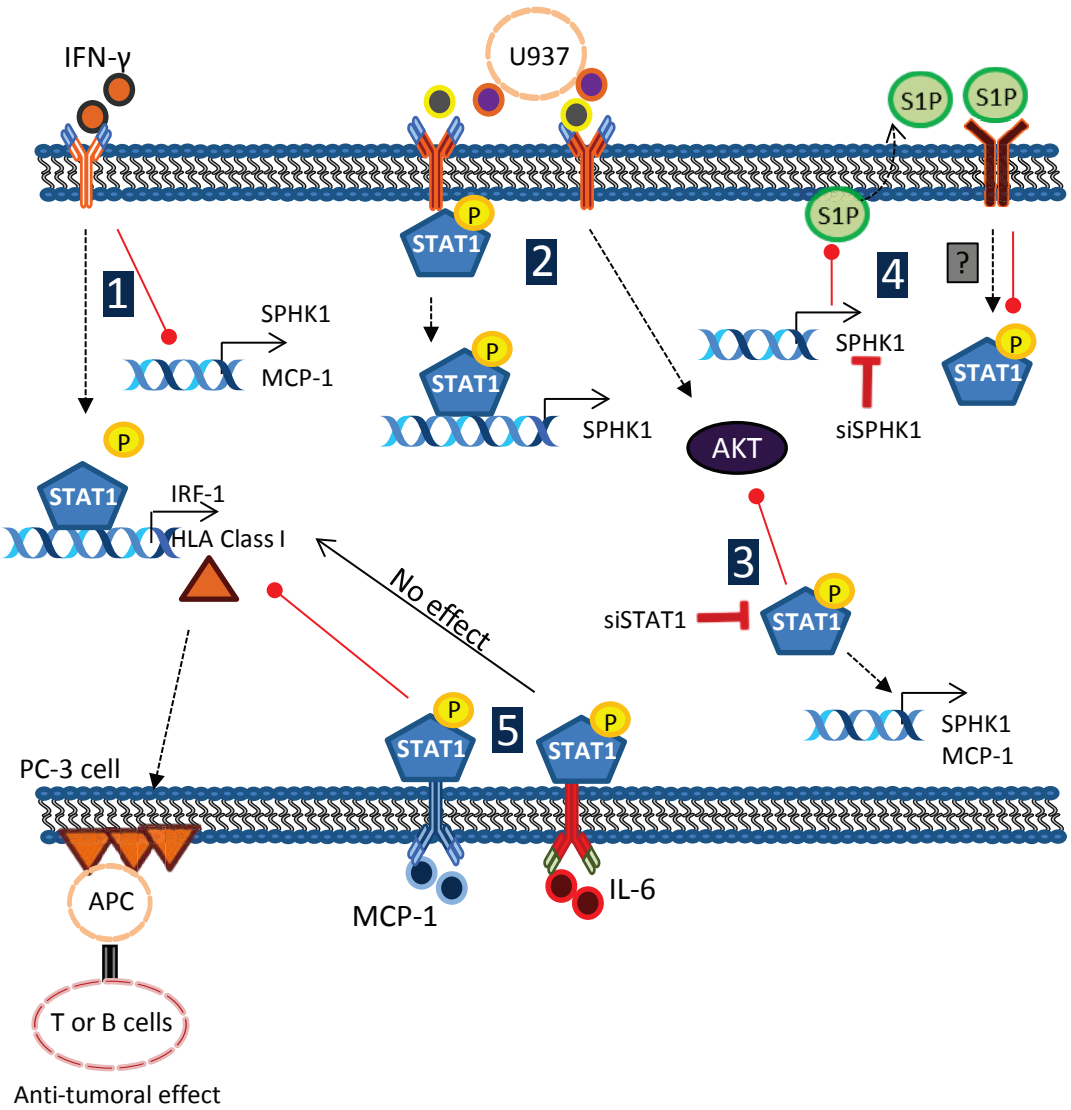


Figure 6.9: Proposed model for STAT1 activation in PC-3 cells. **1)** IFN-γ receptor binding induces STAT1 phosphorylation and translocation to the nucleus where it is involved in the transcription of several genes such as HLA class I and IRF-1. HLA class I is involved in anti-tumoral responses by mediating the interaction with APC and T cells. IFN-γ represses (red line) SPHK1 and MCP-1 transcription; the mechanism of how this repression occurs is not yet understood. **2)** PC-3 co-culture with U937 cells induces STAT1 binding to SPHK1 promoter region increasing its mRNA levels. **3)** STAT1 knockdown increases SPHK1 mRNA expression levels, suggesting that STAT1 can act as a SPHK1 repressor. However, this inhibitory effect appears to be reversed by the interaction with U937 cells. The U937-induced increase in AKT phosphorylation is reduced by STAT1 knockdown, indicating a possible STAT1 regulation on AKT phosphorylation. Interestingly, **4)** SPHK1 knockdown by siRNA reduces STAT1 levels; an effect that could be related with reduced levels of S1P. **5)** Extracellular MCP-1 induces STAT1 phosphorylation and reduces IRF-1 and HLA Class I, supporting the hypothesis that STAT1 can regulate different sets of genes depending on stimulus. In support, IL-6 also induces STAT1 but has no effect on IRF-1 or HLA Class I levels. The sequence of events may not reproduce the real dynamics existing between U937 and PC-3 cells as some of the represented events may occur simultaneously.

7. The role of SPHK1 in PC-3 cells resistance to docetaxel during co-culture

7.1 Introduction

In patients with locally advanced or metastatic prostate cancer, chemotherapy is often given when hormonal therapies have failed. In this context current therapeutics based on taxanes, in particular docetaxel, have shown promising results following demonstration of beneficial effects on patient survival [448-450]. However, this effect proved to be minor as patient survival increase was on average no more than three months; this underlines the importance of finding new targets to re-sensitise prostate cancer and subsequently improve clinical prognosis. With this objective, Pchejetski *et. al* firstly reported that SPHK1 over-expression conferred chemoresistance to docetaxel and camptothecin, whilst the use of a pharmacological SPHK inhibitor re-sensitised the cell to these drugs [216]. This report was followed with a study by Sauer *et. al*, whereby a combination of docetaxel and SPHK1 inhibition led to a 4-fold decrease in the IC50 of docetaxel [78]. Together these findings support the use of SPHK1 inhibitors in combination therapy to overcome cancer resistance to conventional drugs.

Resistance to chemotherapeutic agents can occur due to genetic alterations within the cancer cell [451, 452] or by interactions between the cells and their microenvironment [453]. In this context the tumour microenvironment has been reported to play an important role in chemoresistance, not only in prostate cancer [454] but also in breast [455, 456], pancreatic [457] and colorectal cancers [458]. Of particular relevance to the current project is the observation that apoptotic tumour cells can secrete S1P to attract and polarise macrophages into a tumour-associated phenotype [254, 259]. Considering that the SPHK1/S1P pathway has been implicated in chemoresistance [222] and that monocytes can up-regulate SPHK1, I hypothesised that monocytes can rescue apoptotic tumour cells via a SPHK1/S1P dependent mechanism, and that the signalling pathways involved in this process may represent new therapeutic opportunities for limiting tumour progression associated with inflammation and chemoresistance.

7.2 Effect of U937 monocytic cell line in docetaxel-treated PC-3 cells: the role of SPHK1

To confirm the inhibitory effect of docetaxel (Doc) on prostate cancer cell proliferation an SRB assay was performed. PC-3 cells were treated with two different docetaxel concentrations, 5nM and 20nM, for up to 72 hours. Docetaxel treatment reduced PC-3 cell proliferation at the lower dose of 5nM and completely inhibited proliferation at the higher dose of 20nM (**Fig.7.1a**). As expected from previous work performed in my group, 20nM docetaxel decreased cell proliferation after only 24 hours of treatment; this effect was more evident at 72 hours when a 5-fold difference with respect to non-treated PC-3 cells was observed (**Fig.7.1a**).

Using a transwell model as described previously, the addition of U937 cells to docetaxel treated PC-3 cells proved favourable for cancer cell survival. In a 5nM docetaxel-treated co-culture, U937 cells promoted an increase in PC-3 proliferation; this effect was observed across all three time points, being statistically significant at both 24 and 48 hours (**Fig.7.1b**). Interestingly, the combination of docetaxel treatment and the use of siRNA targeting SPHK1 in PC-3 cells abrogated tumour cell proliferation independent of U937s (**Fig.7.1b**). Of note is the observation that siNT PC-3 cells treated with 5nM docetaxel had a much lower proliferation than the corresponding untransfected controls (**Fig.7.1a**); this reduction was most likely caused by the extra transfection procedure that these cells were subjected to.

With respect to the 20nM co-culture, the addition of U937 cells also had a positive impact on PC-3 cell survival, as characterised by an increased proliferation when compared with cells cultured alone (**Fig.7.1c**). The inhibition of SPHK1 in PC-3 cells by the use of siRNA together with docetaxel treatment (20nM) proved to be a potent inhibitor of proliferation for these cells as U937 cells could not impact on PC-3 growth upon SPHK1 knockdown (**Fig.7.1c**).

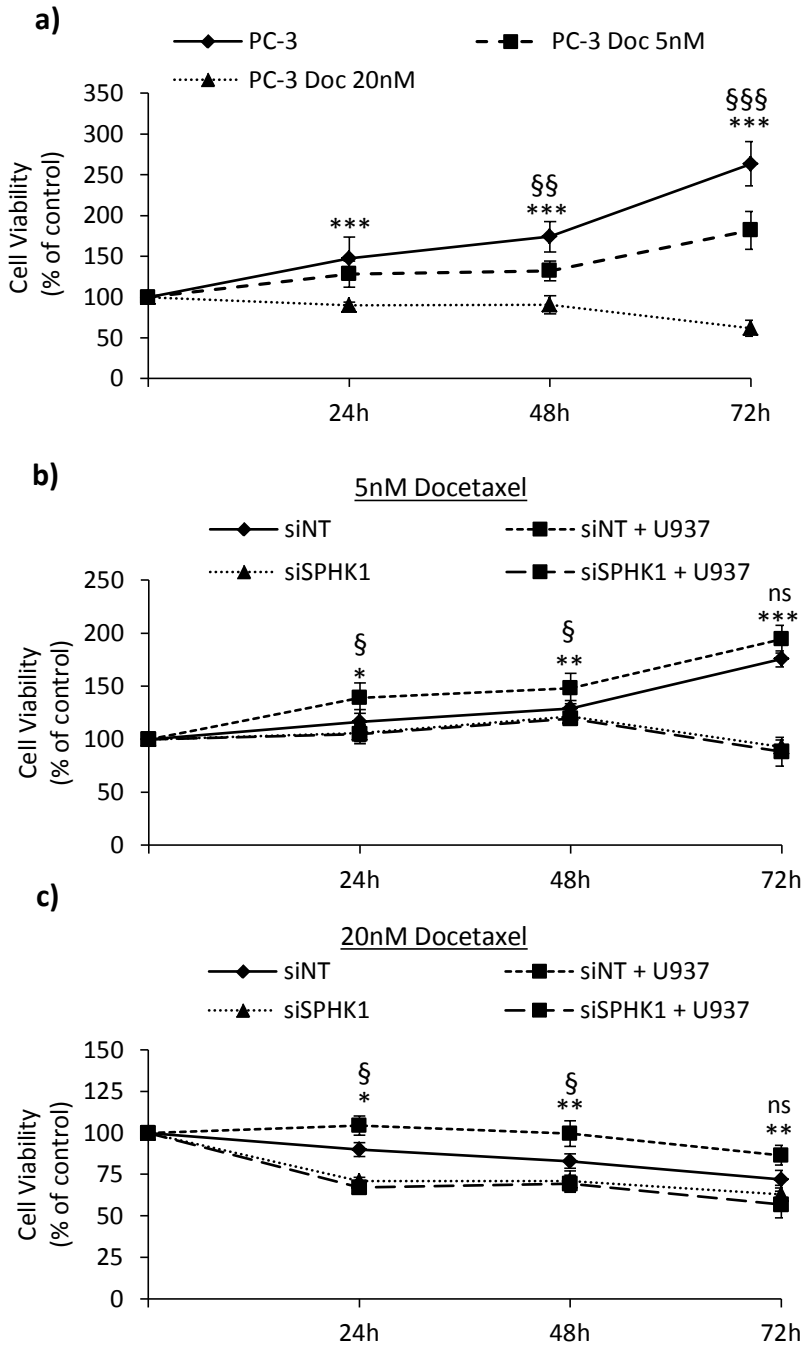


Figure 7.1: Effect of docetaxel in PC-3 cell viability during co-culture. a) PC-3 cells were treated with or without docetaxel (5 or 20nM) in serum-free media. At each indicated time point SRB solution was used to determine PC-3 cell proliferation. **b)** PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1) or non-targeting siRNA (siNT) for 24 hours before cells were washed with PBS and treated with **b)** 5nM or **c**) 20nM of docetaxel for another 24 hours. U937 cells were then added to the upper chamber and left incubating for the indicated time points. SRB solution was added to the bottom of the transwell after the removal of the upper chamber to measure cell proliferation. Data shown is the mean ± SEM of three independent experiments. Statistical analysis was performed using student's t-test relative to each individual time point. **a)*****p<0.001 for PC-3 vs. PC-3 Doc 20nM or §§p<0.01, §§§p<0.001 for PC-3 vs. PC-3 Doc 5nM; **b-c)** ns p>0.05, *p<0.05, **p<0.01, ***p<0.001 for siNT+U937 vs. siSPHK1+U937 or §p<0.05, §§p<0.01, §§§p<0.001 for siNT vs. siNT+U937.

