ROLE OF PLD AND SPHK IN TNFα-INDUCED SIGNALING AND INFLAMMATORY RESPONSES

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TABLE OF CONTENTS

LIST OF FIGURES

SUMMARY

Inflammation is a key homeostatic host response to different stimuli including infections and antigen-antibody reactions, where a tissue responds by initiating repair and eventually leading to the restoration of structure and function of the injured tissue. However, prolonged or disproportionate inflammatory reaction has been a hallmark of inflammatory disorders and it can be managed by dampening the aberrant inflammatory process. One of the major contributing factor in inflammatory disorders is TNFα (Tumor Necrosis Factor alpha), a potent pro-inflammatory cytokine, important in triggering and amplifying inflammatory responses. Though anti-TNF therapies are being used in the management of inflammatory disorders, their side effects and lack of responsiveness in certain situations has made the search for alternatives a necessity. Recently, intracellular signaling mediators like MAPKs and transcription factors, participating in the transduction and amplification of the signaling process have gained attention and are being targeted to dampen inflammation. Lipid mediators like PLD (Phospholipase D) and SphK (Sphingosine kinase) are found to play vital roles in intracellular signaling and immune cell responses, including Fc receptor and complement mediated responses. Therefore, this study investigated the role of PLD and SphK in TNFα induced inflammatory signaling and effector responses. The human monocyte was chosen as the cell model. This study revealed that TNFα-induced effector responses like pro-inflammatory cytokine production and chemotaxis were mediated by PLD. The results also showed that TNFα induces PLD activity and selective translocation of PLD1, from the cytosol to the cell periphery. It was also observed that PLD is essential for TNFα

triggered Raf-1 translocation and subsequent ERK1/2 activation. However, TNFα induced p38-phosphorylation was found to be independent of PLD activity in human monocytes. The results in the study indicate that TNFα triggered activation of NFκB, a key pro-inflammatory transcription factor was dependent on PLD. It was shown earlier that TNFα triggered NFκB activation and responses were mediated by SphK in human monocytes. In this study, it has been shown that TNFα induced SphK activity and cytosolic calcium release were downstream of PLD, indicating that PLD mediates at least some of its effects through SphK and calcium. PLD1 and PLD2 are the two major isoforms of PLD. It was shown that both the PLD isoforms are present in human monocytes. Following up on the selective translocation of PLD1 to TNFα stimuli, our antisense based investigation revealed the coupling of PLD1 to TNFα signaling and responses in human monocytes. Furthermore, we validated the in vivo role of PLD1 and SphK1 in TNFα induced peritonitis in BALB/c mice using short interfering RNA. Collectively, our results showcase a pivotal role for PLD1 and SphK1 in TNFα triggered inflammatory responses and suggests their potential in the therapeutic management of inflammatory disorders.

PRESENTATIONS and PUBLICATION

Conference Presentations

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CHAPTER 1

1. Introduction

1.1 Inflammation

Inflammation is a key homeostatic host response process, where a tissue responds to injury by initiating repair and eventually leading to the restoration of structure and function of the injured tissue (Florey, 1970). This homeostatic process is initiated as a response to different stimuli like infections, antigen-antibody reactions and also to physical, chemical and thermal insults to the tissues. Inflammation, apart from being a beneficial process by facilitating tissue homeostasis and host defense, is also a contributing factor in the pathogenesis of a variety of disease states. Prolonged or disproportionate inflammatory reaction has been a hallmark of inflammatory disorders like, rheumatoid arthritis, chronic bronchitis, asthma, glomerulonephritis, myocardial infarction, inflammatory bowel disease and autoimmune diseases including systemic lupus erythematosus (Boyd, 1992; Stanley L. Robbins, 1987).

Inflammation is a tightly regulated process, well orchestrated by immune cells and inflammatory mediators which include chemokines, cytokines and lipid mediators. Prolonged or aberrant inflammation is mainly due to the disruption in the natural resolution process. There does exist, a natural resolution processes of inflammation which includes the removal of the initial stimuli, reducing the levels of pro-inflammatory mediators and removal of inflammatory cells and debris (Henson, 2005). Dysregulation

in any of the three above mentioned process would result in chronic, persistent inflammation and associated pathology.

One of the major contributing factor in the pathogenesis of inflammatory disorders is TNFα (Tumour Necrosis Factor alpha), a potent pro-inflammatory cytokine. It is important in triggering and amplifying inflammatory responses. Dysregulation in TNFα production or signaling has been associated with septic shock syndrome, fever, acute phase response, cachexia, cerebral malaria, diabetes, cancer, atherosclerosis, liver disease, osteoporosis, allograft rejection and autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus and inflammatory bowel diseases (Aggarwal, 2003; Chen and Goeddel, 2002; Tracey and Cerami, 1993; Tracey and Cerami, 1994). Reviews by Lin et al and Wong et al have a detailed account of the contribution of TNFα in a variety of inflammatory disorders (Lin et al., 2008; Wong et al., 2008). The sheer number of patients receiving anti-TNFα therapies like monoclonal antibodies against TNFα and its receptors, and soluble TNFα receptors for the management of a range of inflammatory disorders will further emphasize the extent of TNFα in inflammation mediated pathologies. Though its success rate is remarkable, there are associated side effects (like infections, Tuberculosis, congestive heart failure, malignancies, demyleinating disorders and even autoimmune diseases) and resistance to therapy in some patients (Lin et al., 2008). These factors have made the search for effective alternatives in the management of immune mediated inflammatory disorders (IMID) a necessity. One of the promising alternative approaches is to target the signaling mediators like p38 kinase, NFκB and matrix metalloproteinases to dampen the aberrant

inflammatory response (O'Neill, 2006). Intracellular signaling mediators regulated events including, phosphorylation, transcription factor activation and translocation among others, in a cell are key reactions to a stimuli, which determines the degree and type of response of the cell to the initial stimuli. Regulating or harnessing these signaling events will influence the inflammatory response and can result in the resolution of the associated pathology. Therefore, in this study, we shall be investigating the effects of phospholipid-modifying enzymes and intracellular signaling regulators: Phospholipase D (PLD) and Sphingosine Kinase (SphK), in TNFα-triggered signaling and cellular responses; hence, evaluating the potential to target these enzymes in the therapeutic management of immune mediated inflammatory disorders. The following sections in this chapter will discuss TNFα, PLD and SphK in detail.

1.2 Tumor Necrosis Factor alpha

TNFα (Tumor Necrosis Factor alpha) is a pleiotropic cytokine extensively studied for its role in the pathogenesis of a variety of disease conditions, which is known to have a wide range of beneficial and deleterious effects in humans (Tracey and Cerami, 1993; Tracey and Cerami, 1994). TNFα is produced by a variety of cells which include: macrophages, monocytes, lymphocytes, NK cells, eosinophils, keratinocytes, langerhan cells, kupffer cells, glial cells, adipocytes and fibroblasts (Baud and Karin, 2001; Tracey and Cerami, 1993; Tracey and Cerami, 1994). This cytokine is known to be produced in response to a wide range of stimuli such as, bacterial toxins (LPS), infections (bacterial, viral, fungal, mycobacterial and parasitic); antigen-antibody complexes; injury; host inflammatory agents (products of the complement activation, auto-antibodies and

cytokines); as well as toxic and non-toxic environmental challenges (Baud and Karin, 2001; Tracey and Cerami, 1994). TNFα elicits a wide spectrum of cellular responses which mediates inflammation, regulates immune response and also induces apoptosis in certain types of cancer cells (Chen and Goeddel, 2002; MacEwan, 2002a). It brings about its immuno-modulatory and inflammatory functions by inducing the production of various cytokines, activation of leukocytes and lymphocytes, enhancing the expression of adhesion molecules, initiating adherence of neutrophils and monocytes to the endothelium, promoting inflammatory cell migration and it is also known to stimulate cellular proliferation and differentiation (Baud and Karin, 2001; Hehlgans and Mannel, 2002; MacEwan, 2002a; Tracey and Cerami, 1993; Tracey and Cerami, 1994). Appropriate levels of TNFα are necessary for homeostatic functions like protection from infection, haematopoiesis, immune response regulation, tumor regression and immune surveillance (Aggarwal, 2003). Dysregulation in TNFα production or signaling has been associated with a range of inflammation based disorders (Aggarwal, 2003; Chen and Goeddel, 2002; Lin et al., 2008; Tracey and Cerami, 1993; Tracey and Cerami, 1994; Wong et al., 2008).

The observations by Dr P Brunes, on spontaneous regression of tumors in patients with acute bacterial infections (Bruns, 1868) and the follow up study by William B Coley on tumor reduction by the use of bacteria-free filtrate preparation from bacterial extracts (Coley, 1891) more than a century ago, have been the real building blocks that led to the discovery of TNF. This factor found in the serum which is capable of causing regression of certain tumors was termed Tumor Necrosis Factor (TNF) in 1975 (Carswell et al., 1975; Old, 1985). A decade later, successful attempts to isolate and characterize this

factor led to the identification of two structurally related factors, which were termed TNF (Tumor Necrosis Factor) or TNFα (Tumor Necrosis Factor alpha) and LT (Lymphotoxin) (Aggarwal et al., 1985; Aggarwal et al., 1984; Clark, 2007). Here, TNFα will be discussed in detail.

1.2.1 TNFα ligand

TNFα brings about its varied functions and responses through ligand-receptor complex formation mechanism, where TNFα is a ligand. It is a homotrimer of 157 amino acid subunits (Chen and Goeddel, 2002) and is a type II transmembrane protein (Hehlgans and Mannel, 2002). It is present as biologically active membrane bound (mTNF) and soluble (sTNF) forms. Soluble TNFα is originally expressed as a 26kDa membrane bound pre-protein which can be cleaved off by a metalloproteinase (TACE – TNF alpha converting enzyme) to be released as a 17kDa mature form of TNFα (Black, 2002; Black et al., 1997; Gearing et al., 1994; Idriss and Naismith, 2000; Moss et al., 1997). The secreted, mature TNFα subunits form non covalently bound homotrimers – sTNFα (Hehlgans and Mannel, 2002; MacEwan, 2002a). This trimerization is necessary for the TNFα to be biologically potent or activate its receptors (Sprang, 1992). The membrane bound form of TNFα ligand is useful in the transfer of signals by cell to cell contact and the effect is said to be localized, whereas the soluble form of TNFα is said to have a more dispersed or systemic effect (MacEwan, 2002a).

1.2.2 TNF receptors

The TNF ligand induces its different cellular responses by binding to TNF receptors TNFR type 1 (TNFR1 / p55TNFR / CD120a) and/or TNFR type 2 (TNFR2 / p75TNFR / CD120b) (Chen and Goeddel, 2002; MacEwan, 2002a; MacEwan, 2002b; Smith et al., 1990; Vandenabeele et al., 1995). TNFRs are single transmembrane glycoproteins with homology in the extracellular domains but not in the intracellular or cytoplasmic domains. The binding process induces receptor trimerization and/or recruitment of signaling proteins to the cytoplasmic domains of the TNFα receptors and the subsequent cellular responses depends on the type of receptor bound to the ligand. Both receptors contain a PLAD (pre ligand binding assembly domain) that pre complexes the receptors and encourages them to trimerize, particularly upon activation by TNF ligand (Chan et al., 2000). TNFR1 can equally be activated by both sTNF and mTNF, where as mTNF is considered to be superior than sTNF in activating TNFR2 (Grell, 1995; Grell et al., 1995). TNFα activates TNFR1 with higher efficiency due to the fact that it binds to TNFR1 with increased affinity (Kd of 19pM) and exhibits a slow rate of dissociation ($t_{½}$ = 33 mins) from the receptor, subsequent to binding (Grell et al., 1998). These kinetics are different for TNFR2, where the affinity of TNFα to TNFR2 is lower and the dissociation rate is faster compared to that of TNFR1 (Grell et al., 1998). TNFR1 is said to be constitutively expressed in a vast majority of cells types and tissues including immune cells, whereas TNFR2 expression is mainly found in immune cells (Chen and Goeddel, 2002; Wajant et al., 2003). TNFR1 is considered to mediate most of the biological effects and responses induced by TNFα (Wajant et al., 2003). TNFR1 is the most widely studied type of TNFR in experimental set up. This is due to the use of

soluble recombinant TNFα, which predominantly activates TNFR1. A novel isoform of TNFR2 (icTNFR2 – intra cytoplasmic TNFR2) reported recently is thought to have originated from TNFR2 gene as a new transcriptional start followed by alternative splicing of the transcript and it was found to be expressed only within the cell (Seitz et al., 2001). Though over expression studies have suggested that icTNFR2 signaling is similar to that of TNFR2 (Seitz et al., 2001), more work has to be done to evaluate the relevance of icTNFR2 compared to that of the well studied TNFα receptors. TNFα receptors lack intrinsic enzymatic activity and bring about their functions by the recruiting different adaptor molecules, which in turn transduce signals for a range of responses like apoptotic cell death, inflammation, cellular survival and proliferation.

1.2.3 TNFα-induced signaling

The binding of the ligand, TNFα to TNFR1 leads to conformational changes in the intracellular domain of the TNFR which initiates the recruitment and subsequent interaction of several adapter proteins to the receptors (Wallach et al., 1999). TNFR1 contains a death domain (DD) motif towards the carboxyl-end of the receptor and is critical in the death- inducing and survival or inflammatory activity of TNFR1 (Tartaglia et al., 1993a). The DD of a protein can associate with the DD of other proteins. SODD (Silencer of Death Domain), an adaptor protein is bound to TNFR1 through its DD, thereby preventing the binding of other molecules with DD to the TNFR (Jiang et al., 1999). In this manner, SODD acts as an endogenous inhibitor of TNFα signaling. Receptor ligation of TNFR1 initiates internalization of the ligand-receptor complex which is another protective mechanism present naturally to disrupt any sustained

signaling subsequent to receptor activation (Higuchi and Aggarwal, 1994; Schutze et al., 1999). Receptor ligation leads to the dissociation of SODD from TNFR1, thereby allowing other DD containing proteins to interact with the DD of TNFR1. This event results in the recruitment of a DD containing signal transduction adapter molecule, TRADD (TNF receptor associated death domain), which recruits a variety of signaling pathways by interacting with other related adaptor molecules. A schematic representation of the signaling pathway is illustrated in (Figure 1.1, page number 26).

1.2.3.1 TRADD dependent signaling

TRADD dependent signaling is unique to TNFR1 and not TNFR2. This is due to the absence of DD in TNFR2. TRADD plays an unique role in directing distinct signaling pathways like, pro-apoptotic or survival pathway by forming a signaling complex with FADD (Fas associated Death Domain) and TRAF2 (TNF receptor associated factor) respectively (Hsu et al., 1996; Liu et al., 1996).

1.2.3.1.1 Pro-apoptotic signaling

Intracellular signaling mediators regulating TNFα–induced pro-apoptotic signaling discussed here are primarly FADD and JNK. The role of RIP and cPLA2 in this process is also discussed.

FADD

TNFR, also known as death receptors, are well known to induce death signals through the extrinsic apoptotic pathway using an initiator caspase (caspase-8) (Denecker et al.,

2001a; Denecker et al., 2001b; Mak and Yeh, 2002; Wyllie, 1997) and TRADD. Subsequent to TNFR1 receptor activation, the TNFR1-TRADD complex recruits another DD containing protein FADD which in turn activates procaspases-8 and 10 which eventually leads to DNA damage and programmed cell death (Ashkenazi and Dixit, 1998; Hsu et al., 1995). FADD recruitment of procaspase-8 is facilitated by protein–protein interaction through their death effector domains (DED), thus leading to the formation of a death inducing signal complex (DISC) along with TNFR1 and TRADD. Procaspase-8 is then autolytically cleaved to release an active form of caspase-8 from the DISC, which then acts on downstream effector caspases, especially caspase-3. These effector caspases cleave or lead to the degradation of a number of substrates necessary for normal cell homeostasis, thus resulting in morphologic and biochemical characteristic of apoptotic cell death. Caspase-8 also cleaves Bid (BH3-interaction death domain agonist, a member of Bcl-2 family) to form t-Bid, which translocates to mitochondria causing the release of cytochrome *c* (Luo et al., 1998). This is followed by the formation of Apoptosome - a complex made up of cytochrome c, Apaf-1 (Apoptosis-activating factor 1) and procaspase-9, which is capable of activating effector caspases like caspase-3 to bring about programmed cell death (Wang, 2001). It was also found that activated caspase-3 can in turn activate the cleavage of procaspases-8 to caspase-8 and thereby creating a positive feed back system (Wieder et al., 2001).

JNK

JNK (c-Jun N-terminal Kinases), an important member of the MAPK (Mitogen activated protein kinase) family influences a variety of cellular functions including

apoptosis (Davis, 2000). JNK is known to be activated by a variety of stimuli like cytokines, UV irradiation, growth factors, serum and DNA damaging agents. TNFα–induced prolonged activation of JNK has long been associated with the induction of apoptosis or necrosis. It is has been reported that TNFR2 activates JNK and not other MAPKs (Jupp et al., 2001). TRAF2 has been identified to be essential in the activation of JNK (Baud et al., 1999; Natoli et al., 1997). Efforts to elucidate the mechanism of TNFα–induced JNK activation has led to the identification of the role of ASK1 in the process (Ichijo et al., 1997). TRAF2 brings about JNK activation by effecting its influence on ASK1 (Apoptosis stimulating kinase / apoptosis signal regulating kinase), a MAPKKK (Nishitoh et al., 1998) and GCK (Germinal center kinase) (Yuasa et al., 1998). ASK1 activates or phosphorylates MKK4 and MKK7 leading to the activation of JNK (Weston and Davis, 2002). TNFα activation of JNK is brought about as two distinct phases. There is TNFα-induced TRAF2 mediated early and transient JNK activation (Lamb et al., 2003) which is followed by a delayed and persistent activation of JNK mediated by reactive oxygen species (ROS) induced by TNFα (Ventura et al., 2004). It was also reported that ROS mediated JNK activation is followed by ROS production mediated by JNK which exhibits a positive feedback of ROS production and JNK activation (Ventura et al., 2004) which would contribute to the induction of apoptosis. TNFα–induced ROS was found to facilitate the activation of ASK1 by oxidizing thioredoxin and its dissociation from ASK1. This is followed by oligomerization and phosphorylation of ASK1 and subsequent JNK activation, leading to apoptosis (Gotoh and Cooper, 1998; Saitoh et al., 1998). A more recent study has shown that, in addition to

activation of ASK1, TNFα–induced increased ROS production inhibited the function of MAPK phosphatase thereby leading to a prolonged or persistent JNK activation resulting in cell death (Kamata et al., 2005). A review by Shen et al can be referred for a detailed discussion on the role of ROS in TNFα–induced JNK activation and associated cell death (Shen and Pervaiz, 2006). All these reports indicate the role of ROS as a co-activator in TNFα-induced JNK mediated cell death. JNK is well known to induce c-Jun dependent transcription leading to AP-1 activation (Karin et al., 1997; Weston and Davis, 2002) and it also phosphorylates transcription factors like ATF2, NF-AT, HSF and STAT3 to bring about it various cellular responses (Kyriakis and Avruch, 2001).

RIP

Receptor interacting protein (RIP) was found to be associated with TNFα–induced apoptosis (Lin et al., 1999). It was found that caspase-8 cleaves RIP to form RIPc and nRIP. It has been reported that RIPc blocks TNFα-induced survival signaling and the association of RIPc with TRADD has been associated with TNFα–induced apoptosis (Lin et al., 1999). Subsequent to cleavage, RIP was found to dissociate from the TRADD, thus enabling the recruitment of FADD and thereby indicating its role in TNFα-induced apoptosis (Lin et al., 1999).

cPLA2

cPLA2 (cytosolic phospholipase A2) has been associated with TNFα-induced apoptosis (Lee et al., 2006a; Pilane and LaBelle, 2002; Pirianov et al., 1999; Wolf and

Laster, 1999). By contrast, some earlier studies reported that cPLA2 and sPLA2 mediated TNFα-induced NFκB activation in human keratinocytes (Anthonsen et al., 2001; Thommesen et al., 1998). However, other studies have clearly shown and emphasized that cPLA2 expression and activation is vital for TNFα-induced cell death in a variety of cell types (De Valck et al., 1998; Jayadev et al., 1997; Wu et al., 1998a; Wu et al., 1998b). cPLA2 activated by TNFRs (Clark et al., 1991; Hoeck et al., 1993; MacEwan, 1996) leads to the generation of arachidonic acid (Jayadev et al., 1997) which is further converted to form eicosanoids (Prostaglandins and Leukotrienes). cPLA2 based eicosanoids induce the generation of ROS which in turn plays a role in determining cell fate (Chang et al., 1992). TNFR1 is thought to play a role in the activation of cPLA2, whereas TNFR2 is said to be involved in regulating the expression of cPLA2 (MacEwan, 1996; MacEwan, 2002a).

1.2.3.1.2 Survival or Inflammatory

TNFR1 facilitates a survival or an anti-apoptotic signal through the recruitment of TRAF to the receptor signaling complex through TRADD. TRAF family of proteins are involved in a range of cellular responses like survival, inflammation and anti-apoptosis (Chung et al., 2002). Of the seven members of the mammalian TRAF family, TRAF2 is well studied and has been found to be associated with TNFR1 and TNFR2 signaling (Xia and Chen, 2005). TRADD was found to be specific for TRAF1 and TRAF2. It has also been reported that TRAF2 has higher affinity for TRADD rather than the TNFRs (Park et al., 2000) and this indicates the predominant role of TRAF2 in TNFR1 signaling. Along with TRAF2, receptor interacting protein (RIP) has also been found to be recruited by

TRADD to the receptor signaling complex. RIP was found to be necessary in TNFα–mediated survival signals (Devin et al., 2000; Hsu et al., 1996) and is also involved in TNFα–induced activation of NFκB (Nuclear Factor kappa B), an important transcription factor (Kelliher et al., 1998). TRAF2 and TRAF5 together are also necessary in TNFα induced NFκB activation (Tada et al., 2001).

Intracellular signaling mediators other than NFκB playing a crucial role in pro-survival and/or anti pro-apoptotic signaling discussed here are IAP (Inhibitor of Apoptotic proteins), PLC (Phospholipase C), PLD (Phospholipase D), PKC (Protein Kinase C), Sphingolipids, p38 kinase and PKB (Protein Kinase B). The other survival mediators like ERK1/2, TNFR2 and FAN (Factor associated with neutral sphingomyelinase) are discussed in section 1.2.3.2 in this chapter.

NFκB

TNFα brings about survival responses predominantly by the activation of NFκB, an important anti-apoptotic and pro-inflammatory transcription factor. NFκB is known to play an important role in regulating the expression of genes responsible for mediating immune responses (Baeuerle and Henkel, 1994; Ghosh et al., 1998). If NFκB signaling is blocked, TNFα can bring about rapid programmed cell death in a variety of cells (Hayden and Ghosh, 2004) which indicates the importance of NFκB in the cellular response specificity, when induced by TNFα. The canonical or the classical NFκB pathway (Pomerantz and Baltimore, 2002) is considered to be the predominantly triggered pathway in most cells and stimuli. Binding of TNFα to TNFR1 is known as the

potent activator of the classical NFκB pathway. In this pathway, NFκB is present in the form of a dimer made up of p50 and p65 subunits. In unstimulated cells or in cells prior to activation, NFκB is sequestered in the cytoplasm in an inactive form and its DNA binding ability is prevented by IκB family of inhibitor proteins (Hoffmann and Baltimore, 2006). On TNFα stimulation, there is a rapid activation of the IKK complex (Hayden and Ghosh, 2004). The IKK complex consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NFκB essential modulator (NEMO) (Ghosh and Karin, 2002). Subsequent to TNFα stimulation TRAF2 is suggested to interact with IKKα and IKKβ recruits the IKK complex to the receptor complex (Devin et al., 2001). RIP is said to help in the shuttling of IKK complex to the receptor complex by binding to NEMO (Zhang et al., 2000) and this role of RIP, independent of TRAF2 is necessary for the activation of the IKK complex (Devin et al., 2001). Activation of IKK complex is followed by the ubiquitination and proteosomal degradation of IκBα within ~10 min (Hayden and Ghosh, 2004). TRAF2 was also found to be capable of interacting with NIK (NFκB inducing kinase) which is reported to phosphorylate IκB and which leads to the latter's degradation (Ling et al., 1998; Malinin et al., 1997). TNFα induced degradation of IκBα triggers activation of NFκB by the release of the dimer to translocate from the cytoplasm to the nucleus and to initiate the activation of its target genes by binding to the DNA (Hoffmann and Baltimore, 2006; Karin and Ben-Neriah, 2000).

It was originally thought that NFκB was a potent inhibitor of JNK activation induced cell death and this was mediated by the activation of NFκB target genes like XIAP (X-chromosome linked inhibitor of apoptosis), GADD45β and cFLIP (De Smaele et al.,

2001; Shu et al., 1997; Tang et al., 2001). This effect was challenged when reports showed that JNK activation was not affected despite the disruption of XIAP and GADD45β (Amanullah et al., 2003; Harlin et al., 2001). This fueled the search for mediator or mediators which enables the crosstalk between NFκB and JNK following TNFα stimulation. This lead to the identification of ROS in the interplay between TNFα induced cell survival and cell death (Papa et al., 2005; Zhang and Chen, 2004). It is now known that NFκB brings about the regulation of ROS through MnSOD (Manganese superoxide dismutase) and FHC (Ferritin heavy chain) (Delhalle et al., 2002; Pham et al., 2004). The antioxidant property of MnSOD and FHC aid in the elimination and prevention of ROS build up in TNFα treated cells (Delhalle et al., 2002; Liochev and Fridovich, 1997). This event prevents the ROS mediated JNK activation. For further information on the role of ROS in TNFα–induced NFκB activation and pro-survival signaling, one can refer a well discussed review by Shen and Pervaiz (Shen and Pervaiz, 2006). PKB activated via PI3K signaling and GSK3β was also found to be necessary in the transcriptional activity of NFκB, thereby preventing TNFα-induced apoptosis (Delhase et al., 2000; Hoeflich et al., 2000; Madrid et al., 2001). Therefore, NFκB brings about its anti-apoptotic, pro-survival signaling by activating key apoptosis inhibitory proteins and also by inhibiting ROS mediated JNK activation and subsequent cell death.

cIAP

TNFR1 also mediates survival by regulating or controlling its pro-apoptotic signaling by the recruitment of Inhibitor of Apopotic Proteins (IAPs), cIAP1 and cIAP2 by TRAF2

to the TNFR1 signaling complex (Rothe et al., 1995; Shu et al., 1996). cIAP1 and cIAP2 are proteins which inhibit apoptosis by inhibiting caspase-3 and caspase-7 (Roy et al., 1997) and proteosomal degradation of caspase-3 and caspase-7 (Huang et al., 2000). Therefore, in TNFR1 signaling, TRAF2 recruited cIAPs can effectively inhibit caspase 8 which has been recruited to the TNFR1 signaling complex by the TRADD-FADD complex (Roy et al., 1997). This would impede the signaling for programmed cell death and facilitate cell survival.

PLC

Lipases are involved in the transduction and regulation of TNFα-mediated signals and responses. Phospholipase C (PLC) is a well known phospholipase that acts on phosphatidylinositol 4, 5-bisphosphate (PIP2) to generate DAG (Diacyl glycerol) and IP3 (Inositol 1, 4, 5-trisphosphate). Activation of Phosphatidyl-Choline specific PLC (PC-PLC) leads to the production of choline and DAG, a potent secondary messenger involved in signal transduction. PLC is known to be activated on TNFα receptor stimulation (Plo et al., 2000; Schutze et al., 1991; Wiegmann et al., 1992) and the resulting second messenger like DAG are extremely relevant in the downstream signal transduction events, like the activation of PKC (Protein Kinase C) (Nishizuka, 1992).

PLD

TNFα signaling was also found to be associated with the activity of another lipase, PLD (Phospholipase D). PLD is a phosphodiesterase which hydrolyzes PC to form PA (Phosphatidic Acid), a potent second messenger and a relatively inert choline. PA can

be further converted to DAG by Phosphatidic Acid Phosphohydrolase (PAP). PLD has been shown to be involved in a range of immune cell signaling and responses. However, there are conflicting reports regarding the apoptotic and survival role PLD in TNFα signaling (Bechoua and Daniel, 2001; De Valck et al., 1993; Kang et al., 1998; Oprins et al., 2001; Oprins et al., 2002; Plo et al., 2000). TNFα–induced PLD was found to influence ERK1/2 phosphorylation and p38 kinase in neutrophil like HL 60 cells (Bechoua and Daniel, 2001). All these findings indicates an antiapoptotic and inflammatory role of PLD in TNFα-induced signaling and responses. Recent studies were more conclusive and detailed about the role of PLD in TNFα signaling. The apoptotic and survival role of PLD in general including those induced by TNFα is well discussed by Nozawa Y (Nozawa, 2002) and recent report has indicated a protective role of PLD against TNFα–induced apoptosis (Birbes et al., 2006). ROS which was found to be a key regulator in TNFα-induced apoptosis and cell survival was also found to play a role in the activation of PLD in endothelial cells (Parinandi et al., 1999). All these facts, strongly urge the fact that future studies should address the need in understanding the role and interaction of PLD with other TNFα signaling mediators.

PKC

The role of PKC (Protein Kinase C) in TNFα–induced signaling and responses in a variety of cells was well summarized by Schutz et al (Schutze et al., 1994). TNFα–induced PKC (Protein Kinase C) activation is considered to be via DAG produced by PLC rather than by PLA2 or DAG produced by PLD. This is because kinetic studies

have shown that TNFα–induced PC-PLC activation precedes PKC activation and TNFα–induced PLA2 and PC-PLD activation is a delayed response, occurring much later than the PKC activation (Schutze et al., 1994). However, a variety of PKC isoforms exist with distinct activation patterns and distributions. Some of the classical isoforms like PKCα, β, δ and atypical isoforms like PKC ζ, λ, τ have been implicated in TNFα-induced responses and signaling events like the activation of MAPKs and NFκB (Bonizzi et al., 1999; Cohen, 1997; Laouar et al., 1999; Lee et al., 2000; Muller et al., 1995; Sanz et al., 1999). An atypical PKC associated protein, p62, interacts exclusively with RIP to facilitate involvement of aPKC to the TNFα–induced NFκB pathway (Sanz et al., 1999).

Sphingolipids

The sphingolipid based sphingomyelin pathway is known to be utilized in TNFα mediated signal transduction. TNFα stimulates both neutral SMase (Sphingomyelinase) and acidic SMase (Chatterjee, 1994; Dressler et al., 1992; Schutze et al., 1992; Wiegmann et al., 1994). TNFα induced NFκB activation is said to be via the endosomal acidic SMase (Schutze et al., 1992). Studies have proven that PC-PLC derived DAG is involved in the activation of acidic SMase (Kolesnick, 1987; Quintern et al., 1987; Schutze et al., 1992). Evidence has shown that sphingomyelin pathway is involved in TNFα–induced NFκB activation in neutrophil like cells (Yang et al., 1993). TNFR1 was found to stimulate membrane associated neutral SMase (sphingmyelinase) (Wiegmann et al., 1992; Wiegmann et al., 1994) either directly or through the activation of another adaptor protein called FAN. This pathway involves the hydrolysis of sphingomyelin, a

major membrane associated sphingolipid to ceramide by action of a neutral sphingomyelinase (NSmase) and subsequently by the stimulation of ceramide-activated protein kinase (Dressler et al., 1992). A study on alveolar epithelial cells has reported that ceramide production by sphingomyelin hydrolysis induced by TNFα activation can activate survival pathway rather than initiating programmed cell death (Mallampalli et al., 1999). Ceramide functions as a selective mediator of the cytotoxic or cytostatic effects of TNFα by playing a positive feedback role in the activation of NFκB (Dbaibo et al., 1993). Ceramide can also be converted to form sphingosine. The latter is phosphorylated by sphingosine kinase (SphK) to form sphingosine 1 phosphate (S1P), a potent second messenger which can act both intracellularly and extracellularly through specific receptors. S1P through its receptor can influence a range of responses like proliferation, differentiation, migration, inflammation and apoptosis. It is also known to play role in the mobilization of intracellular Ca^{2+} (Pyne and Pyne, 2000). The role of SphK in immune cell signaling and responses has been extensively reviewed by Kee et al and Melendez AJ, can be referred for further reading (Kee et al., 2005; Melendez, 2007). TNFα-induced signaling and effector responses are mediated by SphK1 in human monocytes (Zhi et al., 2006). SphK is necessary for TNFα–induced NFκB activation, which is essential for the survival and proinflammatory signaling has also been reported (Zhi et al., 2006). The main effect of SphK is brought about by S1P, which induces mitogenesis, stimulates ERK, survival transcription factors and hindering ceramide mediated JNK activation. Thus, the fate of the cell depends on the tight balance between SIP and ceramide.

p38 kinase

A stress activated protein kinase, p38 MAPK is also well known to be activated by environmental stress and pro-inflammatory cytokines including TNFα. Activation of p38 by TNFα is mediated by its upstream MAPKKKs (ASK1) (Ichijo et al., 1997) and MEKs (MEK-3 and MEK-6) (Enslen et al., 1998) and transduced through TRAF2 (Baud et al., 1999). p38 kinase is activated only by TNFR1 and not by TNFR2 (Jupp et al., 2001). TNFα-induced cellular responses found to be mediated by p38 kinase are considered predominantly to mediate proinflammatory responses (Barone et al., 2001; Nakada et al., 2001). p38 activates the downstream targets like cPLA2, microtubule associated protein Tau, transcription factors like ATF1, ATF2, MEFs, Elk-1 (Kyriakis and Avruch, 2001), AP-1 (Karin et al., 1997) and several MKs (MAPK activated protein kinases) (Roux and Blenis, 2004).

PKB

The importance of PKB (Protein kinase B / AKT) in the regulation of cell survival responses is by the modulation of signaling molecules like IKK, Raf, ERK, Bad and caspase-9 (Scheid and Duronio, 1998). Phosphoinositide 3-kinases (PI3Ks) generate specific inositol lipids like PIP_3 (Phosphatidylinositol-3, 4, 5-triphosphate) found to be relevant in the regulation of cell proliferation, survival and differentiation. PIP_3 recruits PKB to the cellular membrane on stimulation and the latter is subsequently phosphorylated by PDK1 (3'-phosphoinositide-dependent kinase-1) (Vanhaesebroeck and Alessi, 2000). PKB is known to play an important role in TNFα-induced stimulation of NFκB activity, since NIK activation is PKB dependent (Burow et al., 2000; Ozes et al.,

1999). Recently, TNFα-induced PKB and NFκB activation in endothelial cell survival was reported along with the role of integrin-mediated adhesion contributing to the mentioned response (Bieler et al., 2007). Destruction of PKB by caspases and inhibition of PKB by ceramide results in the inhibition of survival signals and favors cell death (Schubert et al., 2000; Widmann et al., 1998; Zinda et al., 2001). The antiapopotic activity of PKB partly contributes to cyclic nucleotides (cAMP and cGMP) mediated suppression of apoptosis (Li et al., 2000). PKB activated via PI3K signaling and GSK3β was also found to be necessary in the transcriptional activity of NFκB, thereby preventing TNFα induced apoptosis (Delhase et al., 2000; Hoeflich et al., 2000; Madrid et al., 2001).

1.2.3.2 TRADD independent signaling

TNFR1 also exhibits TRADD independent signaling, as there are proteins which associate directly to TNFR1. The nature of signaling and resulting responses depends on the type of the associating protein and its influence in the signaling pathways.

ERK1/2

ERK1/2 is a classical member of the MAPK (Mitogen activated protein kinases) family. ERK1/2 activation pathway, like p38 is considered as a potential target for cancer therapeutics, due to its regulatory effect in cellular proliferation (Kohno and Pouyssegur, 2003). ERK1/2 activation associated cellular responses are brought about by the phosphorylation of membrane proteins (including Syk, CD120a), nuclear substrates (including NFAT, Elk-1, c-Fos, c-Myc, STAT3), cytoskeletal proteins and MAPK activated protein kinases (MKs) (Chen et al., 2001; Roux and Blenis, 2004). It is known

that MAPKs are activated by TNFα (Kyriakis and Avruch, 2001; Roux and Blenis, 2004) and ERK1/2 (p44/p42) were identified to be activated through their respective MEK (MEK1 and MEK2) phosphorylations (Van Lint et al., 1992). Like p38 kinase, ERK activation was found to be triggered by TNFR1 only and not TNFR2 (Jupp et al., 2001). Studies have shown the relevance of ERK in modulating TNFα induced survival and apoptotic signals (Cuvillier et al., 1996; Rao, 2001). TNFR1 is thought to bring about its ERK activation through an adaptor protein, MADD. This protein with low homology to DD was found to interact directly with the DD of TNFR1 and activate the ERK signaling pathways (Schievella et al., 1997). TNFR1 induces the activation of ERK also via Grb2, an adaptor protein which interacts with PLAP motif of TNFR1 (Hildt and Oess, 1999). Grb2 is known to interact with SOS which in turn leads to the activation of Ras, followed by c-Raf which leads to the activation of ERK1/2 (Hildt and Oess, 1999). Interestingly, contrary to other reports, a study also reported that TNFα induced ERK activation was absent or lower than the stress activated proteins kinases in most of the cells (Van Lint et al., 1992).

FAN

FAN (Factor associated with neutral sphingomyelinase) is an adaptor protein found to have binding ability to the membrane-proximal region of TNFR1 (Adam-Klages et al., 1996) and it has been found to aid in the stimulation of neutral sphingomyelinase (SMase) (Adam-Klages et al., 1996; Adam-Klages et al., 1998; Kreder et al., 1999). SMase catalyzes the sphingolipids to form ceramide which in turn can influence caspase-3 activation and bring about cell death. It is also capable of activating ceramide activated

protein kinase (CAPK) as well (Dressler et al., 1992). Ceramide can also be converted to sphingosine as well. The sphingolipid pathway is discussed in detail in section 1.4 in this chapter. The signaling events following the activation of SMase and the resulting possibility of transducing apoptotic and survival responses have been discussed earlier (Sphingolipids in section 1.2.3.1.2).

TNFR2

TNFR2 mediated signaling can be categorized as TRADD independent signaling, since TNFR2 is devoid of DD. TRAF2 is directly associated with TNFR2 to initiate its pro-survival signaling and responses. TNFR2 is also known to induce cell death (Medvedev et al., 1994) and this is thought to be brought about by the induction of endogenous mTNF which activates TNFR1 (Grell et al., 1999; Weiss et al., 1998) along with the depletion of TRAF2 by cIAPs. It is also thought that TNFR2 induces apoptotic signals directly by ligand-passing mechanism, where it increases the local TNF concentration in TNFR1 region enabling the latter to initiate the apoptosis (Tartaglia et al., 1993b).

TNF receptor associate proteins (TRAPs) have been found to be associated directly to the regions proximal to the DD in TNFRs to contribute their influence in TNF signaling and responses (Boldin et al., 1995). TRAP1 (Hsp75) localized in the mitochondria (Felts et al., 2000) and TRAP2 (p97) which influences the regulation of proteosomal functions has been found to exhibit DD independent association with TNFRs (Boldin et al., 1995; Dunbar et al., 1997; Felts et al., 2000).

BRE, a stress response protein was found to be interact directly with TNFR1 and it is thought to regulate TNF receptor mediated response by inhibiting and dampening TNFR1 induced signaling (Gu et al., 1998).

An alternative pathway in TNFα–induced phosphatidylinositol signaling via PIP5K-IIβ (Phosphoinositol 4 Phosphate 5 Kinase IIβ) has been reported. TNFR1 but not TNFR2 was found to interact in its juxta-membrane region and activates PIP5K-IIβ (Castellino et al., 1997). PIP5K-IIβ is necessary for the generation of PIP_2, a substrate for PI-PLC mediated IP_3 and DAG (Castellino et al., 1997).

In general, the binding of TNFα to the TNF receptors (TNFR1 and TNFR2) can bring about its varied effects like cellular apoptosis, proliferation or inflammatory responses due to the interplay of signaling mediators, in a TRADD dependent and independent pathway. The differences in the site of action, cell or tissue type, expression patterns and interactions of these signaling molecules play a pivotal role in determining the effect of the wide range of cellular function and responses.

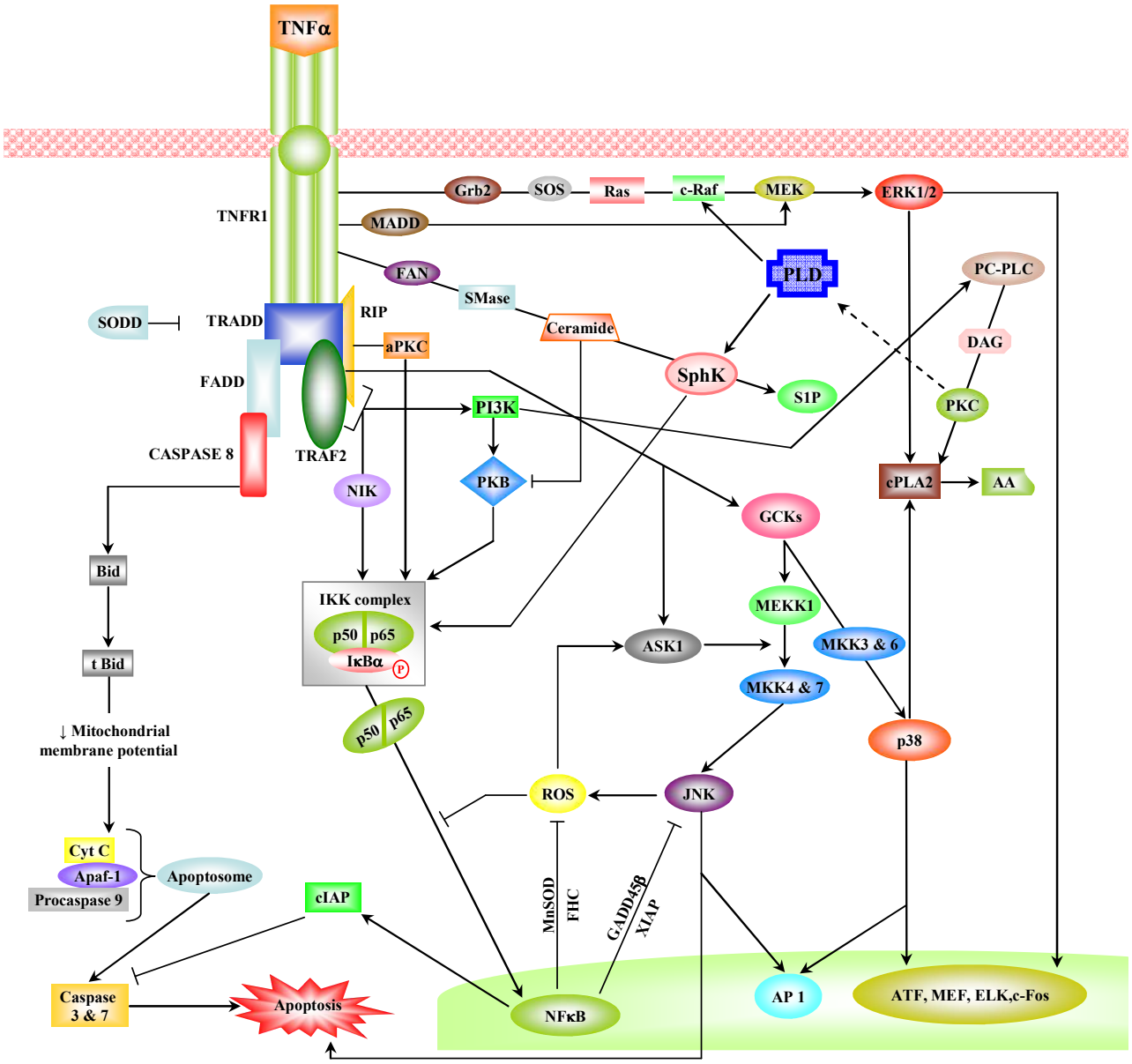


Figure 1.1: TNFα-induced intracellular signaling events
Schematic representation shows TNFR1 mediated intracellular signaling events. The events in the diagram include interaction between pro-apoptotic and pro-survival signaling events triggered by TNFα. The representation also includes TRADD dependent and independent signaling events.

1.2.4 TNFα signaling mediators as therapeutic targets

Inflammation is involved in the maintenance of tissue homeostasis, defense against infection and mediating immune responses. However, dysregulated or prolonged inflammatory process contributes to tissue injury and morbidity, especially in certain chronic diseases and autoimmune conditions. This leads to the necessity to dampen the inflammatory response. TNFα is well known for its role in host defense to bacterial, viral and parasitic infections by mediating and amplifying inflammatory response. Therefore, aberrant TNFα response has been associated with a spectrum of inflammatory disorders as discussed earlier. Biopharmaceutical agents like antibodies and soluble receptors that target TNFα production are being increasingly used in the management of TNFα related disorders. A range of them are currently licensed as TNFα blocking agents and are being used in the management of inflammatory diseases like, rheumatoid arthritis, ankylosing spondylitis and Crohn's disease. The TNFα antagonist exhibits varying degree of efficacy and specificity. The mechanism of action and therapeutic effects in a variety of conditions for most of the currently available TNFα antagonist has been extensively discussed in some recent reviews (Bradley, 2008; Tracey et al., 2008; Valesini et al., 2007; Wong et al., 2008). TNFα blockade has been associated with increase in susceptibility to bacterial, viral and parasitic infections including Listeria, Mycobacteria and granulomatous infections (Pfeffer et al., 1993; Roach et al., 2002; Van Hensbroek et al., 1996; Wallis et al., 2005). It has also been found to be associated with the incidence of opportunistic infection, demyelinating syndromes and autoimmune conditions like lupus. A recent report by Jan Lin et al (Lin et al., 2008) has discussed in detail about the

adverse effects induced by TNFα blockade which clearly indicates the limitations of the use of such biopharmaceuticals. The lack of responsiveness to certain disorders, susceptibility to infections and resistance on long-term use has increased the look out for alternative therapeutic agents.

The search led to the consideration of signaling molecules and mediators regulating inflammatory signals and responses as potential targets. Mediators and molecules involved in various pathways triggered by TNFRs including that of the MAPK and NFκB has gained attention in the recent past. TNFα blockade by inhibiting its production and molecules involved in the TNFα-triggered signaling pathways such as PDE4, p38 MAP-kinase and NFκB inhibitors are currently being explored (Palladino et al., 2003). Especially compounds targeting specific molecules of these pathways such as p38 kinase inhibitors and IKK inhibitors are in various stages of drug development including clinical trials. The present status and future in the treatment of inflammatory diseases by targeting signal transduction has been well discussed by O'Neill LA (O'Neill, 2006). The advancements in the elucidation of signaling pathways have increased the choice of such targets. Identification of suitable and predictable targets for intervention in TNFα-associated inflammatory disorders requires a more detailed understanding of the signaling action and interaction profile of the various signaling mediators.

1.3 Phospholipase D (PLD)

Phospholipase D (PLD) is a membrane associated enzyme. It is a phospholipid specific phosphodiesterase that hydrolyzes phosphaditylcholine (PC) to form phosphatidic acid (PA) and choline. PLD hydrolyzes the terminal diester bond of PC to liberate a bioactive phosphatidic acid and water soluble relatively inert free polar head group, Choline (Exton, 2002a; Exton, 2002b; Pelech and Vance, 1984). PLD superfamily was found to be expressed in bacteria, viruses, plants, yeast, animals and humans (Liscovitch et al., 2000). PLD type activity was first observed in carrot extracts (Hanahan, 1947). It was observed in mammalian system much later in rat brains (Saito et al., 1975). Now, there is accumulating evidence that PLD and its product PA are associated with a range of homeostatic and pathologic processes like inflammatory conditions, diabetes and oncogenesis (Huang and Frohman, 2007).

1.3.1 Phospholipase D metabolic pathway

Phosphatidic Acid (PA) produced by the hydrolysis of PC by PLD can be converted into diacyl glycerol (DAG) by the family of enzymes known as phosphatidic acid phosphohydrolases (PAP) (Brindley and Waggoner, 1996; Brindley and Waggoner, 1998; Sciorra and Morris, 1999). Therefore, activation of PLD led to an increase in the levels of both PA and DAG. The DAG thus produced can be converted back to PA via phosphorylation by diacylglycerol kinases (DGKs). The activities of PAP and DGK tightly control the regulation of PA and DAG levels and their resulting responses. In addition, PA can be converted to form lysoPA (LPA) by the action of phospholipase A (PLA)(Tang et al., 1997). LPA can also be reconverted to PA by lysophosphatidic acid

acyltransferases (LPAAT). Figure 1.2 illustrates the mentioned PLD mediated lipid metabolite conversion pathways. Along with PA, DAG and LPA as potent lipid based second messengers contribute to the effects attributed to PLD. It is well known that intracellular DAG is involved in the activation of both conventional and novel PKC isoforms (Hug and Sarre, 1993). The DAG produced by the PLD pathway is mono or unsaturated. DAG, which is also formed by the hydrolysis of PIP2 by Phospholipase C (Takai et al., 1979), is polyunsaturated, which is the form that activates PKCs in cells (Pettitt et al., 1997). The LPA formed can function as mitogen, thus inducing cell proliferation and survival (Moolenaar et al., 2004). PA is a potent second messenger and it is involved in the activation of PLC (Jackowski and Rock, 1989), P14K (Moritz et al., 1992), Sphinogine kinase (Olivera et al., 1996) and GTPase activating proteins (Tsai et al., 1989). These in turn activate their downstream signaling counterparts.

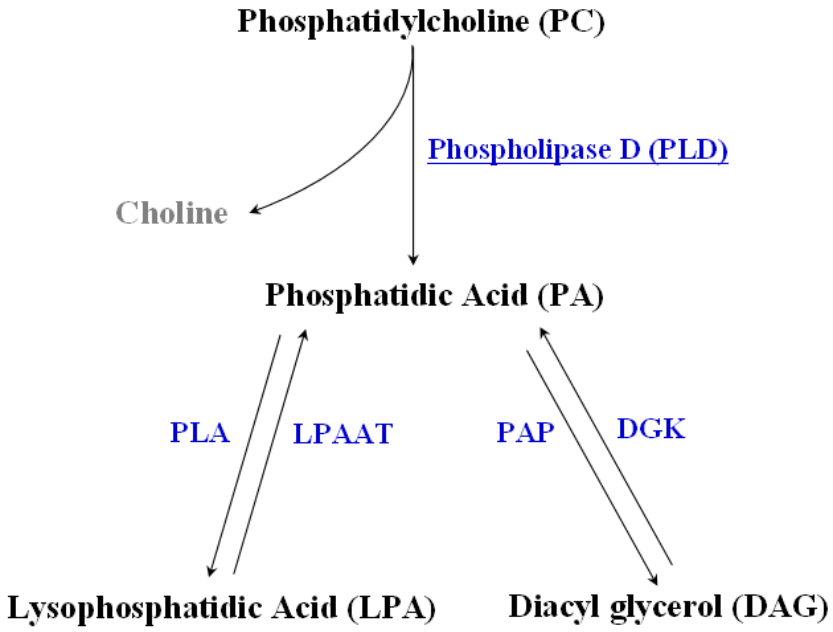


Figure 1.2: Schematic representation of the PLD mediated phospholipid metabolic pathway

1.3.1.1 Transphosphatidylation reaction

A unique property of PLD is its ability to catalyze a transphosphatidylation reaction (Yang et al., 1967). The PLD catalyzed reaction is based on a phosphatidyl group transfer reaction. Normally, the phospholipid substrate acts as a phosphatidyl group donor and water acts as the phosphatidyl acceptor. However, in the presence of a short-chain primary aliphatic alcohol, PLD has the unique ability to utilize the alcohol as the phosphatidyl group acceptor instead of water (Heller, 1978). Thus, generating a phosphatidylalcohol and this reaction is called the transphosphatidylation reaction (Figure 1.3).

In this reaction, the primary alcohol can either be ethanol or butan-1-ol. The preferential utilization of primary alcohol over water is due to the stronger nucleophile nature of the primary alcohol by at least 1000 fold. Due to their steric hindrance both secondary and tertiary alcohols do not have access to the binding pocket of PLD to catalyze this unique reaction. Therefore, they can be used as non-specific effector controls. Phosphatidylalcohols like phosphatidylethanol (PtdEtOH) or phosphatidylbutanol (PtdBut) are produced only by PLDs and the phosphatidylalcohols thus obtained are biologically stable and inactive. As a result this reaction could be used to determine or quantify the enzymatic activity of PLD specifically (Morris et al., 1997). This unique reaction can also be used to study the downstream events triggered by PLD via PA. Since, addition of alcohol shunts the phosphatidyl moieties to the production of biologically inert phosphatidylalcohol instead of the bioactive PA.

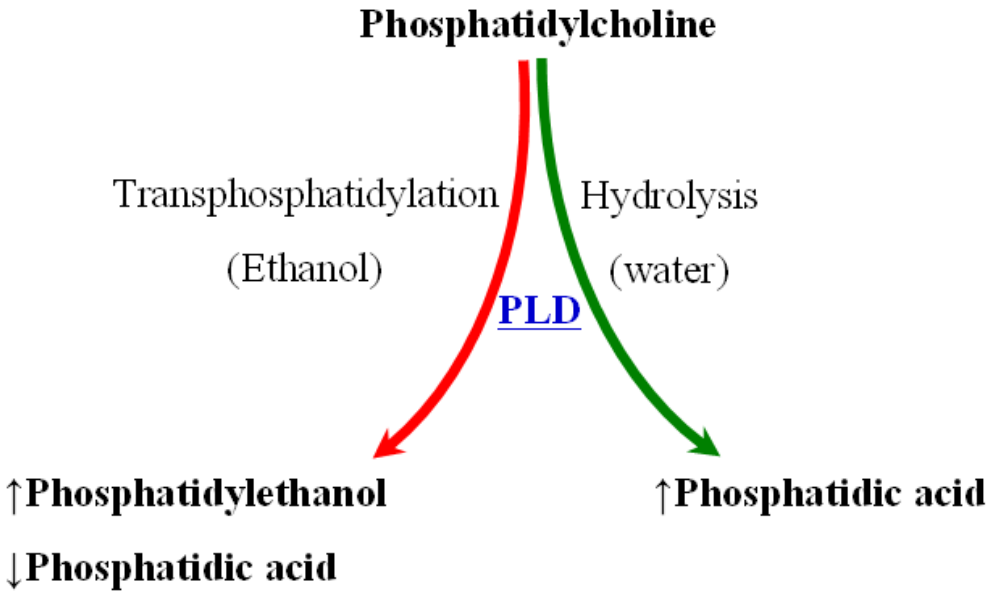


Figure 1.3: Schematic representation of transphosphatidylation reaction unique to PLD

1.3.2 Isoforms and localization of PLD

The classical mammalian PLD enzyme family consists of two PLD isoforms, PLD1 and PLD2 (Hammond et al., 1995; Kodaki and Yamashita, 1997; Lopez et al., 1998). Mammalian PLD1 and PLD2 are widely expressed in most of the tissues and the levels of expression vary based on tissue and cell types. There was a slight difference in the level of expression of the two isoforms in different tissues. PLD1 was found to be highly expressed in the heart, pancreas, spleen, small intestine, ovary, brain, placenta, uterus and liver, and relatively lower levels in lungs, skeletal muscle, kidney, thymus, prostate, testis and colon (Saqib and Wakelam, 1997). Placenta, thymus, prostate, ovary,

uterus, thyroid, spinal cord and trachea showed high PLD2 expression, whereas, liver, skeletal muscle, testis and brain had the lowest (Saqib and Wakelam, 1997).

Structurally both the isoforms contain two HKD catalytic motifs which contribute to the enzymatic activity (Sung et al., 1997). The other conserved domains in PLD genes are the pleckstrin homology (PH) domain, phox consensus sequence (PX) and PI4,5P_2 binding site. The PH domain is necessary for the location of the protein (Sugars et al., 2002) whereas, PX domain mediates protein-protein interaction (Xu et al., 2001). A conserved loop region which functions as a negative regulatory element was found in PLD1 and not in PLD2 (Sung et al., 1999a; Sung et al., 1999b).

Reports on the subcellular distribution of PLD1 and PLD2 are varied. PLD was reported to be in cellular membranes including the ER, Golgi, vesicles, plasma membrane and the nuclear envelope based on biochemical activity (Liscovitch et al., 1999). PLD1 was reported to localized in the perinuclear region, ER, golgi, endosomes, secretory granules and plasma membrane (Brown et al., 1998; Colley et al., 1997; Freyberg et al., 2001; Lucocq et al., 2001; Sung et al., 1999b; Toda et al., 1999). Recent reports have described PLD1 translocating to the plasma membrane subsequent to stimulation (Brown et al., 1998; Du et al., 2003). PLD2 has been reported to be associated with the plasma membrane, cytosol and submembranous vesicles (Colley et al., 1997; Divecha et al., 2000; Du et al., 2004; Honda et al., 1999). It was also found to translocate to the membrane ruffles in response to epidermal growth factor (Honda et al., 1999).

1.3.3 Activation and regulation of PLD

Ligand mediated activation of cell surface receptors are known to generate lipid based intracellular signaling molecules from cellular phospholipids (Divecha and Irvine, 1995; Steed and Chow, 2001). This includes the action of PLD on PC to generated PA and DAG. It is reported that PLD activity is regulated by a group of factors which includes phosphoinositides, Protein kinase C (PKC), ADP-ribosylation factor (ARF), Rho GTPases, protein phosphorylation and lipidation (McDermott et al., 2004). However, the exact mechanism of these factors in the regulation of PLD activity is far from clear.

Phosphoinositides like $PI4,5P_2$ are required for the activation and regulation of both of the PLD isoforms (Colley et al., 1997; Hammond et al., 1995; Hammond et al., 1997; Lopez et al., 1998). $PI4,5P_2$ acts directly on PLD, as it is quite evident that there is a $PI4,5P_2$ binding domain in both the isoforms. It is also said to play a role in the anchoring of PLD to the membranes during activation. Though other phosphoinositides like PIP3 can influence PLD activity, they are not as effective as $PI4,5P_2$ (Hammond et al., 1997).

PKC is another well reported essential regulator for PLD1 isoform. However, the actual mechanism and isoform selectivity is still unclear. It has been suggested that PKC regulated PLD through direct phosphorylation, interaction and also by other indirect mechanisms (Exton, 1997; Exton, 1999). The classical PKC have been found to activate PLD independent of their kinase activity. PLD is directly stimulated by PKCα and PKCβ (Hammond et al., 1997; Singer et al., 1996). However, inhibition of PLD activity via PKCδ has also been reported (Hornia et al., 1999).

PLD activity is regulated by a subfamily of GTPases, the ARF subfamily (Dascher and Balch, 1994; Shome et al., 1998). It has been reported the ARF directly activates both the PLD isoforms (Hammond et al., 1995; Lopez et al., 1998). However, accessory factors like guanine nucleotide exchange factors have been found to play a role suggesting its indirect regulatory role (Caumont et al., 1998; Lambeth et al., 1995). ARF has been strongly implicated in PLD mediated vesicular trafficking (Rothman and Orci, 1992).

PLD is also regulated by the Rho subfamily. RhoGTPases like RhoA, Cdc42Hs and Rac1 have been found to activate PLD (Bowman et al., 1993; Singer et al., 1996). Rho proteins are said to regulate PLD by direct and indirect interactions. Rho is involved PLD mediated cytoskeletal changes (Ridley and Hall, 1992).

The combined regulation of PKC, ARF and Rho has all been shown to have synergistic effect on the regulation of PLD1 activity. Receptor and non receptor tyrosine kinase, serine/threonine kinase, cAMP kinases are suggested to be involved in the regulation of PLD as well (Jenkins and Frohman, 2005).

1.3.4 Cellular responses mediated by PLD

PLD is known to mediate membrane trafficking including exocytosis and endocytosis. It reported to influence the structure of golgi, transport from ER to golgi and the release of secretory vesicles (Bi et al., 1997; Chen et al., 1997; Jenkins and Frohman, 2005). PLD has been reported to be a key player in the exocytosis pathway in adipocytes, pancreatic beta cells, neuroendocrine cells and mast cells (Choi et al., 2002; Huang et al., 2005; Humeau et al., 2001; Waselle et al., 2005). PLD has been associated with

membrane fusion during exocytosis. This action can either be facilitated by PA either by functioning as a lipid anchor or as a fusogenic lipid (Honda et al., 1999; Kooijman et al., 2003).

PLD has been associated with the regulation of endocytosis and phagocytosis as well. This is shown clearly during endocytosis of EGFR. PLD recruitment and binding to dynamin stimulates GTP hydrolysis, accelerates the GTPase activity of dynamin. This in turn triggers membrane fission and clathrin-coated vesicle formation. This potentiates the endocytosis of receptor (Lee et al., 2006b; Shen et al., 2001).

PLD is also associated with cell survival pathways and downregulation of its activity has been observed during apoptosis. The pro-survival is due to the role of PA and its direct downstream targets which includes sphingosine kinase (Olivera et al., 1996), mammalian Target Of Rapamycin (mTOR) – an effector of cell cycle progression and proliferation (Fang et al., 2001) and Raf-1 of mitogen activated protein kinase signaling pathway (Rizzo et al., 2000). mTOR has become a clear target of PA and is proposed to be parallel to the PI3K kinase survival pathway (Jenkins and Frohman, 2005).

1.3.5 Role of PLD in immune and inflammatory responses

PLD has been shown to play an important role in the various effector responses like, NADPH oxidative burst, calcium release, phagocytosis, degranulation, cytokine release and chemotaxis in a variety of immune cells (Melendez and Allen, 2002). PLD activity is also required in signaling events triggered by a wide range of cytokines and chemokines, as well as by chemotactic peptides and chemotactic lipids in immune cells. PLD influences both Fcγ receptor mediated and peptide activated responses like

phagocytosis, oxidative burst and degranulation in neutrophils (Dana et al., 2000; Morgan et al., 1997; Yasui and Komiyama, 2001). A similar effect with PLD regulating these responses were observed in monocytes and macrophage following cell surface receptor activation (Gillooly et al., 1999; Kusner et al., 1999; Melendez et al., 1998b; Melendez et al., 2001). PLD is involved in IgE-mediated functions in mast cells as well (Way et al., 2000). T lymphocytes activation and responses are regulated by PLD activity (Bacon et al., 1995; Mollinedo et al., 1994). It is was also reported that the cross-linking of B cell receptor led to an increase in PLD activity (Hitomi et al., 1999). The activation of Natural Killer cells via CD16 and their triggered responses like degranulation was found to be dependent on PLD as well (Balboa et al., 1992; Milella et al., 1999). This clearly illustrates the functional importance of PLD in immune responses.

The relevance of PLD in health and disease led to the search of PLD inhibitors to harness its activity. Two fungal metabolites, Sch 53823 and Sch 53825 (Chu et al., 1996) and Fodrin, a non-erythroid form of spectrin (Lukowski et al., 1996) was reported to inhibit PLD activity. Rat brain synaptojanin was stated to act as a potent PLD1 inhibitor (Chung et al., 1997). α, β, γ - synuclein was found to inhibit PLD2 (Jenco et al., 1998; Payton et al., 2004). Recently, a new pharmacological inhibitor, FIPI has been identified which inhibits PLD activity and its associated responses (Su et al., 2008). Despite the presence of these inhibitors, a more reliable way currently being followed to characterize the isoform specific role of PLD is by the use of antisense and RNA interference technology both *in vitro* and *in vivo*. However, pharmacological inhibitors against specific isoforms of PLD will certainly be benefical for both research purpose and therapeutics.

1.4 Sphingosine Kinase

Sphingosine Kinase (SphK) is a unique lipid kinase and is a member of the family of sphingolipids, which in the recent past has gained increasing attention as key source of potent signaling molecules. The sphingolipid derived signaling mediators which includes Ceramide and Sphingosine – 1 – phosphate (S1P) are potent bioactive molecules which are being associated with both physiological and pathological responses (Hla, 2004; Hla and Maciag, 1990; Kappos et al., 2006; Kee et al., 2005; Kitano et al., 2006; Melendez, 2008; Sekiguchi et al., 2008). These molecules are major contributors in cellular processes like proliferation, apoptosis and differentiation (Melendez, 2008; Spiegel and Milstien, 1995; Spiegel and Milstien, 2002; Spiegel and Milstien, 2003).

1.4.1 Sphingolipid metabolic pathway

The sphingolipid metabolism is associated with the generation of the above mentioned bioactive molecules. Ohanian and Ohanian in their review has discussed the sphingolipid metabolic pathway very lucidly (Ohanian and Ohanian, 2001). The initial bioactive metabolite generated in this metabolic pathway is ceramide. It is produced via the hydrolysis of sphingomyelin (a major membrane sphingolipid) by sphingomyelinase. Ceramide is also generated by the condensation of serine and palmitoyl-CoA in an alternative pathway. The produced ceramide in turn becomes a precursor to the other bioactive molecule, S1P. Ceramide is hydrolyzed by ceramidases to form sphingosine or phosphorylated to form ceramide – 1 – phosphate or can be converted back to sphingomyelin by sphingomyelin synthase. Sphingosine generated via ceramide is then phosphorylated by sphingosine kinase to form S1P (Spiegel, 1999; Spiegel and Milstien,

2002) and sphingosine on the other hand can also be converted back to ceramide by ceramide synthase. Ceramide – 1 – phosphate can be dephosphorylated back to ceramide by lipid phosphate phosphatase (LPP). Sphingosine-1-phosphate can also be dephosphorylated by S1P phosphatases or LPP to yield sphingosine. S1P can be cleaved by S1P lyase to from hexadecanal and phosphoethanolamine. The sphingolipid metabolism is clearly illustrated in Figure 1.4.

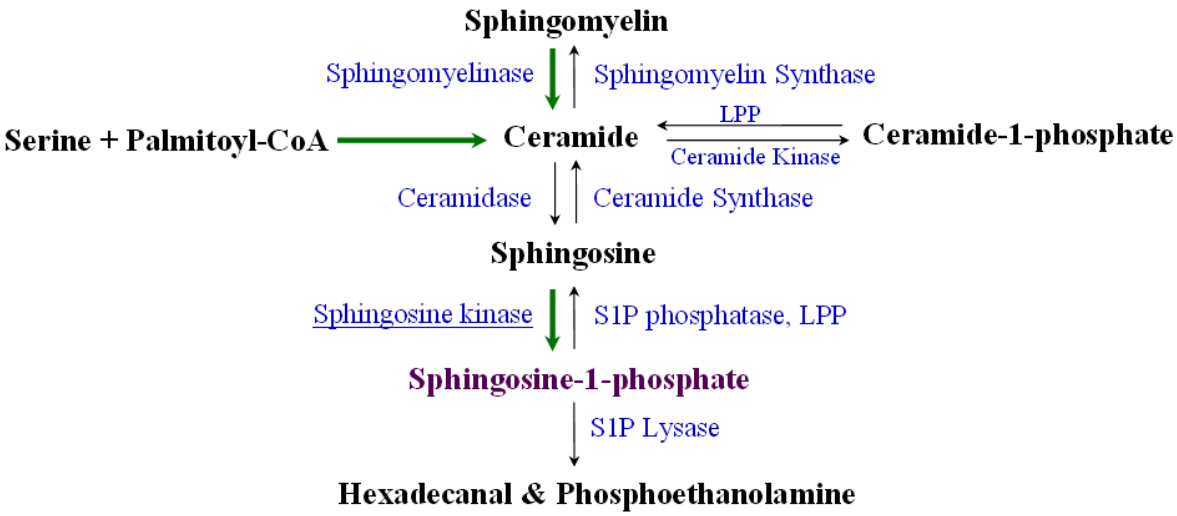


Figure 1.4: Schematic representation of the Sphingolipid metabolic pathway

It is evident that SphK is an important player in the sphingolipid metabolism, because it modulates the relative levels of S1P, sphingosine and ceramide which determines the cell fate. Ceramide has been associated with cellular death, arrest and differentiation (Hannun, 1994; Kolesnick and Kronke, 1998). On the contrary, the other sphingolipid metabolite, S1P is known to be involved in cellular survival, proliferation

and angiogenesis (Spiegel and Milstien, 1995; Spiegel and Milstien, 2002; Spiegel and Milstien, 2003).

1.4.2 Isoforms and localization of SphK

SphK do not share homology with other lipid kinases and are evolutionarily conserved (Labesse et al., 2002; Liu et al., 2002). They are expressed in humans, mice, yeast, bacteria and plants. There are two mammalian isoforms (*SPHK1* and *SPHK2*) that have been cloned, sequenced and characterized (Kohama et al., 1998; Liu et al., 2000; Melendez et al., 2000). They are found to possess a unique kinase catalytic domain, an ATP-binding site and as well as five conserved domains (Kohama et al., 1998; Liu et al., 2000; Melendez et al., 2000). The two isoforms have distinct cellular functions due to the difference in kinetic properties, tissue distribution and temporal expression pattern during development (Spiegel and Milstien, 2003). Erythro-sphingosine, sphinganine and phytosphingosine are the only set of lipids which get phosphorylated by these two isoforms (Kohama et al., 1998; Liu et al., 2000; Melendez et al., 2000; Ozaki et al., 2003). SphK1 has a lower molecular weight and possesses a higher enzymatic activity than SphK2 (Billich et al., 2003).

The localization of SphK was originally thought to be cytosolic but it is shown that the subcellular localization and distribution of SphK would differ based on the organs and tissue type (Olivera et al., 1998). Reports have also shown that on activation SphK can re-localize from the cytosol to the cell membrane (Ibrahim et al., 2004; Johnson et al., 2002; Melendez and Ibrahim, 2004; Melendez and Khaw, 2002). This could be due to the presence of its substrate in the plasma membranes. In addition,

Ca+/Calmodulin has been reported to assist SphK in this translocation process (Young et al., 2003). SphK1 was reported to be highly expressed in the brain, heart, thymus, spleen, kidney and lung (Melendez et al., 2000). The highest expression of SphK2 was reported to be in the kidney and liver (Liu et al., 2000). Reports have also been made that there are different isoforms present in mouse compared to other mammalian isoforms and they possess diverse properties and distribution patterns (Fukuda et al., 2003).

1.4.3 Activation and regulation of SphK

SphK can be activated by a variety of biological effectors like growth factors, hormones, cytokine, G protein couple receptors and other cell surface receptor activation. Ligand binding to the receptors like FcγR1 (Melendez et al., 1998b), FcεR1 (Melendez and Khaw, 2002), fMLP receptor (Alemany et al., 1999), C5aR (Ibrahim et al., 2004; Melendez and Ibrahim, 2004), TNFR (Xia et al., 1998; Zhi et al., 2006) and PDGF receptors (Olivera et al., 1999) resulted in the activation of SphK in immune cells. The activation involves phosphorylation of SphK at Ser225 which results in the translocation of SphK from the cytosol to the cell periphery (Johnson et al., 2002; Pitson et al., 2002). This translocation is necessary because sphingosine, the substrate of SphK is primarily in the cell membrane. S1P is released following the action of SphK on sphingosine. S1P generated is one of the important functional metabolite of the sphingolipid pathway. Other regulatory mechanism in the activation of SphK include PLC induced Ca+ release (Van Koppen et al., 2001), PLD, PKC, protein-protein interaction and proteolysis (Taha et al., 2006). Phosphatidic acid, a bioactive molecule of phospholipid metabolism thought to have direct interaction with SphK to activate the latter (Olivera et al., 1996)

1.4.4 Cellular responses mediated by SphK

The cellular responses regulated by SphK are primarily by its bioactive lysophospholipid metabolite, sphingosine – 1 – phosphate (S1P). The levels of S1P are tightly regulated by SphK, S1P phosphohyrdolase and S1P lyase (Olivera and Spiegel, 2001). S1P acts both as an intracellular second messenger and as an extracellular ligand by binding to its specific receptors, S1P receptors (Pyne and Pyne, 2000; Spiegel and Milstien, 2002). As extracellular ligand, S1P acts by binding to G protein coupled receptor belonging to the endothelial differentiating gene (EDG) family and there are five receptors, $S1P_1$ – $S1P_5$ (Ozaki et al., 2003; Rosen and Goetzl, 2005). S1P and its dihydro analogue can bind to these receptors with great affinity. In mammals $S1P_1$ – $S1P_3$ are found in all tissues, $S1P_4$ is present only in the lungs and lymphoid organs and $S1P_5$ is seen only in skin and brain (Melendez, 2008).

S1P is known to activate diverse signaling transduction pathways to different stimuli in varied cell types. It regulates cell growth and proliferation by expediting the G1-S phase transition in the cell cycle and also by increasing DNA synthesis (Olivera et al., 1999). Its pro-survival profile is also due to its role in Ras and ERK1/2 signaling (Shu et al., 2002; Xia et al., 2000). S1P required for cell growth is either produced by *de novo* synthesis or it is present as a pre-stored form. The latter is reported in the case of platelets with increased store of S1P with high SphK activity. This is due to high basal activity and the absence of degradative enzymes. In the event of tissue injury, the release of S1P gets activated by thrombin and collagen which function in an autocrine and a paracrine manner aiding repair (Yang et al., 1999; Yatomi et al., 1997). S1P aids in cellular survival, proliferation and angiogenesis (Spiegel and Milstien, 1995; Spiegel and

Milstien, 2002; Spiegel and Milstien, 2003). S1P has an anti-apoptotic role by opposing the apoptotic effects of ceramide and sphingosine. Thus, the balance between S1P and ceramide would play a greater role in determining the fate of the cell as shown in the figure 1.5 (Olivera et al., 1999).

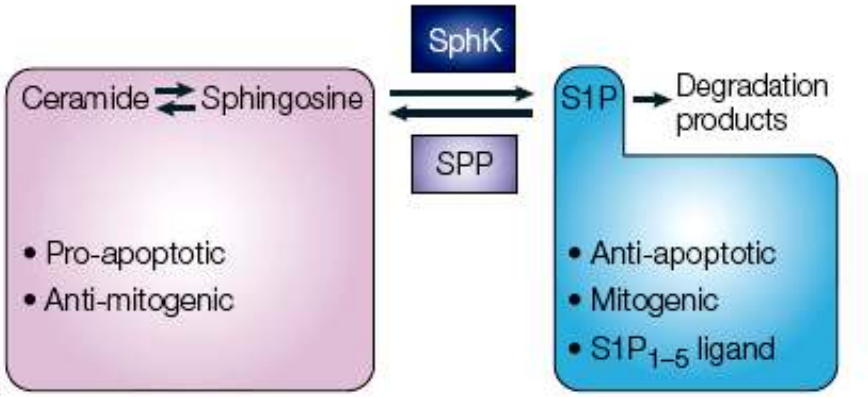


Figure 1.5: The Sphingolipid rheostat
The representation showcases the crucial role SphK in determining cell fate by regulating the balance between S1P and ceramide. Image source (Spiegel and Milstien, 2003).

It is quite evident the calcium plays important roles in cellular processes and S1P has the potential to trigger intracellular calcium signals to render its varied functional responses (Alemany et al., 1999; Melendez et al., 1998b; Melendez and Ibrahim, 2004; Melendez and Khaw, 2002). SphK facilitated receptor mediated mobilization of cytosolic calcium which was dependent on PLC activation and was also found to be independent of IP3 (Choi et al., 1996; Melendez et al., 1998b; Zhi et al., 2006). S1P regulates cell motility and migration both by binding to the cell surface S1P receptors and as well as acting as a chemoattractant (Alemany et al., 1999; Kaneider et al., 2002; Spiegel and Milstien, 2003; Yanai et al., 2000). Therefore, it has been suggested that S1P is involved

in inside and out signaling (Figure 1.6). Since, agonist / ligand binding to receptor induce the increase in intracellular S1P production. This in turn is released out of the cell after rendering its intracellular effects. Once outside the cell, S1P acts on its respective receptors in an autocrine and paracine manner.

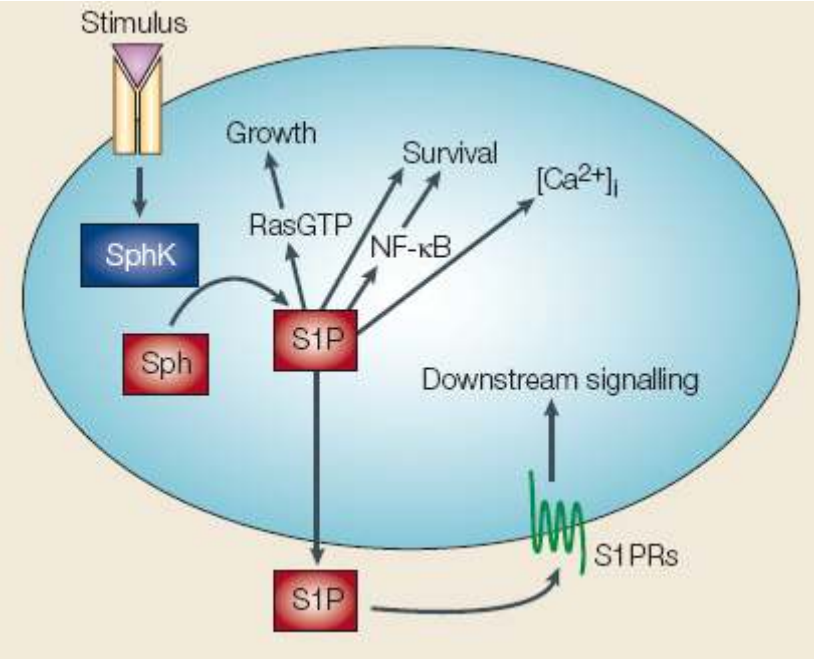


Figure 1.6: S1P signaling
The schematic representation of inside and out signaling by S1P. Image source (Spiegel and Milstien, 2003).