To understand whether the monocyte-induced increase in docetaxel treated PC-3 cell proliferation correlated with a decrease in apoptosis, levels of poly ADP ribose polymerase (PARP), an enzyme implicated in DNA repair and programmed cell death, were determined by western blotting in PC-3 cells at the indicated time points (**Fig.7.2a**). As expected, cleaved PARP levels were significantly increased by the presence of docetaxel, indicating an apoptotic response. The addition of U937 cells decreased cleaved PARP levels in PC-3 cells by approximately 2-fold in docetaxel treated cells (20nM) (**Fig.7.2a-c**). To further investigate whether this event was regulated by SPHK1, an apoptotic co-culture assay was performed with U937 and PC-3 cells transfected with siRNA targeting SPHK1 (siSPHK1), a non-targeting sequence (siNT) and transfection reagent only (Vehicle) control (**Fig.7.2b**). As demonstrated previously, the stimulation of U937 cells led to a decrease in cleaved PARP levels for both vehicle control and siNT PC-3 cells; however, in the absence of SPHK1 this effect was not observed as the cleaved PARP remained constant upon the addition of U937 cells. These findings support the theory that monocytes can confer tumour cell resistance to chemotherapeutic agents through SPHK1.

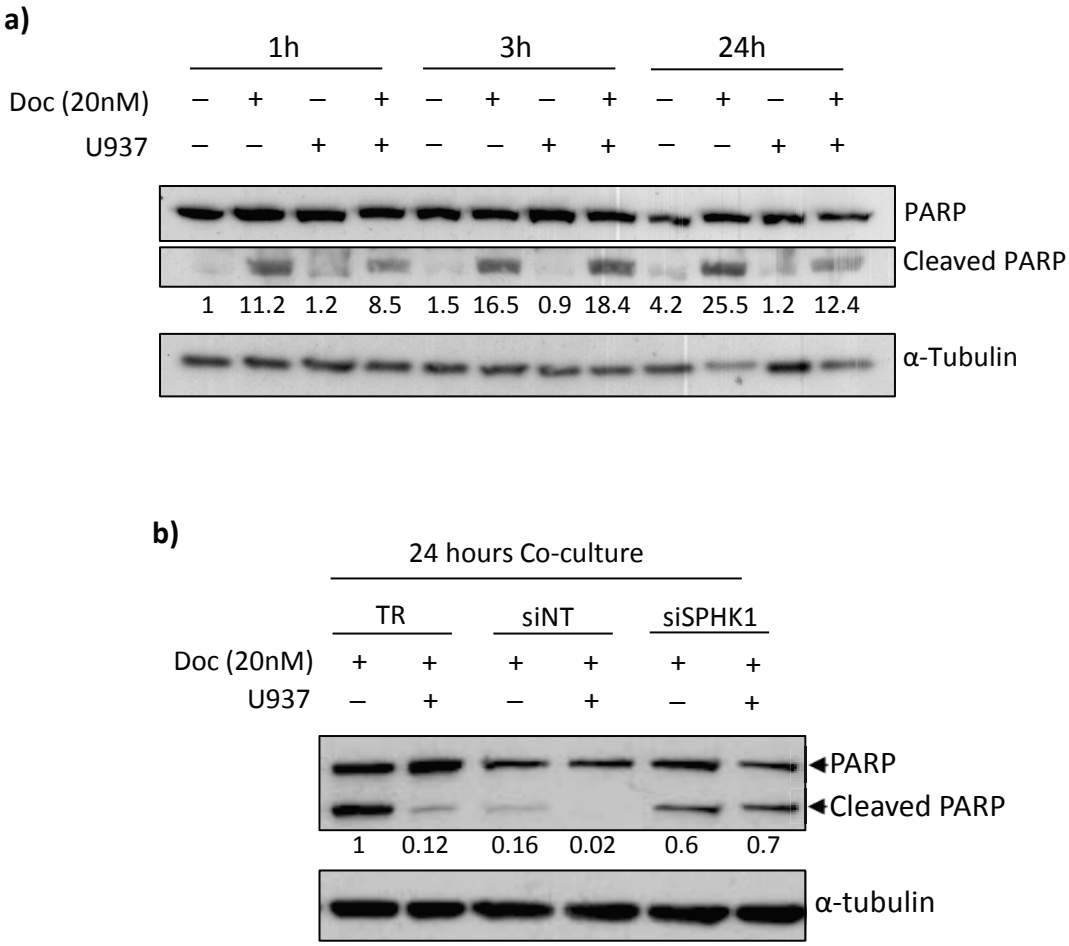


Figure 7.2: Cleaved PARP levels in docetaxel-treated PC-3 cells following their co-culture with U937 cells. a) PC-3 cells were treated with or without docetaxel (20nM) in serum-free media for 24 hours before the addition of U937 cells to the upper chamber for the indicated duration. Cleaved and total PARP levels were determined by western blotting, quantified by Image J software and normalised to α-tubulin. **b**) PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1), non-targeting siRNA (siNT) or transfection reagent (TR) for 24 hours before cells were washed with PBS and treated with 20nM of docetaxel for another 24 hours. U937 cells were then added to the upper chamber and left incubating for 24 hours more. PC-3 cell lysates were collected and Cleaved and total PARP levels were determined by western blotting, quantified by Image J software and normalised to α-tubulin. Data shown represents one independent experiment performed in duplicate.

7.3 SPHK1 regulation in docetaxel-treated PC-3 and U937 cells

To investigate the involvement of SPHK1 in PC-3 cells during apoptotic co-culture with U937 cells, SPHK1 mRNA levels and enzymatic activity were determined at each of the indicated time points. The presence of U937 cells triggered a transient increase in SPHK1 transcriptional levels in PC-3 cells (**Fig.7.3a**); this effect was observed initially after three hours of co-culture when there was an approximate 2-fold increase, whilst a later increase was also detected after 24 hours when SPHK1 expression was up-regulated by 3-fold in PC-3 cultured with U937 cells. At both of these time points the increase proved to be statistically significant when compared to corresponding PC-3 cells cultured alone at each time point.

Similar to SPHK1 mRNA, SPHK1 enzymatic activity in PC-3 cells was clearly increased by the presence of U937 cells (**Fig.7.3b**). Docetaxel treatment of PC-3 cells induced a constant response from monocytes that are, in turn, promoting the activation of SPHK1. The highest levels of activation were observed following three hours of co-culture whereby an approximate 100% increase was observed; indeed this increase in activation proved to be statistically significant not only at three hours but also following just one and 24 hours of co-culture.

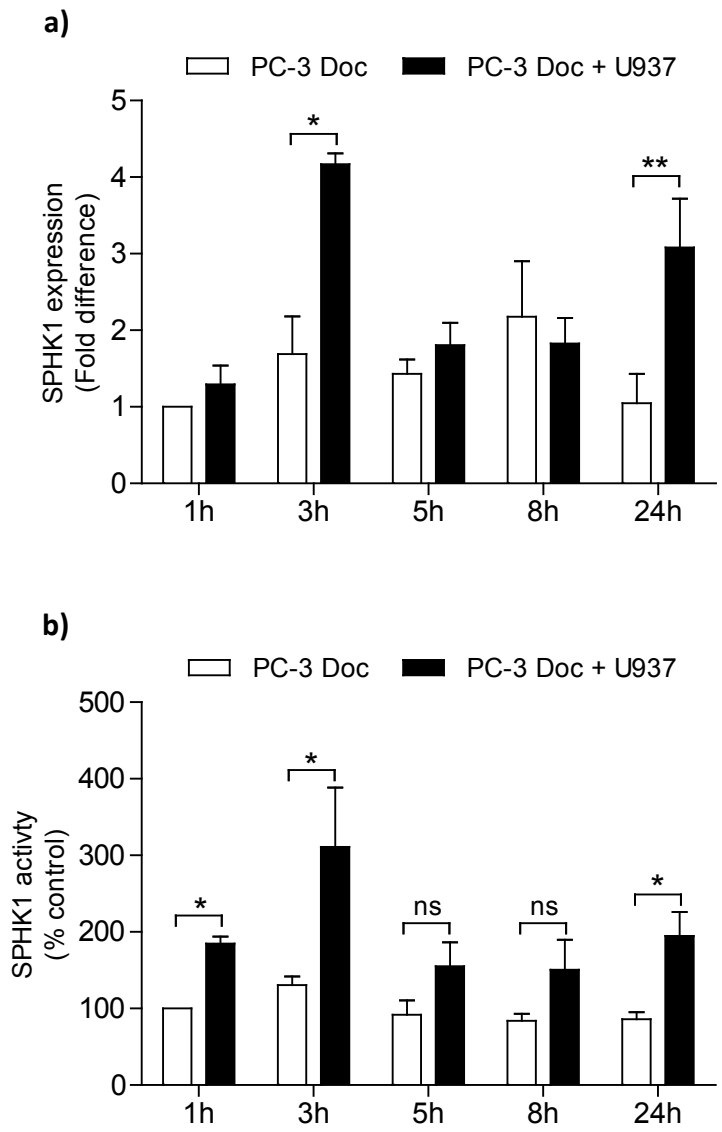


Figure 7.3: SPHK1 activity and expression in docetaxel-treated PC-3 cells following their co-culture with U937 cells. a) PC-3 cells were treated with docetaxel (20nM) in serum-free media for 24 hours before the addition of U973 cells to the upper chamber for the indicated duration. **a)** SPHK1 expression and **b)** SPHK1 activity were determined in PC-3 cell lysates containing equal amounts of mRNA and protein using qRT-PCR and radiolabelling techniques respectively. For qRT-PCR, SPHK1 mRNA expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. Data shown is the mean ± SEM of three independent experiments. Statistical test was done using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$, **$p<0.01$, for PC-3 Doc vs. PC-3 Doc + U937 cells.

Following these initial results, it was important to understand whether the increase in SPHK1 levels in PC-3 cells (**Fig.7.4**) was paralleled in U937 cells. Indeed, there was an up-regulation of SPHK1 mRNA expression in U937 upon stimulation with apoptotic PC-3 cells for all the time points under investigation (**Fig.7.4a**). Docetaxel alone did not affect SPHK1 expression in U937 cells over the time-course. It must be noted that docetaxel was not added directly to the monocytes, instead, and in order to mimic the co-culture model, docetaxel was added for 24 hours to the bottom chamber of the transwell before the addition of U937 cells into the top chamber.

Using the same experimental design as previously described, the enzymatic activity of SPHK1 was also measured in U937 cells (**Fig.7.4b**). Following a similar pattern to that observed in PC-3 cells, SPHK1 activity was increased in U937 cells upon the presence of apoptotic PC-3 cells. This effect was observed across all the studied time points and proved significant up to eight hours of co-culture. Altogether these results indicate an important role for SPHK1 during apoptotic co-culture, as its activation in both cell lines appears to be important for tumour cell protection against docetaxel.

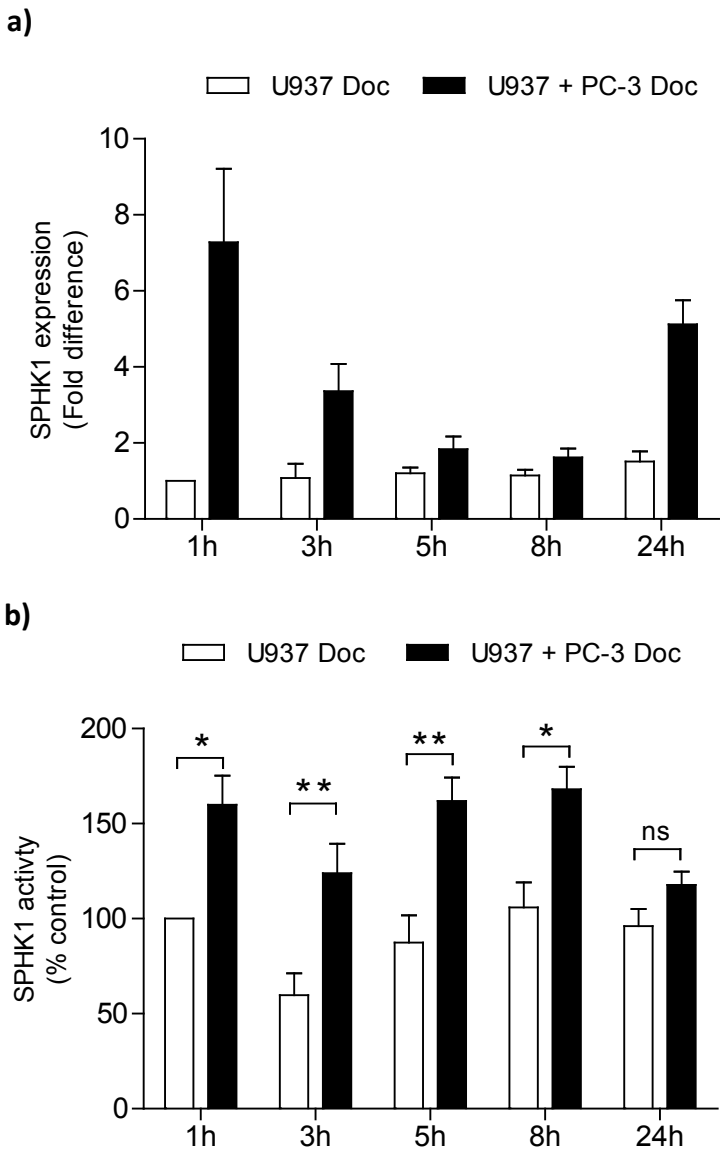


Figure 7.4: SPHK1 activity and expression in U937 cells co-culture with docetaxel treated PC-3 cells. a) PC-3 cells were treated with docetaxel (20nM) in serum-free media for 24 hours before the addition of U973 cells to the upper chamber for the indicated duration. As a control docetaxel was added to the bottom chamber for 24 hours before the addition of U937 cells to the upper chamber (U937 Doc). **a)** SPHK1 expression and **b)** SPHK1 activity were determined in U937 cell lysates containing equal amounts of mRNA and protein using qRT-PCR and radiolabelling techniques respectively. For qRT-PCR, SPHK1 mRNA expression levels were normalised using three housekeeping genes, GAPDH, YWHAZ and UBC. Data shown is the mean ± SEM of **a)** two and **b)** three independent experiments. **b)** Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$, **$p<0.01$, for U937 doc vs. U937 + PC-3 doc cells.