1.4.5 Role of SphK in immune and inflammatory responses

Increase in SphK activity with S1P production are associated with responses like intracellular calcium release, cytokine – chemokine production, chemotaxis, super-oxide production, degranulation and increase in the expression of cell adhesion molecule in a variety of immune cells (Melendez, 2008). Extensive studies have implicated the role of SphK in intracellular signaling and responses in leukocytes such as, neutrophils (Ibrahim et al., 2004; Niwa et al., 2000; Vlasenko and Melendez, 2005), mononuclear cells like monocytes, macrophages (Melendez et al., 1998b; Melendez and Ibrahim, 2004; Zhi et al., 2006), mast cells (Choi et al., 1996; Melendez and Khaw, 2002) and T lymphocytes (Lai et al., 2008a; Matloubian et al., 2004; Pappu et al., 2007).

SphK plays a role in neutrophil priming, activation and chemotaxis (Ibrahim et al., 2004; MacKinnon et al., 2002). SphK has also been reported to regulate the expression of various cell adhesion molecules both in leukocytes and endothelial cells which is necessary in immune cell infiltration (Xia et al., 1998). Consistent with this, Fc receptor and anaphylatoxin, C5a mediated responses in monocytes and macrophages were reported to depend on SphK1 activity (Melendez et al., 1998a; Melendez and Ibrahim, 2004). Similarly, IgE receptor mediated activation of mast cells, including its calcium release and its responses are mediated by S1P (Melendez and Khaw, 2002; Prieschl et al., 1999). A more detailed study on the role of SphK isoform on mast cell activation has reported that SphK2 regulates mast cell activation and SphK1 increases the susceptibility to antigen challenge (Olivera et al., 2007). SphK2 positively modulates the effect of IL-12 in T cell responses resulting in the production of IFNγ (Yoshimoto et al., 2003). Moreover, S1P receptors play a role in the egress of lymphocytes from the

lymphoid organs and inhibition of S1P leads to the sequestration of lymphocytes in secondary lymphatic tissues (Brinkmann et al., 2001; Mori et al., 2007).

Studies have also shown that blockade of SphK reduced the severity of inflammatory responses by inhibiting the production of proinflammatory cytokines (Ibrahim et al., 2004; Lai et al., 2008a; Lai et al., 2008b; Melendez et al., 1998b; Melendez and Ibrahim, 2004; Zhi et al., 2006) and chemotaxis (Ibrahim et al., 2004; Melendez and Ibrahim, 2004). The role of SphK and its isoform specificity in inflammation and immune responses in *in-vivo* has been well documented with use of both SphK inhibitors and RNA interference technology (Lai et al., 2008a; Lai et al., 2008b; Lee et al., 2004a; Vlasenko and Melendez, 2005).

SphKs can be inhibited by inhibitors which are analogues of sphingosine, such as N,N-dimethylsphingosine (DMS) and DL-threo-dihydrosphingosine (DHS) (Buehrer and Bell, 1993; Edsall et al., 1998). However, these inhibitors are not very specific for SphKs and they also inhibit other enzymes at different concentrations. A latest inclusion in the list is FTY720, sphingosine analogue which is effective in improving graft survival and dampening the severity of autoimmune conditions in animals (Mansoor and Melendez, 2008; Yanagawa et al., 1998). SphK2 specifically plays a role in the phosphorylation of FTY720 (Allende et al., 2004; Zemann et al., 2006) which is currently in the clinical trials for the management of renal transplantation and multiple sclerosis (Kieseier et al., 2007; Tedesco-Silva et al., 2005). More analogues of sphingosine are being developed to obtain isoform specific inhibition of SphK for its potential therapeutic application.

1.5 Rationale & Aims

Inflammation is an important and highly relevant homeostatic host response. Dysregulation in this physiologic process contributes to the pathogenesis of inflammatory disorders with significant morbidity. TNFα has been identified as one of the major factors associated in such immune mediated inflammatory conditions. Alternate or additional therapies like harnessing the role of signaling mediators have been proposed to overcome certain set backs reported in the current anti-TNF therapies. Phospholipase D (PLD) and Sphingosine Kinase (SphK) have been proposed to be useful candidates in this approach. This is due to the fact that PLD and SphK are important lipid based signaling mediators whose role in the immune cell signaling and responses are being extensively explored.

In this research study, we sought to elucidate the relevance of PLD in certain key TNFα-induced intracellular signaling events and responses. Antisense based *in vitro* studies will be performed to understand the isoform specific role PLD in the TNFα-induced signaling events and responses *in vitro*. The study will also include studying the signaling hierarchy between PLD and SphK. The *in vivo* roles of these inflammatory mediators (PLD & SphK) will be studied using RNA interference in a TNFα–induced peritonitis model in mice.

The working hypothesis and strategy is that inhibition or knock down of the mentioned lipid based signaling mediators would reduce the degree of TNFα-induced inflammatory responses. Targeting these mediators can be potentially used in the

dampening of aberrant inflammatory response and hence can be used in the management of TNFα-associated inflammatory conditions (Figure 1.7).

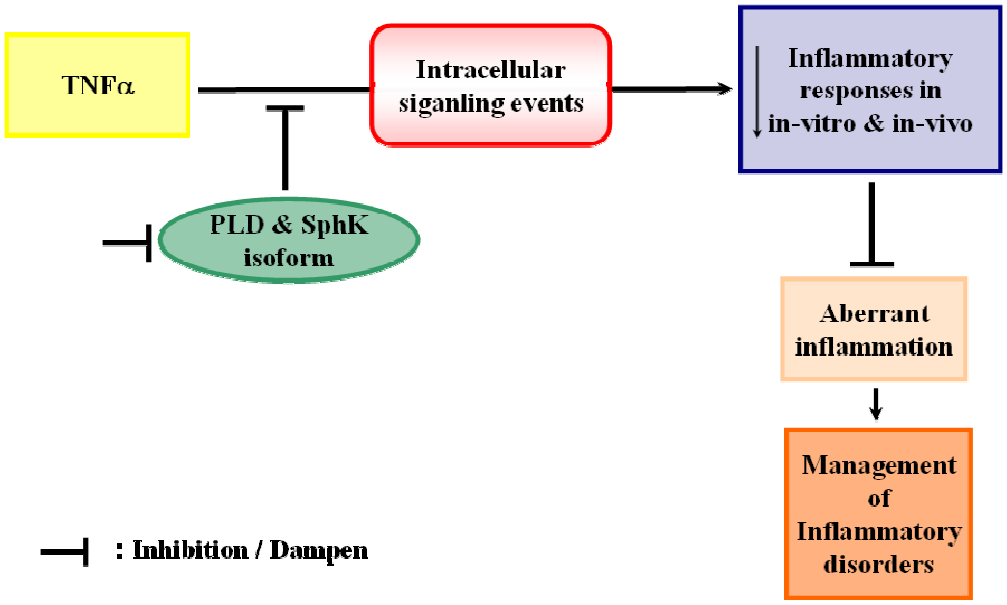


Figure 1.7: Schematic representation of the rationale and strategy of the proposed study

CHAPTER 2

2. Materials and Methods

2.1 Chemicals and Reagents

The chemicals and reagents listed were purchased from the following companies.

- Sigma-Aldrich, Singapore
    - RPMI 1640, IFNγ, L-glutamine, Iodine crystals, Leupeptin, Pepstatin, Aprotinin, PMSF, Penicillin/Streptomycin antibiotic cocktail, Sphingosine, Sphingosine-1-phosphate, BSA, Tris-Base, HEPES, Glucose, Sodium Citrate, Sodium deoxycholate, Glycine, Glycerol, NaCl, $MgCl_2$, $NaHCO_3$, KCl, H_2SO_4, EDTA, EGTA, $MgSO_4$, NaF, $ZnCl_2$, H_2O_2, Bromophenol blue, Trypan blue dye, Bisindolylmaleimide I, rabbit polyclonal anti-PLD2 antibody, HRP or FITC conjugated antibodies, 4-deoxypyridoxine, SB203580.
- NUMI (National University Medical Institutes) Media Preparation Facility
    - 10 x PBS, SDS, Absolute ethanol, Acetic acid, Ethyl acetate, 1-Butanol, Chloroform, Methanol, β-Mercaptoethanol.
- GE Healthcare Bio-Sciences, Singapore
    - ECL Western Blotting detection system, [H^3] Palmitic acid, [γ^{32}-P]ATP, Ficoll-Paque™.

- Santa Cruz Biotech, Santa Cruz, CA, USA.
    - Primary antibodies – mouse monoclonal anti-GAPDH (6C5), mouse monoclonal anti-Raf-1 (E10), mouse monoclonal anti-PLD1 (F12), rabbit polyclonal anti-PLD1 (H160), rabbit polyclonal anti-PLD2 (H133), rabbit polyclonal anti-ARF (H50), rabbit polyclonal anti-p38 (C20), mouse monoclonal anti-α tubulin (B7), rabbit polyclonal anti-VCAM (H276) and goat polyclonal anti-ICAM1 (E15).
    - Secondary antibodies – goat anti-mouse IgG-HRP, , goat anti-rabbit IgG-HRP, goat anti-mouse IgG-FITC, donkey anti-goat IgG-FITC and goat anti-rabbit IgG-FITC.
- Cell Signaling Technology, Danvers, MA, USA.
    - Primary antibodies – mouse monoclonall anti-phospho ERK1/2 (Thr 202/Tyr204; E10), mouse monoclonal anti-phospho p38 (Thr180/Tyr182; 28B10), mouse monoclonal anti-IκBα (L35A5).
- Exalpha Biologicals, Inc, MA, USA.
    - Primary antibodies – Rabbit polyclonal anti-SphK1.
- Calbiochem, Merck, Singapore
    - *N,N*-dimethylsphingosine, Na_3VO_4, PD98059, t-butanol, Protein A-agarose.
- Bio-Rad Laboratories, Singapore
    - Triton-X 100, Tween-20, Ammonium persulphate, 30% Bis-acrylamide, TEMED, Kaleidoscope protein standard.

- Molecular Probes, Invitrogen, Singapore
    - FURA2-AM and FBS
- Roche Molecular Biochemicals, Singapore
    - Complete, Mini – Protease Inhibitor Cocktail Tablets
- MP Biomedicals Inc, Singapore
    - Phosphatidylethanol and Phosphatidyl-1-butanol standard
- Chemicon International, Temecula, CA, USA
    - QCM^{TM} Chemotaxis 3μM 96-well cell migration assay kit
- The following were purchased from PeproTech Inc.Rocky Hill, NJ, USA
    - Recombinant human TNF and recombinant mouse TNF
- The following were purchased from Pierce Biotech, Rockford, IL, USA
    - EZ-Detect^{TM} NFκB p65 transcription factor kit
- The following were purchased from BD Biosciences, San Jose, CA
    - OptEIA^{TM} Cytokine kits and BD Mercury^{TM} TransFactor Profiling Kit (NFκB p50 Kit and NFκB p65 Kit)
- The following were purchased from Qiagen, Valencia, CA
    - siRNAs (mouse SphK1 and mouse PLD1)
- The following were purchased from Avanti Polar Lipids, Inc., Alabaster, AL
    - Sphingosine-1-phosphate standard

2.2 Solutions and Buffers

- RIPA buffer for total cell lysate preparation
    - 50 mM Tris-HCl (pH 7.4), 150 nM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1%NP-40, 1 mM PMSF, 1mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin
- Sphingosine kinase assay buffer
    - 20 mM Tris-HCl (pH7.4), 1 mM β-mercaptoethanol, 1mM EDTA, 20% glycerol, phosphatase inhibitors (20mM $ZnCl_2$, 1 mM sodium orthovanadate and 15 mM sodium fluoride), protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin and 1 mM PMSF) and 0.5 mM 4-deoxypyridoxine
- Sphingosine kinase assay running buffer
    - 1-butanol, ethanol, acetic acid and distilled water in the ratio of 80:20:10:20) (v:v:v:v)
- PLD assay running buffer
    - Ethyl acetate, iso-octane, acetic acid and distilled water in the ration of 65:10:15:50 (v:v:v:v)
- Lysis buffer for nuclear extract preparation
    - 10 mM HEPES (pH7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 1 mM PMSF.

- Extraction buffer for nuclear extract preparation
    - 10 mM HEPES (pH7.9), 0.4M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF.
- Buffers used in ELISA
    - Coating buffer – 1M NaHCO3 in 1 x PBS
    - Assay buffer – 1% FBS in 1 x PBS
    - Washing buffer – 0.05% Tween-20 in 1 x PBS
    - Substrate solution – 1 tablet of TMB in 10ml of 0.05M citrate phosphate buffer (in 1xPBS) and 2 µl of 30% H_2O_2.
    - Stop solutions – 1M H_2SO_4
- Buffers used in SDS-PAGE Gel electrophoresis and Western Blot
    - Resolving gel – Distilled water, 30% bis-acrylamide, 1.5M Tris (pH 8.8), 10% SDS, 10% APS, TEMED.
    - Stacking gel – Distilled water, 30% bis-acrylamide, 1M Tris (pH 6.8), 10% SDS, 10% APS, TEMED.
    - Running buffer – 25mM Tris base, 250mM glycine(pH 8.3), 0.1% SDS
    - Loading buffer – 50mM Tris-HCl (pH 6.8), 100mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol.
    - Transfer buffer – 48mM Tris base, 39mM glycine (pH 8.3), 0.037% SDS, 20% Methanol.
    - Washing buffer – 0.1% Tween-20 and 1 x PBS
- HEPES Ca2+ supplemented buffer for calcium assay

- 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10mM HEPES, 0.18% Glucose, 0.2% BSA.

- MACS buffer
  - 2 mM EDTA in 1 x PBS.

2.3 Cells

The U937 cell line was chosen as the monocytic cell model for the study. U937 cells (pro-monocytic cells) were cultured in RPMI 1640 (Gibco, Rockville, MD) supplemented with 10% Fetal Calf Serum (FCS), 1% glutamine, 150 units/ml penicillin, and 150 μg/ml streptomycin at 37°C in 5% carbon dioxide in a water-saturated atmosphere. Differentiation of these pro-monocytic cells to attain a monocytic phenotype was established by treating the cells with Interferon-γ (200 ng/ml) for 16 hours prior to experiments. Cell viability was determined using Trypan blue and morphology was observed using a light microscope to ensure the state of the cells prior to all experiments.

2.4 Isolation of human peripheral blood monocytes

Human peripheral blood monocytes were isolated from heparinized venous blood obtained from fasting healthy donors. Initially peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Hypaque density gradient centrifugation. During this process, owing to the density differences between mononuclear cells and other cells in whole blood, the mononuclear cells would collect on top of the Ficoll-Hypaque layer due to their lower density. In contrast, red blood cells and granulocytes which have higher density would collect at the bottom of the Ficoll-

Hypaque layer. This was done by carefully layering heparinized whole blood on Ficoll-Paque™ (GE Healthcare Bio-Sciences, Singapore) gradient (density – 1.077 g/liter) and centrifuging at 500 x g, without brakes for 30 minutes at room temperature. The PBMC layer above the Ficoll was gently aspirated and washed three times with sterile 1 x PBS and the number of cells were counted using a hemocytometer.

To meet the demands of our objective to conduct functional studies, including cytokine secretion in primary monocytes, required obtaining untouched or monocytes that are not already activated. Therefore, we also used the Monocyte Isolation Kit II human (Miltenyi Biotec Inc, Auburn CA, USA.) which uses the magnetic cell sorting (MACS) principle to isolate human monocytes from the PBMCs. This kit involved the isolation of monocytes by depleting non-monocytes (negative selection) from PBMCs. Non-monocyte PBMCs are indirectly magnetically labeled with a mixture of biotin conjugated monoclonal antibodies specific for lineage marker and anti-biotin monoclonal antibodies conjugated to the microbeads. When such magnetically labeled PBMCs are passed through the column in a magnetic field, the magnetically labeled non monocytes are retained in the column and only the unlabeled monocytes will pass through the column to be collected in the eluate. The step by step protocol and sample preparation was performed by strictly following the procedures and instruction provided by the manufacturer along with the kit. This technique is considered to be more appropriate to our experimental design as it provides highly pure monocyte population. It also prevents the activation of monocytes compared to those isolated by MACS assisted positive selection of monocytes or by adherence.

The isolated monocytes were then washed three times with plain RPMI 1640 medium and resuspended in the RPMI culture medium (mentioned in section 2.3) and incubated at 37°C in 5% carbon dioxide in a water-saturated atmosphere. Cell viability was determined by Trypan Blue prior to all the experiments.

2.5 TNFα stimulation

The required numbers of cells based on the experiments planned were suspended in culture RPMI 1640 medium prior to stimulation. The cells were either kept untreated or pretreated with various inhibitors as per the experimental design. The cells were then stimulated by the addition of recombinant human TNFα 10 ng/ml (PeproTech Inc.Rocky Hill, NJ, USA.) and incubated at 37°C in 5% carbon dioxide in a water-saturated atmosphere for the various time points indicated.

2.6 Measurement of Phospholipase D activity

The characteristic feature of PLD is a transestrification reaction – transphosphatidylation, in the presence of a primary alcohol. This reaction yields a biologically stable and inert phosphatidyl-alcohol instead of the second messenger, PA (Jenkins and Frohman, 2005; McDermott et al., 2004; Steed and Chow, 2001). A detailed description of this reaction can be seeen chapter 1, section 1.3.1.1. PLD activity measurement in intact cells can be done by overnight radiolabeling of endogenous substrates, followed by the addition of an extracellular stimulus in the presence of a primary alcohol. Subsequent to this process, detection and quantification of the

metabolically stable phosphatidyl alcohols produced by PLD catalyzed reaction can be performed (Morris et al., 1997).

Briefly, the cells were labeled (10^6 cells/ml) with [^{3}H] Palmitic acid (5 μCi/ml) (GE Healthcare Bio-Sciences, Singapore) in the cell culture medium for 16 hours. Following washing with ice cold medium, the cells were incubated at 37°C for 15 minutes in culture medium containing 0.3% of either one of the primary alcohols (1-Butanol or Ethanol) and incubated with inhibitors if any, based on the experiments planned. The cells were then stimulated by TNFα over the desired time indicated at 37°C. The cellular lipids were extracted by Bligh-Dyer phase separation (Bligh, 1959). An aliquot of the lower organic phase was obtained from all the samples to determine the content of radioactivity in the total lipid fraction and equal amount of aliquot was also taken from the phase to quantify the product generated. Phosphatidylalcohol (PtdOH) like Phosphatidylethanol (PtdEtOH) or Phosphatidyl-1-butanol (PtdBut-1-ol) standard was added to all the latter samples and dried under vacuum (Savant ISS110). The lipid containing samples were then re-dissolved in 50 μl of 19:1 (v:v) chloroform/methanol and spot dropped on pre-run, heat-activated thin-layer chromatography plates – TLC silica gel G60 plates (Whatman, Maidstone, UK). The separation was initiated by placing the plates in the organic phase of the solvent (ethyl acetate/ iso-octane/acetic acid/water) for around 90 minutes and the separated phosphatidylalcohol product was detected by visualizing the standard using iodine vapour staining. [^{3}H] phosphatidylalcohol ([^{3}H]PtdOH) containing silica was scraped into vials containing scintillation fluid and the amount of radioactivity was quantified by liquid scintillation spectrometry using a scintillation counter (Wallace 1414 WinSpectral Liquid Scintillation Counter,

PerkinElmer). The amount of PLD activity is measured as the ratio of [^{3}H]PtdOH to [^{3}H]total lipids.

2.7 Fluorescent microscopy

Cellular re-localization or translocations of PLD isoforms and Raf-1 in response to TNFα were analyzed using fluorescent microscopy. After TNFα stimulation, the suspended cells were fixed in 4% paraformaldehyde and deposited on microscope slides using a cytospin centrifuge. They were then permeabilised for 5 min in 0.1% Triton X-100 in PBS. The permeabilised cells were blocked for non-specific binding with 5% Fetal Calf Serum for 10 min at room temperature. Fluorescent labeling was performed by incubating the cells with relevant primary antibodies specific for the targets of interest for 1 hour at room temperature. The cells were washed with PBS and then incubated with appropriate FITC conjugated IgG based secondary antibodies. To a set of cells, only the secondary antibodies were added as control. Stainings were visualized with an inverted fluorescence Leica DM IRB microscope and the images were captured using a Leica DC 300F digital camera. The images were analyzed with the Leica IM500 Image Manager software

2.8 Measurement of cytokine production.

A total of 2 x10^6 cells/ml per category of differentiated U937 (monocytic cells) and human peripheral blood monocytes pretreated with appropriate inhibitors were stimulated with TNFα 10 ng/ml for 24 hours. At the end of the time point the cell suspension were centrifuged and the supernatants were collected and stored in -20°C until

further use. Human IL-6 and human IL-1β were measured using OptEIA^{TM} Kit (BD Biosciences, San Jose, CA, USA) and by strictly adhering to manufacturer's instructions and protocol. The levels of mouse IL-6, mouse MIP-1α and mouse MIP-1β in the peritoneal lavage and serum were measured using mouse IL-6, mouse MIP-1α and mouse MIP-1β OptEIA^{TM} Kit (BD Biosciences, San Jose, CA, USA) and by strictly adhering to manufacturer's instructions and protocol.

To briefly explain the procedure, a 96-well ELISA plate was initially coated with the relevant or specific capture antibody in coating buffer and incubated at 4°C overnight. The following day, the plate was washed three time with ELISA wash buffer (0.5% TWEEN in 1 x PBS) and tapped dried to remove the remaining wash buffer from the wells. This was followed by two hour incubation with ELISA blocking buffer at room temperature. The plate was washed as described earlier. Relevant standards were added by serial dilution to the designated wells and finally 50 µl of the supernatant is added to the marked well and incubated for 2 hours at room temperature. The plate was again washed as described earlier and incubated with detection antibody for 1 hour. This is followed by another round of washing and then enzyme concentrate; avidin-HRP added to all of the wells and incubated in the dark for half an hour. The plate was then washed six times and incubated in the dark for another half hour with 100 µl of TMB substrate. This reaction was quenched by the addition of 100 µl of 1M H_2SO_4. The OD (Optical Density) was then measured using a spectrophotometer (TECAN SpectrophorPlus) at 450 nm. Based on the raw OD data obtained, a standard curve was plotted and the concentrations of each cytokine were then calculated.

2.9 Cell migration assay

Chemotaxis of monocytes were assayed using the Chemicon QCM^{TM} Chemotaxis 3 μm 96-well Cell Migration Assay Kit (Cat no. ECM 515; Chemicon International, Temecula, CA, USA) following manufacturers instructions. A total of 2 x 10^5 cells were used per sample and TNFα at a concentration of 10ng/ml was used for the migration assays. To explain the principle of the experiment briefly, the assay utilizes 3 μm pore size Boyden chambers. The cells were loaded in the upper chamber and the TNFα was placed in the lower chamber and then the cells were incubated for 24 hours at 37°C. Following manufacturer's instruction the cells in the lower chamber, considered to have migrated from the upper chamber were collected, lysed and detected using the CyQuant GR dye provided. This dye when bound to cellular nucleic acid is said to exhibit a strong green fluorescence which can be measured by a fluorescence plate reader using 480/520 nm filter set. To convert the OD values to number of migrated cells, known number of cells were lysed and OD values detected using the dye. These OD values were used to plot the standard curve and by plotting the OD values of the sample along the standard curve, the number of migrated cells can be determined.

2.10 Cell viability assay

The viability of cells following incubation with the various inhibitors and TNFα were determined using MTT; 3-(4, 5-Dimethylthiazol-2-yl)-2, 5 Diphenyltetrazolium assay (Sigma Aldrich, Singapore). Briefly, 1 × 10^4 viable cells were added to each well of a 96 well culture plate (Nunc, Denmark) containing 100 μl medium with relevant

inhibitors or TNFα and incubated for 24 hours at 37°C in a 5% CO_2. After the incubation period, 50 µl of the MTT solution (4 mg/ml in plain RPMI) was added to each well and the cells were incubated at 37°C for 2 hours in the dark. The cells are attached to the floor of the wells by centrifugation and the supernatant is aspirated and discarded. The resulting formazan crystals were dissolved by the addition of 190µl of dimethylsulfoxide – DMSO (Sigma Aldrich, Singapore) and 10µl of Sorenson's glycine buffer and the absorbance of each well was measured at 570 nm in a plate reader (TECAN SpectrophorPlus) Cell death or survival was estimated based on the difference in the OD values between the controls and the treated categories.

2.11 Gel electrophoresis and Western blot analysis

This technique was used to assess the level of PLD isoforms, SphK1, TNFα induced phosphorylation of ERK1/2, p38 and TNFα induced IκBα degradation and to investigate the role of PLD in these events. Briefly, the treated and their control cells were lysed with 1 x RIPA buffer and then the amount of protein quantified using Bradford assay. Subsequently 40µg of the lysate for each category was then resolved in polyacrylamide gels (SDS-PAGE). Lower molecular weight proteins were resolved on 12% Polyacrylamide gels (SDS-PAGE) and higher molecular weight proteins were resolved in lower percentage gels, under denaturing conditions and then transferred to PVDF membranes. The PVDF membranes (Millipore, Billerica, MA, USA) were handled as per the manufacturer's instructions. The membranes were incubated with the relevant primary antibodies for 2 - 4 hours at room temperature and appropriate horseradish peroxidase–conjugated secondary antibody at room temperature for another 2 - 4 hours.

The membranes were washed with washing buffer (1% Tween 1x PBS) three time for 5 minutes after primary and secondary antibody incubation. The bands were visualized by autoradiography using ECL Western Blotting Detection System (Amersham, Buckinghamshire, United Kingdom).

2.12 Immunoprecipitation

Phospholipase D isoforms were immunoprecipated to evaluate its knock down efficiency. PLD isoform specific antibodies were made to form complexes with Protein A-agarose. In order to achieve these complexes, the specific antibodies were incubated with 50% slurry agarose and rocked at 4°C for 2 hours. Cell lysates were prepared from the cells as descirbed earlier. The cell lysates were precleared by incubating with the protein A-agarose for 30 minutes under the rocking condition and the agarose was subsequently removed. The collected lysates were then incubated with the PLD isoform specific antibody-Protein A-agarose complex (prepared earlier), for 4 hours at 4°C. The unbound complexes were removed and washed three times with ice-cold 1 x PBS before resolving the precipitated complexes using SDS-PAGE gel electrophoresis and western blotting.

2.13 Measurement of Sphingosine kinase activity

SphK activity was measured in monocytes after TNFα stimulation at the indicated time points. The cells were either untreated or pretreated with inhibitors or antisense oligonucleotides prior to TNFα stimulation and SphK activity was measured in cell extracts by an assay which is based on the SphK catalyzed transfer of the γ-phosphate group of ATP to a specific substrate. This was done by using a mixture of cold ATP (non

radio-labeled ATP) and radio-labeled [γ^{32}P] ATP (GE Healthcare Biosciences, Singapore) (Melendez et al., 1998b; Olivera et al., 1994).

Subsequent to TNFα stimulation, the reaction was stopped by the addition of ice-cold PBS. The cells were centrifuged and resuspended in ice cold SphK buffer. This was followed by the lysing of the cells using repeated freeze thaw cycles. The supernatant collected after high speed centrifugation was used to measure the sphingosine kinase activity. The assay was performed by incubating with Sphingosine (Sigma-Aldrich, Singapore) and [γ^{32}P] ATP (1 μCi/sample) as indicated (Olivera et al., 1994). 80 μg of cell or the sample lysate was placed in glass tubes and kept on ice, 10 μl of Sphingosine–BSA complexes were added. The reaction mixture was then supplemented with SphK buffer to a final volume of 190 μl and 10 μl of ATP mixture (Radio-labeled ATP/Mg^{2+} mixture – [^{32}P] γ-ATP with unlabeled ATP-$MgCl_2$) was added. Reactions were initiated by placing the samples in 37°C for 30 minutes. The lipids were extracted by adding of chloroform: methanol : HCl (100 : 200 : 1, v/v) to the sample mixture and incubated at room temperature for 5-10 minutes. The organic and aqueous phases were separated by the addition of chloroform and KCl. This mixture was mixed well and incubated at room temperature for 5-10 minutes followed by a 5-10 minutes centrifugation at 400g. 50 μl of samples from the organic phase, which contain radio-labeled S1P, were spotted onto a thin-layer chromatography plates – TLC silica gel G60 plates (Whatman, Maidstone, UK). The products were separated on TLC plates in the presence of Sphingosine kinase running buffer. The radioactive spots were analyzed using autoradiography by exposing

the plate to a Tyhoon scanner and Typhoon phosphor imager was used to measure the intensity of the radioactive signals which would reflect Sphingosine kinase activity.

2.14 Measurement of NFκB activity

NFκB activation was determined directly by measuring the levels of p65 and p50 subunits of the NFκB complex in the nucleus, as it is well known that, these subunits translocate from the cytosol to the nucleus on activation. NFκB activity was analyzed in the cells subsequent to TNFα (10 ng/ml) stimulation for 30 minutes at 37°C, in resting cells and in those pretreated with various inhibitors prior to TNFα stimulation. NFκB activity was analyzed using the EZ-Detect™ NFκB p65 Transcription Factor Kit (Pierce Biotechnology, Rockford, IL, USA) and BD Mercury™ TransFactor Kits (BD Biosciences, San Jose, CA, USA) for both p50 and p65 subunits following the manufacturer's instructions. The determination of the presence or increase in the amounts of NFκB subunits in the nucleus were analyzed using an ELISA based technique. The kit is provided with a 96-well format with the bound biotinylated-consensus sequence for the respective subunits. The biotinylated-consensus duplexes will bind only to the active forms of the transcription factor subunits of interest. This system improves the specificity.

Subsequent to stimulations the cells were washed with 1 x PBS and then lysed using the cell lysis buffer and centrifuged at 10,500 rpm for 20 mins at 4°C. The supernatant is the cytosolic fraction, is collected in a separate tube. The remaining crude nuclear pellet is then treated with nuclear extraction buffer and centrifuged at 13,000 rpm

for 5 minutes at 4°C. The supernatant obtained is the nuclear extract is collected in a fresh tube and stored at -20°C prior to its use. Bradford assay was done to estimate the protein concentration in the nuclear extract. 20 μg of the nuclear extract was used for the assay by adding it to the pre-coated wells and incubated at room temperature for an hour. The plate was then washed using the wash buffer and the appropriate primary antibody (anti-p65 or anti-p50) was added to the wells and incubated for an hour on a shaker at room temperature. This is followed by another round of washing and incubated with HRP conjugated secondary antibody for another hour at room temperature. After washing, a luminol based chemiluminescent substrate or TMB provided with the relevant kits was added to the wells and the signals were detected using a luminometer or a spectrophotometer at the recommended OD.

Another indirect method to assess NFκB activation was by determining the IκBα degradation after stimulation. Since, the phosphorylation and subsequent degradation of IκBα in the cytsol is essential for the translocation of the NFκB subunits from the cytosol into the nucleus. NFκB activity was also determined in the cells subsequent to TNFα (10 ng/ml) stimulation by analyzing IκBα degradation after stimulation. The cells were stimulated up to various time points indicated and lysates obtained. Subsequent to protein estimation, western blot procedure was done using specific antibodies to observe the TNFα–induced IκBα degradation patterns, which is an upstream event to NFκB subunit translocation into the nucleus, indicative of NFκB activation.

2.15 Use of antisense oligonucleotides

The cells were incubated with 10 μM antisense oligonucleotides in presence of a transfection reagent (Superfect – Qiagen, Valencia, CA, USA) for a total of 36 hours (20 hours prior to the addition of IFN-γ and then for the another 16 hours in culture after the addition of IFN-γ for differentiation). Antisense oligonucleotides were purchased from 1st Base, Singapore. 24-mers were synthesized, capped at either end by the phosphothiorate linkages (first two and last two linkages) and corresponded to the reverse complement of the first 8 amino acids for either PLD1 or PLD2. The sequences of the oligonucleotides were, 5`CCGTGGCTCGTTTTTCAGTGACAT 3` for human PLD1 and 5`GAGGCTCTCAGGGGTCGCCGTCAT 3` for human PLD2. The knock down efficacy and specificity was determined using western blotting procedure as described in the earlier sections.

2.16 Measurement of cytosolic calcium

Untreated monocytic cells or cells pretreated either with antisense to human PLD1 or human PLD2 prior to TNFα stimulation were incubated with 1 μg/ml of Fura2-AM (Molecular Probes, Invitrogen, Singapore) in PBS, 1%BSA and 1.5 mM Ca^{2+} for 30 minutes in the dark. Calcium was added to the medium to prevent the depletion of calcium from the intracellular stores prior to stimulation or onset of the experiments. 2 x 10^6 cells/ml was used per category. After incubation, excess reagents were removed and cells were suspended in the cuvette with calcium assay buffer and warmed to 37°C in the spectrofluorophotometer (Shimadzu RF-5301 PC). The measurements of calcium level at

basal was measured for a minute and then the stimulus (TNFα 10 ng/ml) was added to the cell suspension in the cuvette with a magnetic stirrer. The calcium levels were measured using ratiometric measurements. Since, the probe used exhibits significant fluorescence with differene in spectrum (on either side of isoemissive point) both before and after binding with Ca^{2+}. The ratiometeric fluorescent measurements were obtained from signals recorded at 340 and 380 nm over few minutes before the reaction is stopped. The maximum (R_{max}) and minimum (R_{min}) fluorescence measurements necessary for the calculating the ratiometric difference indicate of Ca2+ release were obtained by the addition of ionomycin (to cause Ca2+ saturation) and EGTA (to remove Ca2+ from the probe) respectively.

2.17 Mice

All the *in vivo* experiments were carried out on male BALB/c mice (8–10 weeks old) weighing 20-25gms. The animals were obtained from the National University of Singapore, Sembawang Laboratory Animals Centre and housed in the animal holding unit at NUS prior to and during the experiments. The animals were housed in appropriate cages with free access to food and water. All the animal experiments performed in this study were conducted by strictly adhering to the guidelines stated by NUS Institutional Animal Care and Use Committee (IACUC).

2.18 TNFα–induced peritonitis model in mice

Acute inflammation in the peritoneal cavity of BALB/c mice was induced by the intraperitoneal (i.p) administration of recombinant mouse TNFα (PeproTech Inc.Rocky

Hill, NJ, USA). Based on dose response studies, the dose of rmTNFα of 5 μg/mouse in a final volume of 100 μl was chosen for the experiments. Three mice were used for each category (n=3) per experiment. All the basal category or control mice were injected (i.p) with 100 μl of sterile 1 x PBS. Experiments investigating TNFα response over time, lead to the choice of 2 hours after TNFα injection to assess the acute inflammatory responses. When the effect of SphK1 and PLD1 were investigated, TNFα intra peritoneal injections were administered after 72 hours subsequent to the onset of siRNA treatment, as described in the following section. The various factors analyzed were rectal temperature; Interleukin-6, MIP-1α and MIP-1β levels in the serum and peritoneal lavage; neutrophils infiltration into the peritoneal lavage; cellular infiltration pattern and the expressions of VCAM and ICAM1 in the peritoneal tissues adjacent to and at the site of inflammation or TNFα injection. All the mice were completely anaesthetized by 100μl intra peritoneal administration of ketamine and medetomidine mixture (Animal Holding Unit, NUS) prior to any painful or terminal procedure.

2.19 siRNA administration and gene knock down of mouse SphK1 and mouse PLD1 *in vivo*

The mice were anesthetized and siRNAs were administered via intravenous tail vein injections. Prior to intravenous injections, the tail of the mouse was immersed in sterile warm water for few seconds to dilate the veins for efficient administration of the siRNA. Based on earlier invivo siRNA optimization experiments (Pushparaj et al., 2008), the dose of synthetic siRNAs injected was chosen as 4 μg/mouse in 100 μl of siRNA

diluting buffer and repetitive administration of a specific siRNA (three doses at 24 hours interval) was performed. The intravenous administration was done over 60 seconds using a sterile 30 gauge, 1 inch long needles. siRNA for mouse PLD1(mPLD1) were administered separately to knock down PLD1 in mice. siRNAs and the relevant negative controls were obtained from Qiagen, Valencia, CA, USA. The siRNA sequence used to knock down mouse PLD1 were (AGAGGUGGUUGAUAGUAAA)dTdT and (UUUACUAUCAACCACCUCU)dTdT and a negative siRNA control (Allstar negative control) was used. siRNA for mouse SphK1(mSphK1) were administered separately to knock down SphK1 in mice. siRNAs and the relevant negative controls were obtained from Qiagen, Valencia, CA, USA. The siRNA sequence used to knock down mouse SphK1 were (GGGCAAGGCUCUGCAGCUC)d(TT) and GAGCUGCAGAGCCUUGCCC)d(TT) and scrambled siRNA sequences (GACUCCAAUGGACUGGCAU)d(TT) and (AUGCCAGUCCAUGGAGUC)d(TT) were used as negative controls. The siRNAs were prepared and stored as per the technical instructions provided by the manufacturer.

2.20 Rectal temperature measurement in mice

Temperature changes in mice as a result of the induced inflammatory response were measured rectally using a digital rectal thermometer (Natsume Seisakusyo Co., Tokyo, Japan). The thermometer probe was dipped in oil prior to measurements. The mice were held in a custom built restrainer during the measurements. The probe was inserted into the rectum up to 2 cm deep and held in the same position for 15 seconds until a stable temperature read out was obtained. Rectal temperature changes were

recorded in untreated mice and in mice pretreated with or without siRNA prior to TNFα intra peritoneal injection at the time points indicated.

2.21 Collection of peritoneal lavage in mice

The peritoneal lavage was obtained to analyze the cytokine levels and cellular infiltrations, if any. The mice were fully anaesthetized at specific time points after the intended treatments and their peritoneal cavity was washed with 1.5 ml of ice cold PBS with 0.1% BSA. The peritoneal lavage collected were either stored in -20°C for cytokine or chemokine concentration measurements by ELISA or analyzed immediately for cellular infiltration. Neutrophil infiltration in the peritoneal lavage was analyzed by using Turks solution (which stains the nucleus), which enables the visualization of the polymorphic nucleus of neutrophils under a light microscope. Hemocytometer was used to quantify the number of neutrophils infiltrated into the peritoneal lavage for the various test categories.

2.22 Blood collection procedure in mice

Considerable amount of good quality blood samples from mice were required for the collection of serum for cytokine level analysis and for the isolation of PBMCs. In order to meet this requirement, the cardiac puncture technique was chosen to collect whole blood from mice. Since, it is a terminal procedure; the mice were fully anaesthetized prior to the procedure and were held in a dorsal recumbent position. A sterile 1 ml syringe with a 25 gauge needle was used for the procedure. The needle was inserted 0.5cm to 1cm deep under the xyphoid cartilage slightly to the left of midline.

The needle was inserted at a 25° to 30° angel from the horizontal axis to the sternum to enter the heart. The blood flow from the heart was checked by slightly aspirating and when successful, the blood was drawn slowly taking care to prevent the sudden collapse of the heart. The drawn blood samples were collected in heparinized tubes and placed on ice for further processing.

2.23 Collection of serum from mice blood

In order to collect serum samples from mice, whole blood was collected in non heparinized microfuge collection tubes and allowed to clot spontaneously on ice. Once the clot is formed at the bottom of the tube, the serum was gently pipetted out to a fresh tube. This collected serum was further clarified by centrifugation at 3000rpm for 10 minutes and the supernatant (serum) was collected gently without disturbing the pellet and stored at -20°C until use.

2.24 Isolation of peripheral blood leukocytes from mice

Peripheral blood leukocytes from mice were isolated by Ficoll-Hypaque density gradient centrifugation. The whole blood from mice was collected by cardiac puncture technique, as described earlier. The blood was collected in a heparinized collection tube to prevent clotting. Equal volumes of sterile 1 x PBS and whole blood were mixed at room temperature and layered carefully on Ficoll-Paque^{TM} (GE Healthcare Bio-Sciences, Singapore) gradients (density – 1.077 g/liter) and centrifuged at 500 x g, without brakes for 30 minutes at room temperature. The PBMC layer above the ficoll or at the interface was gently aspirated and washed three times with sterile 1 x PBS. The pellet obtained at

the bottom contained a mixture of RBCs and neutrophils. RBCs were lysed using hypotonic lysis procedure, based on the susceptibility of RBC to hypotonicity. The whole blood was subjected to hypotonic lysis by resuspending whole blood cells (obtained after centrifugation of whole blood) in ice-cold 0.2% NaCl for exactly 30 sec. At the end of this period, isotonicity was restored by the addition of equal volume of ice-cold 1.6% NaCl. This suspension was centrifuged and the neutrophils pellet was obtained. The PBMCs along with neutrophils were then lysed to obtain protein lysates for western blot analysis as described earlier.

2.25 Cellular infiltration pattern in peritoneal tissue

Hematoxylin and Eosin (H and E) staining was done on peritoneal tissues to analyze cellular infiltration pattern in response to induced the inflammation. After specific treatments, the mice were fully anaesthetized and euthanized. The peritoneal tissues at the site of induced inflammation was dissected and stored in formaldehyde. The peritoneal tissues were embedded in paraffin and the sections were mounted on slides. The sectioning and staining was performed at the core facility in NUMI (National University Medical Institutes), NUS. On the day of staining, the sections mounted on slides were dewaxed and cleared using Xylene and decreasing percentage of Ethanol. After washing the slides with deionized water the sections were stained using Harris's Hematoxylin for 2 minutes. The sections were then washed with water prior to alkaline solution dips for few seconds and rinsed with water. This is followed by counter staining the sections with Eosin for 2-3 seconds. The sections were then rinsed with water and dehydrated with ascending percentage of ethanol. The sections were passed through

Xylene and mounted using DEPEX mounting medium. Once dried the sections were observed using a light microscope (Leica DM IRB microscope) and the images were captured using a Leica DC 300F digital camera. The images were analyzed with the Leica IM500 Image Manager software.

2.26 Immunohistochemistry

Immunohistochemistry (IHC) was performed on peritoneal tissues to assess the changes of expression of cell adhesion molecules (CAMs) during induced peritonitis. Subsequent to siRNA treatment and TNFα induced peritoneal inflammation; the mice were fully anaesthetized and euthanized. The peritoneal tissues at the site of induced inflammation was dissected and stored in formaldehyde. The peritoneal tissues were then embedded in paraffin and the sections were mounted on slides. The sectioning and staining was performed at the core facility in NUMI (National University Medical Institutes), NUS. At the time of staining the sections mounted on slides were dewaxed using Xylene the slides were washed with deionized water. The excess water was removed from the slide, taking care that the sections should not left to dry or devoid of moisture until ready for mounting with mounting medium. The tissue section was permeablized using 0.2% Triton X-100 for 10 min and washed with 1 x TBS, three times for 5 minutes. The sections were blocked with the relevant serum (based on the primary antibody) for 30 minutes are room temperature. The excess serum were then drained and incubated with the primary antibody against VCAM or ICAM1 over-night in a humidity chamber at 4°C. After washing as described earlier, the sections were incubated with the relevant FITC conjugated secondary antibody for 1 hour at room temperature. The

sections were washed and the mounted with cover slips using FluorSave™ Reagent (Calbiochem, San Diego, CA, USA) and allowed to dry. The sections were visualized with an inverted fluorescence Leica DM IRB microscope and the images were captured using a Leica DC 300F digital camera. The images were analyzed with the Leica IM500 Image Manager software

2.27 Statistical analysis

Statistical differences between control and treated cells or samples were calculated using unpaired two tailed Student's *t*-test. A statistical difference of at least 95% ($p < 0.05$) was considered significant.

CHAPTER 3

Phospholipase D1 mediates TNFα-induced intracellular signaling events and responses *in vitro*

3.1 Introduction

Tumor Necrosis Factor alpha (TNFα), is one of the most potent and pleiotropic pro-inflammatory cytokines, and has been associated with a wide range of diseases, including acute and chronic infections, as well as in inflammatory, allergic and autoimmune diseases (DeVries et al., 1999; Matsumoto and Kanmatsuse, 1999; Palladino et al., 2003; Plo et al., 2000; Tracey and Cerami, 1993; Tracey and Cerami, 1994). TNFα is produced by many cells, but in higher amounts by phagocytes and mast cells; it is also produced by lymphocytes, NK cells, Kupffer cells, glial cells and adipocytes (Tracey and Cerami, 1993). TNFα binding to its transmembrane receptors (TNFR I and TNFR II), expressed on a wide variety of cell-types, leads to the stimulation of multiple signaling cascades (Vilcek and Lee, 1991), including the MAPK and NFκB cascades, leading to the generation of proinflammatory cytokines and other proinflammatory molecules. However, the mechanisms that regulate TNFα-induced MAPK and NFκB activation in human monocytes are still poorly understood.

Receptor-coupled signaling transduction mechanisms are tightly regulated by a series of protein phosphorylation and dephosphorylation events, and MAPK is a key one among them. MAPKs play a crucial role in amplifying signals and regulating signal

transduction cascades leading to cell proliferation, chemotaxis and the translation of diverse extracellular stimuli to the nucleus, resulting in cellular functions like gene expression, mitosis, differentiation, survival and apoptosis (Roux and Blenis, 2004). Five distinct MAP kinase subfamilies have been identified to date: these are the Extracellular-Regulated Kinases (p44/42 - ERK1/2), the 38 KDa MAPKs (p38 α, β, γ and δ), the c-Jun amino-terminal kinases (JNK1, 2 and 3), and the ERK 3 and 4 and ERK5 kinases (Roux and Blenis, 2004). ERK1/2 are activated by growth factors, phorbol esters, serum, cytokines, certain stresses and ligands of GPCRs (Lewis et al., 1998; Pearson et al., 2001): MAPKs are found to be key regulators of cell proliferation (Roux and Blenis, 2004). p38 kinase activity is triggered by osmotic, physical and chemical stress, UV irradiation, pro-inflammatory cytokines and hormones (Chen et al., 2001; Pearson et al., 2001; Whitmarsh et al., 1995) and is found to be vital in regulating immune and inflammatory responses (Roux and Blenis, 2004).

Cell surface receptor engagement by a ligand leads to phospholipase-mediated hydrolysis of cellular phospholipids, leading to the generation of lipid-derived products, which in turn play critical roles in a wide range of intracellular signaling pathways (Divecha and Irvine, 1995; Steed and Chow, 2001). Intracellular signaling mediated by Phospholipase D (PLD) has been a target of interest in inflammation and tumor metastases for more than two decades (Steed and Chow, 2001). It is well-known that PLD is a major source of second messengers, including Phosphatidic Acid (PA) (Hanahan, 1947), which can be converted to another important messenger DAG (Diacylglycerol) (Sciorra and Morris, 1999). PLD is considered to regulate cellular responses that contribute to inflammation, and tumor cell migration (Steed and Chow,

2001). In mammalian cells, activation of phosphocholine-specific PLD has been proposed to control signal transduction pathways regulating a wide range of physiological processes, including membrane trafficking (Siddhanta and Shields, 1998) and cytoskeletal reorganization (Colley et al., 1997), phagocytosis (Kusner et al., 1999), phagocytic cell NADPH-oxidase-respiratory burst (Dana et al., 2000; Melendez et al., 2001), and mast cell degranulation (Hammond et al., 1995).

Some studies have suggested a role for PLD in TNFα-induced cellular cytotoxicity (De Valck et al., 1993; Kang et al., 1998). However, these early studies have not been followed or validated and the functional significance of these observations remains controversial (MacEwan, 2002b). The role of PLD in TNFα-induced intracellular signaling events remains largely unknown. Hence, in this study, we sought to elucidate the relevance of PLD in key TNFα-induced intracellular signaling events and responses *in vitro*. Antisense based *in vitro* studies were performed to understand the isoform specific role PLD in the TNFα-triggered response and signaling, including the hierarchy between PLD and SphK in human monocytes. Here, it is shown that phospholipase D1 is activated by TNFα, plays a key role in the signaling events like TNFα–triggered sphingosine kinase, NFκB and ERK1/2 activation, but not for p38 phosphorylation. Furthermore, the physiological importance of PLD1 in the TNFα-induced signaling pathways is emphasized by its role in mediating proinflammatory cytokine production.

3.2 Results

3.2.1 TNFα-induced effector responses in monocytes are dependent on its PLD activity

3.2.1.1 TNFα induces PLD activity in human monocytes

The ability of TNFα to induce the activity of PLD in the human monocytic cell line used was investigated by performing PLD activity assay. In this assay, the production of [^{3}H]PtdOH was measured and it served as a means for quantitative measurement of PLD activity following stimulation. The monocytic cells were triggered with TNFα (10 ng/ml) and PLD activity was measured at the 2, 5, 10, 30 and 60 minutes. Subsequent to TNFα stimulation there was an increase in [^{3}H]PtdOH generation indicating an increase in PLD activity (Figure 3.1A).

TNFα stimulated a rapid increase in PLD activity, which was detectable as early as 2 minutes after TNFα stimulation and maximum PLD activity was observed at the end of 30 minutes after stimulation (Figure 3.1A). There was a dose dependent response in PLD activation following stimulation with a maximum activity at 10ng of TNFα (Figure 3.1B). However, activity with 2 ng and 5 ng of TNFα was quite robust as well. Therefore, TNFα concentration of 10 ng/ml and PLD activity at 30 minutes after TNFα stimulation was used to assess PLD activity in the subsequent experiments. Similar experiments carried out on primary monocytes isolated from human peripheral blood exhibited a significant increase in PLD activity in 30 minutes, subsequent to TNFα (10 ng/ml) stimulation (Figure 3.2). These observations indicate that the enzymatic activity of Phospholipase D is indeed induced in human monocytes following TNFα stimulation.

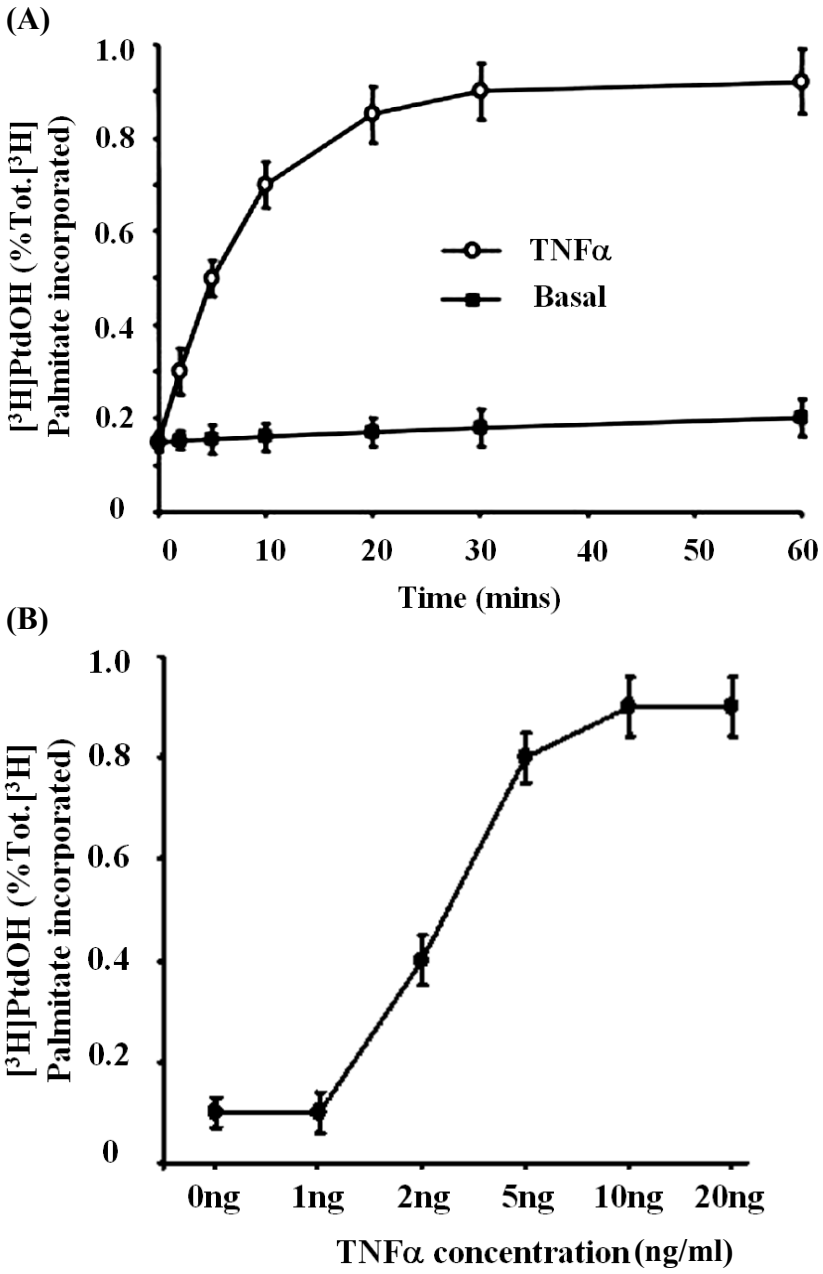


Figure 3.1: Induction of PLD activity by TNFα in human monocytic (U937) cells (A). PLD activity was measured in unstimulated cells (Basal) and in cells stimulated with TNFα 10 ng/ml (TNFα) for 0, 2, 5, 10, 20, 30, and 60 minutes. **(B)**. PLD activity was measured following stimulation with different concentrations of TNFα for 30 minutes. Results shown are the mean ± the standard deviation of triplicate measurements and are the representative of three separate experiments

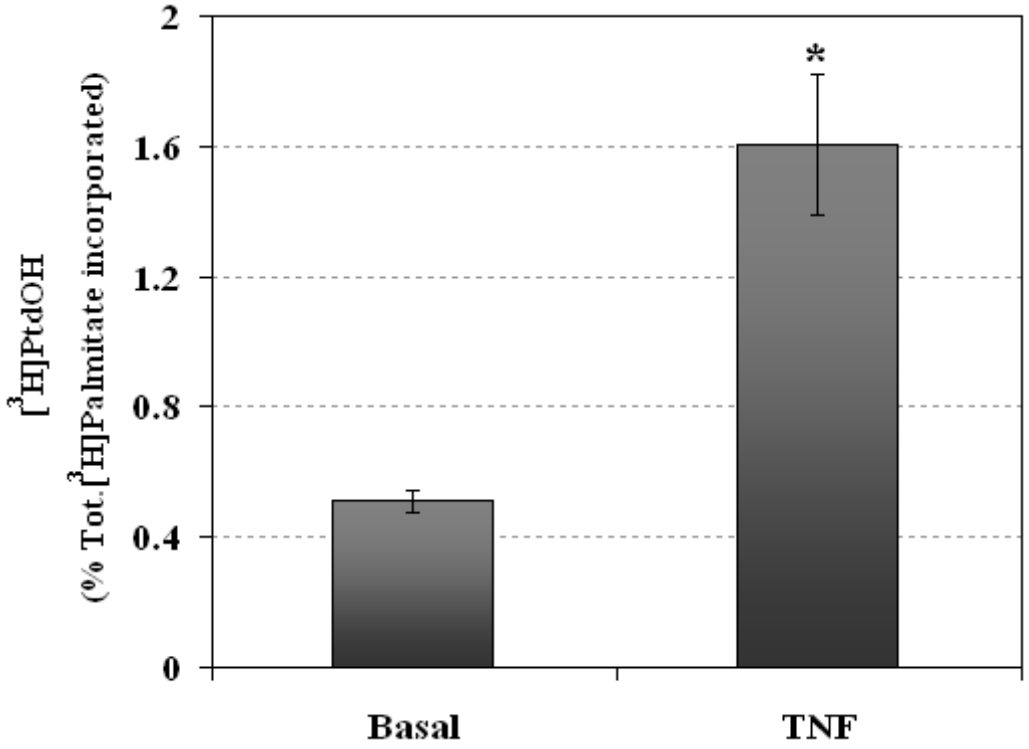


Figure 3.2: Induction of PLD activity by TNFα in primary human monocytes

Basal PLD activity in un-stimulated primary monocytes in culture medium (Basal) and PLD activity in cells stimulated with TNFα 10 ng/ml for 30 minutes (TNF). Results shown are the mean ± the standard deviation of triplicate measurements and are the representative of three separate experiments. (*) indicates Student's *t* test *p* value <0.05.

3.2.1.2 PLD mediates TNFα-induced effector responses in human monocytes

The functional relevance of PLD in inflammatory responses was studied by investigating the role of PLD in TNFα-induced pro-inflammatory cytokine (IL-6 and IL-1β) production and cellular migration. These responses were chosen as representatives of TNFα-triggered pro-inflammatory effector responses in this study. Cytokines and chemokines are soluble regulatory proteins which play vital roles in the onset, propagation and regulation of immune and inflammatory responses. Disproportionate levels of pro-inflammatory cytokines are associated with inflammatory disorders.

The role of PLD in cellular functional responses was investigated by taking advantage of the characteristic transestrification reaction – transphosphatidylation of PLD, in the presence of a primary alcohol. This reaction yields an inert and stable phosphatidyl alcohol instead of a biologically active second messenger, PA (Phosphatidic acid), thereby inhibiting its downstream signaling events and responses. Since this reaction is specific for primary alcohol, tertiary alcohol was used as an added control. This ensures that, the effect observed was indeed due to the inhibition of downstream signaling of PLD and not a non specific effect of alcohol used.

One of the potent and important physiological and pathophysiological roles for TNFα is to induce the release of other pro-inflammatory cytokines. In our experiments, we have shown that TNFα stimulates the release of IL-6 and IL-1β from human monocytic cells and pre-treatment of cells with 1-Butanol (primary alcohol) but not t-Butanol (tertiary alcohol), prior to TNFα stimulation, inhibited TNFα -induced production of IL-6 and IL-1β (Figure 3.3).

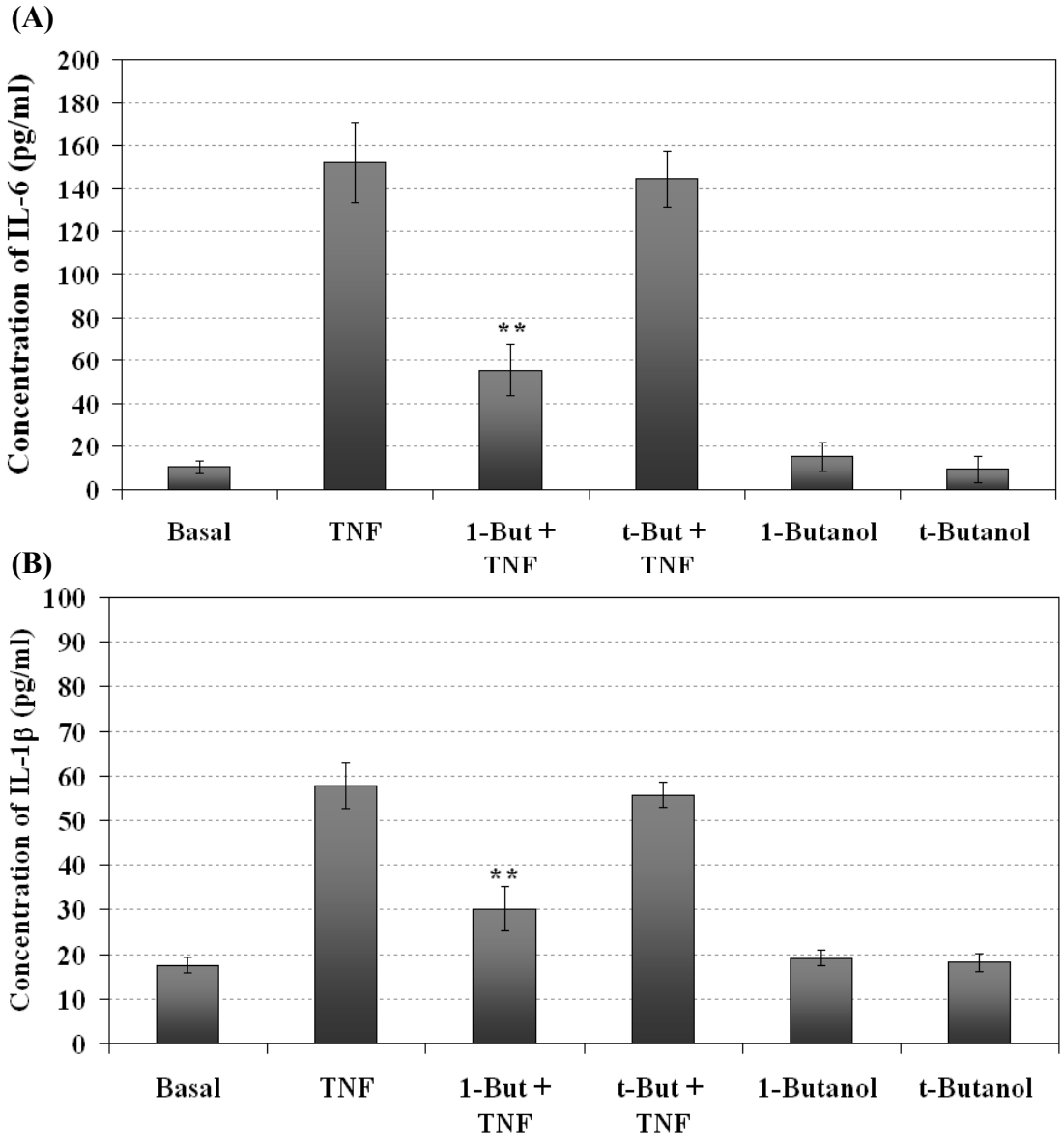


Figure 3.3: IL-6 and IL-1β release triggered by TNFα is dependent on PLD in human monocytic cells

The graphs indicate the IL-6 (A) and IL-1β (B) production in 24 hours. The categories include the cytokine levels in unstimulated cells (Basal); in cells stimulated with TNFα (10 ng/ml) only (TNF), in cells pre-treated with 0.3% 1-Butanol prior to TNFα (10 ng/ml) stimulation (1-But + TNF); in cells pre-treated with 0.3% t-Butanol prior to TNFα (10 ng/ml) stimulation (t-But + TNF) and in cells incubated with 0.3% 1-Butanol and 0.3% t-Butanol only (1-Butanol and t-Butanol respectively). Results shown are the mean ± the standard deviation of triplicate measurements and are the representative of three separate experiments. (**) indicates Student's *t* test *p* value <0.01.

In order to validate the functional importance of PLD in inflammatory responses in a more relevant cell model, primary monocytes from human peripheral blood were isolated and similar experiments were done. The results obtained also exhibited the inhibition of TNFα-induced IL-6 and IL-β production in primary monocytes pre-incubated with 1-Butanol but not t-Butanol (Figure 3.4).

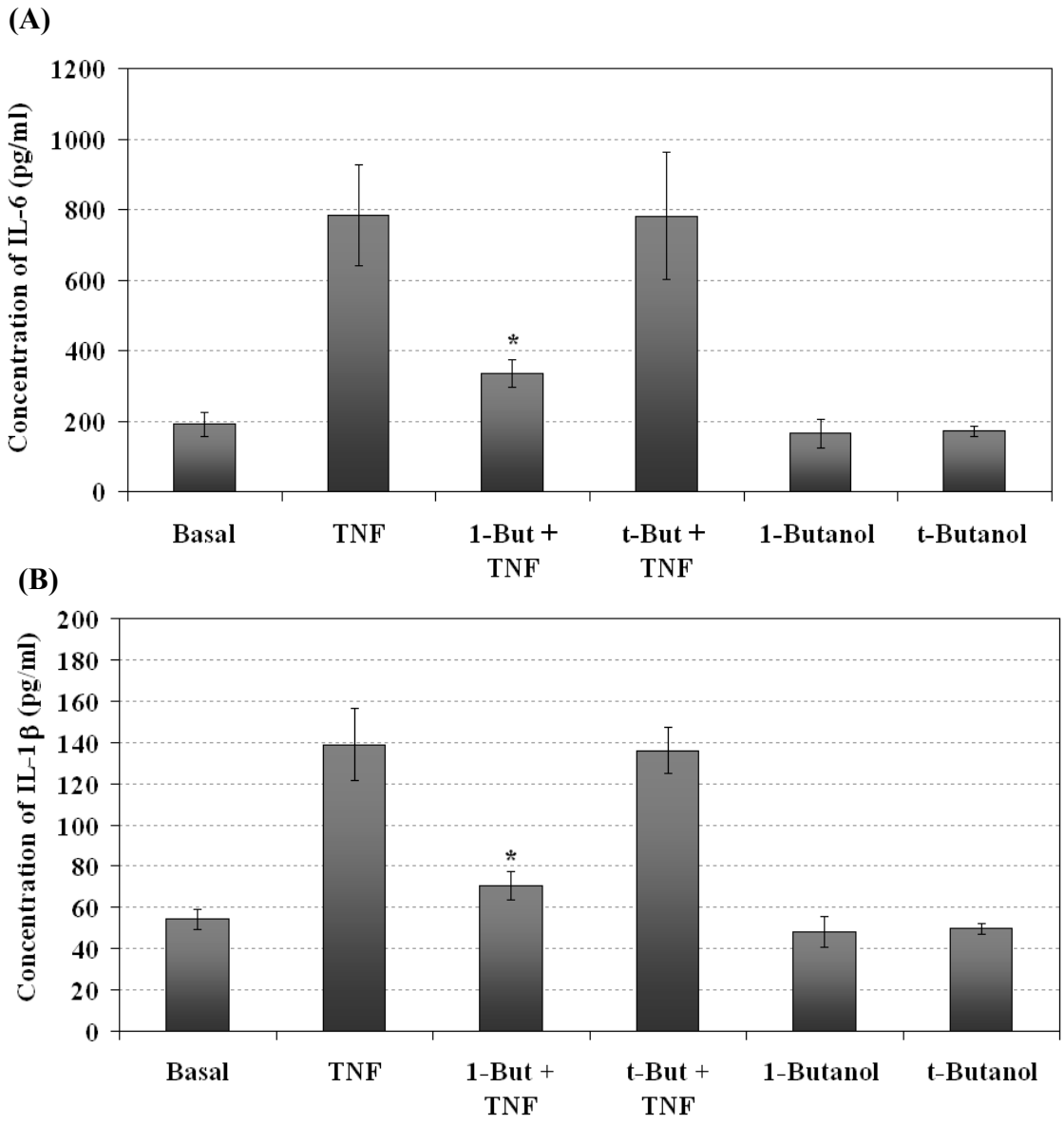


Figure 3.4: IL-6 and IL-1β release triggered by TNFα is dependent on PLD in human peripheral blood monocytes

The graphs indicate the IL-6 (A) and IL-1β (B) production in 24 hours. The categories include the cytokine levels in unstimulated cells (Basal); in cells stimulated with TNFα (10 ng/ml) only (TNF), in cells pre-treated with 0.3% 1-Butanol prior to TNFα (10 ng/ml) stimulation (1-But + TNF); in cells pre-treated with 0.3% t-Butanol prior to TNFα (10 ng/ml) stimulation (t-But + TNF) and in cells incubated with 0.3% 1-Butanol and 0.3% t-Butanol only (1-Butanol and t-Butanol respectively). Results shown are the mean ± the standard deviation of triplicate measurements and are the representative of three separate experiments. (*) indicates Student's *t* test *p* value <0.05.

Immune cell migration to the site of inflammation is another important response event during inflammation. PLD has been involved in chemoattractant mediated chemotaxis in leukocytes and this is reported to be brought about the regulation of polarization of the cells by PLD (Lehman et al., 2006). Therefore, we investigated the role of TNFα as a chemo-attractant in monocytic cells and the role of PLD in this response. TNFα (10 ng/ml) triggered a chemotactic response in human monocytes. However, pre-treatment of cells with the 1-Butanol but not t-Butanol, substantially inhibited the chemotactic or the migratory response of monocytic cells (Figure 3.5). The measurements obtained were in RFU (Relative Fluorescence Units) as shown in Figure 3.5A. In order to have a more relevant data representation, a standard curve was plotted with known number of cells and the RFU was noted. Substitution of RFU values obtained in the experiments on standard curve indicated the total number cells migrated for the categories indicated (Figure 3.5B).

These set of experiments as shown in Figures 3.3 – 3.5 vividly portrays the functional necessity of PLD in the propagation of TNFα-induced inflammatory responses.

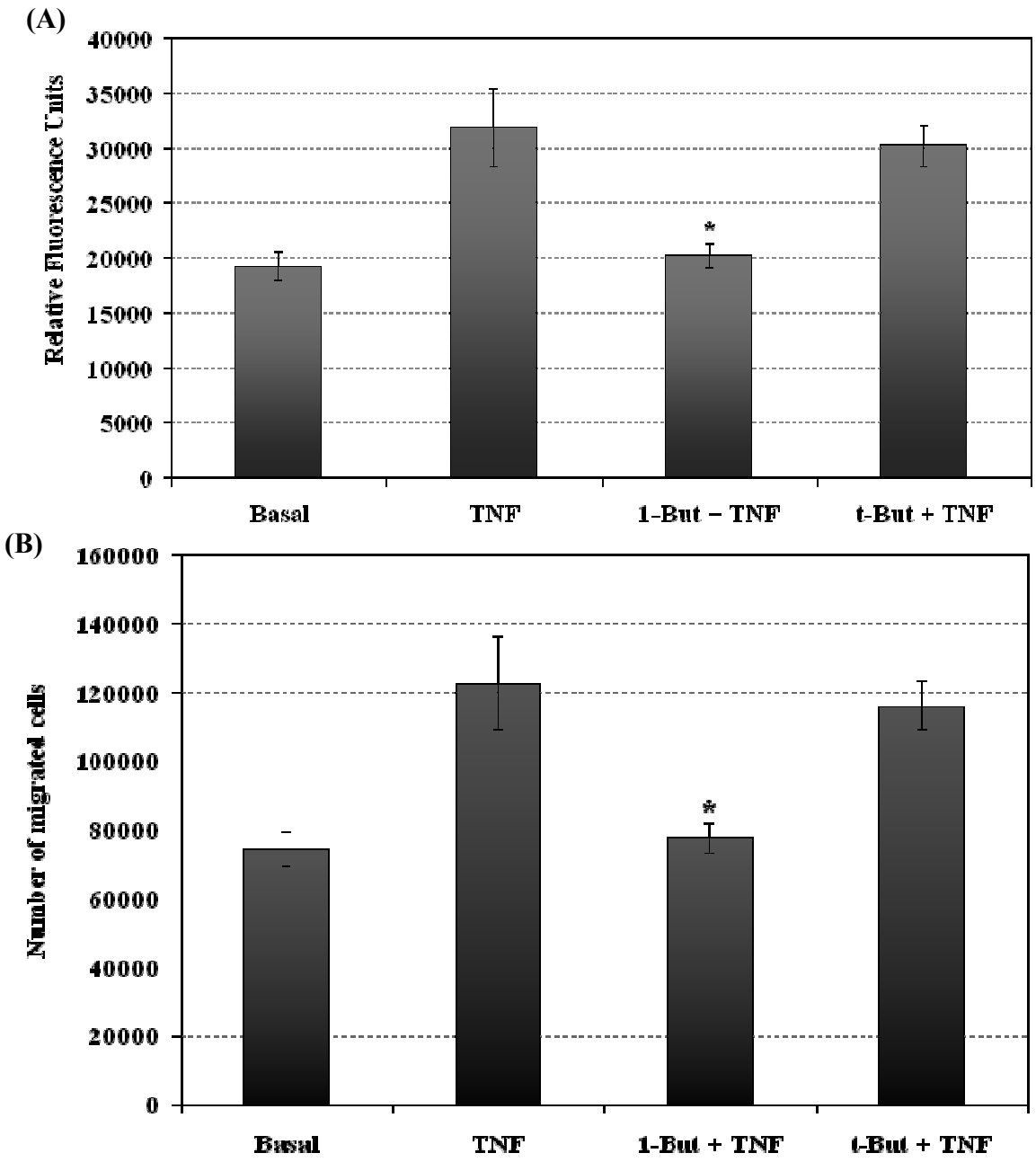


Figure 3.5: TNFα-induced chemotaxis is PLD dependent in human peripheral blood monocytes

The graph (A) indicates indirect measurement of number of migrated cells in Relative fluorescence units (RFU) and graph (B) indicates the absolute cell number of migrated cells based on standard curve obtained with absolute cell numbers and their respective RFU. In graph (B) the Y axis indicates the number of migrated cells towards TNFα. The graphs shows basal migration of untreated cells (Basal); migration of untreated cells toward the TNFα 10 ng/ml containing chamber (TNF); migration of cells pretreated with 0.3 % 1-Butanol (1-But + TNF) and migration of cells pretreated with 0.3% t-Butanol (t-But + TNF) towards the TNFα 10 ng/ml containing chambers. Results shown are the mean ± the standard deviation of triplicate measurements and are the representative of three separate experiments. (*) indicates Student's *t* test *p* value <0.05.

In order to ascertain that the inhibitory effects were indeed due to inhibition of the enzymatic reaction's downstream products and not because of cell death induced by the reagents used, a cell viability assay was performed. MTT assay was done to investigate the cell viability after incubating the cells with TNFα, primary and tertiary alcohol for 24 hours. Measurements at the end of the time point failed to show any significant decrease in cell viability induced by the mentioned reagents (Figure 3.6). This indicates that the inhibition in responses was due to the transphosphatidylation reaction causing the inhibition of production of the bioactive downstream product (like PA) of the enzymatic reaction.

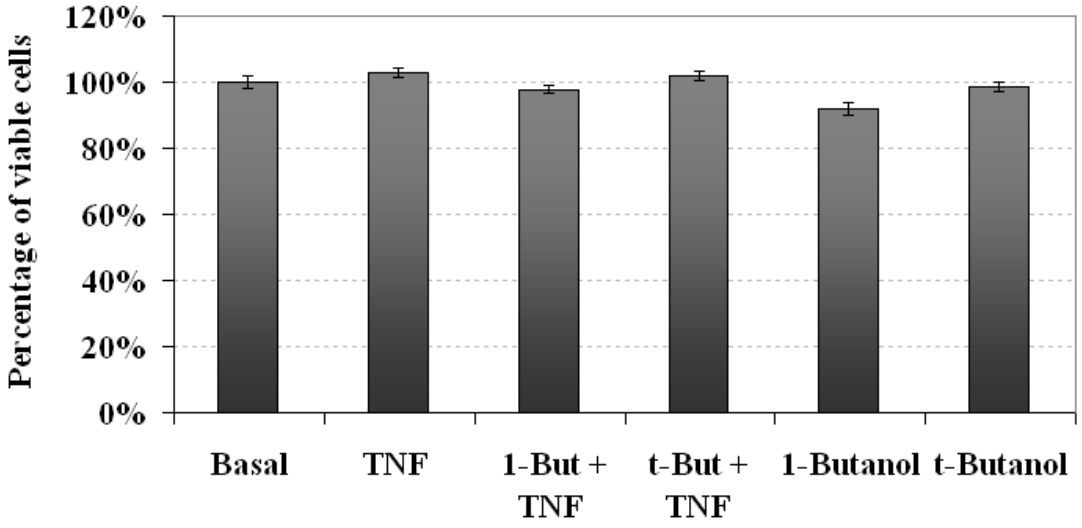


Figure 3.6: Percentage of cell viability in human monocytic cells after 24 hours incubation with TNFα, a primary (1-Butanol) and a tertiary alcohol (t-Butanol)
The graphs shows the percentage of viable untreated cells (Basal); cells incubated with TNFα 10 ng/ml (TNF); cells incubated with 0.3 % 1-Butanol and TNFα 10 ng/ml (1-But + TNF); cells incubated with 0.3 % t-Butanol and TNFα 10 ng/ml (t-But + TNF) and cells incubated with 0.3% 1-Butanol and 0.3% t-Butanol only (1-Butanol and t-Butanol respectively). Results are the mean and ± SD of triplicate measurements and are the representative of three separate experiments.

Based on the results in this section, we can suggest that, Phospholipase D mediates TNFα– triggered pro-inflammatory responses in human monocytes.

3.2.2 Role of PLD in TNFα–induced intracellular signaling events

3.2.2.1 Role of PLD in TNFα-induced MAPKs activation

3.2.2.1.1 TNFα-triggered effector responses are regulated by ERK1/2 and p38 kinase.

MAPKs are key intracellular signaling mediators which regulate a variety of cellular processes and responses. Since it is already evident from our experiments, that PLD influences TNFα–induced release of IL-6 and IL-1β in monocytic cells (Figure 3.7), we evaluated the functional relevance of MAPKs like ERK1/2 and p38 kinase in TNFα-induced effector response prior to the evaluation of the signaling hierarchy between PLD and the MAPKs in TNFα-triggered signaling. In order to investigate this objective, we pre-treated the cells with a MEK inhibitor (PD98059) and a p38 inhibitor (SB203580), before stimulating them with TNFα for 24 hours and quantified the release of IL-6 and IL-1β. It was observed that, MEK and p38 inhibitors decreased the level of TNFα induced IL-6 and IL-1β production in human monocytic cells (Figure 3.7). Based on the results obtained, it can be inferred that including PLD, TNFα-triggered IL-6 and IL-1β production in monocytes are also regulated by ERK1/2 and p38 kinase. Therefore, our experiments focused on the signaling events or signaling hierarchy if any, between PLD and MAPKs when stimulated by TNFα in human monocytic cells.