7.4 SPHK1 impact upon cell signalling transduction pathways during co-culture of docetaxel-treated PC-3 and U937 cells

The next step was to understand the molecular mechanisms underpinning SPHK1 activation. Previous findings indicated that SPHK1 was an important regulator of Akt (chapter 4, **Fig.4.2**) and an increase in Akt phosphorylation was accompanied by an increase in SPHK1 levels, therefore it was important to determine Akt phosphorylation levels during apoptotic co-culture. For this assay analysis was only performed for the two time points at which SPHK1 expression in PC-3 cells was shown to be statistically significant, namely three and 24 hours (**Fig.7.3a**). SPHK1 knockdown efficiency was measured by real time PCR (**Fig.7.5ai-ii**) and showed a 60-70% reduction in SPHK1 mRNA levels; an effect that was independent of U937 cell addition at both time points (**Fig.7.5ai-ii**). Western blotting indicated that no increase in Akt phosphorylation levels was apparent in PC-3 cells upon stimulation with U937 cells following three hours of co-culture (**Fig.7.5bi**). SPHK1 knockdown had a negative effect on Akt phosphorylation, as demonstrated by a decrease of 50% in identified levels, however with the addition of U937 cells these levels were restored to parity with non-transfected PC-3 cells (**Fig.7.5bi**). At a later stage of co-culture, Akt phosphorylation levels were increased by the presence of U937 cells; an effect that was abrogated when PC-3 were transfected with SPHK1 siRNA (**Fig.7.5bii**). Indeed, the reduced levels of SPHK1 led to a reduction in Akt phosphorylation which could not be restored by the presence of U937 cells, indicating once more a key role for SPHK1 in Akt regulation.

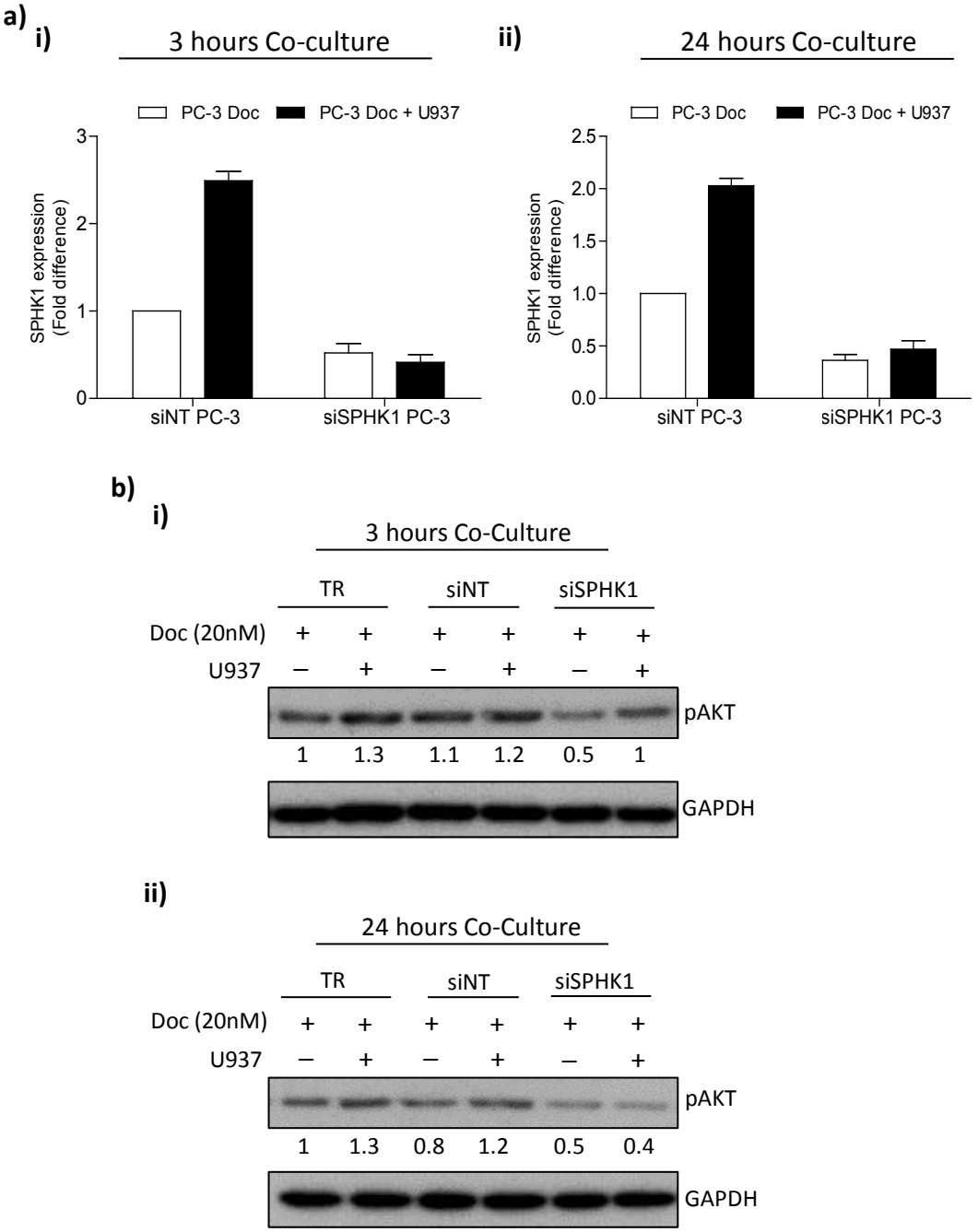


Figure 7.5: SPHK1 expression and AKT phosphorylation levels in docetaxel treated PC-3 cells following their co-culture with U937 cells. a) PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1), non-targeting siRNA (siNT) and **b)** transfection reagent (TR) for 24 hours before cells were washed with PBS and treated with 20nM of docetaxel for another 24 hours. U937 cells were then added to the upper chamber and left incubating for further three and 24 hours. **a)** SPHK1 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. **b)** AKT phosphorylation levels were determined by western blotting, quantified by Image J software and normalised to GAPDH. Data shown is the mean ± SEM of **a)** two independent experiments and **b)** represents one experiment performed in duplicate.

Next it was important to understand what molecular regulators were involved in the U937 signalling response to PC-3 cells, and if these regulators were dependent on SPHK1 activation in PC-3 cells. Using the same co-culture model, an analysis of Akt, ERK1/2 and NF-KB phosphorylation levels in U937 cells was determined by western blotting (**Fig.7.6a**). The levels of Akt phosphorylation in U937 cells were clearly increased by the presence of docetaxel-treated PC-3 cells at both time points, with an initial increase observed at three hours (**Fig.7.6ai-** and **7.6bi**) increasing further at 24 hours (**Fig.7.6aii** and **7.6bii**). ERK1/2 phosphorylation was mildly increased by the presence of docetaxel-treated PC-3 cells after three hours of co-culture (**Fig.7.6ai-** and **7.6bi**); however, following 24 hours of co-culture a larger increase was observed (**Fig.7.6aii** and **7.6bii**). P65, a subunit of the NF-KB complex, was also shown to be up-regulated by PC-3 cells at three (**Fig.7.6ai-** and **7.6bi**) and 24 hours (**Fig.7.6aii** and **7.6bii**).

To investigate whether SPHK1 inhibition in docetaxel-treated PC-3 cells would affect previously identified U937 signalling pathways such as Akt, ERK1/2 and NF-kB, western blotting was performed on protein extracts of U937 cell pellets following their co-culture with PC-3 cells transfected with SPHK1 siRNA and treated with docetaxel (**Fig.7.7a**). Interestingly, following three hours of co-culture there was a clear decrease in Akt, ERK1/2 and NF-KB phosphorylation levels in U937 cells when SPHK1 was knocked down in PC-3 cells (**Fig.7.7ai** and **7.7bi**). At 24 hours, there was small increase in Akt and NF-KB phosphorylation whereas ERK1/2 was not affected by SPHK1 knockdown in PC-3 cells (**Fig.7.7aii** and **bii**). These results come in support of the notion that SPHK1 is a major regulator of the tumour microenvironment and its activation is vital for other important molecular regulators that could ultimately lead to tumour resistance.

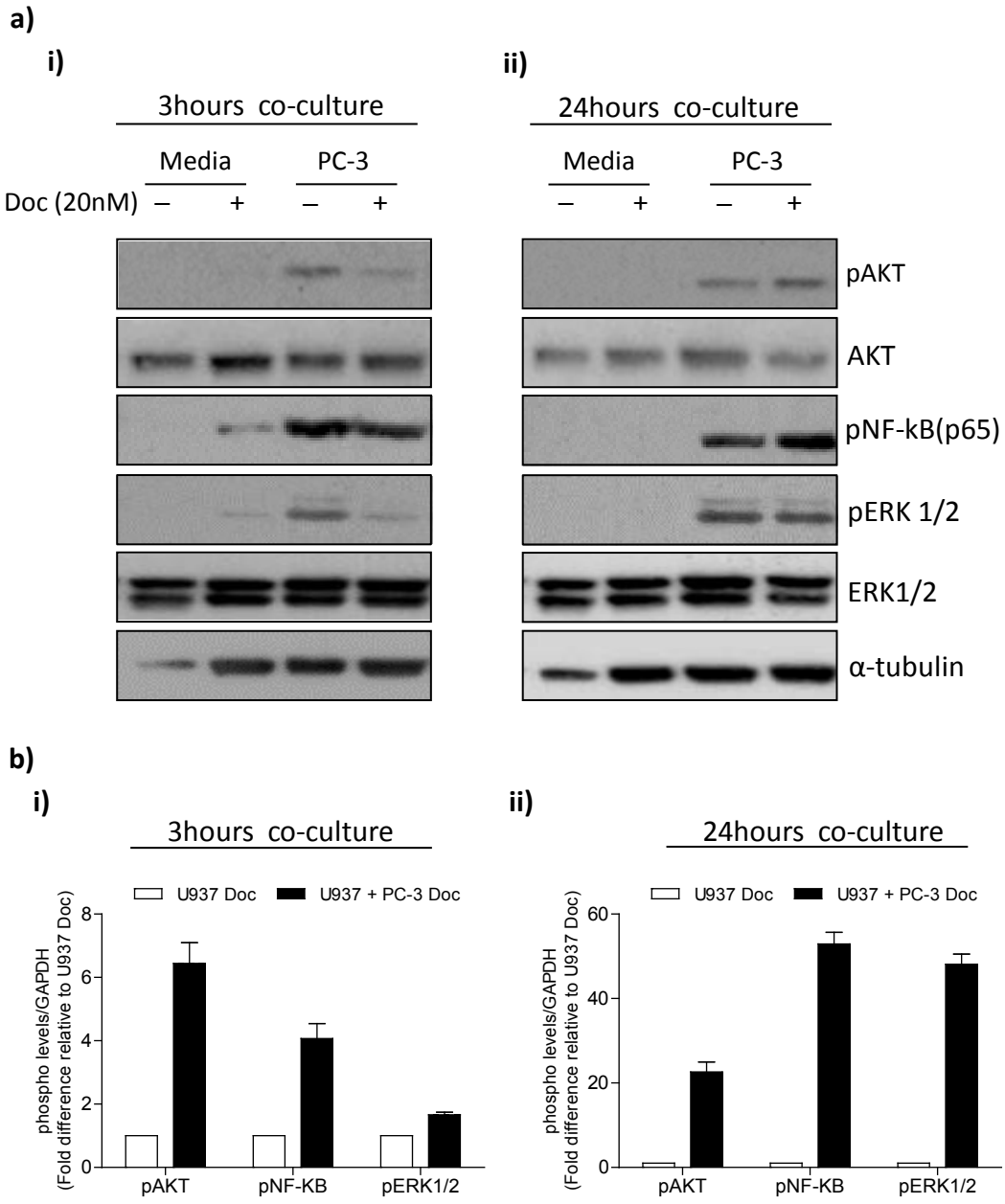


Figure 7.6: Signalling transduction pathways regulation in U937 cells following their co-culture with docetaxel-treated PC-3 cells. PC-3 cells were treated with or without docetaxel (20nM) in serum-free media for 24 hours before the addition of U973 cells to the upper chamber for the indicated duration. As a control docetaxel was added to the bottom chamber for 24 hours before the addition of U937 cells to the upper chamber (U937 Doc). **a)** AKT, NF-kB and ERK1/2 phosphorylated levels in U937 cell lysates was determined by western blotting, **b)** quantified by Image J software and normalised to α-tubulin. Data shown is the mean ± SEM of two independent experiments.