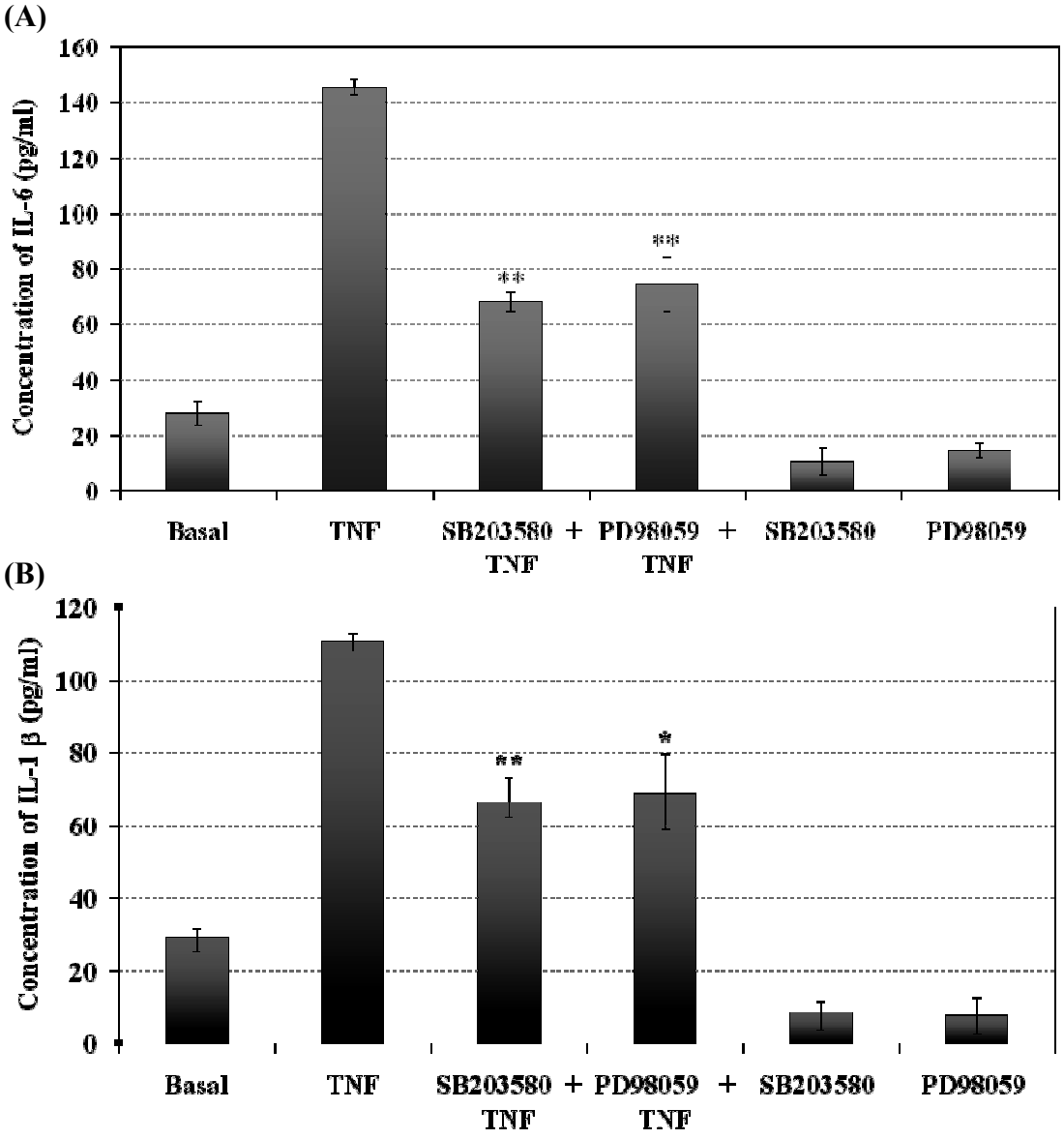


Figure 3.7: Cytokine production triggered by TNFα is dependent on ERK1/2 and p38 kinase in human monocytic cells

Graph (A) indicates the IL-6 production and graph (B) indicates IL-1β production in 24 hours. Cytokine production in unstimulated cells (Basal); in cells stimulated with TNFα (10 ng/ml) only (TNF); in cells pre-treated with 10 μM of SB203580 (p38 inhibitor) prior to TNFα (10 ng/ml) stimulation (SB203580 + TNF); in cells pre-treated with 50 μM of PD98059 (MEK1/2 inhibitor) prior to TNFα (10 ng/ml) stimulation (PD98059 + TNF) and in cells incubated with 10 μM of SB203580 and 50 μM of PD98059 only (SB203580 and PD98059 respectively). Results shown are the mean ± the standard deviation of triplicate measurements and are the representative of three separate experiments. (** and *) indicates Student's *t* test *p* value <0.01 and <0.05 respectively.

3.2.2.1.2 TNFα–induced ERK1/2 activation pathway is mediated by PLD

To evaluate the significance of PLD and ERK1/2 (p44/42) in TNFα-triggered signaling, ERK1/2 phosphorylation by TNFα in cells pre-treated with and without 1-Butanol and t-Butanol were investigated. TNFα was found to activate ERK1/2 (dually phosphorylate ERK1/2) in human monocytic cells and this phosphorylation, was inhibited when the cells were pre-treated with 1-Butanol (Figure 3.8). This inhibitory effect was absent in cells pre-treated with t-Butanol, which indicates the specific role of PLD in this process (Figure 3.8). This suggests that TNFα-mediated ERK1/2 phosphorylation is indeed dependent on PLD in human monocytic cells.

Subsequently, investigation was done to evaluate the role of ERK1/2 in TNFα–induced PLD activity. To meet this objective PLD activity was measured in cells following TNFα stimulation and was compared to that of resting cells and cells pre-incubated with PD98059 (50 μM), a potent, selective and cell permeable inhibitor, which inhibits the phosphorylation of ERK1/2 (p44/42) by MAP Kinase Kinase (MEK). The TNFα-induced PLD activation was not affected by PD98059 pretreatment (Figure 3.9).

These results (Figure 3.8 and 3.9) suggest that PLD is upstream of ERK1/2 and PLD is essential in TNFα-triggered ERK1/2 activation cascade in human monocytic cell model.

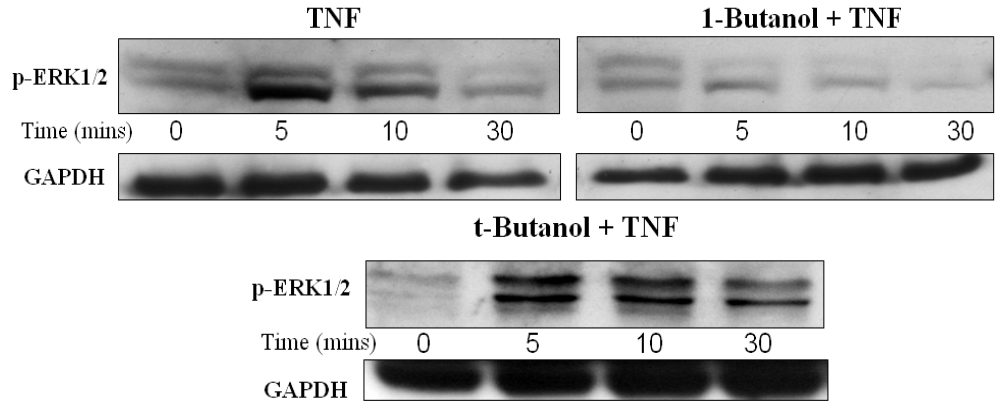


Figure 3.8: TNFα–triggered ERK1/2 phosphorylation is PLD dependent in human monocytic cells

Western blot analysis shows phosphorylation of ERK1/2 (p-ERK1/2) over time (0, 5, 10 and 30 mins) following TNFα (10 ng/ml) stimulation. The figure exhibits untreated-control cells following TNFα stimulation (TNF); cells pretreated with the 0.3% 1-Butanol prior to TNFα stimulation (1-Butanol + TNF) and cell pretreated with 0.3% t-Butanol prior to TNFα stimulation (t-Butanol + TNF). GAPDH was probed for loading control. Results shown are a representative of three separate experiments.

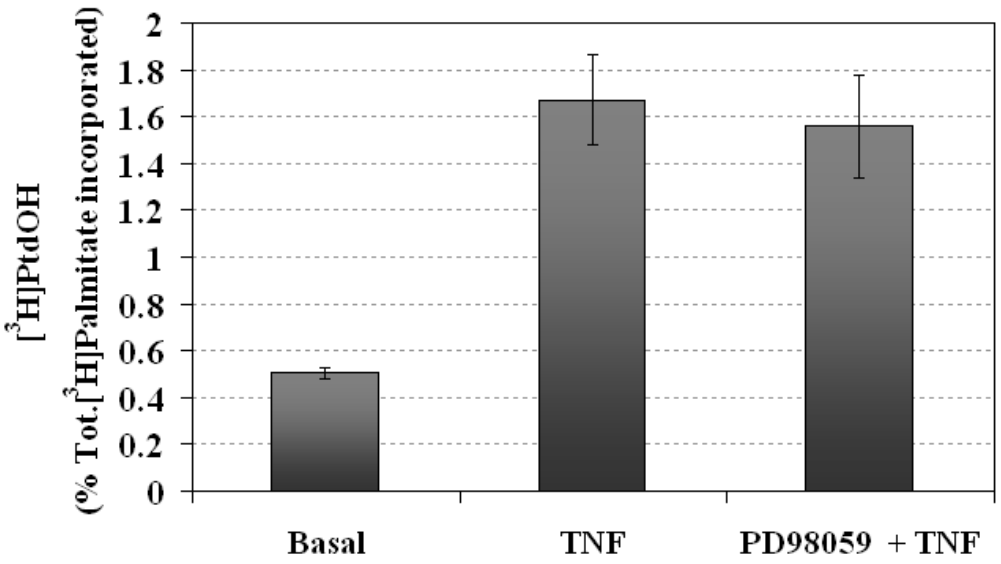


Figure 3.9: TNFα-triggered PLD activity is independent of ERK1/2 in human monocytic cells

Basal PLD activity in un-stimulated cells (Basal); PLD activity in cells stimulated with TNFα 10 ng/ml (TNF) and PLD activity in cells pre-incubated with MEK inhibitor, PD98059 at 50 μM prior to stimulation with TNFα 10 ng/ml (PD98059 + TNF). Results shown are the mean ± S.D. of triplicate measurements and are a representative of three separate experiments.

We also investigated Raf-1 translocation from the cytosol to the cell periphery on stimulation, an upstream event of agonist induced ERK1/2 phosphorylation. Fluorescence microscopic results show that in resting cells Raf-1 is primarily cytosolic, and after TNFα (10 ng/ml) stimulation Raf-1 has been found to translocate to the cell periphery (Figure 3.10). TNFα-induced translocation of Raf-1 was inhibited when the cells were pre-treated with 1-Butanol and not when the cells were pre-treated with t-Butanol (Figure 3.10). This indicates that TNFα–induced Raf-1 translocation is mediated by PLD as well. Therefore, we can suggest that PLD mediates TNFα-induced ERK1/2 activation cascade by influencing the translocation of Raf-1 in human monocytic cells**.**

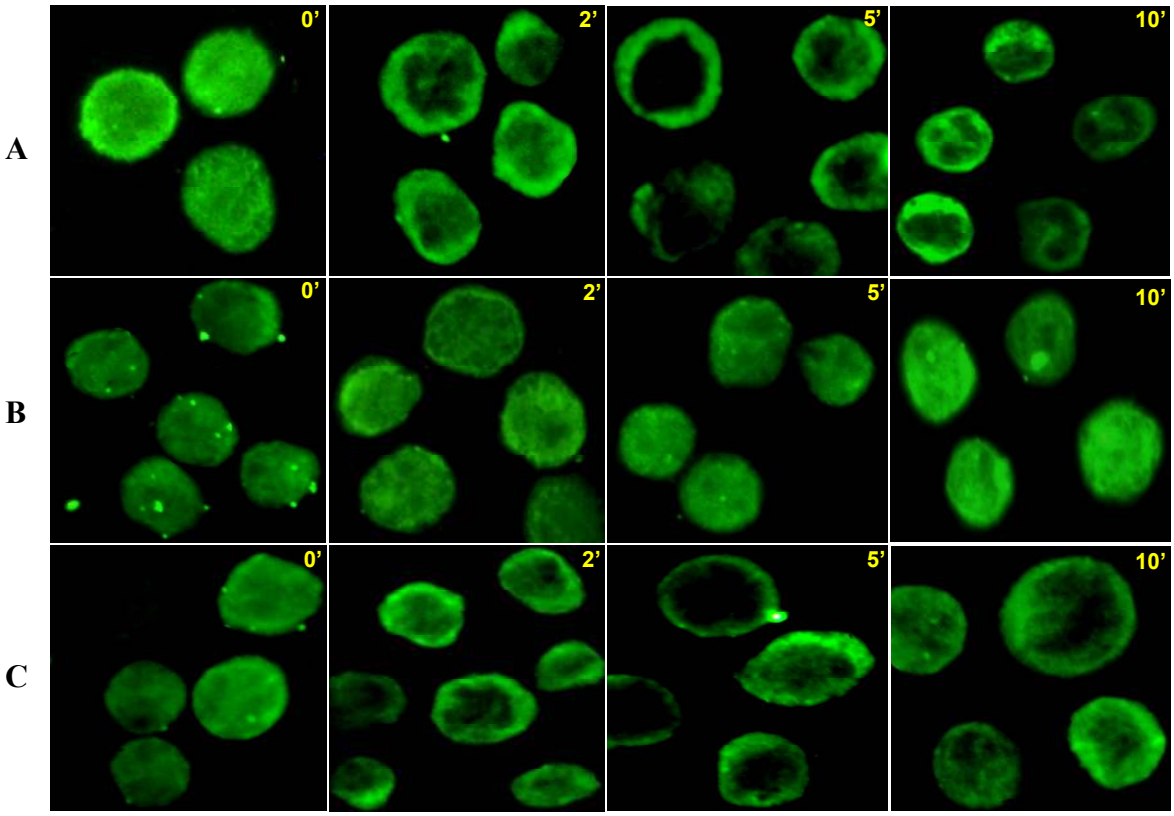


Figure 3.10: TNFα triggered Raf-1 translocation is PLD dependent in human monocytic cells

Fluorescence microscopic images of cells stimulated with TNFα 10 ng/ml and subsequently immune-stained for Raf-1 are exhibited. Untreated-control cells stimulated by TNFα are shown in row (A); cells pre-treated with 0.3% 1-Butanol prior to TNFα stimulation are shown in row (B) and cells pre-treated with 0.3% t-Butanol prior to TNFα stimulation are shown in row (C). The panels includes cells at various time points (0 minute or Basal; 2 minutes; 5 minutes and 10 minutes) after TNFα stimulation.

3.2.2.1.3 PLD and p38 kinase are independent of each other in TNFα–induced signaling

To evaluate the relevance of PLD to p38 kinase in TNFα-triggered signaling pathway, we looked at the phosphorylation of p38 induced by TNFα. It was found that TNFα activated p38 kinase in human monocytic cells and this phosphorylation of p38 kinase was not inhibited when the cells were incubated with 1-Butanol, prior to TNFα stimulation (Figure 3.11). This result indicates that TNFα-mediated phosphorylation of p38 is independent of PLD.

Since, TNFα-induced p38 kinase phosphorylation was independent of PLD; investigation was done to find out whether p38 kinase was necessary for TNFα–induced PLD activity. As a result, TNFα-induced PLD activity was measured in normal resting cells and in those cells pre-incubated for 20 minutes with SB203580 (10 μM), a potent and highly selective p38 kinase inhibitor. PLD activity showed a significant increase subsequent to 30 minute of TNFα stimulation over the basal and this activity was not affected or inhibited by SB203580 (Figure 3.12). Thus suggesting that, TNFα-induced PLD activity and p38 phosphorylation are independent of each other in human monocytic cells.

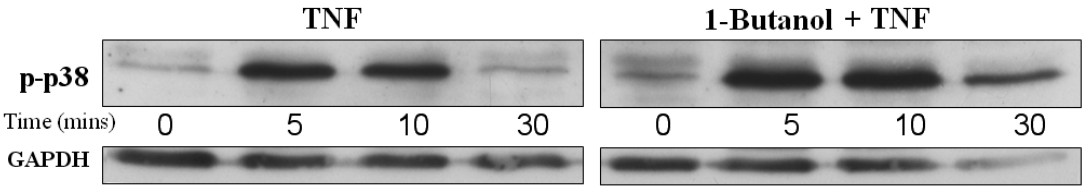


Figure 3.11: TNFα-triggered p38 phosphorylation is PLD independent
Western blot analysis shows phosphorylation of p38 kinase (p-p38) over time (0, 5, 10 and 30 mins) following TNFα (10 ng/ml) stimulation. The figure indicates untreated-control cells following TNFα stimulation (TNF) and cells pretreated with the 0.3% 1-Butanol prior to TNFα stimulation (1-Butanol + TNF). GAPDH was probed for loading control. Results shown are a representative of three separate experiments.

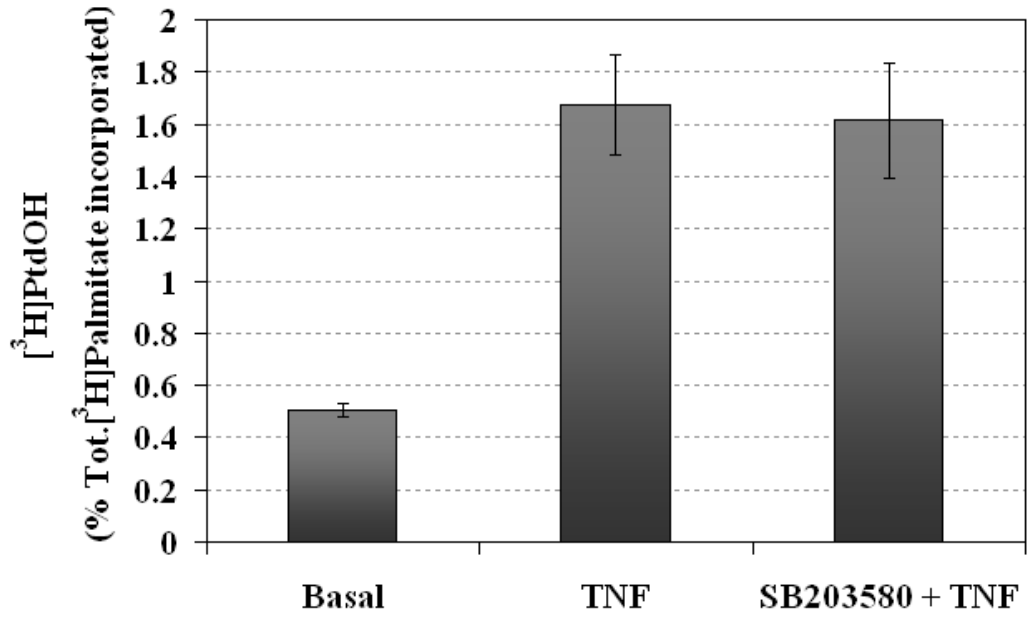


Figure 3.12: TNFα-triggered PLD activity is independent of p38
Basal PLD activity in un-stimulated cells (Basal); PLD activity in cells stimulated with TNFα 10 ng/ml (TNF) and PLD activity in cells pre-incubated with p38 inhibitor, SB203580 at 10μM prior to stimulation with TNFα 10 ng/ml (SB203580 + TNF). Results shown are the mean ± S.D. of triplicate measurements and are a representative of three separate experiments.

3.2.2.2 Role of PLD in TNFα-induced SphK activity

3.2.2.2.1 TNFα -triggered effector response is regulated by SphK.

We evaluated the functional relevance SphK in TNFα-induced effector response prior to the evaluation of the signaling hierarchy between PLD and SphK in TNFα-triggered signaling. In order to investigate this objective, we pre-treated the cells with SphK inhibitor – *N,N*-dimethylsphingosine (DMS), an analogue of sphingosine, before stimulating them with TNFα for 24 hours and quantified the release of IL-6 and IL-1β. It was observed that SphK inhibitor decreased the level of TNFα -induced IL-6 and IL-1β production (Figure 3.13). This indicates a role of SphK in TNFα-induced pro-inflammatory cytokine release in human monocytic cells.

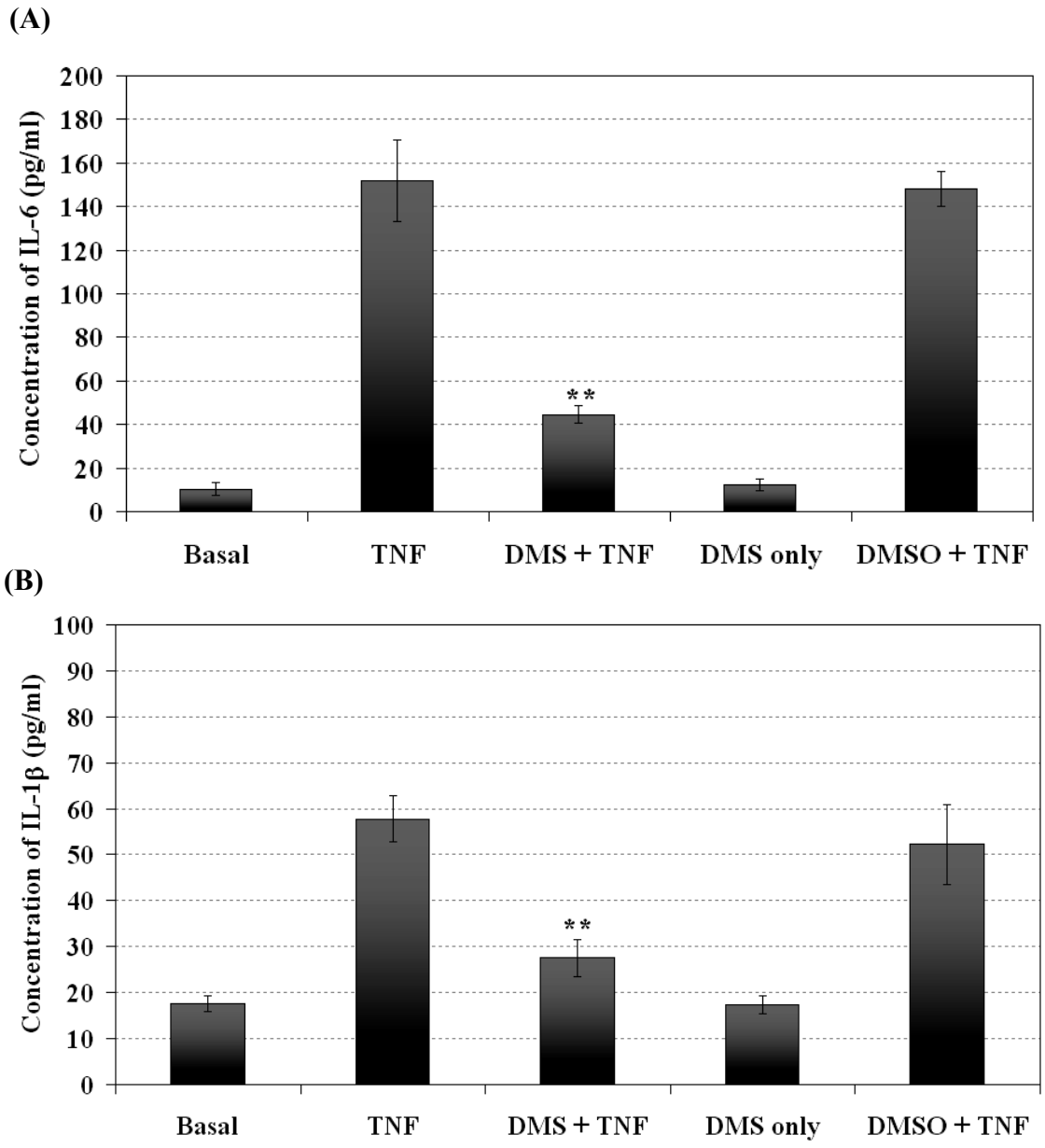


Figure 3.13: Cytokine production triggered by TNFα is dependent on SphK in human monocytic cells

The graph (A) indicates the IL-6 production and graph (B) indicates IL-1β production within 24 hours. Cytokine production in unstimulated cells (Basal), in cells stimulated with TNFα (10 ng/ml) only (TNF), in cells pretreated with 10 μM of DMS (SPHK inhibitor) prior to TNFα (10 ng/ml) stimulation (DMS + TNF), in cells pretreated with 10 μM of DMS only (DMS only) and in cells incubated with DMSO prior to TNFα stimulation (DMSO + TNF) as an added control. Results shown are the mean ± the standard deviation of triplicate measurements and are the representative of three separate experiments. (**) indicates Student's *t* test *p* value <0.01.

3.2.2.2.2 TNFα–induced SphK activity is downstream of PLD

To evaluate the significance of PLD and SphK in TNFα-triggered signaling, investigation was done to determine the role of SphK in TNFα-induced PLD activity. To evaluate this, PLD activity measured in cells following TNFα stimulation was compared to that of resting cells and cells pre-incubated with DMS, a potent SphK inhibitor for 20 minutes. The TNFα (30 minutes) induced PLD activation was not affected by DMS pre-treatment (Figure 3.14). This indicates that TNFα–induced PLD activity is not influenced by SphK

Experiments were then performed to determine whether PLD influence TNFα–stimulated SphK activity. TNFα was found to activate SphK in human monocytic cells and this activation was inhibited when the cells were pre-treated with 1-Butanol (Figure 3.15). This inhibitory effect on SphK activity was not observed in cells pre-treated with t-Butanol (Figure 3.15). These two findings clearly suggest that TNFα–mediated SphK activity is downstream of PLD in human monocytic cells.

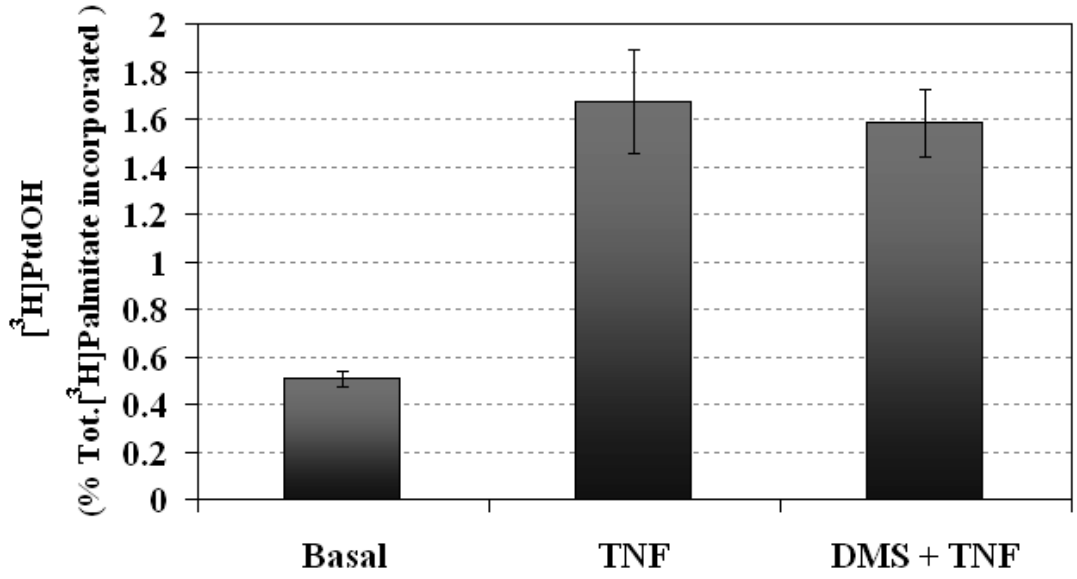


Figure 3.14: TNFα–triggered PLD activity is independent of SphK
Basal PLD activity in un-stimulated cells (Basal); PLD activity in cells stimulated with TNFα 10 ng/ml (TNF) and PLD activity in cells pre-incubated with SphK inhibitor, DMS at 10 μM prior to stimulation with TNFα 10 ng/ml (DMS + TNF). Results shown are the mean ± S.D. of triplicate measurements and are a representative of three separate experiments.

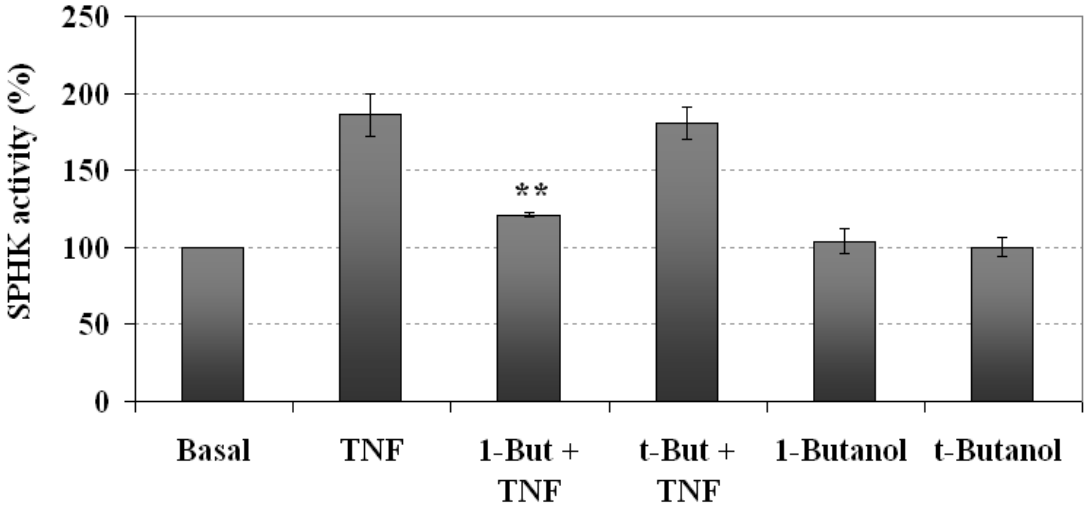


Figure 3.15: TNFα–triggered SphK activity is dependent on PLD
Basal SphK activity in un-stimulated cells (Basal); SphK activity in cells stimulated with TNFα 10 ng/ml (TNF); SphK activity in cells pre-incubated with 0.3% 1-Butanol prior to stimulation with TNFα 10 ng/ml (1-But + TNF); SphK activity in cells pre-incubated with 0.3% t-Butanol prior to stimulation with TNFα 10 ng/ml (t-But + TNF); SphK activity in cells treated with 0.3% 1-Butanol and 0.3% t-Butanol only (1-Butanol and t-Butanol respectively). Results shown are the mean ± S.D. of triplicate measurements and are a representative of three separate experiments. (∗ ∗) indicates Student's *t* test *p* value ≤0.01.

3.2.2.3 Role of PLD in TNFα-induced NFκB activation

NFκB (Nuclear Factor kappa B) is one of the important transcription factors that contributes in the amplification of inflammatory responses by being responsible for the production of pro-inflammatory mediators and also signals for cell survival. NFκB is responsible for the transcription of inflammatory genes which includes that of cytokines, chemokines, cell adhesion molecules and other immune response mediators. Therefore, NFκB has been associated with the pathogenesis of inflammatory disorders (Yamamoto and Gaynor, 2001). In the following set of experiments, we have investigated the association of PLD and NFκB in TNFα-triggered inflammatory signaling and responses.

3.2.2.3.1 TNFα -induced inflammatory response is mediated by NFκB

The direct role of NFκB in TNFα-triggered inflammatory response like pro-inflammatory cytokine (IL-6 and IL-1β) production in human monocytic cells was investigated by the use of an NFκB inhibitor (Ro106-9920). This inhibitor is known to interfere with NFκB activity by inhibiting IκBα ubiquitination by E3 ligase, thereby preventing IκBα degradation and thus preventing NFκB activation (Swinney et al., 2002). Results indicate that TNFα-induced IL-6 and IL-1β production was found to be inhibited in cells pre-treated with Ro106-9920 (Figure 3.16). This suggests the direct involvement of NFκB in TNFα-mediated inflammatory response.

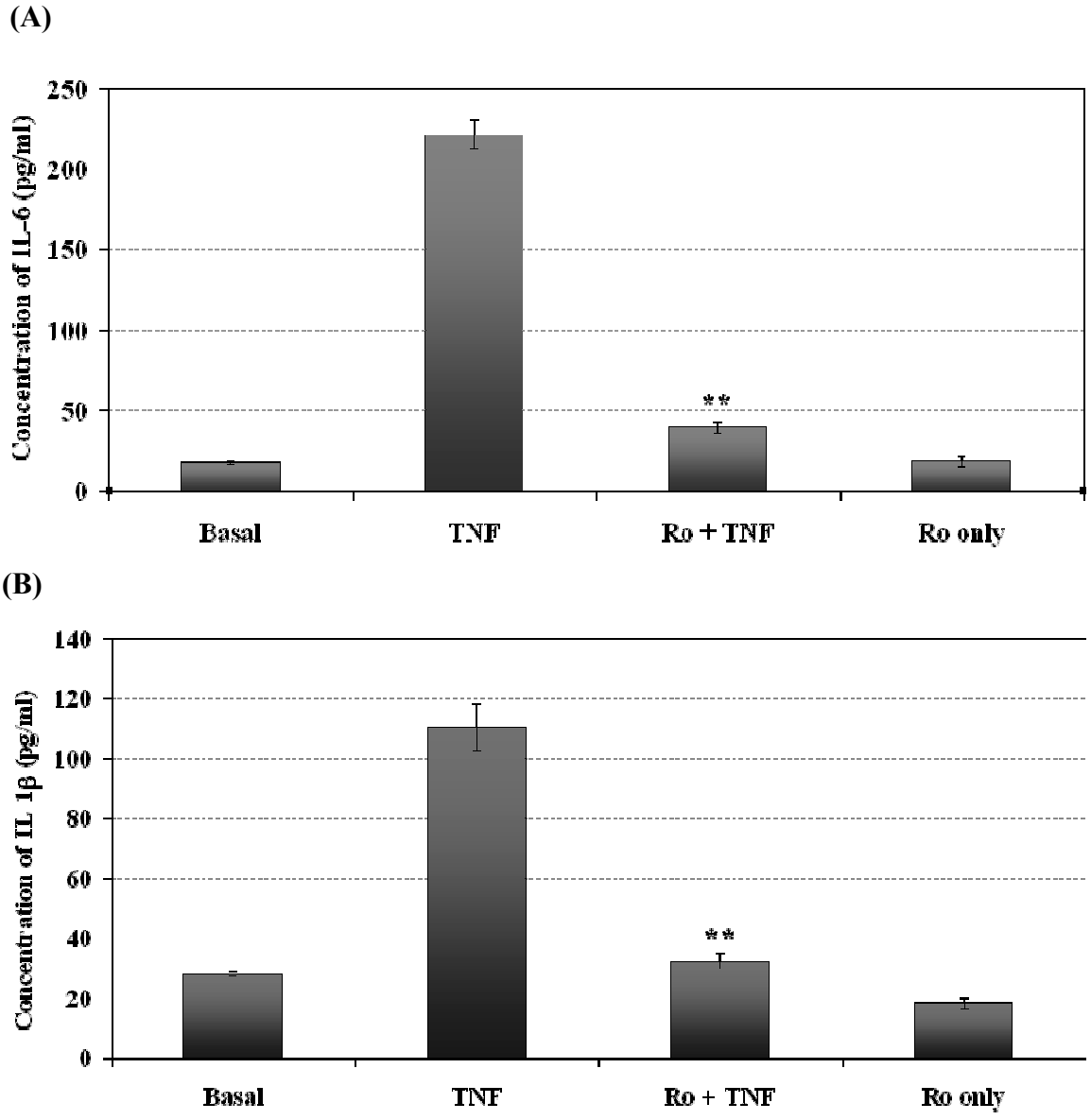


Figure 3.16: Cytokine production triggered by TNFα is dependent on NFκB in human monocytic cells

The graph (A) indicates the IL-6 production and graph (B) indicates IL-1β production within 24 hours. Cytokine production in unstimulated cells (Basal), in cells stimulated with TNFα (10 ng/ml) only (TNF), in cells pretreated with 10 μM of Ro106-9920 (NFκB inhibitor) prior to TNFα (10 ng/ml) stimulation (Ro + TNF) and in cells pretreated with 10 μM of Ro106-9920 only (Ro only) Results shown are the mean ± the standard deviation of triplicate measurements and are the representative of three separate experiments. (∗∗) indicates Student's *t* test *p* value <0.01.

3.2.2.3.2 TNFα–triggered NFκB activity in human monocytic cells requires PLD

Since, our earlier experiments have shown that TNFα–induced responses were also influenced by PLD, we investigated whether PLD influenced TNFα–induced NFκB activity. We evaluated or quantified TNFα-triggered NFκB activity by measuring the amount of p65 subunits of NFκB in the nuclear fraction after stimulation. p65 subunit is primarily cytsolic in unstimulated cells and on activation, it translocates into the nucleus to bring about its transcription function by binding to DNA. An ELISA based assay was done to quantify the amount of p65 subunits in the nuclear fraction. Based on the current results, there was an increase in the amount of p65 subunits in untreated cells stimulated by TNFα (Figure 3.17). This increase in p65 levels in the nucleus was inhibited when the cells were pre-treated with 1-Butanol and not t-Butanol prior to TNFα stimulation (Figure 3.17). This strongly suggests a role of PLD in TNFα–induced NFκB activation in monocytic cells.

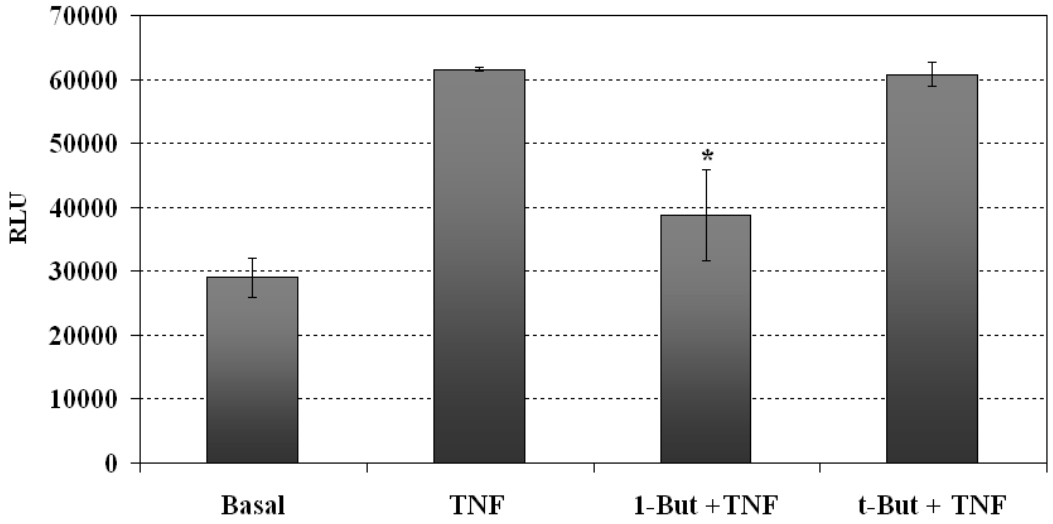


Figure 3.17: TNFα-triggered NFκB activity is dependent on PLD in human monocytic cells

NFκB activity was measured based on the presence of p65 subunit of the NFκB heterodimer in the nuclear fraction, 30 minutes after TNFα 10 ng/ml stimulation. Activity in control resting cells (Basal); activity in control cells after TNFα stimulation (TNF); activity in cells pretreated with 0.3% 1-Butanol prior to TNFα stimulation (1-But + TNF) and activity in cells pretreated with 0.3% t-Butanol prior to TNFα stimulation (t-But + TNF). Results shown are the mean ± S.D of triplicate measurements. (*) indicates Student's *t* test *p* value <0.05.

Experiments were conducted further to investigate as to how PLD prevented the translocation of p65 subunit into the nucleus. It is well known that IκBα degradation is a crucial event in the process of NFκB activation, as the latter's degradation is essential for the release of p50 and p65 subunits of NFκB from the inhibitory complex in the cytosol and facilitate its translocation into the nucleus to bring about its transcriptional activity (Ghosh and Karin, 2002; Moynagh, 2005). Therefore, we looked into the IκBα degradation pattern induced by TNFα and the role of PLD in this process. We have shown by western blotting procedure that in untreated cells, TNFα induces IκBα degradation (Figure 3.18). TNFα-triggered IκBα degradation was either inhibited or delayed in cells pre-treated with 1-Butanol prior to TNFα stimulation (Figure 3.18), thus explaining the reduction in the p65 subunit levels in the nucleus. However, this inhibition of IκBα degradation was absent in cells pre-treated with t-Butanol, indicating the role of PLD in TNFα–induced IκBα degradation or NFκB activation in human monocytic cells. These results suggest that NFκB is downstream of PLD and the latter is required for TNFα-induced NFκB activation and associated responses. Ro106-9920 selectively inhibits essential ubiquitination activity associated TNFα-induced IκBα degradation. This prevents the translocation of both the p65 & p50 subunits into the nucleus which essential for the activation of the subunits. However, only the levels of p65 subunits in the nucleus were investigated as an indicator of TNFα induced NFκB activity.

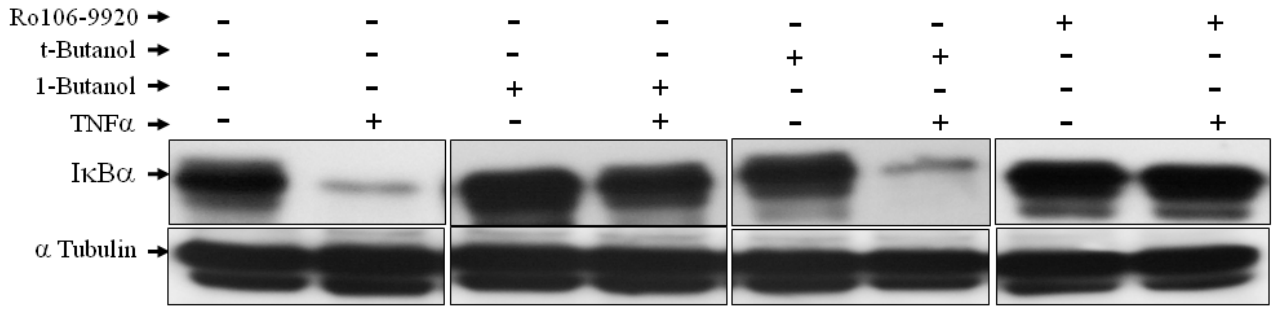


Figure 3.18: TNFα–triggered IκBα degradation is influenced by PLD
Western blot analysis shows degradation of IκBα in 20 minutes following TNFα (10 ng/ml) stimulation. The figure indicates untreated-control cells following TNFα stimulation; cells pretreated with the 0.3% 1-Butanol prior to TNFα stimulation; cells pretreated with the 0.3% t-Butanol and cell pretreated with Ro106-9920 prior to TNFα stimulation. α Tubulin was probed for loading control. Results shown are a representative of three separate experiments.

Similarly, studies were done to evaluate whether PLD dependent ERK1/2 or PLD independent p38 kinase influences TNFα–induced NFκB activation. Since it has been reported that members of the MAPK family can regulate the activation of NFκB by phosphorylating the IκB alpha kinase complex (Lee, 1997; Tergaonkar, 2006). An ELISA based experiment described earlier was used to quantify the levels of p65 in the nuclear fraction after following TNFα stimulation. Cells incubated with MEK inhibitor and p38 inhibitor failed to exhibit a decrease in p65 levels in the nuclear fraction after TNFα stimulation (Figure 3.19). Western blot analysis has shown that these above mentioned inhibitors also failed to alter or affect the IκBα degradation patterns after TNFα stimulation (Figure 3.20). These set of results indicates that TNFα–induced NFκB activation was not influenced by ERK1/2 and p38 kinase in human monocytic cells.

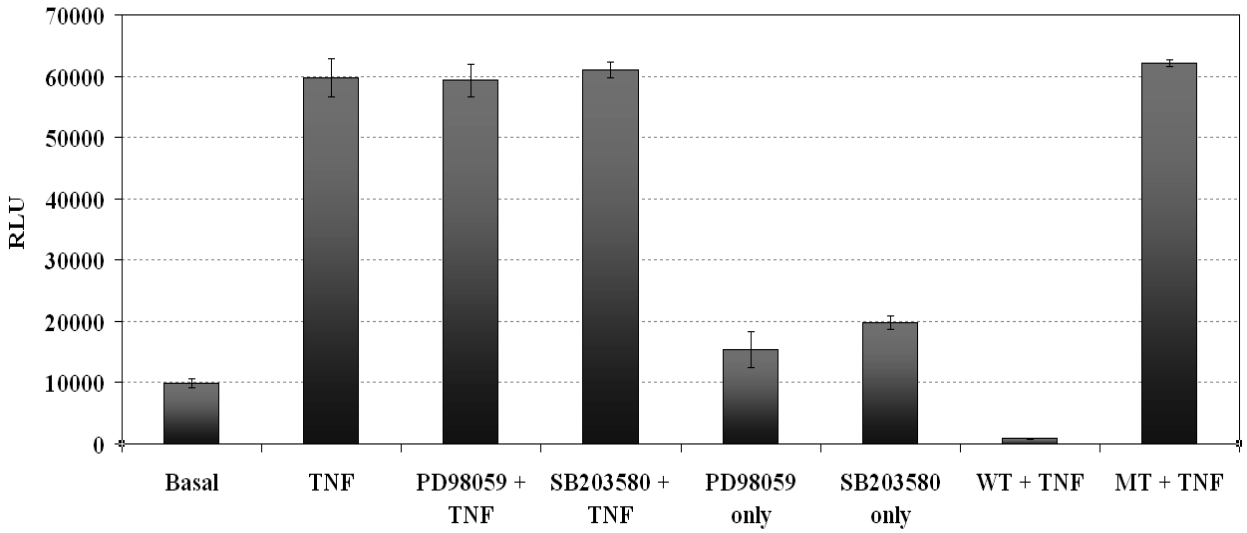


Figure 3.19: TNFα–triggered NFκB activity is not dependent on ERK1/2 and p38 kinase in human monocytic cells

NFκB activity was measured based on the presence of p65 subunit of the NFκB heterodimer in the nuclear fraction, 30 minutes after TNFα 10 ng/ml stimulation. Activity in control resting cells (Basal); activity in control cells after TNFα stimulation (TNF); activity in cells pretreated with the 50 μM of PD98059 (MEK inhibitor) prior to TNFα stimulation (PD98059 + TNF) and activity in cells pretreated with the 10 μM of SB203580 (p38 kinase inhibitor) prior to TNFα stimulation (SB203580 + TNF). Wild type duplex (WT + TNF) and mutant duplex (MT + TNF) in cells stimulated with TNFα was used added controls to prove specificity. Wild Type and Mutant NFκB Competitor Duplex is provided along with the detection system is to ensure signal specificity. Adding the Wild Type NFκB Competitor Duplex in the reaction prior to the addition of the sample extracts will prevent NFκB subunit in the extracts from binding to the sequence attached to the plate and thus eliminating specific signals. However, if a Mutant NFκB Competitor Duplex was added to the reaction mixture instead, it will not affect specific NFκB binding, thus exhibiting the specific signal. Results shown are the mean ± S.D of triplicate measurements.

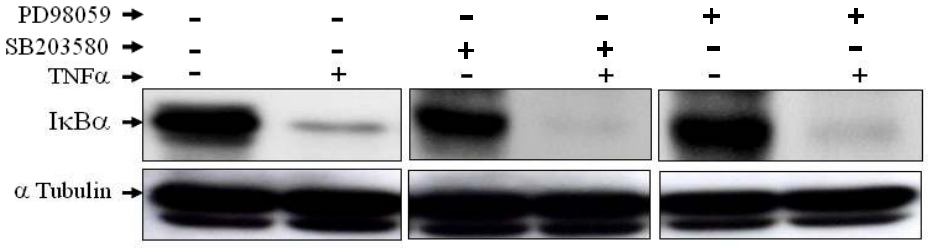


Figure 3.20: TNFα-triggered IκBα degradation is not dependent on ERK1/2 and p38 kinase in human monocytic cells

Western blot analysis shows degradation of IκBα in 20 minutes following TNFα (10 ng/ml) stimulation. The figure indicates untreated-control cells following TNFα stimulation; cells pretreated with the 10 μM of SB203580 (p38 kinase inhibitor) prior to TNFα stimulation and cells pretreated with the 50 μM of PD98059 (MEK inhibitor) prior to TNFα stimulation. α Tubulin was probed for loading control. Results shown are a representative of three separate experiments.

MTT assay was done to investigate the cell viability after incubating the cells with the various inhibitors used in the study mentioned in this section. This is to ensure that the inhibitory effects are not due to cell death / decrease proliferation and are indeed due to the inhibition of activity of the specific proteins of interest. Measurements at the end of the time point failed to show any significant cell death or changes in the number of viable cells following incubation with the different inhibitors used earlier (Figure 3.21).

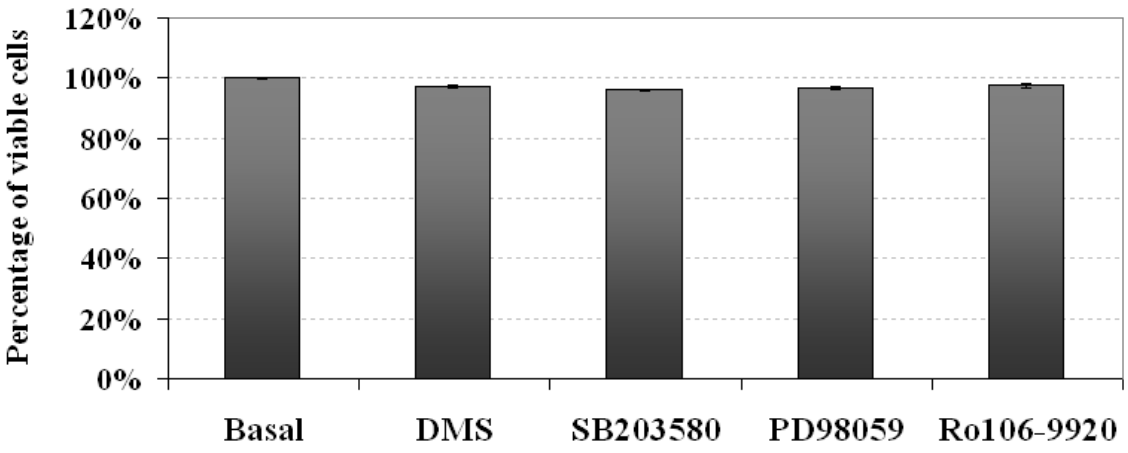


Figure 3.21: Percentage of cell viability in human monocytic cells after 24 hours incubation with various inhibitors used

The graphs shows the percentage of viable untreated cells (Basal); cells incubated with 10 μM of Sphingosine kinase inhibitor, DMS (DMS); cells incubated with 10 μM of SB203580, p38 kinase inhibitor (SB203580); cells incubated with 50 μM of PD98059, MEK inhibitor (PD98059) and cells incubated with 10 μM of Ro106-9920, NFκB inhibitor (Ro106-9920). Results are the mean and ± SD of triplicate measurements.

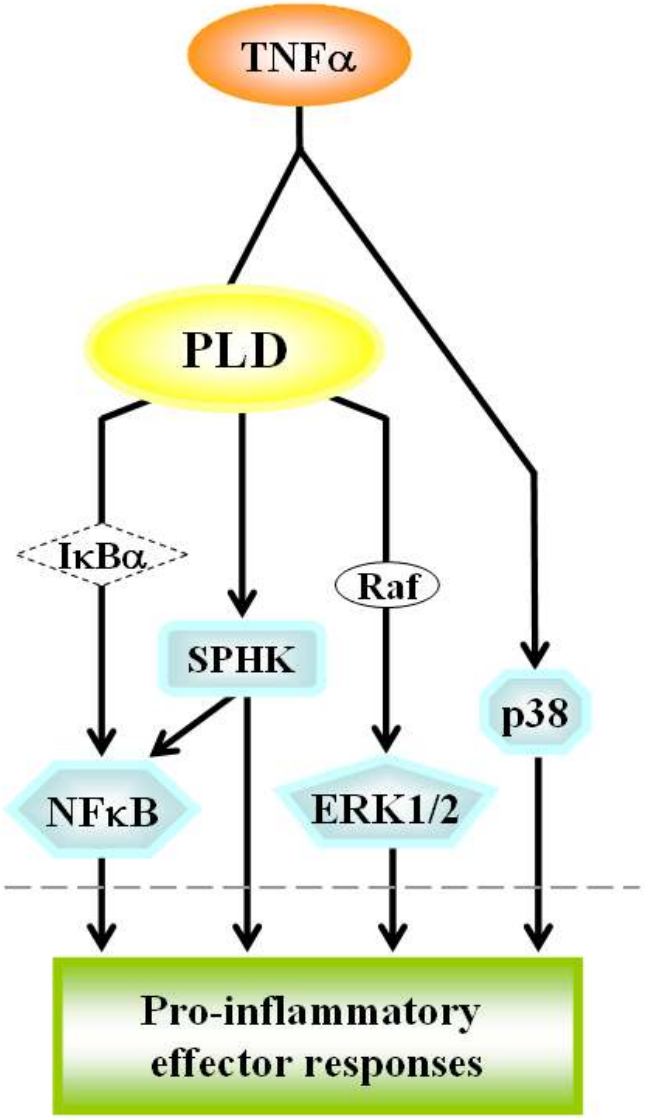


Figure 3.22: Schematic representation of the role of PLD in TNFα-induced intracellular signaling events and responses

3.2.3 Isoform specific function of PLD1 in TNFα–induced signaling and responses

3.2.3.1 TNFα induces sub cellular re-localization of PLD1 in human monocytic cells

PLD isoforms (PLD1 and PLD2) were found to be expressed in the human monocytic cells used in this study (Melendez et al., 2001). Therefore, we investigated the localization of these two isoforms and whether TNFα stimulated their re-localization, as a means to start dissecting the specific isoform(s) activated by this cytokine. Fluorescence microscopy derived results, in this study, reveals that in resting cells both PLD isoforms have a general cytosolic localization and that TNFα induces the re-localization of PLD1, but not PLD2, to the cells' periphery (Figure 3.23). This would suggest that the PLD isoform activated by TNFα is potentially, PLD1.

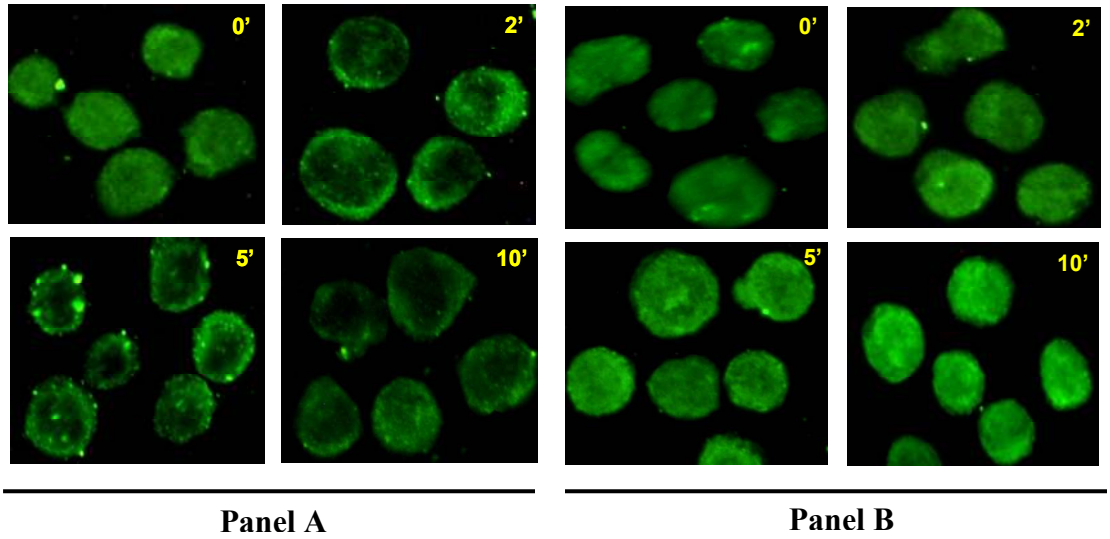


Figure 3.23: PLD1 re-localization subsequent to TNFα stimulation in human monocytic cells
Fluorescence microscopic images of cells stimulated with TNFα 10 ng/ml and subsequently immune-stained for PLD1 and PLD2 are shown in Panel A and Panel B respectively. The panels includes cells at various time points (0 minute or Basal; 2 minutes; 5 minutes and 10 minutes) after TNFα stimulation.

3.2.3.2 Specific knockdown of PLD isoforms using antisense oligonucleotides

Since it is known that U937 cells express both the PLD isoforms (PLD1 and PLD2), experiments were performed to identify the PLD isoform which specifically plays a role in TNFα induced signaling and responses in human monocytic cells. To address this, the expression of the PLD isoforms were specifically knocked down using specific antisense oligonucleotides designed against each of the PLD isoforms (i.e. antisense to PLD1 and antisense to PLD2). It has been previously shown that U937 cells are sensitive to antisense manipulation and the latter can be used to study the isoform specific role of PLD (Melendez et al., 2001).

IFN-γ primed cells were treated with antisense oligonucleotides specific against the two PLD isoforms. The knock down specificity of the antisense oligonucleotides on the respective PLD isozymes expression was analyzed by western blot analysis of immunoprecipates of PLD1 and PLD2 cell lysates (Figure 3.24). Cells treated with antisense against PLD1 isoform showed a reduction in PLD1 immunoreactivity or protein expression, without affecting PLD2 expression. Likewise, in cells treated with antisense to PLD2, there was a reduction in PLD2 expression without affecting the expression of PLD1. This indicates the isoform specific knock down of antisense oligonucleotides.

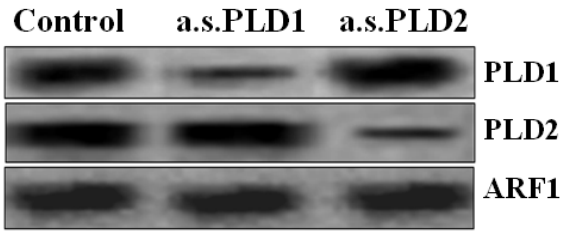


Figure 3.24: Specific knockdown of PLD isoforms (PLD1 and PLD2) using antisense oligonucleotides in human monocytic cells
Western blot analysis of immunoprecipitates of either PLD1 or PLD2 to assess expression of either isozymes in human monocytic cells, following 36 hours treatment with antisense oligonucleotides (10 μM) specific for either PLD1 (a.s.PLD1) or PLD2 (a.s.PLD2) and untreated cells severed as controls (Control). ARF1 (ADP-ribosylation factor 1) was used as loading control and the samples were obtained from whole cell lysates. The results shown are typical from three separate experiments.

3.2.3.3 TNFα-stimulated PLD activity is coupled to PLD1 isoform

Unstimulated cells treated with antisense oligonucleotides were assayed for PLD activity to measure basal levels of activity and it was found that treatment of cells with the antisense oligonucleotide to PLD1 resulted in no change in basal PLD activity. However, following TNFα stimulation, the increase in PLD activity was significantly reduced compared to the control cells (Figure 3.25). In contrast, treatment of cells with the antisense oligonucleotide to PLD2 slightly reduced basal PLD activity, but TNFα induced PLD activity was only marginally reduced in cells treated with the antisense to PLD2 (Figure 3.25). This reduction was accounted for by the reduction in basal levels. Therefore, this experiment demonstrates that, at least in the present system, TNFα-stimulated PLD activity is specifically contributed by PLD1 isoform.

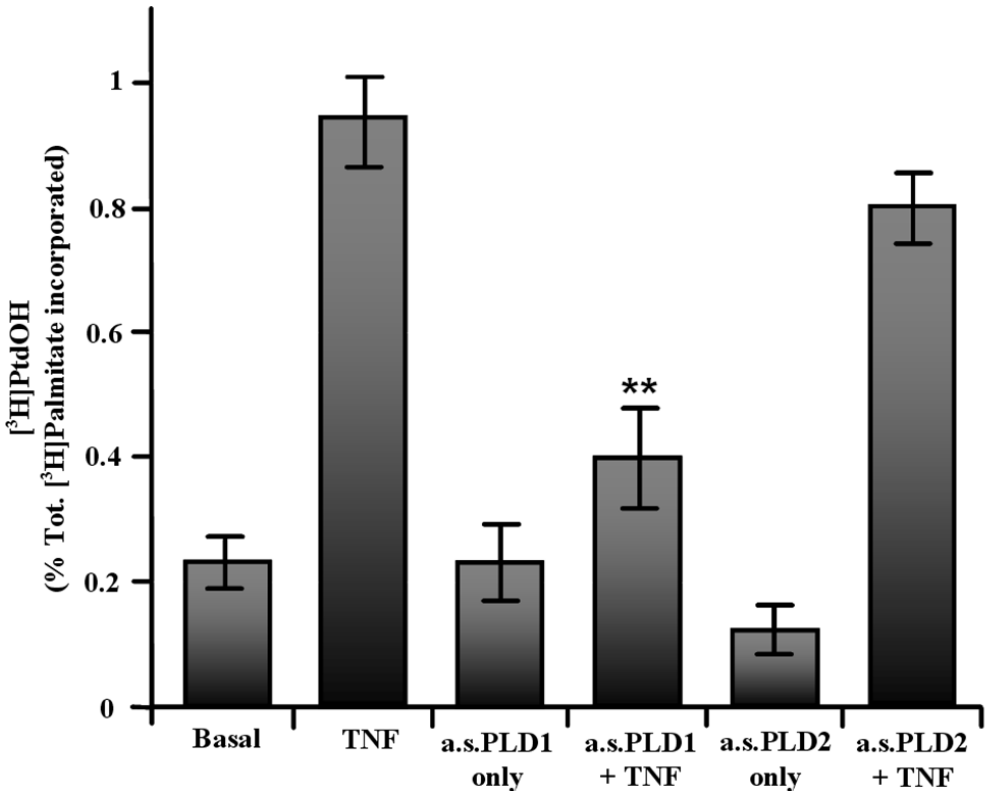


Figure 3.25: PLD1 isoform was found to be coupled with TNFα signaling
PLD activity following TNFα (10 ng/ml) stimulation in cells pretreated with 10 μM antisense oligonucleotides for either PLD1 (a.s.PLD1) or PLD2 (a.s.PLD2). PLD activity in untreated cells (Basal); in cells stimulated with TNFα (TNF); in cells pretreated with a.s.PLD1 only (a.s.PLD1 only); in cells pretreated with a.s.PLD1 prior to TNFα stimulation (a.s.PLD1 + TNF); in cells pretreated with a.s.PLD2 only(a.s.PLD2 only) and in cells pretreated with a.s.PLD2 prior to TNFα stimulation a.s.PLD2 + TNF). Results are the mean ± S. D. of triplicate measurements from three separate experiments. (**) indicates Student's *t* test *p* value <0.01.

3.2.3.4 TNFα-triggered intracellular signaling events is coupled to PLD1

TNFα–induced intracellular signaling events like ERK1/2 phosphorylation, Sphingosine kinase activity, calcium release from intracellular stores and transcription factor – NFκB activation in human monocytic cells were dependent on PLD1 isoform.

TNFα-triggered ERK1/2 phosphorylation is coupled to PLD1, but TNFα induced p38 phosphorylation is independent of PLD activity

TNFα coupled to the activation of MAPKs have important functional consequences for several functional cellular responses, including in the generation of proinflammatory cytokines. This study demonstrates a critical role for PLD1 in mediating the TNFα-induced phosphorylation of ERK1/2. Treatment of cells with the antisense oligonucleotide to PLD1 resulted in the inhibition of the phosphorylation levels of ERK1/2 triggered by TNFα (Figure 3.26). In contrast, treatment of cells with the antisense oligonucleotide to PLD2 did not alter TNFα-induced ERK1/2 phosphorylation, compared to control cells (Figure 3.26).

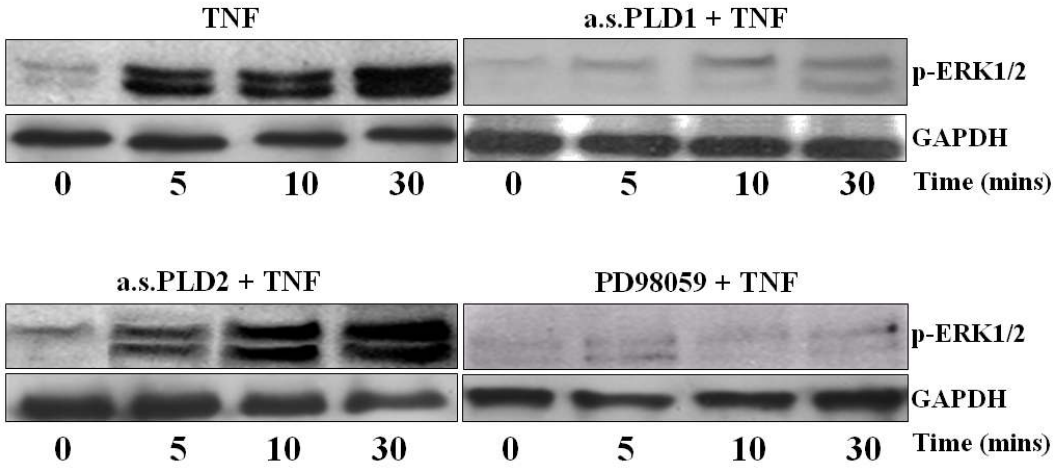


Figure 3.26: TNFα–triggered ERK1/2 phosphorylation is PLD1 dependent in human monocytic cells

Western blot analysis shows TNFα (10 ng/ml) triggered ERK1/2 phosphorylation over the time points indicated. Categories include TNFα induced phosphorylation status of ERK1/2 in untreated cells (TNF); in cells pretreated with the PLD1 antisense prior to TNF stimulation (a.s.PLD1 + TNF); in cell pretreated with the PLD2 antisense prior to TNF stimulation (a.s.PLD2 + TNF) and in cells pretreated with the MEK inhibitor, PD98059 at 50 μM (PD98059 + TNF). Results shown are typical of three separate experiments. GAPDH was probed for loading control.

To evaluate the relevance of PLD1 in p38 kinase-activity in the TNFα-triggered signaling pathway, we looked at the phosphorylation of p38 induced by TNFα. We found that TNFα did indeed trigger p38 phosphorylation, in the human monocytic cells. However, in contrast to ERK1/2 phosphorylation, the phosphorylation of p38 was not inhibited when the cells were pretreated with antisense oligonucleotide to PLD1 (Figure 3.27). Similarly, pretreatment of cells with the antisense oligonucleotide to PLD2 did not alter the TNFα-induced p38 phosphorylation as well (Figure 3.27).

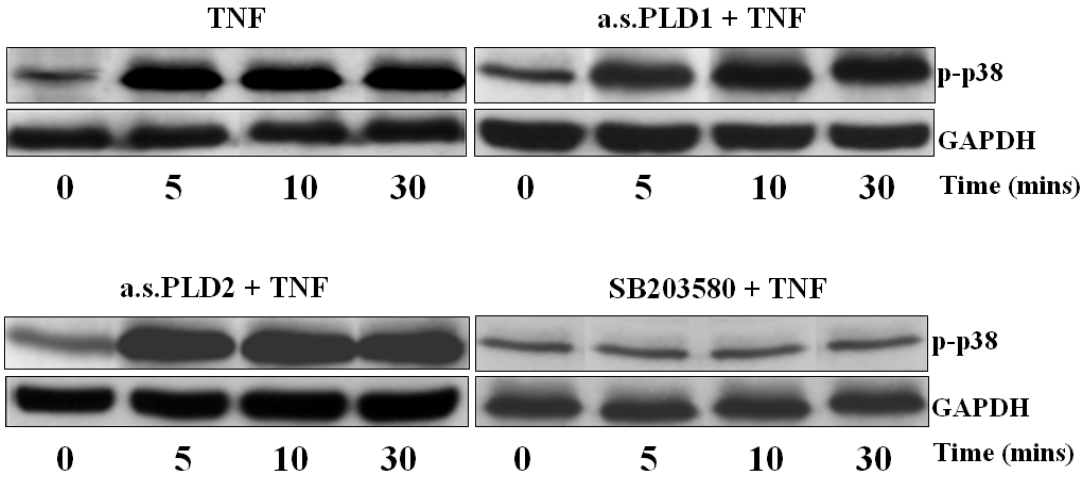


Figure 3.27: TNFα-triggered p38 phosphorylation is not dependent on PLD1 and PLD2 in human monocytic cells
Western blot analysis shows TNFα (10 ng/ml) triggered p38 kinase phosphorylation over the time points indicated. Categories include TNFα–induced phosphorylation status of p38 in untreated cells (TNF); in cells pretreated with the PLD1 antisense prior to TNF stimulation (a.s.PLD1 + TNF); in cell pretreated with the PLD2 antisense prior to TNF stimulation (a.s.PLD2 + TNF) and in cells pretreated with the p38 kinase inhibitor, SB203580 at 10 μM, (SB203580 + TNF). Results shown are typical of three separate experiments. GAPDH was probed for loading control.

Results in the earlier section (Figure 3.9 and 3.12) have clearly shown that TNFα-induced PLD activity is not mediated by ERK1/2 and p38 kinase. Together, with the results seen in this section (Figure 3.26 and 3.27), we can suggest that PLD1 mediates TNFα-induced ERK1/2 activation, but TNFα-mediated p38 phosphorylation is independent of both PLD1 and PLD2.

TNFα-induced sphingosine kinase activity; cytosolic calcium release and NFκB activation are dependent on PLD1.

SphK is known to be an important signaling event which is necessary for signal transduction and various effector responses. Intracellular or cytosolic calcium release has also been a key regulator in intracellular signaling and responses. Here, we have shown that SphK activity, cytosolic calcium responses and NFκB activity triggered by TNFα depend on PLD1.

Pretreating cells with the antisense oligonucleotide to PLD1 to knock-down this specific isozyme/isoform expression significantly reduced the activation of sphingosine kinase, following TNFα stimulation. Reduction in expression of PLD2 isozyme had no effect on the ability of TNFα to activate the enzymatic activity of sphingosine kinase (Figure 3.28).

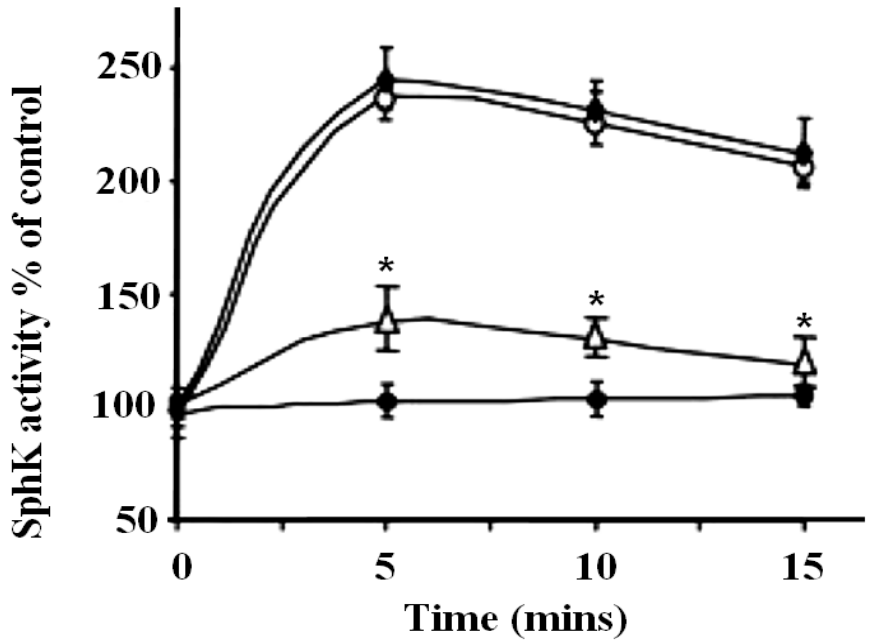


Figure 3.28: TNFα-induced sphingosine kinase activity is dependent on PLD1
Sphingosine kinase activity following TNFα (10 ng/ml) stimulation is shown. Cells were harvested at given time points indicated in the figure to measure sphingosine kinase activity. Sphingosine kinase activity, in untreated control cells (● – Basal); in untreated cells following TNFα stimulation (○ – TNF) and in cells pretreated with antisense oligonucleotides (10 μM) for either PLD1 (Δ – a.s.PLD1 + TNF) or PLD2 (♦ – a.s.PLD2 + TNF) prior to TNFα stimulation. Results are the mean ± S.D of triplicate measurements and from three separate experiments. (*) indicates Student's *t* test *p* value <0.01.

Similarly, reduction in expression of PLD1 by pre-treatment of cells with antisense PLD1 oligonucleotide resulted in the attenuation of the cytosolic calcium response following TNFα stimulation (Figure 3.29). Reducing expression of PLD2 had no effect on the calcium transients compared to that of the TNFα control.

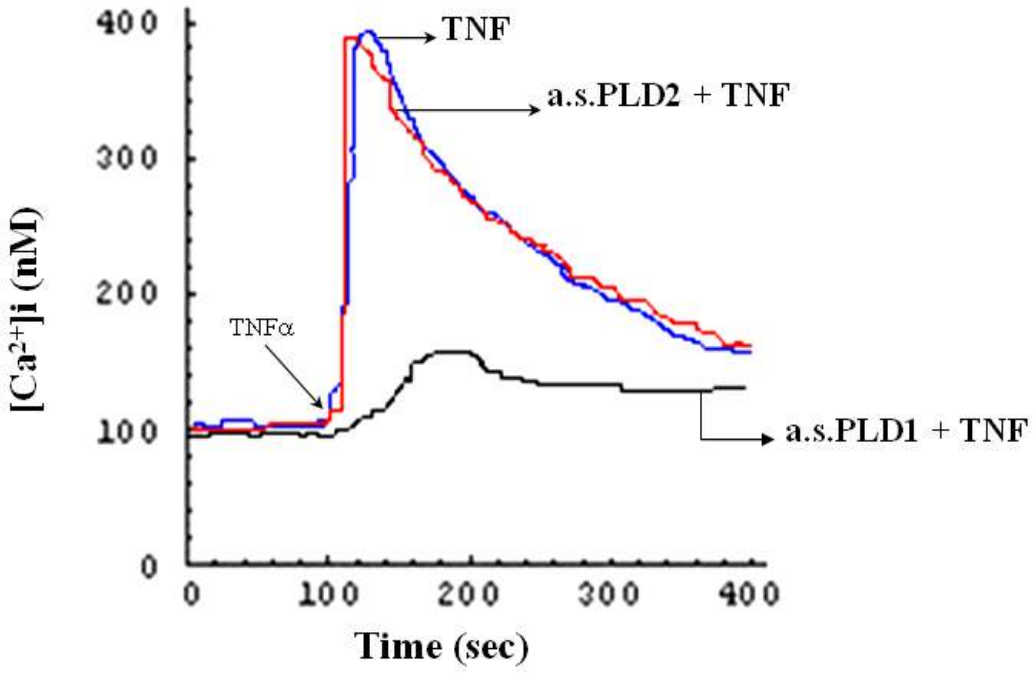


Figure 3.29: Intracellular cytosolic calcium changes following TNFα stimulation is PLD1 dependent
Intracellular cytosolic calcium responses after TNFα stimulation were compared in control cells and cells pretreated with antisense oligonucleotides (10 μM) to either PLD1 or PLD2. Traces shown are: **blue**, control cells treated with TNFα 10 ng/ml (TNF); **black,** cells pretreated with antisense to PLD1 (a.s.PLD1 + TNF) and **red,** cells pretreated with antisense to PLD2 (a.s.PLD2 + TNF). The arrow marks the addition of TNFα. Traces are typical from three separate experiments.

NFκB is one of the key transcription factors that trigger the generation of many proinflammatory mediators and aid in the onset and propagation of inflammatory response. It is shown here that the TNFα triggered activation of NFκB in human monocytes is mediated by PLD1. This is substantiated by the reduction in the translocation of both p50 and p65 subunits from the cytoplasm into the nucleus subsequent to TNFα stimulation in cells pretreated with the antisense oligonucleotide against PLD1, whereas pretreatment with a PLD2 antisense oligonucleotide had no such observed effect on NFκB subunits after TNFα stimulation in human monocytic cells (Figure 3.30).

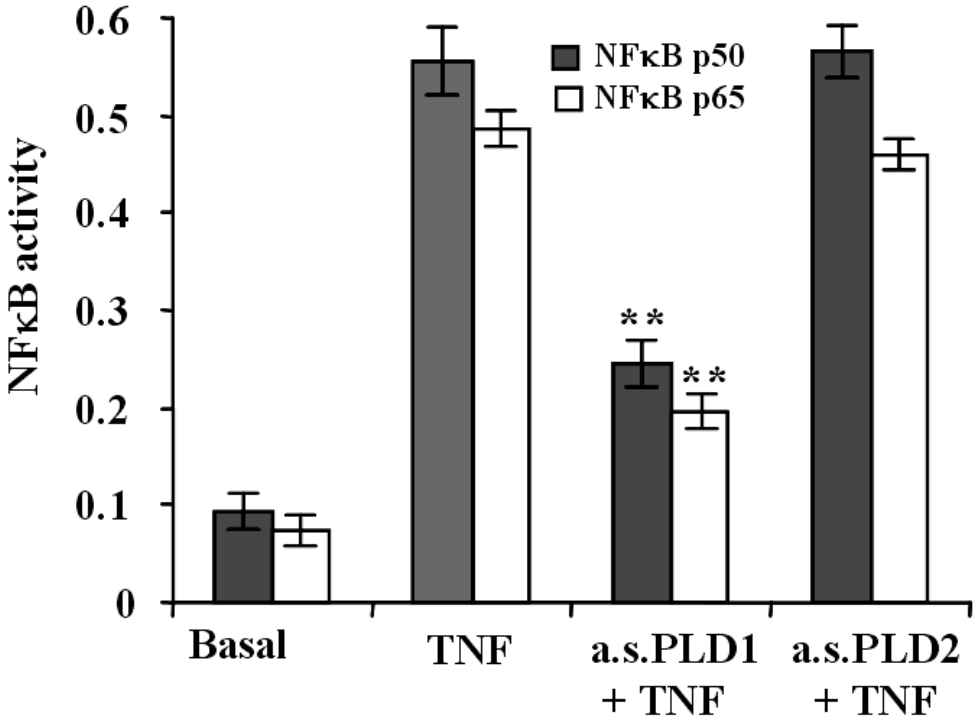


Figure 3.30: PLD1 mediates TNFα–induced NFκB activity in human monocytic cells
Activities of p50 and p65 subunits of NFκB following TNFα 10 ng/ml stimulation for 30 minutes were measured. Activities in normal untreated resting cells (Basal); in untreated cells following TNFα stimulation (TNF); in cells pretreated with antisense for PLD1 prior to TNFα stimulation (a.s.PLD1 + TNF) and in cells pretreated with antisense for PLD2 prior to TNFα stimulation (a.s.PLD2 + TNF). Results shown are the mean ± S.D. of triplicate measurements. (**) indicates Student's *t* test *p* value <0.01.