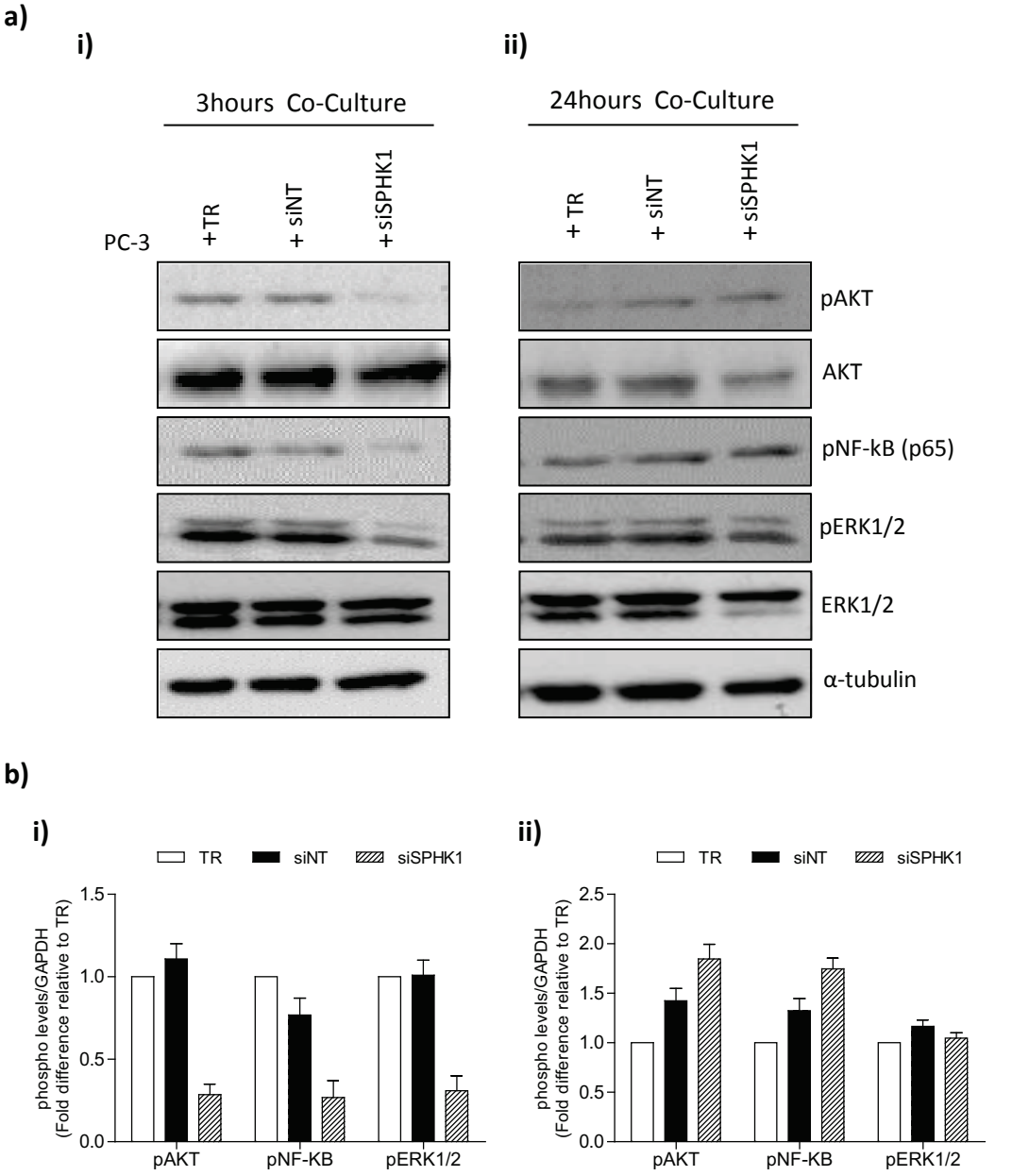


Figure 7.7: SPHK1 knockdown in docetaxel-treated PC-3 cells. Effect on AKT, NF-KB and ERK1/2 phosphorylation levels in U937 cells following their co-culture. PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1), non-targeting siRNA (siNT) or transfection reagent (TR) for 24 hours before cells were washed with PBS and treated with 20nM of docetaxel for another 24 hours. U937 cells were then added to the upper chamber and left incubating for further three and 24 hours. **a)** AKT, NF-kB and ERK1/2 phosphorylated levels in U937 cell lysates was determined by western blotting, **b)** quantified by Image J software and normalised to α-tubulin. Data shown is the mean ± SEM of two independent experiments.

7.5 MCP1 secretory levels during co-culture of docetaxel-treated PC-3 and U937 cells: the role of SPHK1

Following the observed increases in MCP-1 and IL-6 expression and secretion during non-apoptotic co-culture (as discussed in **chapters 5** and **6**), it was important to understand if these two molecules were also playing a role during apoptotic co-culture between PC-3 and U937 cells. For this purpose the media from previous co-cultures (**Fig.7.4** and **Fig.7.5**) was collected and MCP-1 and IL-6 levels were analysed by ELISA. Interestingly MCP-1 secreted levels and mRNA were increased by the presence of U937 cells; this effect was more significant at 24 hours (**Fig.7.8ai-ii**). The use of siRNA targeting SPHK1 in PC-3 cells did not affect the overall levels of MCP-1 present in the media (**Fig.7.8b**).

A similar effect was observed in U937 cells proximity media, as the extracellular levels and mRNA expression of MCP-1 were higher when PC-3 cells were also present (**Fig.7.9ai-ii**), however no difference was noted when SPHK1 was knocked down in PC-3 cells (**Fig.7.9b**). Interestingly, the MCP-1 levels were higher in the media proximal to U937 than in the media adjacent to PC-3 cells (**Fig.7.8**). This comes as a surprise as in the non-apoptotic co-cultures the PC-3 cell media was shown to have higher levels than the U937 media (discussed previously in **chapters 4** and **5**).

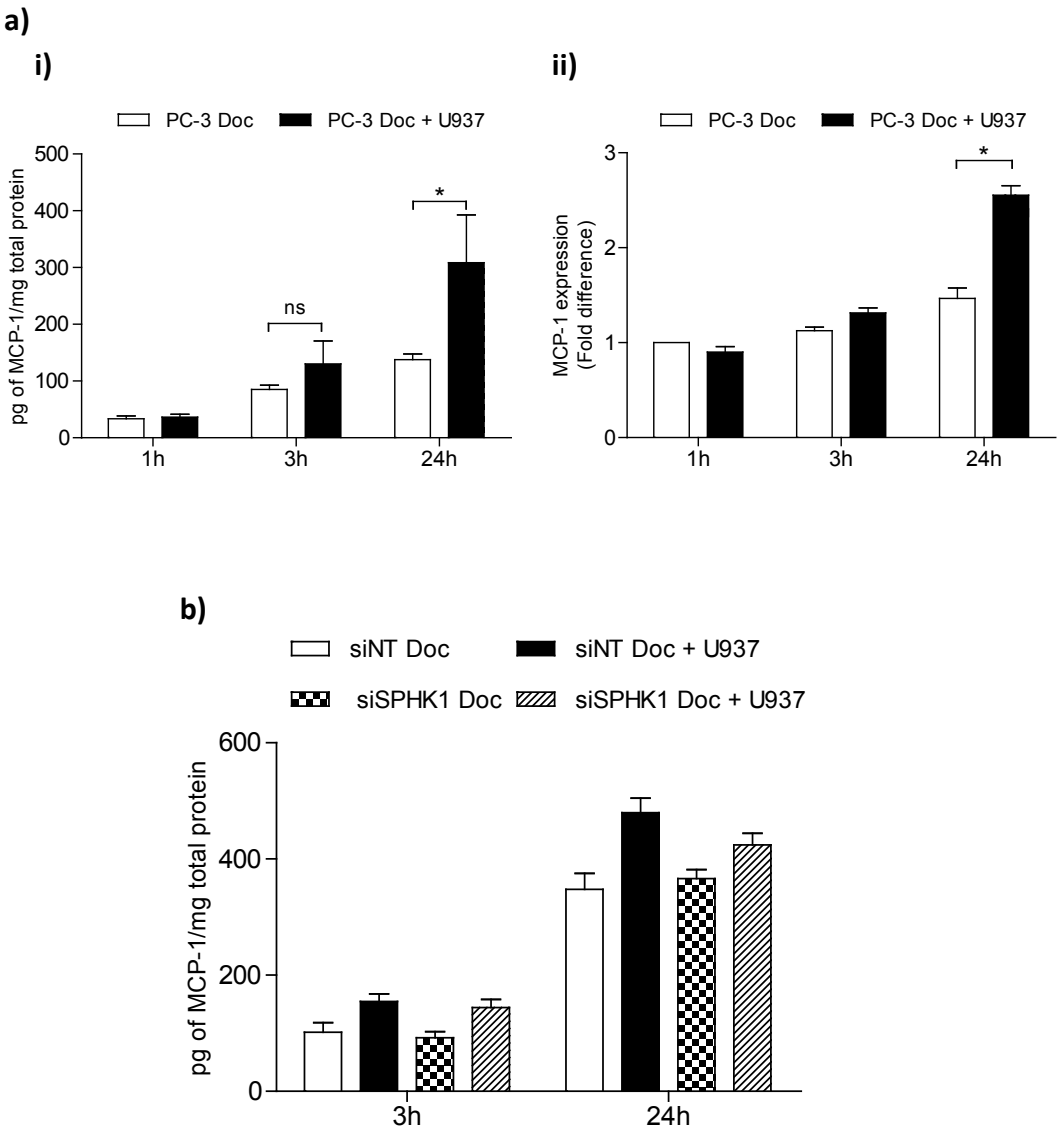


Figure 7.8: MCP-1 levels in docetaxel treated PC-3 cells during co-culture with U937 cells. a) PC-3 cells were treated with docetaxel (20nM) in serum-free media for 24 hours before the addition of U973 cells to the upper chamber for the indicated duration. **b)** PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1) or non-targeting siRNA (siNT) for 24 hours before cells were washed with PBS and treated with 20nM of docetaxel for another 24 hours. U937 cells were then added to the upper chamber and left incubating for further three and 24 hours. **ai,b)** MCP-1 concentration in the bottom chamber media was determined by ELISA. Media was incubated with MCP-1 specific antibodies and a standard curve was used to quantify the relative amount of MCP-1 in each sample. Protein concentration of each cell lysate was used for normalisation. **aii)** MCP-1 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. Data shown is the mean ± SEM of **a)** three and **b)** two independent experiments. **a)** Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$ for PC-3 Doc vs PC-3 Doc +U937.