3.2.3.5 PLD1 is required for TNFα-triggered inflammatory responses like proinflammatory cytokine generation

TNFα is capable of amplifying the inflammatory response by promoting the generation and release of several proinflammatory cytokines and chemokines. Here, the results indicate that TNFα stimulates IL-6 and IL-1β production and release from human monocytic cells and this TNFα–induced response was inhibited in cells pretreated with the antisense PLD1 oligonucleotide (Figure 3.31). In contrast, pretreatment with a PLD2 antisense had no inhibitory effect on these TNFα-induced proinflammatory cytokine productions (Figure 3.31). Thus, suggesting that PLD1 mediates the TNFα–triggered response like cytokine production in human monocytic cells.

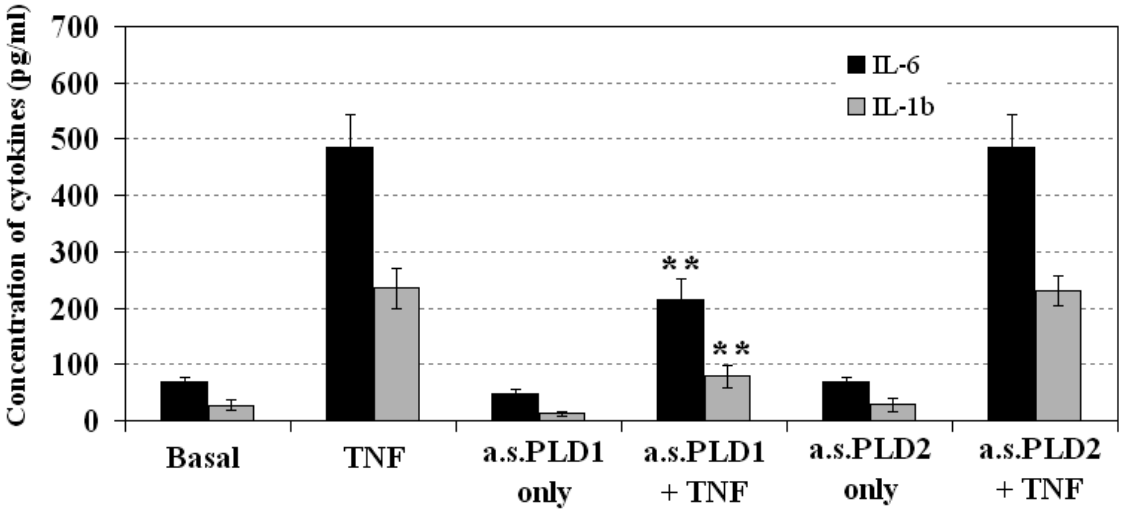


Figure 3.31: TNFα-triggered cytokine release is inhibited in cells pretreated with the PLD1 antisense
Measurements of IL-6 and IL-1β in unstimulated cells (Basal) and after stimulation with TNFα 10 ng/ml (TNFα), pretreated or not with the antisense oligos: Basal (Basal control); TNFα stimulation (TNF); Basal in cells pretreated with a.s.PLD1 (a.s.PLD1); TNFα stimulation in cells pretreated with a.s.PLD1 (a.s.PLD1 + TNF); Basal in cells pretreated with a.s.PLD2 (a.s.PLD2) and TNFα stimulation in cells pretreated with a.s.PLD2 (a.s.PLD2 + TNF). Results shown are the mean ± S.D. of triplicate measurements and from three separate experiments. (**) indicates Student's *t* test *p* value <0.01.

3.2.3.6 TNFα activated the PLD1 pathway in primary human monocytes to mediate its inflammatory response

The experiments discussed earlier were carried out in a human monocytic cell line (U937 cells) differentiated to a mature monocyte phenotype with IFNγ. In order to validate these findings in a more relevant model, it was then decided to investigate whether TNFα is also capable of activating the PLD1 pathway in primary human monocytes.

It has been shown in the earlier section that TNFα stimulated PLD activity in primary monocytes as well (Figure 3.2), similar to that observed for U937 cells (Figure 3.1). PLD1 and PLD2 were also present in primary monocytes and have a general cytosolic localization in resting cells (Figure 3.32). Similar to the monocytic cell model, TNFα triggered the re-localization of PLD1, but not PLD2, to the cells' periphery or cell membrane (Figure 3.32).

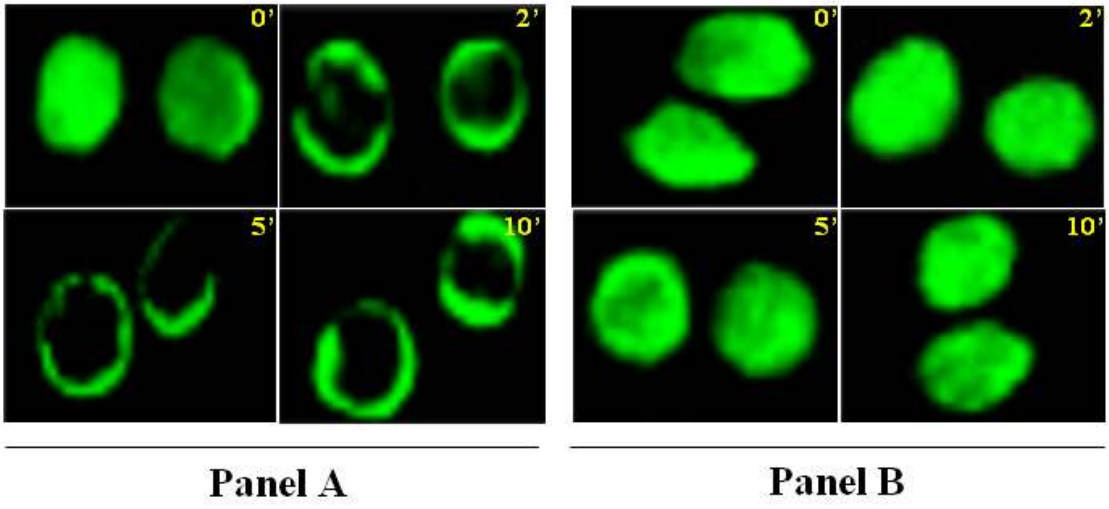


Figure 3.32: PLD1 re-localization subsequent to TNFα stimulation in primary human monocytes
Fluorescence microscopic images of cells stimulated with TNFα 10 ng/ml and subsequently immune-stained for PLD1 and PLD2 are shown in Panel A and Panel B respectively. The panels include cells at various time points (0 minute or Basal; 2 minutes; 5 minutes and 10 minutes) after TNFα stimulation.

The primary monocytes obtained were pretreated with antisense oligonucleotides against the two PLD isoforms. The levels of PLD1 and PLD2 expression has been significantly downregulated following the respective antisense oligonucleotide treatment can be seen in Figure 3.33. Based on the figure mentioned it is evident that there is an isoform specific reduction in the expression of PLD following antisense treatment. This is similar to the results in the earlier section dealing with human monocytic cell line.

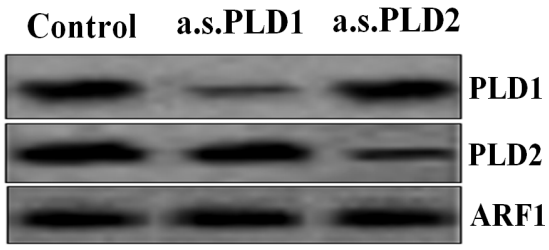


Figure 3.33: Specific knockdown of PLD isoforms (PLD1 and PLD2) using antisense oligonucleotides in primary human monocytes
Western blot analysis of immunoprecipitates of either PLD1 or PLD2 to assess expression of either isozyme in human monocytic cells, following 36 hours treatment with antisense oligonucleotides (10 μM) specific for either PLD1 (a.s.PLD1) or PLD2 (a.s.PLD2) and untreated cells severed as controls (Control). ARF1 (ADP-ribosylation factor 1) was used as loading control and the samples were obtained from whole cell lysates. The results shown are typical from three separate experiments.

Treatment of cells with the antisense oligonucleotide to PLD1 resulted in no change in basal PLD activity. However, following TNFα stimulation, the increase in PLD activity was significantly reduced, compared to the control cells (Figure 3.34). In contrast, treatment of cells with the antisense oligonucleotide to PLD2 only had an effect on the basal PLD activity and not on the TNFα–induced increase in PLD activity (Figure 3.34).

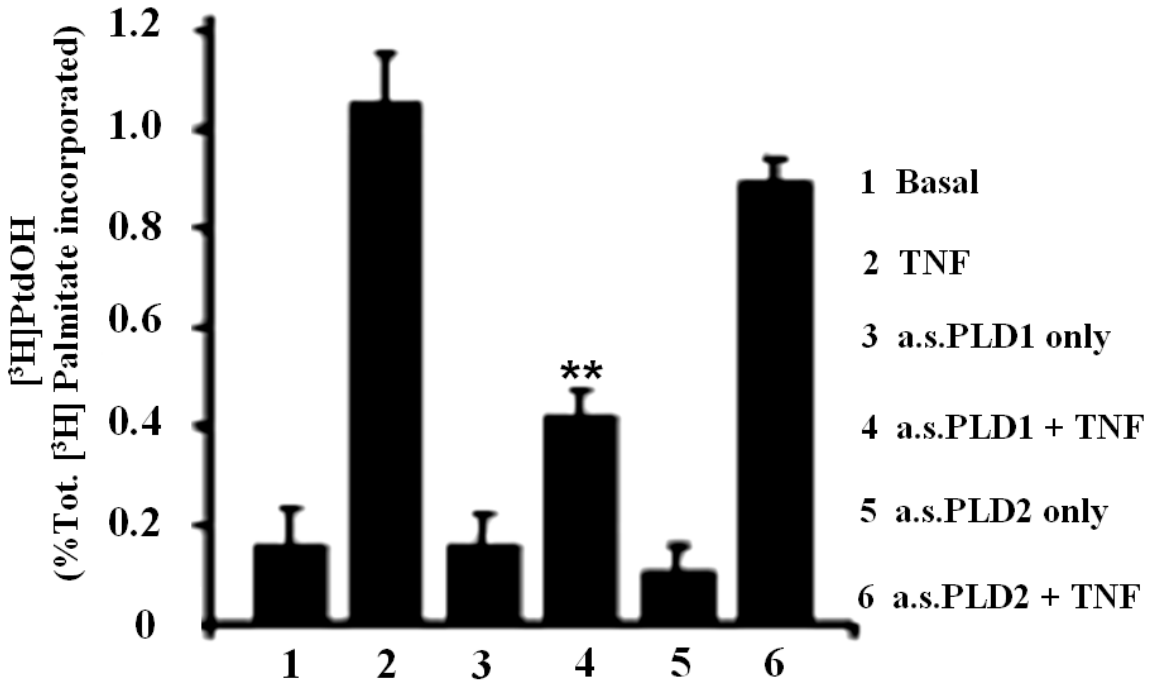


Figure 3.34: PLD1 isoform was found to be coupled with TNFα signaling in primary human monocytes
PLD activity following TNFα (10 ng/ml) stimulation in cells pretreated with 10 μM antisense oligonucleotides for either PLD1 (a.s.PLD1) or PLD2 (a.s.PLD2). PLD activity in untreated cells (Basal); in cells stimulated with TNFα (TNF); in cells pretreated with a.s.PLD1 only (a.s.PLD1 only); in cells pretreated with a.s.PLD1 prior to TNFα stimulation (a.s.PLD1 + TNF); in cells pretreated with a.s.PLD2 only (a.s.PLD2 only) and in cells pretreated with a.s.PLD2 prior to TNFα stimulation a.s.PLD2 + TNF). Results are the mean ± S. D. of triplicate measurements from three separate experiments. (**) indicates Student's *t* test *p* value <0.01.

Furthermore, it is also shown here that, as observed in the U937 cells, PLD1 is coupled to TNFα–triggered intracellular signaling events like the activation of cytosolic calcium release and SphK activity in peripheral blood derived primary human monocytes (Figures 3.35 and Figure 3.36 respectively).

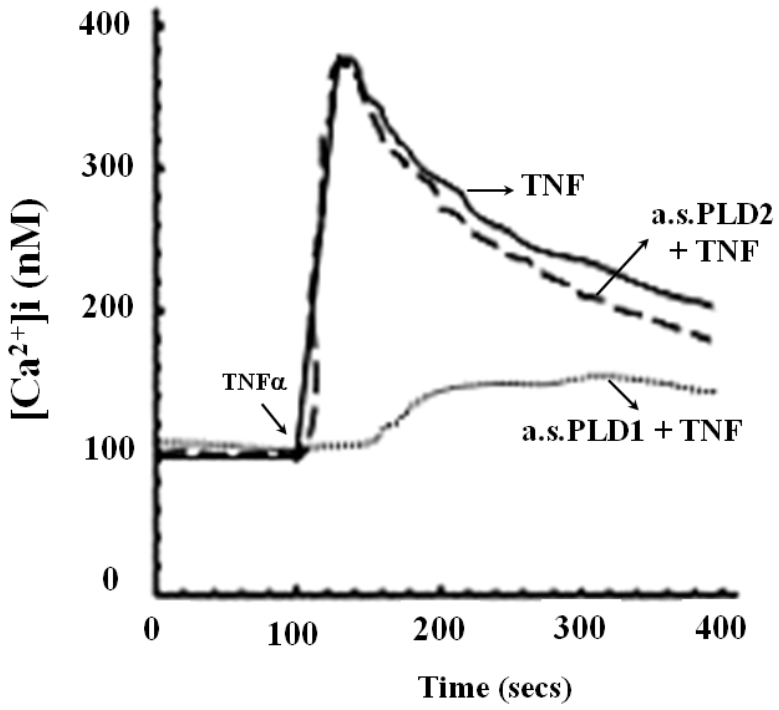


Figure 3.35: Intracellular cytosolic calcium changes following TNFα stimulation is PLD1 dependent in primary human monocytes
Intracellular cytosolic calcium responses after TNFα stimulation were compared in control cells and cells pretreated with antisense oligonucleotides (10 μM) to either PLD1 or PLD2. Traces shown are, control cells treated with TNFα 10 ng/ml (TNF); cells pretreated with antisense to PLD1 (a.s.PLD1 + TNF) and cells pretreated with antisense to PLD2 (a.s.PLD2 + TNF). The arrow marks the addition of TNFα. Traces are typical from three separate experiments.

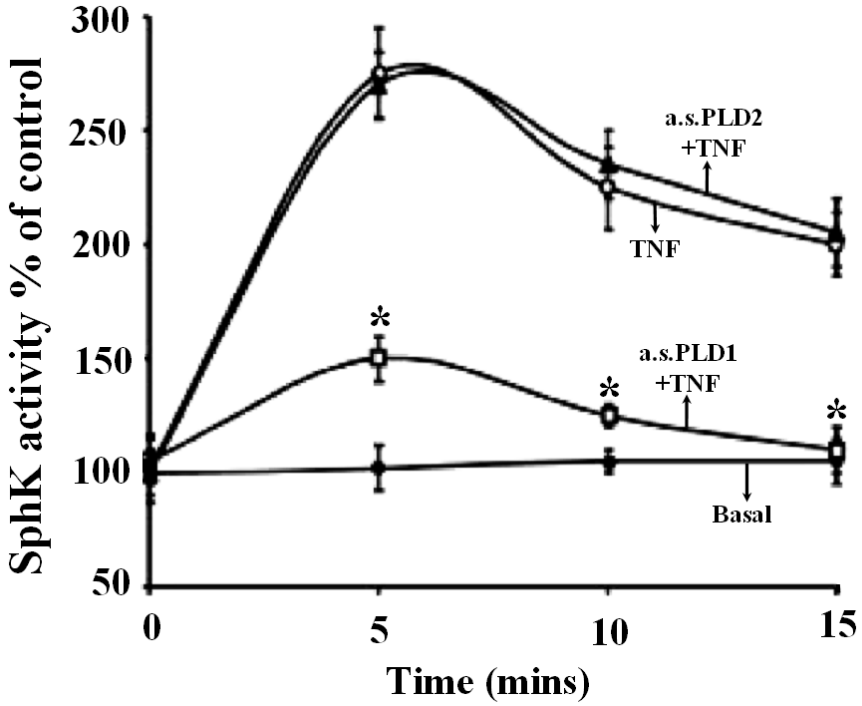


Figure 3.36: TNFα-induced sphingosine kinase activity is dependent on PLD1 in primary human monocytes

Sphingosine kinase activity following TNFα (10 ng/ml) stimulation is shown. Cells were harvested at given time points indicated in the figure to measure sphingosine kinase activity. Sphingosine kinase activity, in untreated control cells (● – Basal); in untreated cells following TNFα stimulation (○ – TNF) and in cells pretreated with antisense oligonucleotides (10 μM) for either PLD1 (◊ – a.s.PLD1 + TNF) or PLD2 (▲ – a.s.PLD2 + TNF) prior to TNFα stimulation. Results are the mean ± S.D of triplicate measurements and from three separate experiments. (*) indicates Student's *t* test *p* value <0.01.

In addition to the intracellular signaling events, PLD1 was also found to be essential for TNFα–induced effector responses like proinflammatory cytokine (IL-6 and IL-1β) production in peripheral blood derived primary human monocytes as well (Figure 3.37).

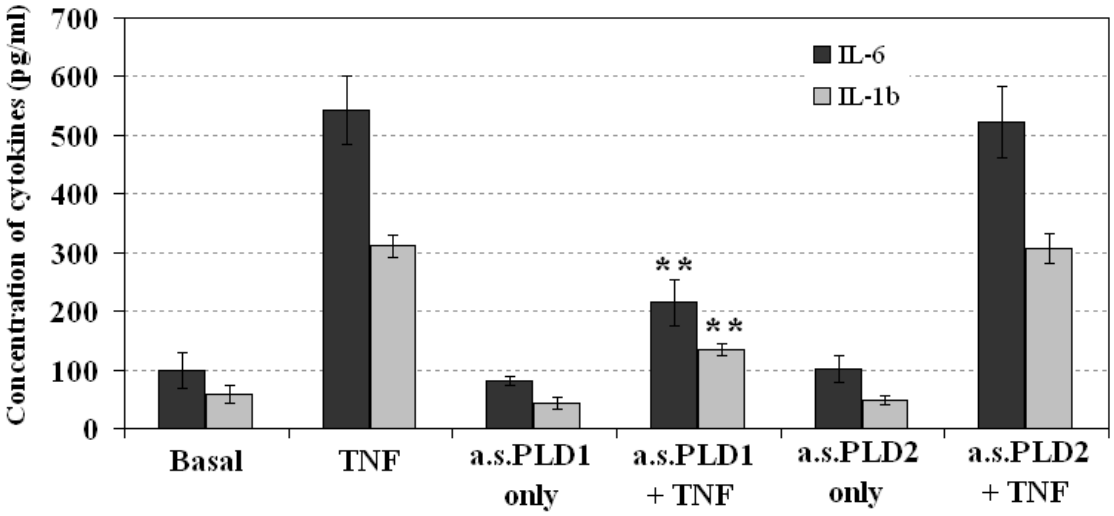


Figure 3.37: TNFα-triggered cytokine release in primary human monocytes are mediated by PLD1
Measurements of IL-6 and IL-1β in unstimulated (Basal) and after stimulation with TNFα 10 ng/ml (TNFα), pretreated or not with the antisense oligos: Basal (Basal control); TNFα stimulation (TNF); Basal in cells pretreated with a.s.PLD1 (a.s.PLD1); TNFα stimulation in cells pretreated with a.s.PLD1 (a.s.PLD1 + TNF); Basal in cells pretreated with a.s.PLD2 (a.s.PLD2) and TNFα stimulation in cells pretreated with a.s.PLD2 (a.s.PLD2 + TNF). Results shown are the mean ± S.D. of triplicate measurements and from three separate experiments. (**) indicates Student's *t* test *p* value <0.01.

Based on the results discussed in this chapter, it can be comprehended that PLD in general and PLD1 in particular mediates TNFα-induced intracellular signaling events and pro-inflammatory responses both in the chosen cell line and primary cell model.

3.3 Discussion

In this study, it has been shown that TNFα is functionally coupled to PLD1, but not PLD2, in IFN-γ primed U937 cells, as well as in human primary monocytes, even though both the enzymes were expressed in these cells. We further show that PLD1, and not PLD2, is required for TNFα–induced activation of the sphingosine kinase, intracellular calcium responses, the activation of a key transcription factor and cytokine generation.

Understanding the intracellular signal transduction mechanisms that regulate TNFα-mediated responses has profound implications, not the least of which is to identify novel molecules as potential therapeutic targets. There is overwhelming evidence for believing that TNFα is associated with a variety of inflammatory conditions in several diseases (Aderka et al., 1985; Bolling et al., 1992; Feldmann, 2002; Feldmann et al., 1995; Lahdevirta et al., 1988; Lang et al., 1992; Maury and Teppo, 1987; Oliff et al., 1987; Remick et al., 1987; Roux-Lombard et al., 1989; Selmaj et al., 1991; Stephens and Pekala, 1991; Tracey et al., 1986; Tracey et al., 1987; Tracey et al., 1988; Ulich et al., 1991). Efforts are being made to find novel ways to balance TNFα levels or TNFα-induced responses in several diseases, including rheumatoid arthritis and SLE. Regulation of molecules involved in the TNFα-triggered signaling pathways has gained attention in this regard, as a variety of signaling molecules such as PDE4, p38 MAP-kinase and NFκB inhibitors are being studied in clinical trials (Palladino et al., 2003). More efficient therapies may become available by elucidation of the molecular mechanisms, and the role of key molecules, involved in the TNFα-triggered signaling events. Thus, the role of

PLD isoforms in TNFα-induced intracellular and effector responses in human monocytes was investigated.

Results in this study show that TNFα triggers PLD activity and that PLD1 (but not PLD2) is rapidly translocated from a cytosolic distribution to the plasma-membrane periphery. The cells used in this study expresses both PLD isoforms (PLD1 and PLD2) (Melendez et al., 2001). The selective re-localization of PLD1 to the cell membrane after TNFα stimulation suggests that TNFα signaling might be associated with the selective activation of PLD1. However, as there are many reports indicating the stimulation of isoform-specific PLDs in different cells, responding to distinct stimuli (Bechoua and Daniel, 2001; Jenkins and Frohman, 2005; McDermott et al., 2004; Melendez et al., 2001; Paruch et al., 2006). Therefore, this study was designed to further the investigation of the specific isoform activation of PLD by TNFα. Antisense oligonucleotides were utilized to specifically knockdown the expression of either PLD1 or PLD2. The results from the antisense experiments demonstrate that TNFα specifically activates PLD1 but not PLD2.

To improve our understanding, studies were conducted to elucidate the role of PLD1 in TNFα–induced intracellular signaling events. It has been previously shown that PLD plays a role in inflammatory signals (Jenkins and Frohman, 2005) and may stimulate MAPK phosphorylation events (Fang et al., 2001; Ghosh et al., 1996; Jones and Hannun, 2002; Rizzo et al., 1999) or prevent protein de-phosphorylation (Jones and Hannun, 2002). MAPKs are vital regulators and/or amplifiers of extracellular stimuli leading to cellular functions. They are regulated by sequential phosphorylation of their

preceding kinase-family members (Roux and Blenis, 2004). Regulation of cell survival, growth and differentiation are some of the important roles of ERK1/2 (Cowley et al., 1994; Hill and Treisman, 1995). In this study, the results indicate that PLD is upstream of ERK1/2. It is quite well known that Raf-1 translocation subsequent to an agonist activation is an upstream event prior to ERK1/2 phosphorylation (Chen et al., 2001; Roux and Blenis, 2004). The results show that PLD mediates TNFα induced Raf-1 translocation via phosphatidic acid. Previous studies have also shown that Raf-1 can be activated by phosphatidic acid as the latter has been suggested to possess a binding site for PA (Ghosh et al., 1996; Rizzo et al., 1999). The current finding that ERK1/2 is downstream of PLD is, therefore, consistent with these other previous *in vitro* studies (Ghosh et al., 1996; Rizzo et al., 1999). This study also shows that the TNFα stimulated ERK1/2 phosphorylation is dependent on PLD1. However, the phosphorylation of p38 is not. Interestingly, several studies have reported receptor-coupled activation of ERK1/2 to be not only independent of PLD activity, but that PLD activity was actually dependent on ERK1/2 activity (Paruch et al., 2006). It is shown here that, at least in human monocytic cells, TNFα signals in a very different way, i.e., TNFα stimulated PLD activity is upstream of ERK1/2 phosphorylation, while p38 phosphorylation is independent of PLD activity. This is in contrast to an elegant report by Bechoua and Daniel, that showed that fMLP-triggered p38 activation in HL-60 cells was dependent on PLD activity, whereas ERK1/2 phosphorylation was not (Bechoua and Daniel, 2001). In order to clarify these potential contradictions, and to establish the molecular specificity in our system, we utilized MEK and p38 inhibitors and looked at PLD activity triggered by TNFα. Our results showed that neither inhibition of MEK nor p38 had any effect on TNFα–mediated

PLD activity and the inhibitors were shown to be effective and functioning appropriately as they inhibited their respective targets and their associated effector responses.

To further our studies on the role of PLD1 in TNFα-triggered intracellular signaling events, we looked at its role in SphK activation triggered by TNFα. It has been previously shown that, in these cells, TNFα activates Sphingosine kinase to mediate its responses (Zhi et al., 2006). Here, the results show that in cells pretreated with the antisense against PLD1, the TNFα stimulated SphK activity was substantially inhibited, whereas in the cells pretreated with the antisense against PLD2, the TNFα–mediated SphK activity remained intact. This is further proof for the specificity of PLD1 in the TNFα–mediated signaling events. The finding the PLD is upstream of SphK in this model reiterates the fact suggested by Olivera et al that SphK is directly activated by phosphatidic acid (Olivera et al., 1996). Therefore, we can suggest that TNFα-triggered PLD derived PA regulates the activation of SphK and its down stream mediators and responses.

Intracellular calcium is well known as an important intracellular second messenger and is known to regulate cellular responses. Cytosolic calcium influences cellular responses by participating in a variety of biological process like the activation of kinases, phosphatases, degranulation and activation of transcription factors. Increase in the level of cytsolic calcium and release from internal stores following immune receptor activation has been reported (Floto et al., 1995; Mandeville and Maxfield, 1996). Here it is shown that TNFα induces an increase in cytosolic calcium which dependent on PLD1. It has already been shown in a similar model that TNFα–induced calcium release is

SphK1 dependent (Zhi et al., 2006). Consistent with the previous study, our results are clear that TNFα-induced SphK regulated intracellular calcium release is mediated by PLD1. Based on the current study and the earlier study (Zhi et al., 2006) we can propose that TNFα mobilizes intracellular calcium through the coupling of PLD1 to SphK1. This is the first example of this pathway to be shown in cytokine signaling.

Results in this study clearly showcase the importance of PLD in TNFα–induced NFκB activation and the consequent inflammatory response. This was demonstrated by the decrease in the levels of p65 subunit in the nucleus when PLD activity was blocked prior to stimulation. This current study also indicated that PLD activity was responsible for regulating p65 by targeting the upstream event of IκB alpha degradation subsequent to agonist stimulation. This regulatory event is of primary importance, as it facilitates the release of the active NFκB subunits which translocate from the cytoplasm into the nucleus. As mentioned earlier, this study demonstrates that PLD1 is required for the phosphorylation/activation of ERK1/2 MAPKs. Interestingly, it has long been established that members of the MAPK family can phosphorylate the IκB alpha kinase complex, which in turn leads to the activation of NFκB (Lee, 1997; Tergaonkar, 2006). However, the results in this investigation clearly shows that TNFα induced IκB alpha degradation and NFκB activity is dependent on PLD but TNFα–induced IκB alpha degradation and NFκB activity are not dependent on ERK1/2 and p38 kinase. The fact that TNFα needs to couple to PLD1 to trigger calcium release from internal stores may, at least in part, explain the role of PLD1 in TNFα stimulated NFκB activation, as it has been shown that calcium amplitude and/or modulation is required for NFκB activation (Aoki, 1997;

Dolmetsch R. E, 1997; Tergaonkar, 2006). Moreover, it has also been shown that the product of SphK activity, S1P can activate NFκB in U937 cells (Shatrov V. A., 1997).

As the NFκB and ERK1/2 pathways appear to be dependent on PLD activity, we went on to measure the TNFα-triggered proinflammatory cytokine production and cellular migration, and showed that the antisense to PLD1 substantially blocked the responses measured. Taken together, the data presented here suggest that TNFα stimulation in cells triggers a number of effector functions, including the generation of several cytokines and chemotaxis. The novel intracellular signaling pathway demonstrated here appears to be functionally interactive / associated with these. In the study reported here, silencing PLD1 reduced the ability of TNFα to mobilize calcium from intracellular stores and, significantly reduced the activation of signal ampifiers like ERK1/2 and key transcription factors, such as NFκB. It is of interest that these transcription factors play major roles in the inflammatory responses, by triggering cytokine and chemokine genes, as well as cycloxigenases and other genes involved in the inflammatory responses (Shaffer et al., 2001). The finding that TNFα triggers the rise in cytosolic calcium and cytokine production, via a novel pathway that utilizes the sequential activation of PLD1 and SphK, has profound implications for the development of strategies for therapeutic intervention against aberrant inflammation.

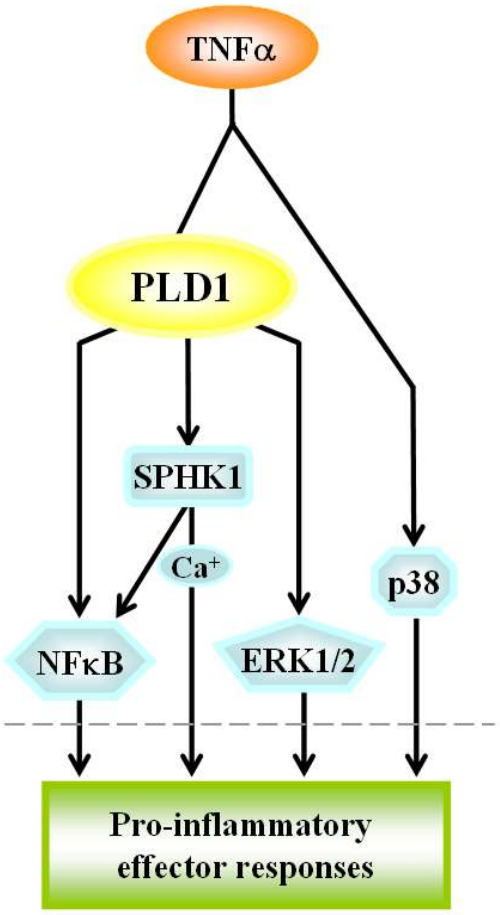


Figure 3.38: Schematic representation of the isoform specific role of PLD1 in TNFα–induced intracellular signaling events and responses

CHAPTER 4

TNFα–induced inflammatory response *in vivo* is mediated by Phospholipase D1

4.1 Introduction

Ligand mediated activation of cell surface receptors is known to generate lipid based intracellular signaling molecules from cellular phospholipids (Divecha and Irvine, 1995; Steed and Chow, 2001). Phosphatidylcholine specific phospholipase D (PLD) is an enzyme which hydrolyzes phosphatidylcholine, a membrane bound cellular phospholipid to generate phosphatidic acid (PA) and choline (Heller, 1978; Yang et al., 1967). PA, a potent second messenger by itself, can be further converted to other mediators like diacylglycerol (DAG) and Lysophosphatidic acid (LPA) by Phosphatidic acid phosphohydrolases and Phospholipase A2 respectively (English, 1996; English et al., 1996; Exton, 2002a; Exton, 2002b; Sciorra and Morris, 1999). Since, PLD is a major source of these bioactive molecules, it is considered to be an important lipid based intracellular signaling mediator. It thereby regulates a variety of homeostatic cellular functions like membrane trafficking, vesicular transport, cytoskeletal re-organization, cellular migration, proliferation and survival (Colley et al., 1997; Exton, 2002a; Exton, 2002b; Siddhanta and Shields, 1998). The role of PLD in immune cell responses and inflammation has been well documented. It is known to mediate receptor activated effector responses like phagocytosis (Gewirtz and Simons, 1997; Kusner et al., 1999),

NADPH-oxidative burst (Dana et al., 2000; Melendez et al., 2001), immune cell migration (Atta ur et al., 1999; Lehman et al., 2006; Sethu et al., 2008), immune cell degranulation (Hammond et al., 1995; Morgan et al., 1997) and cytokine production (Sethu et al., 2008; Singh et al., 2006). Therefore, PLD has been a potential target for managing aberrant inflammation (Steed and Chow, 2001).

PLD comprises of two major isoforms, PLD1 and PLD2 (Colley et al., 1997; Hammond et al., 1995). They are expressed in a wide range of almost all the mammalian tissues. The isoforms have been reported to mediate specific immune responses based on their differential expression profiles on immune cells. The PLD isoforms are reported in neutrophils (Di Fulvio and Gomez-Cambronero, 2005), monocytes (Melendez et al., 2001), macrophages (Iyer et al., 2004), lymphocytes (Diaz et al., 2005), NK cells (Milella et al., 1999) and dendritic cells (Lee et al., 2004b). PLD1 has been associated with inflammatory and immune responses in monocytes, macrophages and neutrophils (Cadwallader et al., 2004; Iyer et al., 2006; Locati et al., 2001; Melendez et al., 2001; Sethu et al., 2008) and PLD2 has been reported to mediate responses in T lymphocytes (Hamdi et al., 2008; Mor et al., 2007). However, both PLD1 and PLD2 together have also been found to mediate phagocytosis in macrophages as well (Iyer et al., 2004; Lehman et al., 2006). Studies have also reported the stimuli or agonist specific activation of selective PLD isoforms in various cells types (Bechoua and Daniel, 2001; Jenkins and Frohman, 2005; McDermott et al., 2004; Melendez et al., 2001; Paruch et al., 2006). Therefore, PLD isoforms are potential targets to dampen specific immune responses.

Tumour Necrosis Factor alpha (TNFα), a potent pleiotropic pro-inflammatory cytokine is associated with a variety of inflammatory disorders (DeVries et al., 1999;

Matsumoto and Kanmatsuse, 1999; Palladino et al., 2003; Plo et al., 2000; Tracey and Cerami, 1993; Tracey and Cerami, 1994). It is produced by a variety of cells including immune cells, glial cells, keratinocytes and fibroblasts. In an attempt to identify strategies to reduce TNFα-associated morbidity, molecules involved in the TNFα-triggered signaling pathways such as PDE4, p38 MAP-kinase and NFκB inhibitors are being explored (Palladino et al., 2003). PLD has gained attention in this regard as it is a major player in receptor mediated signaling cascade. However, the studies have reported the role of PLD in TNFα-mediated signaling and responses with contrasting findings (De Valck et al., 1993; Kang et al., 1998; MacEwan, 2002b). In our recent study we have reported that PLD1 is coupled to TNFα signaling and associated inflammatory responses *in vitro* (Sethu et al., 2008). *In vivo* studies will be useful to demonstrate the potential therapeutic implication of PLD1 in inflammation. *In vivo* validation of the function of PLD1 in general and its role in inflammation in specific is yet to be explored. The peritonitis model in mice is a dependable method to study the inflammatory process *in vivo*. TNFα can induce an inflammatory response with the associated characteristic signs and symptoms when injected directly into the peritoneal cavity. The detailed description of the model has been described in section 2.18 in chapter 2. Therefore, in this study the role of PLD1 in TNFα-induced peritonitis in mice was evaluated. The results indicate that PLD1 mediates responses associated with peritonitis, implying that PLD1 is coupled with TNFα associated inflammatory responses *in vivo*.

4.2 Results

The dose of TNFα and time of maximum inflammatory response following intra-peritoneal injection of TNFα was to be determined in BALB/c mice.

4.2.1 Determination of TNFα dosage

Classical inflammatory responses like changes in body temperature and increase in the levels of proinflammatory cytokine like IL-6 were chosen as parameters to determine the appropriate dose and time of maximum response. Therefore, we evaluated the changes in body temperature of mice following intra-peritoneal administration of varying doses (2.5 μg/mouse, 5 μg/mouse and 10 μg/mouse) of TNFα using a rectal thermometer. Mice injected with different doses of TNFα exhibited an increase in body temperature (Figure 4.1). The temperature changes were initially monitored every hour for 6 hours. This increase in temperature peaked at two hours of TNFα administration followed by gradual drop in the increase in temperature in the subsequent hourly measurements. There was approximately a 2°C increase in temperature from that of the observed basal temperature with TNFα doses of 5 μg/mouse and 10 μg/mouse.

Another parameter evaluated was the levels of IL-6, an important proinflammatory cytokine useful in the amplification of TNFα-induced responses. Therefore, we measured the levels of IL-6 in both the peritoneal lavage and in the serum following 2 hours and 6 hours subsequent to TNFα administration via i.p injection. There was a marked increase in the levels of IL-6 both in the serum and peritoneal lavage, 2 hours after TNFα treatment (Figure 4.2). However, this increase in levels dropped at the

end of 6 hours (Figure 4.2). Though there was an increase in IL-6 levels in all the doses tested, 5 μg/mouse and 10 μg/mouse of TNFα showed a more marked response.

Based on this data set (Figure 4.1 and 4.2), TNFα of 5 μg/mouse was chosen as the dose to induce the required experimental inflammatory response. The choice is due to the observation that this dose was the lowest dose tested which initiated a marked response among the doses tested. In other words, 5 μg/mouse of TNFα induced a response greater than 2.5 μg/mouse but similar to 10 μg/mouse. 2 hours post intra-peritoneal administration was chosen as the time point to evaluate the various acute inflammatory responses associated with intra-peritoneal TNFα administration.