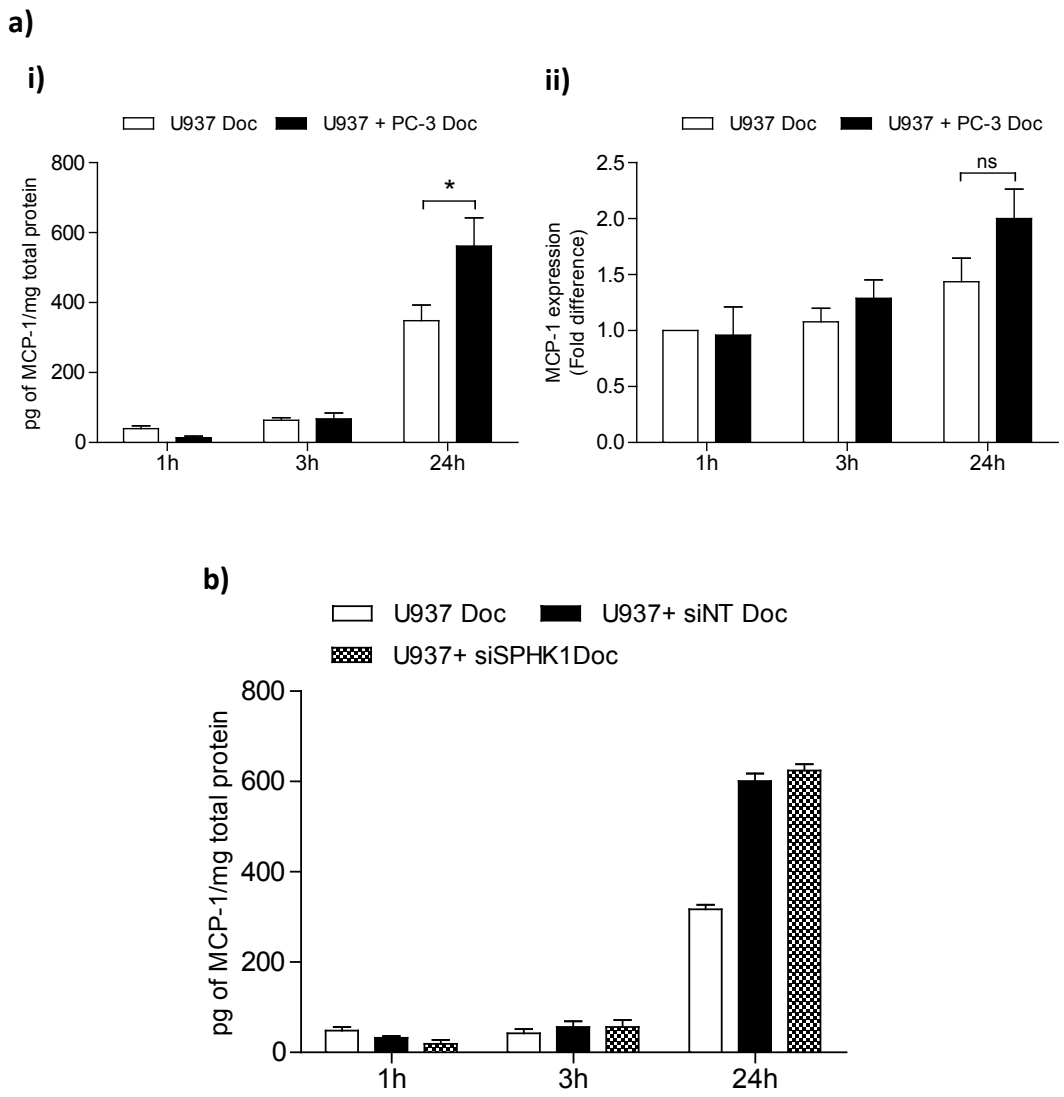


Figure 7.9: MCP-1 levels in U937 cells during co-culture of docetaxel treated PC-3 cells. a) PC-3 cells were treated with docetaxel (20nM) in serum-free media for 24 hours before the addition of U973 cells to the upper chamber for the indicated duration. **b)** PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1) or non-targeting siRNA (siNT) for 24 hours before cells were washed with PBS and treated with 20nM of docetaxel for another 24 hours. U937 cells were then added to the upper chamber and left incubating for further three and 24 hours. As a control docetaxel was added to the bottom chamber for 24 hours before the addition of U937 cells to the upper chamber (U937 Doc). **ai,b)** MCP-1 concentration in the upper chamber media was determined by ELISA. Media was incubated with MCP-1 specific antibodies and a standard curve was used to quantify the relative amount of MCP-1 in each sample. Protein concentration of each cell lysate was used for normalisation. **aii)** MCP-1 expression in U937 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. Data shown is the mean ± SEM of **ai)** two, **aii)** three and **b)** one independent experiment. **aii)** Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$, *$p<0.05$ for U937 Doc vs U937 + PC-3 Doc.

7.6 IL-6 secretory levels during co-culture of docetaxel-treated PC-3 and U937 cells: the role of SPHK1

The same ELISA procedure was performed to measure IL-6 extracellular levels during co-culture. In PC-3 proximity media, IL-6 secretory levels (**Fig.7.10ai**) and mRNA expression (**Fig.7.10aii**) were mildly increased by the presence of U937 cells, however the increase did not prove to be statistically significant (**Fig.7.10aii**). Importantly, the use of SPHK1 siRNA did not affect IL-6 secretion in PC-3 cells cultured alone, however during co-culture SPHK1 proved to be necessary as IL-6 secretion was reduced by approximately 2-fold when PC-3 cells were transfected with SPHK1 siRNA (**Fig.7.10b**).

An approximate 60% increase in IL-6 was detected in U937 cells media upon co-culture with PC-3 cells for 24 hours (**Fig.7.11ai**). IL-6 mRNA was also increased in U937 cells co-cultured with docetaxel-treated PC-3 cells after one hour (**Fig.7.11aii**). Interestingly, IL-6 secretion from U937 cells was slightly reduced by the absence of SPHK1 signalling from PC-3 cells after 24 hours in co-culture (**Fig.7.11b**). It must be noted that experiments **7.10b** and **7.11b** were performed only once; therefore it is important that these data are analysed and considered with caution as further repeats are required to gain confidence in the results and allow the drawing of more assertive conclusions.

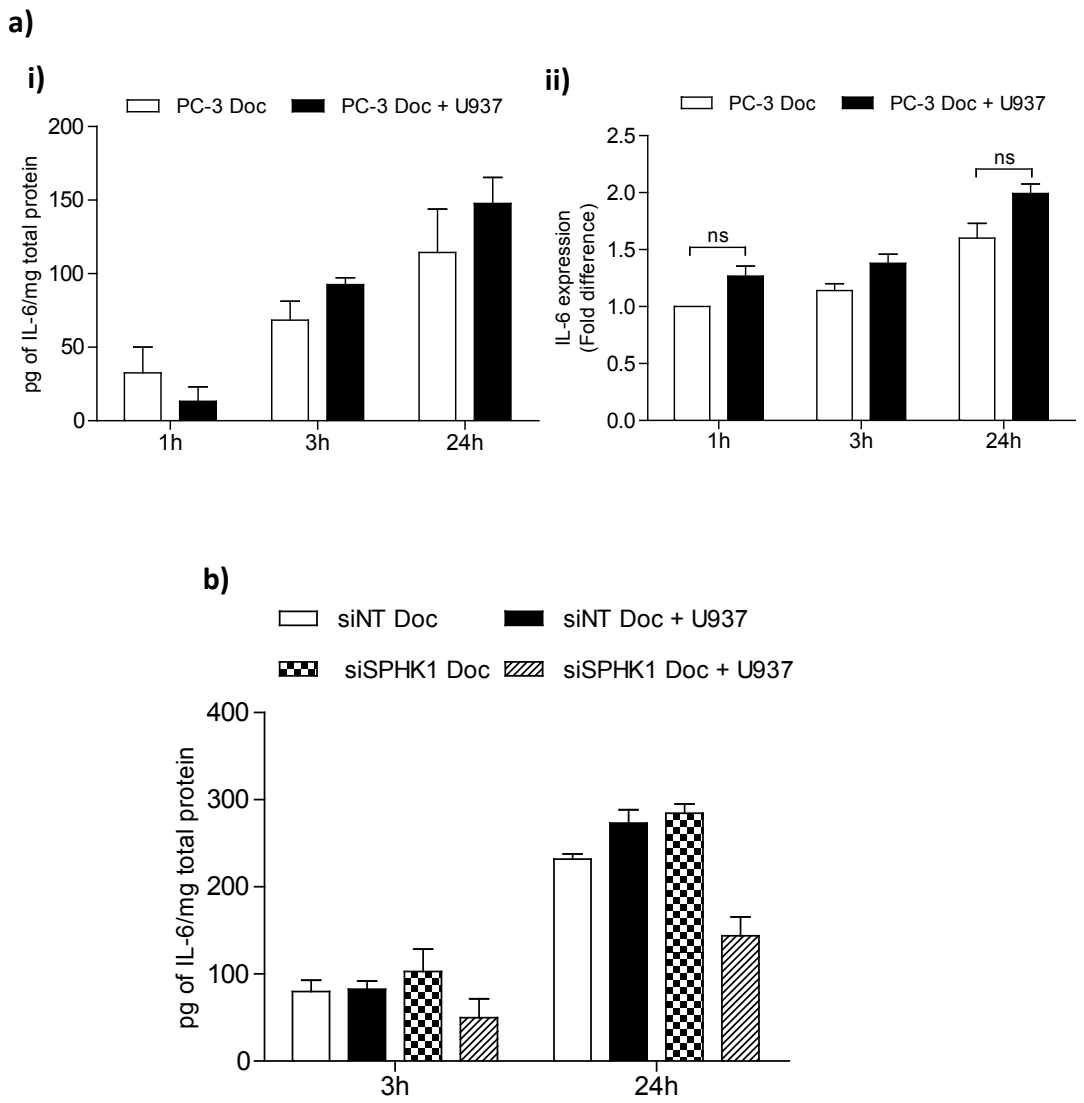


Figure 7.10: IL-6 levels in docetaxel treated PC-3 cells during co-culture with U937 cells. a) PC-3 cells were treated with docetaxel (20nM) in serum-free media for 24 hours before the addition of U973 cells to the upper chamber for the indicated duration. **b)** PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1) or non-targeting siRNA (siNT) for 24 hours before cells were washed with PBS and treated with 20nM of docetaxel for another 24 hours. U937 cells were then added to the upper chamber and left incubating for further three and 24 hours. **ai,b)** IL-6 concentration in the bottom chamber media was determined by ELISA. Media was incubated with IL-6 specific antibodies and a standard curve was used to quantify the relative amount of IL-6 in each sample. Protein concentration of each cell lysate was used for normalisation. **aii)** IL-6 expression in PC-3 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. Data shown is the mean ± SEM of **a)** three and **b)** one independent experiments. **aii)** Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$ for PC-3 Doc vs PC-3 Doc +U937.

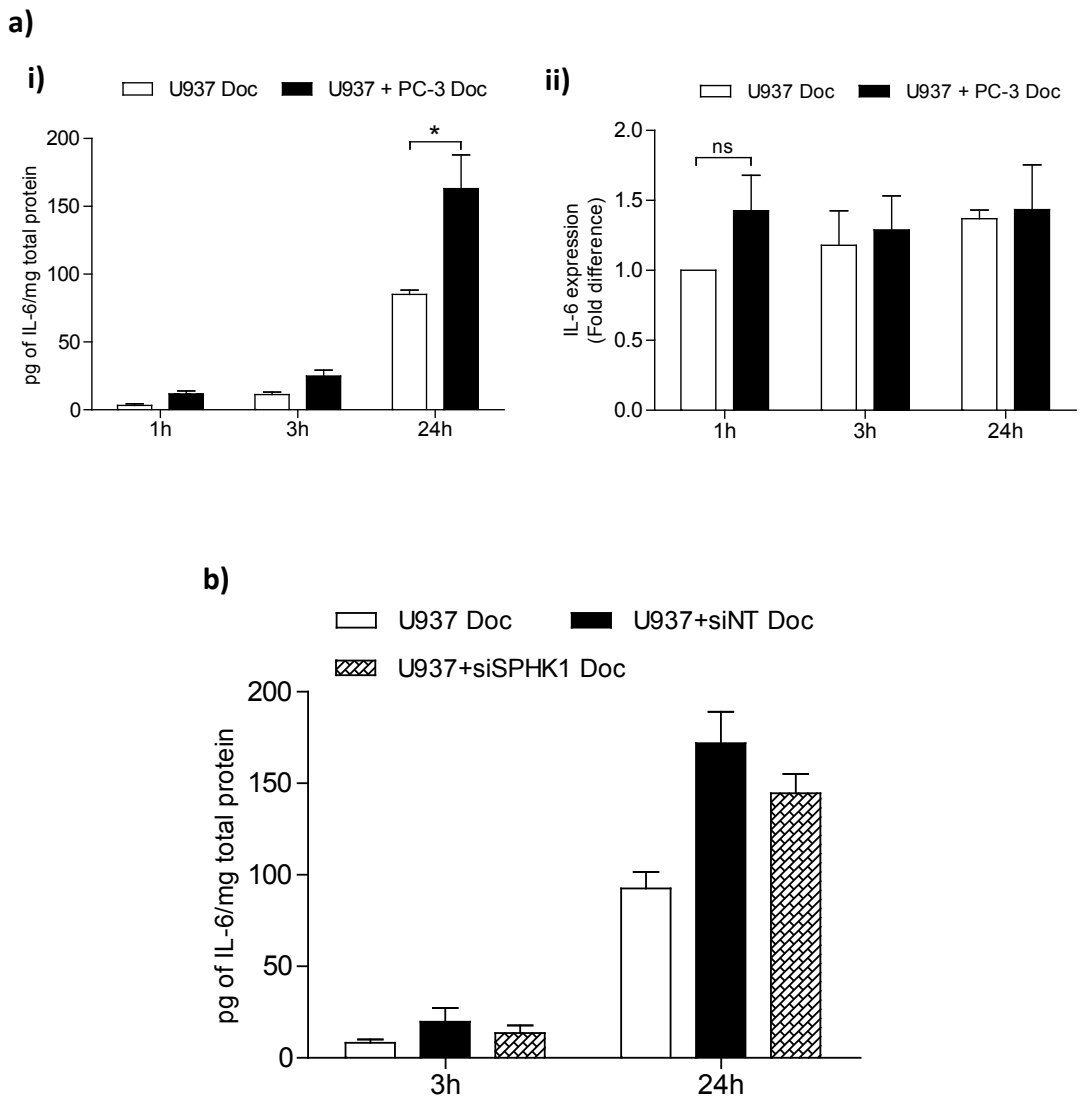


Figure 7.11: IL-6 levels in U937 cells during co-culture of docetaxel treated PC-3 cells. a) PC-3 cells were treated with docetaxel (20nM) in serum-free media for 24 hours before the addition of U973 cells to the upper chamber for the indicated duration. **b)** PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1) or non-targeting siRNA (siNT) for 24 hours before cells were washed with PBS and treated with 20nM of docetaxel for another 24 hours. U937 cells were then added to the upper chamber and left incubating for further three and 24 hours. As a control docetaxel was added to the bottom chamber for 24 hours before the addition of U937 cells to the upper chamber (U937 Doc). **ai,b)** IL-6 concentration in the upper chamber media was determined by ELISA. Media was incubated with IL-6 specific antibodies and a standard curve was used to quantify the relative amount of IL-6 in each sample. Protein concentration of each cell lysate was used for normalisation. **aii)** IL-6 expression in U937 cell lysates was determined by qRT-PCR. Three housekeeping genes, GAPDH, YWHAZ and UBC were used to normalise. Data shown is the mean ± SEM of **ai)** two, **aii)** three and **b)** one independent experiment. **aii)** Statistical analysis was performed using student's t-test relative to each individual time point. ns $p>0.05$ for U937 Doc vs U937 + PC-3 Doc.

7.7 Identification of soluble mediators involved in the co-culture of docetaxel-treated PC-3 and U937 cells

In order to understand what other factors may be involved in the apoptotic co-culture of PC-3 with U937 cells, a proteome profiler assay was performed. Media from PC-3 cells cultured with docetaxel for 48 hours, PC-3 treated with docetaxel for 24 hours and then cultured with U937 for another 24 hours, and media from U937 cells cultured in the presence of docetaxel for 24 hours were added separately to individual membranes pre-coated with 36 different antibodies (**Fig.7.12a**). The media from PC-3 co-cultured with U937 cells showed an increase in CD40L, siCAM-1, IL-16 and IL-32α in comparison with either PC-3 or U937 cells cultured alone (**Fi.7.12b**). CD40L secretion was mildly increased by the presence of U937 cells, whilst as for siCAM-1 secretion there was an increase of approximately 4-fold in comparison with PC-3 cells cultured alone and a 2-fold increase in comparison with U937 cells cultured alone. IL-16 and IL-32α also showed similar increases when compared with PC-3 cells cultured alone, however the secretion of these molecules by U937 cells alone could explain this and may suggest that these effects are rather a consequence of media diffusion than of the presence of PC-3 cells.

Based upon the initial findings from the previous assay, a more detailed investigation of CD40L and siCAM-1 with respect to their regulation by SPHK1 was proposed. For CD40L it was not possible to validate the previous results or explore further its role in SPHK1 regulation due to technical difficulties with the ELISA for this cytokine, despite several attempts to resolve the issue. As for siCAM-1, its secreted levels were increased during co-culture when compared with PC-3 cells cultured alone (**Fig.7.13a**). This effect was observed at all the studied time points and proved to be independent of SPHK1 as its knockdown did not inhibit siCAM-1 secretion (**Fig.7.13b**). A similar effect was observed in U937 cell media, were siCAM-1 secretion was increased by the presence of U937 cells but was not affected the absence of SPHK1 in PC-3 cells (**Fig.7.13c**). The relative values of siCAM-1 suggest PC-3 cells as the main secretors and most likely responsible for the increase observed in U937 cell media, through the natural diffusion that occurs during co-culture. A possible role for siCAM-1 in chemoresistance could be hypothesised from these findings, however further investigation is required to validate this preliminary data.

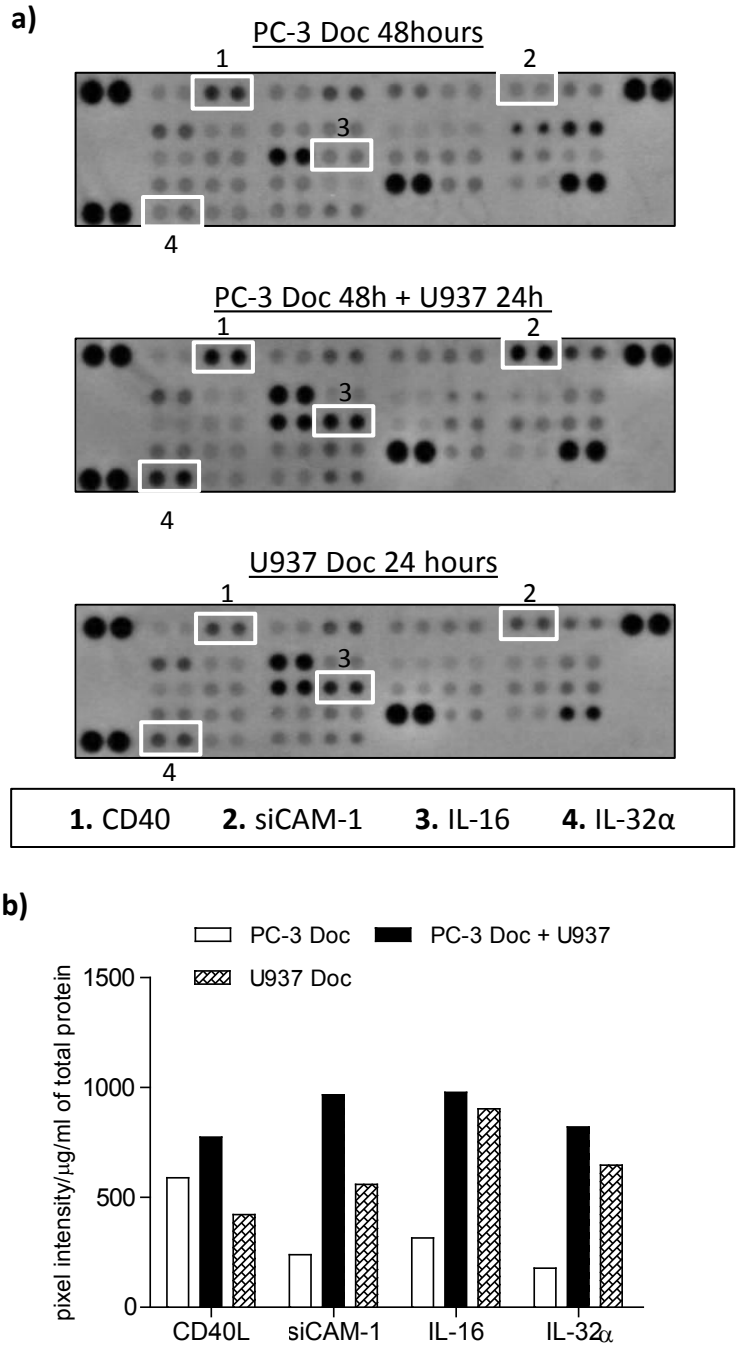


Figure 7.12 Proteome Profiler Assay in apoptotic co-culture. a) PC-3 cells were treated with docetaxel (20nM) in serum-free media for 24 hours before the addition of U973 cells to the upper chamber for further 24 hours. As a control docetaxel was added to the bottom chamber for 24 hours before the addition of U937 cells to the upper chamber (U937 Doc). After collection, media was centrifuged in order to remove non-adherent cells. Each media was incubated individually with a pre-probed membrane. Signal intensity for each of the 36 molecules on the membrane was measured using Image J software and the average intensity from the duplicate samples calculated. **b)** The results obtained for selected molecules are presented graphically and were normalised to the protein content; data is representative of a single experiment.

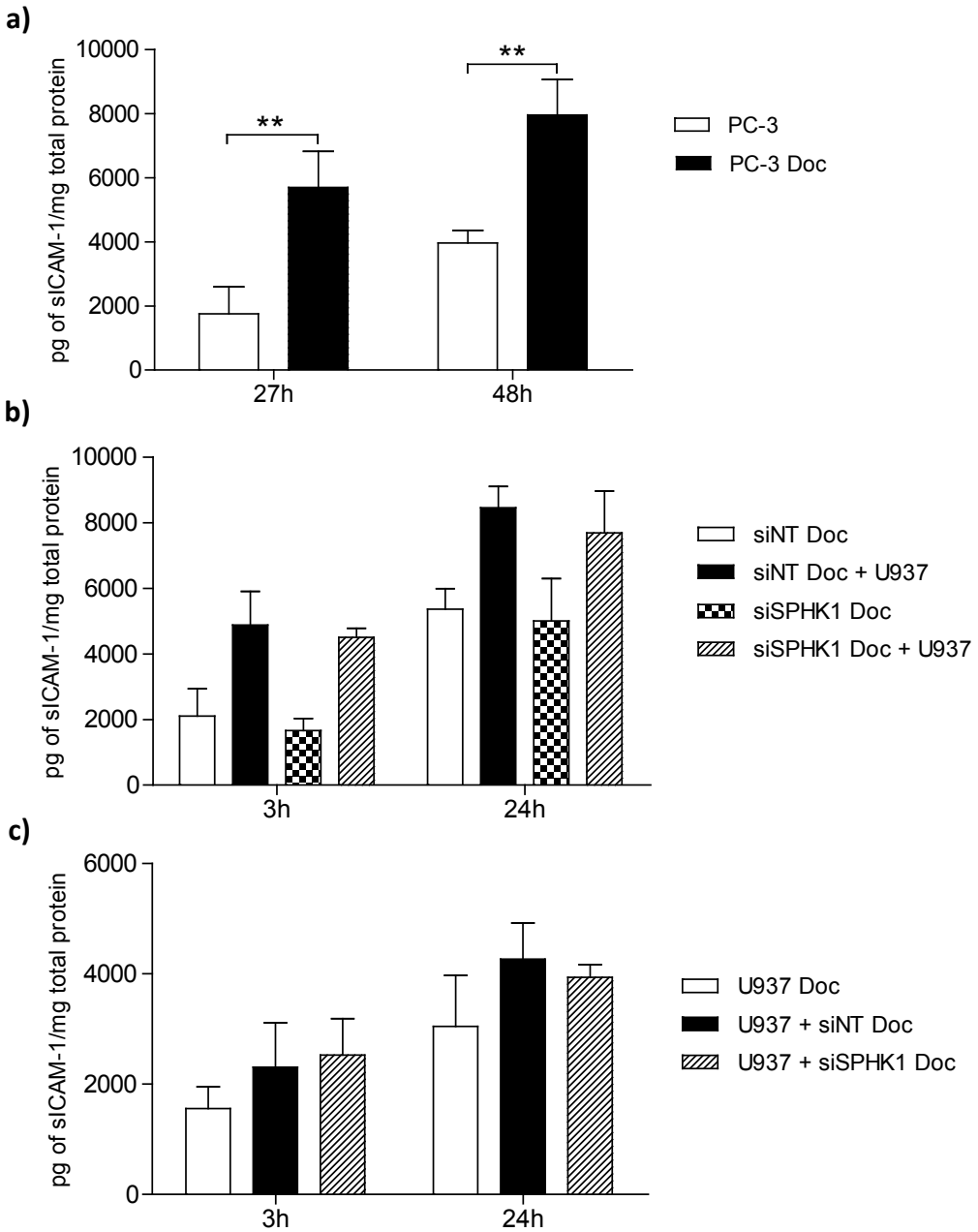


Figure 7.13: soluble ICAM-1 levels during co-culture of docetaxel treated PC-3 with U937 cells. a) PC-3 cells were treated with or without docetaxel (20nM) in serum-free media for 24 hours. sICAM-1 concentration in the upper chamber media was determined by ELISA. Media was incubated with siCAM-1 specific antibodies and a standard curve was used to quantify the relative amount of sICAM-1 in each sample. Protein concentration of each cell lysate was used for normalisation. **b-c)** PC-3 cells were transfected with siRNA targeting SPHK1 (siSPHK1) or non-targeting siRNA (siNT) for 24 hours before cells were washed with PBS and treated with 20nM of docetaxel for another 24 hours. U937 cells were then added to the upper chamber and left incubating for further three and 24 hours. As a control docetaxel was added to the bottom chamber for 24 hours before the addition of U937 cells to the upper chamber (U937 Doc). sICAM-1 concentration in the **b)** upper and **c)** lower chamber was determined was described in **(a)**. Data shown is the mean ± SEM of **a)** three and **b-c)** two independent experiments. **a)** Statistical analysis was performed using student's t-test relative to each individual time point. **p>0.01 for PC-3 vs PC-3 Doc.

7.8 Discussion

A dysregulation in the balance of sphingolipid metabolism towards the activation of the SPHK1/S1P pathway has been linked with the acquisition of several oncogenic characteristics such as cancer cell proliferation, metastasis and chemoresistance [459-461]. SPHK1/S1P inhibition was synergistic with camptothecin [216], docetaxel [78] and radiation therapy [144] and led to a re-sensitisation of cancer cells to these therapies. In a tumour microenvironment context S1P is known to act as a strong chemoattractant for the recruitment of immune cells [459, 462]. S1P has been shown to be secreted from apoptotic leukemic cells; consequently monocytes and macrophages were attracted to these dying cells via S1P [259]. In this context I investigated the involvement of SPHK1/S1P in monocyte-induced resistance to chemotherapy in apoptotic tumour cells. I found that U937 cells increased proliferation in docetaxel-treated PC-3 cells; this effect was significant either with a lower (**Fig.7.1b**) or higher (**Fig.7.1c**) docetaxel concentration. In support, cleaved PARP levels were also reduced in docetaxel-treated PC-3 cells after co-culture with U937 cells (**Fig.7.2a**) indicating a protective effect of this co-culture. Interestingly SPHK1 levels in PC-3 cells were crucial for monocytes to rescue apoptotic cells, as the use of siRNA targeting SPHK1 in these cells abrogated U937-induced proliferation (**Fig.7.1b** and **Fig.7.1c)** and inhibited the decrease in cleaved PARP levels in PC-3 cells (**Fig.7.2b**). Supporting the importance of SPHK1, PC-3 (**Fig.7.3**) and U937 cells (**Fig.7.4**) both showed an increase in SPHK1 transcription and enzymatic activity following their co-culture; in fact SPHK1 activity in both cells was consistently increased when in co-culture (**Fig.7.3b** and **Fig.7.4b**). These results are clear indicators that monocytes are responding to apoptotic PC-3 cells and *vice versa*, with SPHK1 being one of the main regulated molecules.

Due to its autocrine and paracrine action, S1P emerges as a possible regulator and activator of SPHK1 in both cell types. I previously showed that S1P can induce SPHK1 transcriptional activation in both PC-3 and U937 cells (**chapter 4** and **5**). Other groups have also suggested that S1P is involved in cancer cell resistance via a “cross-talk” interaction with macrophages, for instance Weigert *et al.* showed that apoptotic breast cancer cells (MCF-7) induced macrophage polarisation towards an alternative phenotype, leading to an increase in their viability. More interestingly the use of the apoptosis resistant MCF-7-Bcl2 cell line failed to induce an alternative activation of macrophages leading to a loss of cell viability in

MCF-7; a similar effect was also seen when S1P production was inhibited, suggesting a role for apoptosis-released S1P in induction of macrophage polarisation [463]. In support, Gude *et al.* showed that S1P was released from U937 cells upon doxorubicin treatment; interestingly this increase was accompanied by an increase in SPHK1 expression and led to an attraction of other monocytes and macrophages [259].

The use of siRNA against SPHK1 in PC-3 cells not only affected Akt regulation in these cells (**Fig.7.5**) but also affected important signalling molecules such as ERK1/2, NF-KB and Akt in U937 cells (**Fig.7.7**). In fact, for the first time it is shown that SPHK1 levels in cancer cells can regulate the activation of ERK1/2, NF-KB and Akt in monocytes. Indeed, these pathways were activated in U937 cells upon the presence of apoptotic PC-3 cells (**Fig.7.6**), but to a lesser extent when SPHK1 was repressed in PC-3 cells. This comes in support of the concept that SPHK1 is involved in the secretion of soluble molecules from apoptotic tumour cells that in turn lead to the activation of pro-survival pathways in monocytes. Reports in the literature indicate Akt and ERK1/2 as important regulators of myeloid survival. Marfe *et al.* showed that Akt inhibition in resistant chronic myeloid leukaemia cells (Ki562) led to an increase in cell sensitivity to imatinib (tyrosine kinase inhibitor). With particular relevance to this work, a decrease in SPHK1 expression and activity was observed when Akt was inhibited [187]. In accordance, another study showed human primary monocyte-derived macrophages increased their resistance to TNF-α and Cycloheximide (CHX) induced-apoptosis when cultured in the presence of apoptotic cell media or S1P. This protection was found to be dependent on Akt, and to a less extent ERK1/2, activation as the use of pharmacological inhibitors for Akt and ERK1/2 increased their sensitivity to TNF-α and CHX [254]. These findings suggest an important role of Akt and ERK1/2 in macrophage resistance to apoptosis and modulation of their response to cancer cells. Importantly, my work shows that Akt and ERK1/2 can be indirectly inhibited in monocytes by SPHK1 inhibition in apoptotic cancer cells, supporting the concept of SPHK1/S1P involvement in apoptotic cross-talk between cancer cells and their surroundings.

As for other possible mediators in the apoptotic tumour-monocyte interaction, MCP-1 and IL-6 were considered as their levels were previously found to be high during co-culture of untreated PC-3 (chapter 4, **Fig.4.7** and **Fig.4.10**) with U937 cells (**chapter 5**, **Fig.5.10** and **Fig.5.15**). Despite their increase during co-culture in the presence of docetaxel, secreted

levels of both MCP-1 (**Fig.7.8** and **Fig.7.9**) and IL-6 (**Fig.7.10** and **Fig.7.11**) did not prove to be affected by SPHK1 knockdown in PC-3 cells, with the exception of IL-6 at 24 hours (**Fig.8.10b**). It should be noted that the experiments shown in figures **7.8b**, **7.9b**, **7.10b** and **7.11b**, were performed only once; it is therefore important that analysis of these data is considered with caution as further repeats are required to gain confidence in the results. However, and together with the previous chapters, these results suggest a SPHK1-independent regulation of these cytokines during apoptotic co-culture and indicate the activation of other pathways during this interactive process. Nonetheless in previous chapters IL-6 was shown to regulate SPHK1 expression and activity in PC-3 (chapter 4, **Fig.4.12a-b)** and U937 cells (chapter5, **Fig.5.17**), as well as MCP-1 expression in U937 (chapter 5, **Fig.5.19**). It is certainly possible that the same phenomenon is occurring during apoptotic co-cultures and it is therefore important to study their role in greater detail. An interesting study showed that MCP-1 mRNA levels were increased upon docetaxel treatment in two different prostate cancer cell lines (LNCaP and LAPC4), whilst the inhibition of MCP-1 re-sensitised cells to docetaxel inhibitory effect upon cell proliferation. In addition, it was shown that in patients that underwent chemotherapy a significant increase in gene expression of MCP-1 and IL-6 was observed in tumours. [464].

Supporting the role of IL-6 in tumour chemoresistance, Castells *et al.* proposed that IL-6 secreted by macrophages led to an increase in chemoresistance in ovarian cancer cells, however the factor triggering IL-6 secretion was not identified [465]. Furthermore, Wang *et al.* showed that IL-6 treatment of multiple myeloma cells led to an activation of SPHK1, resulting in protection from apoptosis, and SPHK1 siRNA inhibited the IL-6 effect on cell protection, leading to an increase in apoptosis [294].

The proteome profiler assay indicated other potential targets such as siCAM-1, CD40L and IL-32α (**Fig.7.12**). siCAM-1 is a soluble form of Intracellular Adhesion Membrane-1 (ICAM-1) that is shed from cells, and high levels have been found in serum of patients with inflammation, infection and cancer [319]. *In vivo* it has been reported to promote tumour growth [466] and in patients with colorectal cancer siCAM-1 levels were significantly higher than in control patients, with high levels correlating with formation of metastasis. A more recent study showed the involvement of S1P/S1PR2 in ICAM-1 expression through an NF-KB dependent mechanism [467]. In this study I show for the first time that siCAM-1 is secreted

from PC-3 cells upon docetaxel treatment (**Fig.7.13a**) and this effect is even more accentuated in the presence of U937 cells (**Fig.7.13b**), but does not appear to be mediated by SPHK1 (**Fig.8.13c**). Altogether, siCAM-1 appears to play a role in cancer development and could be an important mediator in the acquisition of drug-resistance by cancer cells. The exact effect on monocyte activation and recruitment as well as its involvement in SPHK1 regulation is not known yet and should be studied in further detail.

CD40L and IL-32α levels during apoptotic co-culture were not possible to measure, however reports in the literature suggest a possible role in cancer-immune cell interactions for these two molecules. Futagami *et al.* showed that CD40L and MCP-1 stimulated macrophages and induced the expression of COX-2 correlating with an increase in PGE_2 and VEGF production [468]. On the contrary, CD40L has been reported to have anti-tumour activity; however its soluble form sCD40L contributes to systemic inflammatory diseases [469]. Another interesting molecule that was up-regulated in the apoptotic PC-3 co-culture was IL-32α, a pro-inflammatory cytokine that has been shown to be activated by other cytokines such as TNF-α and IL-1β through PI3K and NF-κB activation in myofibroblasts [470]. Nishida *et al.* showed an increase in IL-32α expression in pancreatic cancer cells when compared with normal pancreatic cells; moreover, the use of IL-32α-specific siRNA suppressed the expression of Bcl-2 and Mcl-1, known anti-apoptotic proteins, leading to an increase in apoptosis [471]. Together with siCAM-1 these two molecules are likely to play a role in tumorigenesis and inflammation, so their role in SPHK1 regulation and prostate cancer progression should be assessed.

Taken together these data re-enforce the idea that SPHK1 should be considered as a possible target as part of a combined therapy with chemotherapeutic agents, in particular when monocytes/macrophages are highly present as part of the stromal microenvironment. The ability of SPHK1 to influence other important signalling pathways via autocrine or paracrine actions of its regulatory molecules should be considered as an important therapeutic target against cancer and its stromal microenvironment cross-talk.

8. General Discussion

8.1 SPHK1 and cancer development

Sphingosine Kinase-1 (SPHK1) is a highly conserved lipid kinase that is ubiquitously expressed and catalyses the formation of sphingosine-1-phosphate (S1P), an important mediator with intra and extracellular functions. S1P and SPHK1 can regulate a wide range of cellular processes, including cell proliferation, survival, migration and immune cell trafficking. A deregulation in SPHK1 levels has been described in many cancers such as prostate, breast, lung and pancreatic, with increased levels associated with poor clinical prognosis. Conversely, SPHK1 down-regulation by siRNA or pharmacological inhibition markedly reduced cell proliferation in both an *in vitro* and *in vivo* setting [144, 203, 222]. Of particular relevance to this study, the SPHK1 inhibitor SKi-II was previously described to have *in vivo* anti-tumoral activity; French et al showed an approximate 60% reduction in JC mammary adenocarcinoma mouse model after 10 days with dose of 100mg/kg [472]. In this study, my data upholds these findings and shows that *in vivo* SKi-II significantly impairs tumour growth in a xenografted prostate cancer model. This effect was accompanied by a decrease in SPHK1 mRNA, supporting SPHK1 as a valid target for cancer therapeutics. It is important to note that future therapies using SPHK1 inhibitors should consider each type of cancer as individual cases as supported by recent reports indicating that some tumours appear to respond better to different sphingosine kinsase inhibitors. This is demonstrated in a recent study that shows only SKi-I, and not SKi-II, can act as a tumour growth inhibitor in melanoma tumours [351].

In a cancer related event, it is thought that tumour cells are able to recruit inflammatory cells to the tumour site and orchestrate their response towards a pro-tumorigenic effect **[389, 473]**. Previous studies have demonstrated the capabilities of monocytes in promoting tumour development in both *in vitro* and *in vivo* models **[246, 248, 250]**. In this study, and using a co-culture system, my data supports the positive effect of monocytes on cancer cell proliferation, more specifically in prostate (PC-3) and breast cancer cells (MDA-231 and BT549). This effect extends to PC-3 cells treated with docetaxel, as monocytes are capable of reducing the inhibitory effect of this drug on cancer cell

proliferation. This is of particular relevance as the microenvironment has been suggested to play an active role in supporting cancer against such drugs, as exemplified by the tendency of CRPC tumours to become unresponsive to chemotherapeutic agents, resulting in a poor clinical prognosis for the patient. My group have previously shown that targeting SPHK1 sensitises prostate cancer cells to docetaxel, whilst SPHK1 overexpression induces an opposite effect **[216]**. My data upholds these findings and shows that SPHK1 targeted inhibition not only reduces tumour cell growth but can also reduce the positive impact of monocytes on resistance to current chemotherapeutic drugs such as docetaxel.

In addition, I show for the first time that SPHK1 enzymatic activity and transcriptional regulation is up-regulated in both cancer cells and monocytes during co-culture indicating the involvement of SPHK1 in the signalling mechanisms driving this interaction. In support, I showed that SPHK1 inhibition in PC-3 cells not only abrogates the monocyte-induced effect on PC-3 cell proliferation but also decreases SPHK1 levels in monocytes. Furthermore, SKi-II pre-treatment of monocytes reduces SPHK1 activation in PC-3 cells, re-enforcing the existence of a bidirectional signalling loop mediated by SPHK1. Indeed the existence of a positive signalling loop mediated by SPHK1 has been proposed, with two recent papers indicating the SPHK1/S1P/S1PR1 loop as being persistently activated in tumours and acting as a main driver for tumour development and persistent inflammatory conditions [299, 474]. According to my results, S1P can indeed lead to the activation of SPHK1 in both cancer cells and monocytes and therefore be considered a possible mediator in this interaction. The role of S1P in tumour development has been demonstrated in several xenograft models where the use of an S1P targeting antibody leads to a significant reduction in tumour growth [214]. Further investigation is required to assess S1P levels as well as the importance of S1P receptors during this interaction between different cell types. In an inflammatory context, the secretion of S1P from apoptotic cells was shown to induce macrophage polarization [463] and secretion by macrophages of prostaglandin E(2) (PGE2), a known inducer of cancer migration and proliferation [475], to support cancer progression . Interestingly, my data shows a higher SPHK1 activation in monocytes when PC-3 cells were treated with docetaxel, which in part could be mediated by the S1P released from apoptotic cancer cells.

Altogether these data reveals the importance of SPHK1 during cancer-monocyte interaction and reveals the existence of a paracrine signalling loop between these two cell

types. This loop is mediated by soluble molecules that appear to be under SPHK1 regulation being S1P a very plausible and natural candidate. More importantly I show that SPHK1 target therapy could not only affect targeted cells but also its surrounding and therefore impact on the cancer microenvironment which is a known promoter of cancer progression.

8.2 Mechanism of SPHK1 regulation during co-culture

SPHK1 occupies a powerful position in sphingolipid metabolism and consequently in many biological processes with implications in several malignancies such as cancer. A better understanding of SPHK1 regulatory pathways is of great interest as it could explain the mechanism through which SPHK1 exerts its pro-survival actions in cancers. In this context, accumulating evidence suggests AKT, an oncogenic protein often activated in prostate cancer, to be under SPHK1 regulation [340, 342, 476]. In my study AKT activation follows a similar pattern as observed for SPHK1; the increase in phosphorylation levels in both PC-3 and U937 cells during co-culture suggests a cross-regulation between these two oncogenic proteins. A further evidence of this regulation arises from the findings in which AKT phosphorylation is reduced upon siRNA targeting of SPHK1 in cancer cells; this effect was monocyte independent as the addition of monocytes did not affect SPHK1-induced AKT inhibition. Interestingly, SPHK1 inhibition in PC-3 cells reduced AKT phosphorylation in monocytes supporting once more the existence of a SPHK1 regulatory loop during co-culture. Indeed, in other studies it has been shown that AKT activation was mediated by a SPHK1/S1P signalling loop [187, 406, 477], however these studies were focused on the autocrine loop and did not investigate the involvement of a paracrine loop within a stimulatory microenvironment. Here I propose a bidirectional model whereby SPHK1 activation leads to the production and secretion of S1P that in turn can induce AKT and SPHK1 activation in neighbouring cells, creating favourable conditions for tumour development. The ability of S1P to increase AKT phosphorylation in either PC-3 or U937 cells supports of this model and accords with other studies showing a similar effect on AKT phosphorylation by S1P [477-479].

ERK1/2 is another important signalling molecule that also appears to play a role in this interaction; interestingly the increase in ERK1/2 phosphorylation levels in U937 follows a similar trend to that observed for AKT and SPHK1. The capability of ERK1/2 to phosphorylate and activate SPHK1 is well described in the literature [166, 182], and I propose that ERK1/2 activation could be an early event in the SPHK1/S1P/AKT signalling loop between cancer cells and monocytes. Evidence in this work support this model, with particular reference to the decrease of SPHK1 expression and AKT phosphorylation in monocytes when ERK1/2 was silenced by siRNA in PC-3 cells. This indicates the ability of ERK1/2 to impact upon molecules that are able to activate SPHK1 and AKT in U937 cells, and suggests a more detailed analysis of S1P levels in ERK1/2 knockdown co-culture would be informative. The increased levels and/or phosphorylation of these important signalling molecules (AKT, ERK1/2 and SPHK1) in both cancer cells and monocytes indicates an activation of pathways involved in essential cellular functions that underpin the biology of cancer and supports the important role of monocytes in its development.

The transcriptional regulation of SPHK1 appears to be directly mediated by STAT1 when PC-3 cells are in co-culture with U937 cells. STAT1 binding to the up-stream genomic region of SPHK1 suggests a possible binding to the promoter, leading to increased transcription of SPHK1. However, this activation appears to be modulated by monocytes, more specifically by their secreted molecules. Most studies attribute a pro-apoptotic and tumour-repressive role to STAT1 activation [354, 367], however a few reports indicate that STAT1 activation is associated with oncogenic events [374, 438]. Here I propose that the repressive role of STAT1 in SPHK1 transcription is overcome by U937 cell stimulation; the mechanisms by which this switch may occur remain unclear.

8.3 Signalling molecules

MCP-1 is a known inflammatory mediator involved in cancer development [309]. In this study, my data shows an increase in MCP-1 mRNA and secreted protein levels in both prostate cancer cells (PC-3) and U937 cells when in co-culture, reinforcing the importance of this chemokine in prostate cancer growth and proliferation [323] and of the inflammatory state in prostate cancer development [304, 309, 322]. The ability of SPHK1 to regulate inflammatory mediators such as MCP-1 has been previously reported in lung carcinoma cells [326], mast cells [324] and epithelial cells [293]; here I show that SPHK1 knockdown can partially abrogate U937-induced increase of MCP-1 levels in PC-3 cells. This is significant as it has been shown that MCP-1 secreted from prostate cancer cells acts as a driver for macrophage infiltration that in turn benefits tumour cell growth and survival [322]. These data deem it reasonable to propose that SPHK1 modulation could provide a novel approach in treating prostate cancer associated with a highly inflammatory state.

Another important inflammatory mediator with a proven effect on prostate cancer development is IL-6 [290]. In this study, SPHK1 inhibition in PC-3 cells did not result in a decrease in IL-6 mRNA or secreted levels suggesting that IL-6 is not under SPHK1 regulatory control in PC-3 cells as has been previously demonstrated in other cell types [294, 480, 481]. However, the ability of S1P and MCP-1, which are under SPHK1 regulation, to increase IL-6 mRNA expression levels in monocytes could explain the increase observed in the U937 proximity media. Interestingly ERK1/2 and AKT levels in PC-3 cells appear to be important for the increase in IL-6 mRNA in U937 cells, indicating that in PC-3 cells ERK1/2 and AKT activation can also mediate the secretion of soluble factors in a SPHK1-independent manner. Alterations of these pathways during co-culture appears to dictate the secretory profile of each cell type, with the dynamic balance between these factors varying with time as well as the requirements of the cells. The bidirectional activation of these strong pro-tumorigenic mediators [327, 401] supports the model in which cancer cells benefit from the interaction with monocytes, with SPHK1 having an important role in their activation. A recent study deemed the role of SPHK1 in macrophages as negligible for the formation of inflammatory responses [482]; in the study murine macrophages lacking SPHK1 and SPHK2 did not decrease cytokine expression after treatment with inflammatory mediators such as LPS and

TNF-α. This contradicts previous studies, which have shown that SPHK1 inhibition in macrophages leads to reduction of pro-inflammatory cytokines such as IL-6 and MCP-1 [297, 483, 484]. It is possible that macrophages without SPHK1 from an early stage of development could acquire other mechanisms that compensate for SPHK1 absence. Despite the observation that an absence of SPHK1 results in a reduction of intracellular S1P [482] it would be interesting to look at other SPHK1 intracellular regulated molecules in the murine knockout cells, such as AKT or ERK1/2, to understand if the basal levels remain the same as those of the control macrophages.

Other soluble factors were detected in this study that could play an important role in cancer-monocyte signalling interactions. In the case of GM-CSF, my study reveals for the first time a possible regulation by SPHK1 of secreted GM-CSF protein levels. This is particularly relevant as GM-CSF is a known inducer of prostate cancer proliferation [415] and has been described to have a role in monocyte and macrophage proliferation [410]. The targeted inhibition of SPHK1 in cancer cells could reduce GM-CSF release from such cells, resulting in possible disruption of one of the signalling molecules that cancer cells use to recruit immune cells. The ability of GM-CSF to activate AKT and ERK1/2 through the RAS pathway [485] reinforces its potential as one of the mediators involved in ERK1/2 and AKT phosphorylation in monocytes in my co-culture model.

Similarly, GROα association with the SPHK1/S1P pathway has not been described in literature. Evidence exists that GROα inhibition leads to a reduction in both tumour growth and formation of metastases in breast cancer [486], and in prostate cancer its inhibition leads to a reduction of NF-KB and COX-2, two important inflammatory mediators [487], suggesting its involvement in maintaining a favourable inflammatory environment. In my study neither of these mediators were focused upon in depth and therefore no firm conclusions can be inferred from this preliminary data; however, taken together, my data and evidence in literature suggest that a more detailed analysis of the factors in a cancer-inflammation event could prove beneficial for future cancer therapies.

Intercellular Adhesion Molecule 1 (ICAM-1) is a known effector of inflammation, ensuring coordinated interactions that allow leukocytes to home in on sites of injury. Since

both endothelial and myeloid cells express this transmembrane protein it is of no surprise that ICAM-1 has been shown to be highly present in the serum of cancer patients [488] and at inflammatory sites [489]. A direct correlation between ICAM-1 expression levels in cancer and immune cell infiltration has not yet been described, however my initial findings indicate that ICAM-1 may play a role in cancer-monocyte interactions during chemotherapy. Here I show that docetaxel treatment of PC-3 cells significantly increases secreted ICAM-1 levels, with the effect further increased when U937 cells are present, suggesting ICAM-1 as an active mediator of these interactions. The fact that ICAM-1 levels are not affected by siRNA-target inhibition of SPHK1 in cancer cells indicates an SPHK1-independent regulatory mechanism. Nonetheless, the role of ICAM-1 in chemoresistance should be taken into consideration in further studies. The secreted molecules and intracellular mechanisms which I have deduced from my studies are involved in monocyte-tumour cell interaction are summarised in Figure **8.1**.

Future directions of this work comprise the clarification of S1P levels and characterisation of S1P receptors in both cancer cells and monocytes, this could be achieved through the analysis of mRNA and protein levels for these receptors as well as measuring S1P levels by HPLC. In order to understand how monocyte recruitment is affected by SPHK1, an in vivo assay could be performed whereby U937 cells are stably transfected with GFP and injected into PC-3 xenograft mice in the presence or absence of SKi-II inhibitor. U937 infiltration and migration to the tumour site could then be detected by live imaging and immunohistochemistry analysis of the tumour and other organs. Understanding the role of SPHK1 in the recruitment of monocytes to the tumour site could provide new insights as to why tumours may benefit from this interaction and further support the development of SPHK1/S1P inhibitors that in conjugation with current drugs could ultimately benefit PCa patients with tumours containing high levels of inflammatory cells.

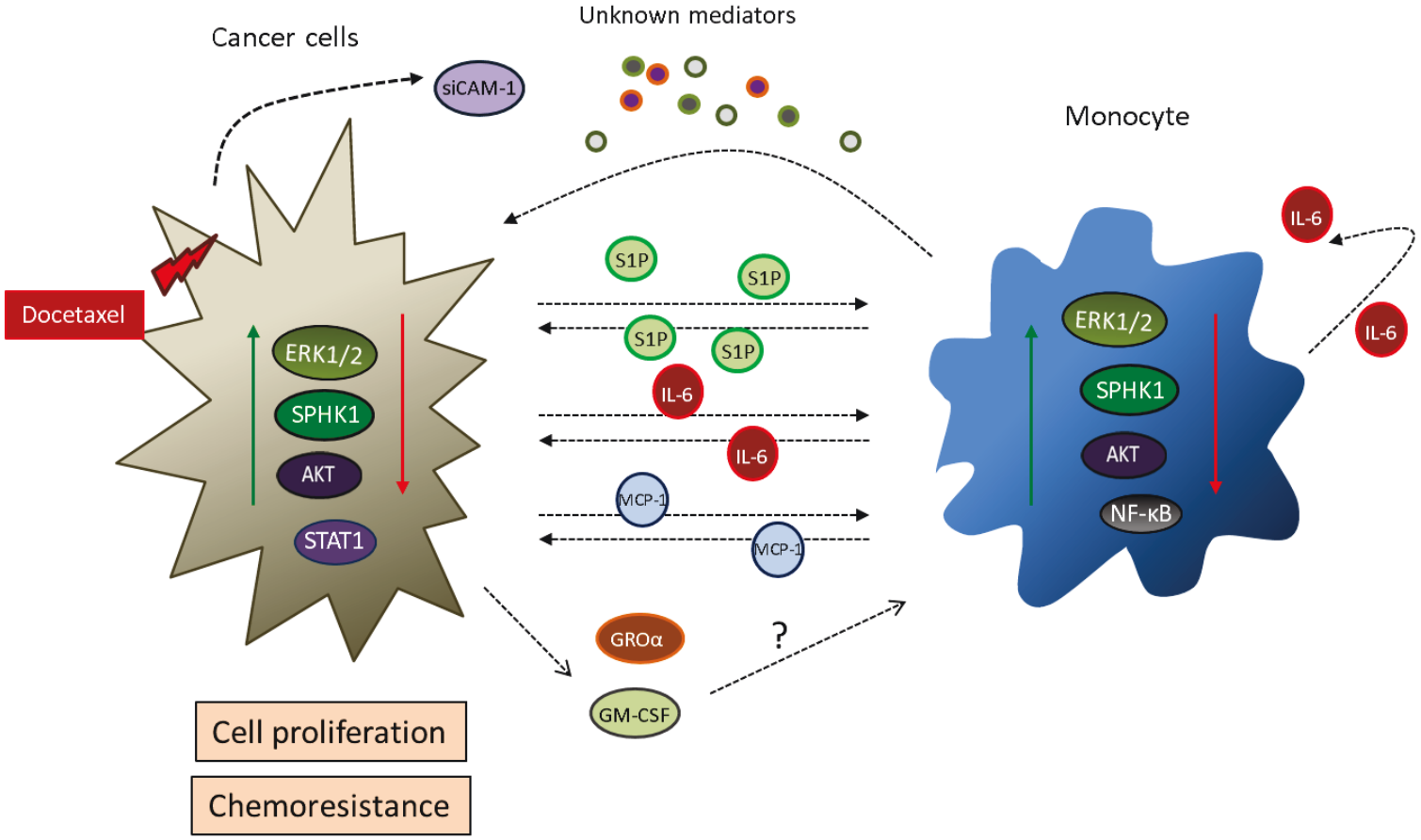


Figure 8.1 Schematic representation of the molecules and intracellular pathways involved in the interaction between prostate cancer cell (PC-3) and monocytes (U937) during co-culture. Monocytes are able increase PC-3 cell proliferation and protect cancer cells from docetaxel induced apoptosis. The activation of SPHK1 in both cells together with the ability to regulate several soluble molecules and intracellular pathways such as ERK1/2 and AKT re-enforces the crucial role of these oncogene in cancer development.

9. Conclusion

The tumour microenvironment encompasses a myriad of molecules, cell types and interactions that could open the door to many potential therapeutic agents, offering the opportunity to augment or halt the progression of cancer to a metastatic state. The work in this study has identified SPHK1 as a key regulator of monocyte-cancer cell interactions and indeed supports the key role that the proximity of these cells within the microenvironment plays in the activation of this key regulator. The true extent to which SPHK1 orchestrates important signalling pathways remains to be seen, although its importance is demonstrated by the manifestation of cancer cell proliferation and resistance to chemotherapeutics upon its activation. Indeed a highly inflammatory environment, as characterised by immune cell infiltration, is associated with elevated levels of SPHK1; consequently, targeting of this molecule offers new therapeutic approaches to reducing this inflammatory state and subsequent disease progression.

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