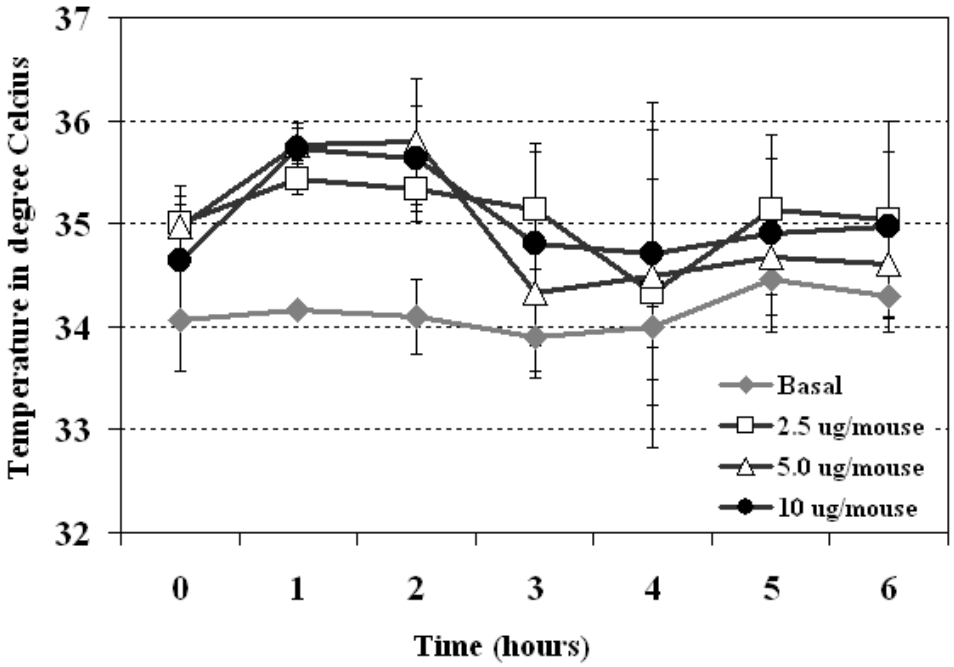
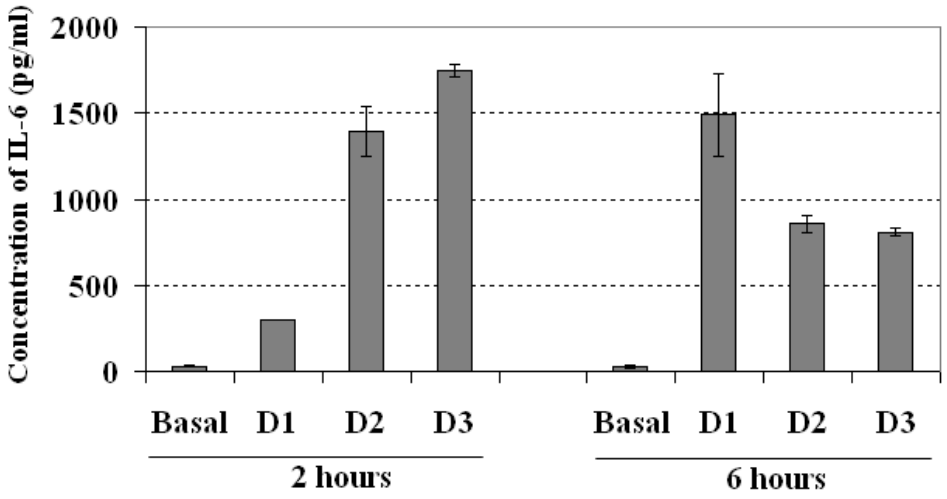


Figure 4.1: Rectal temperature pattern triggered by TNFα in BALB/c mice
The graphs indicate TNFα-induced rectal temperature changes observed over the time points indicated. Different doses of TNFα (2.5 μg/mouse; 5 μg/mouse and 10 μg/mouse) as indicated were administered via intra-peritoneal (i.p) injections. Equal volume of sterile 1xPBS of was also administered (i.p) for the Basal controls. Results shown are the mean ± the standard deviation of measurements from three different mice for each category.

(A)



(B)

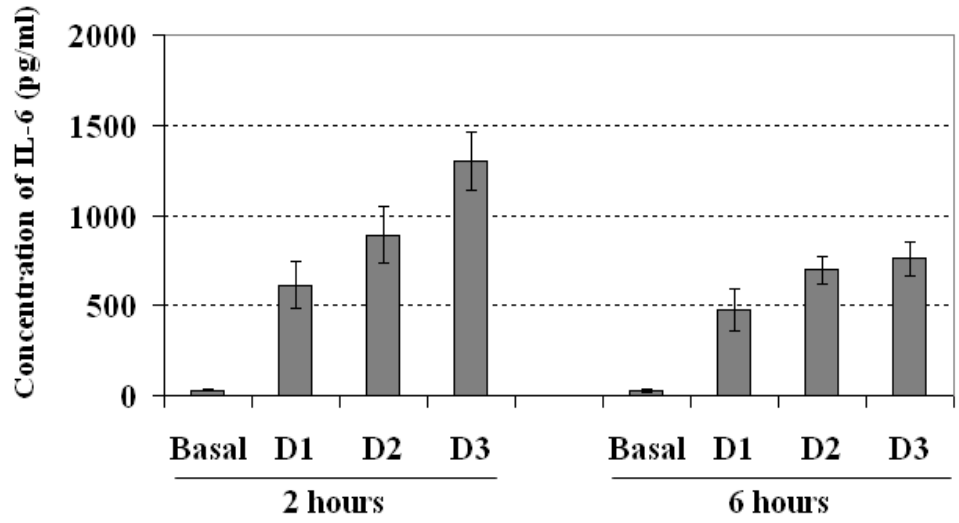


Figure 4.2: IL-6 release response pattern triggered by TNFα in BALB/c mice
The graphs indicate TNFα–induced IL-6 release in the peritoneal lavage **(A)** and in the serum **(B)**. IL-6 production was observed over the time points indicated. Three different doses of TNFα (D1 – 2.5 μg/mouse; D2 – 5 μg/mouse and D3 – 10 μg/ mouse) as indicated were administered via intra-peritoneal (i.p) injections. Equal volume of sterile 1xPBS of was also administered (i.p) for the Basal controls. Results shown are the mean ± the standard deviation of triplicate measurements of samples obtained from three different mice for each category.

4.2.2 *In vivo* knock down of mPLD1

Intravenous administration of a siRNA is known to result in a significant knockdown of the gene product in the mice. Figure 4.3A, shows a significant decrease in the protein level of murine phospholipase D1 (mPLD1) in peripheral blood leukocytes of mice, following intravenous administration of siRNA (4 μg/mouse) to knock down mPLD1. siRNA administration strategy was described in chapter 2. Since both the PLD isoforms are expressed in BALB/c mice (Kim et al., 2007), we determined the isoform specific knock down of PLD1 by evaluating the expression of PLD2 as well, following the above mentioned siRNA intravenous administration. The level of mPLD2 expression remained unaffected subsequent siRNA treatment specific for PLD1 (Figure 4.3B). Thus, it clearly indicates the efficiency of the isoform specific knock down of PLD1 *in vivo*.

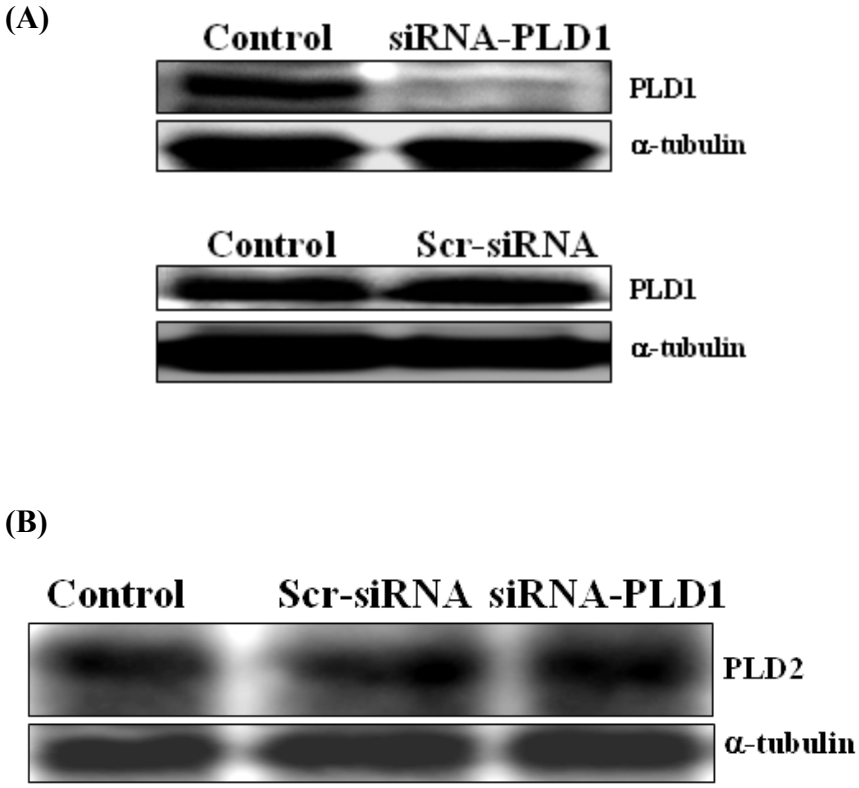


Figure 4.3: Specific knock down of PLD1 in BALB/c mice using siRNA
(A) Western blot analysis shows the knockdown of murine phospholipase D1 (mPLD1) in BALB/c mice peripheral blood leukocytes, by the use of specific siRNA for PLD1 (4 μg/mouse) administered via intravenous tail vein injection regimens as described earlier. **(B)** Western blot analysis shows the expression of murine phospholipase D2 (mPLD2) in the same set of samples as indicated. Lysates probed for PLD1 and PLD2 from peripheral blood leukocytes were obtained from, untreated mice which severed as control (Control); from mice injected with siRNA-PLD1 (siRNA-PLD1) and from mice injected with scrambled siRNA (Scr-siRNA). In addition, α-tubulin was probed for loading control. The immunoblot shown here is a representative of three different replicate experiments done. This was performed by obtaining peripheral blood leukocytes lysates from three different mice for each category.

4.2.3 Role of PLD1 in TNFα-induced acute peritonitis in mice

The functional contribution of PLD1 in TNFα–induced acute peritonitis model was determined by studying the responses in both control mice and in mice with decreased PLD1 expression in peripheral blood leukocytes following siRNA treatment. The responses studied include changes in body temperature, proinflammatory cytokine and chemokines production, cellular infiltration and/or migration and expression of cell adhesion molecules in the vascular endothelium following TNFα administration (intra peritoneal injection).

4.2.3.1 Temperature response

One of the characteristic systemic responses related to TNFα administration is pyrexia or increase in body temperature. Therefore, the change in body temperature of mice following TNFα intra-peritoneal administration and the role of PLD1 in this response was evaluated. Mice, injected with TNFα developed marked increase in body temperature in an hour (Figure 4.4). This increase in body temperature was considerably reduced in mice pretreated with the PLD1 siRNA. However, the temperature changes in mice pretreated with the negative control siRNA the temperature changes were identical to that of the TNFα treated mice.

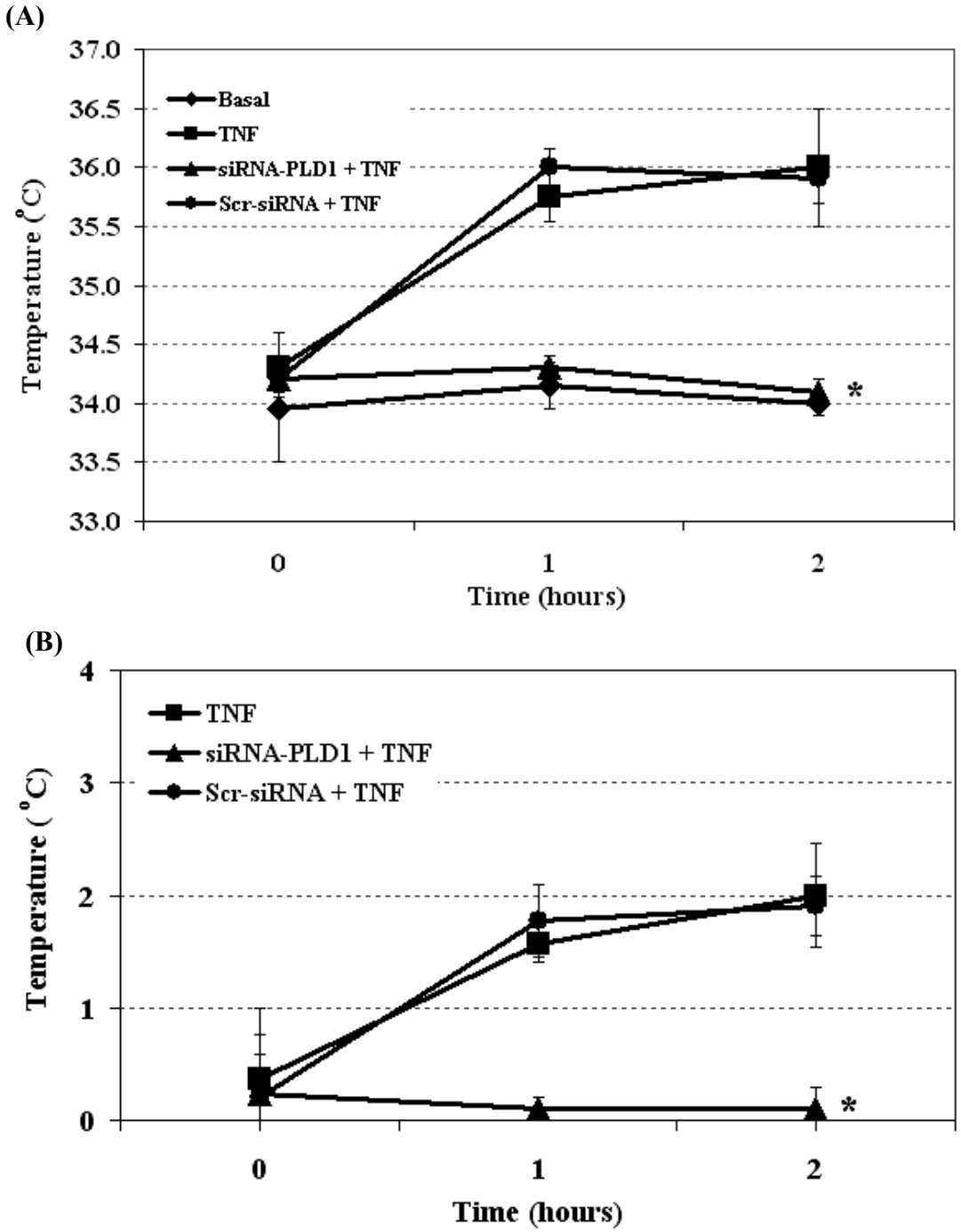


Figure 4.4: PLD1 knock down alters rectal temperature changes triggered by TNFα in BALB/c mice.

The graphs indicate TNFα (5 μg/mouse) induced rectal temperature changes observed over the time points indicated. TNFα was administered via intra-peritoneal (i.p) injections. The above indicates, temperature pattern in control mice (♦); temperature changes after TNFα administration (■); temperature changes in mice injected with siRNA-PLD1 (▲) and scrambled siRNA (●) prior to TNFα administration. Results shown are the mean ± the standard deviation of measurements from three different mice for each category. (*) indicates Student's *t* test *p* value <0.05.

4.2.3.2 Proinflammatory cytokine production

A key event in the inflammatory process is the production of proinflammatory cytokines. TNFα is capable of amplifying its inflammatory response by promoting the generation and release of several proinflammatory cytokines and chemokines. Therefore we investigated the levels of IL-6, MIP-1α (Macrophage inflammatory protein – 1 alpha) and MIP-1β (Macrophage inflammatory protein – 1 beta) in both the peritoneal lavage and in the serum following TNFα administration. We found there was a significant rise in the levels of these proinflammatory cytokine in the serum and peritoneal lavage of mice treated with TNFα (Figure 4.5 – 4.7). In contrast, an inhibition in this response was observed when the mice were treated with siRNA-PLD1 prior to TNFα injection (Figure 4.5 – 4.7). This indicates the necessity of PLD1 in this response.

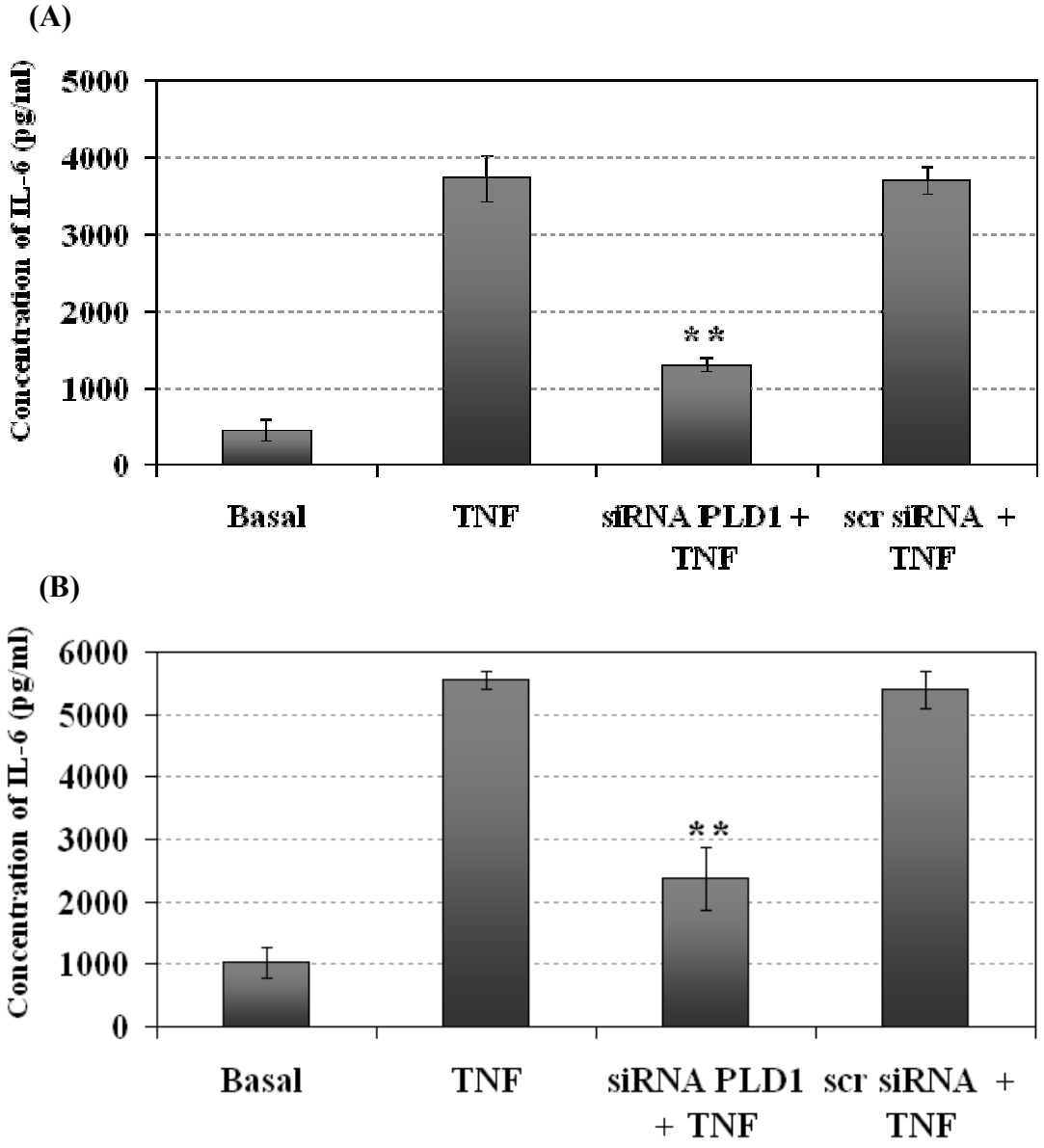


Figure 4.5: TNFα-induced IL-6 release is dependent on PLD1 in BALB/c mice
The graphs indicate the TNFα (5 μg/mouse) induced (i.p) IL-6 release in 2 hours in peritoneal lavage (A) and in serum (B). IL-6 levels in control mice (Basal); in mice injected with TNFα (TNF); in mice injected with siRNA-PLD1 prior to TNFα administration (siRNA PLD1 + TNF) and in mice injected with scrambled siRNA prior to TNFα administration (scr siRNA + TNF). Results shown are the mean ± the standard deviation of triplicate measurements of individual samples collected from three different mice for each category. (∗ ∗) indicates Student's *t* test *p* value <0.01.

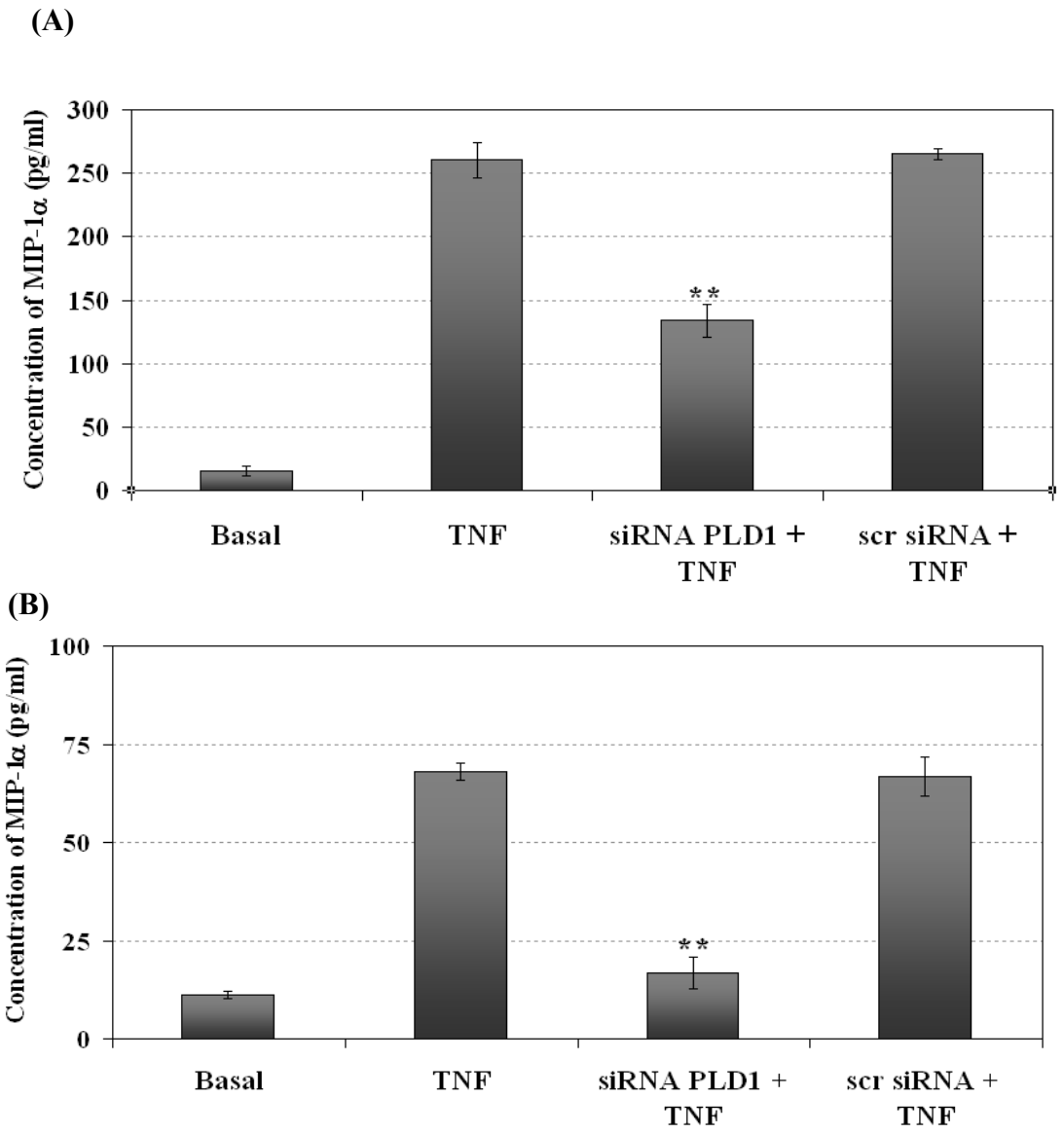


Figure 4.6: TNFα–induced MIP-1α release is dependent on PLD1 in BALB/c mice
The graphs indicate the TNFα (5 μg/mouse) induced (i.p) MIP-1α release in 2 hours in peritoneal lavage (A) and in serum (B). MIP-1α levels in control mice (Basal); in mice injected with TNFα (TNF); in mice injected with siRNA-PLD1 prior to TNFα administration (siRNA PLD1 + TNF) and in mice injected with scrambled siRNA prior to TNFα administration (scr siRNA + TNF). Results shown are the mean ± the standard deviation of triplicate measurements of samples collected from three different mice for each category. (**) indicates Student's *t* test *p* value <0.01.

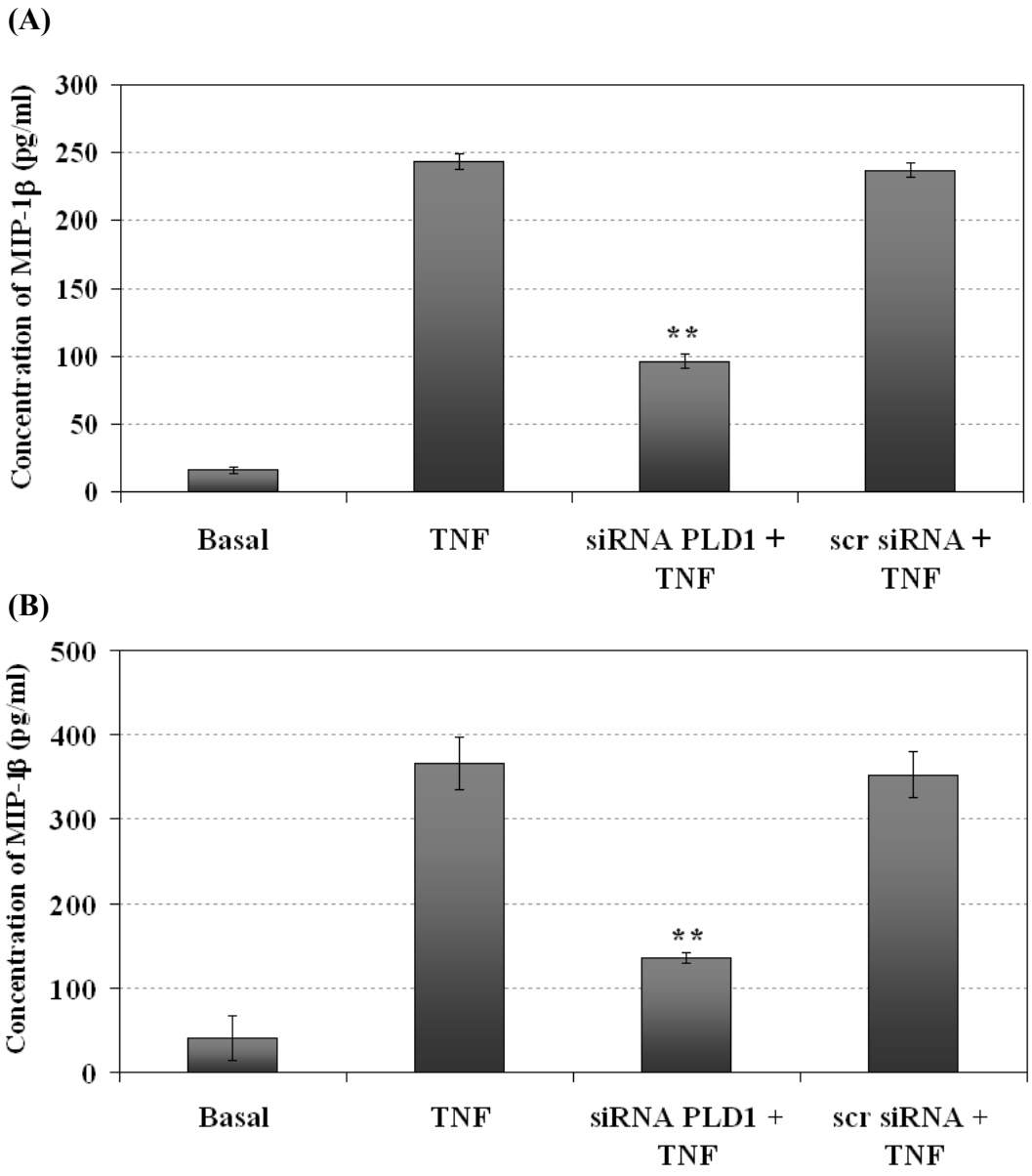


Figure 4.7: TNFα–induced MIP-1β release is dependent on PLD1 in BALB/c mice

The graphs indicate the TNFα (5 μg/mouse) induced (i.p) MIP-1β release in 2 hours in peritoneal lavage (A) and in serum (B). MIP-1β levels in control mice (Basal); in mice injected with TNFα (TNF); in mice injected with siRNA-PLD1 prior to TNFα administration (siRNA PLD1 + TNF) and in mice injected with scrambled siRNA prior to TNFα administration (scr siRNA + TNF). Results shown are the mean ± the standard deviation of triplicate measurements of samples collected from three different mice for each category. (**) indicates Student's *t* test *p* value <0.01.

4.2.3.3 Cellular infiltration/migration

One of the characteristic events in an inflammatory response is the increase in vascular permeability, which leads to the influx of immune cells to the site of inflammation. This was confirmed by investigating the number of neutrophils in the peritoneal lavage at the end of two hours. Intra-peritoneal administration of TNFα-induced a significant influx of neutrophils into the peritoneal cavity compared to that of the untreated controls (Figure 4.8). This increase in the neutrophils counts in the peritoneal lavage following TNFα administration was significantly inhibited in those mice pretreated with PLD1 siRNA (Figure 4.8).

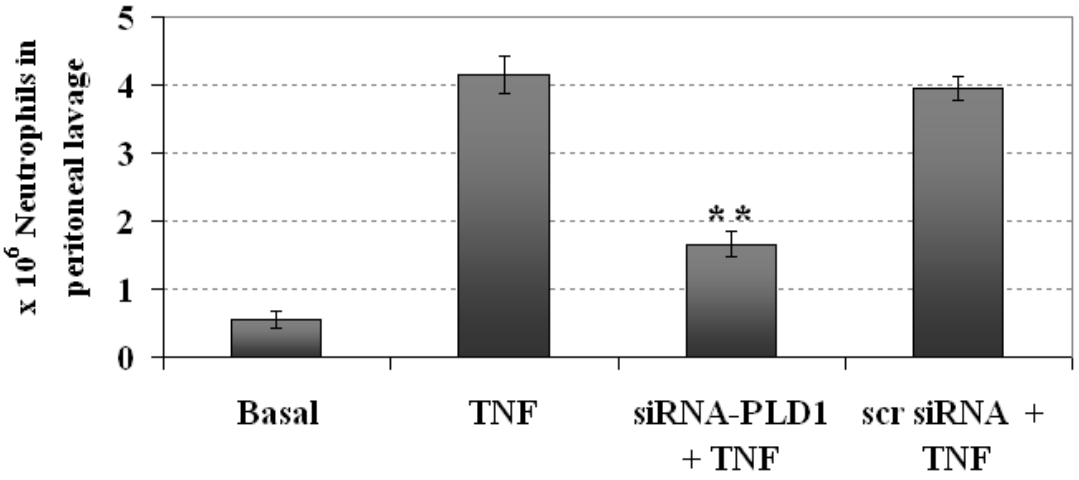


Figure 4.8: Neutrophils infiltration into the peritoneum lavage induced by TNFα is dependent on PLD1 in BALB/c mice

The graphs indicate the TNFα (5 μg/mouse) induced (i.p) neutrophil infiltration in 1.5ml of peritoneal lavage after 2 hours of TNFα administration. Number of neutrophils, in peritoneal lavage of control mice (Basal); in the peritoneal lavage of mice injected with TNFα (TNF); in the peritoneal lavage of mice injected with siRNA-PLD1 prior to TNFα administration (siRNA-PLD1 + TNF) and in the peritoneal lavage of mice injected with scrambled siRNA prior to TNFα administration (scr siRNA + TNF). Results shown are the mean ± the standard deviation of triplicate measurements of lavage samples collected from three different mice for each category. (**) indicates Student's *t* test *p* value <0.01.

The role of PLD1 in TNFα-triggered migration of neutrophils or immune cells to the site of inflammation was further evaluated using H and E staining of the inflamed peritoneal tissues. The peritoneal tissues from mice treated with TNFα intra-peritoneal injection exhibited an increase in cellular infiltration pattern following H and E staining (Figure 4.9). However, these changes in the cellular infiltration pattern were markedly inhibited in the peritoneal tissues of mice pretreated with siRNA against PLD1 (Figure 4.9).

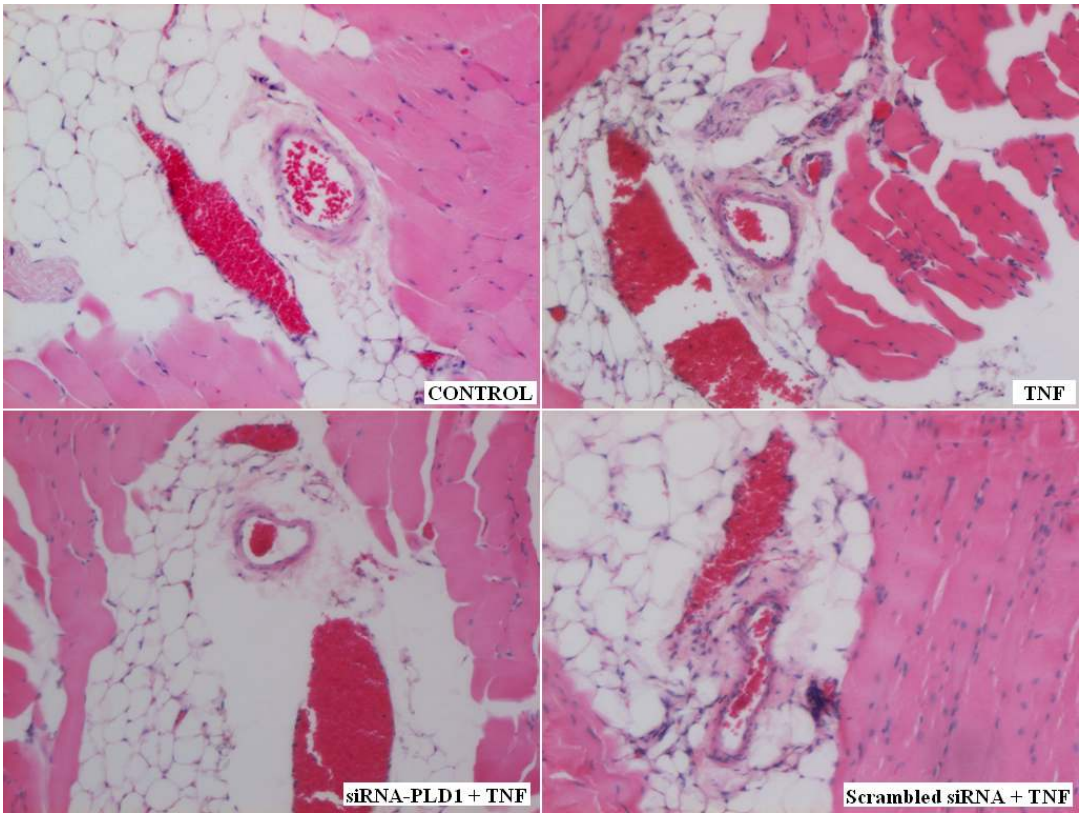


Figure 4.9: Cellular infiltration pattern in peritoneal tissue induced by TNFα is dependent on PLD1 in BALB/c mice

The panels show TNFα (5 μg/mouse) induced (i.p) cellular infiltration pattern using H and E staining in the peritoneal tissues after 2 hours of TNFα administration. The panels indicate the peritoneal tissues from control mice (CONTROL); from mice injected with TNFα (TNF); from mice injected with siRNA-PLD1 prior to TNFα administration (siRNA-PLD1 + TNF) and from mice injected with scrambled siRNA prior to TNFα administration (scrambled siRNA + TNF). Results shown are representatives of multiple sections and fields from three different mice for each category.

4.2.3.4 Expression of cell adhesion molecules

It is well known that cell adhesion molecules are important mediators in the cellular migration and infiltration. Therefore, we evaluated the expression of ICAM1 and VCAM on the vascular endothelium using immunohistochemistry in the peritoneal tissues of mice in our study model. There was an increase in the expression of VCAM and ICAM1 (Figure 4.10 and 4.11 respectively) on the vascular endothelial cells following intra-peritoneal TNFα injection. On the contrary, there was a decrease in the expression of the cell adhesion molecules investigated in the tissues samples obtained from mice injected with siRNA – PLD1 prior to TNFα administration (Figure 4.10 and 4.11).

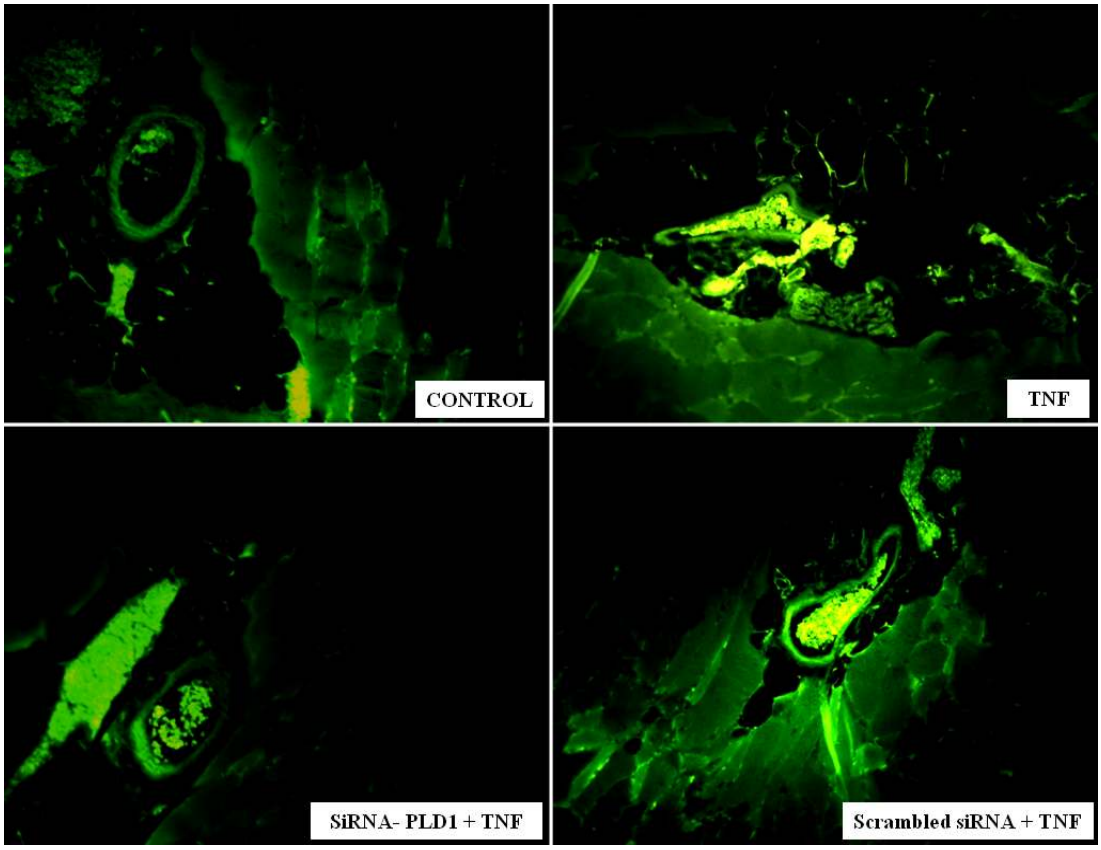


Figure 4.10: TNFα-induced VCAM expression in peritoneal tissues is dependent on PLD1 in BALB/c mice

The panels show TNFα (5 μg/mouse) induced (i.p) VCAM expression pattern using immunohistochemistry in the peritoneal tissues after 2 hours of TNFα administration. The panels indicate the peritoneal tissues from control mice (CONTROL); from mice injected with TNFα (TNF); from mice injected with siRNA-PLD1 prior to TNFα administration (siRNA-PLD1 + TNF) and from mice injected with scrambled siRNA prior to TNFα administration (scrambled siRNA + TNF). Results shown are representatives of multiple sections and fields from three different mice for each category.

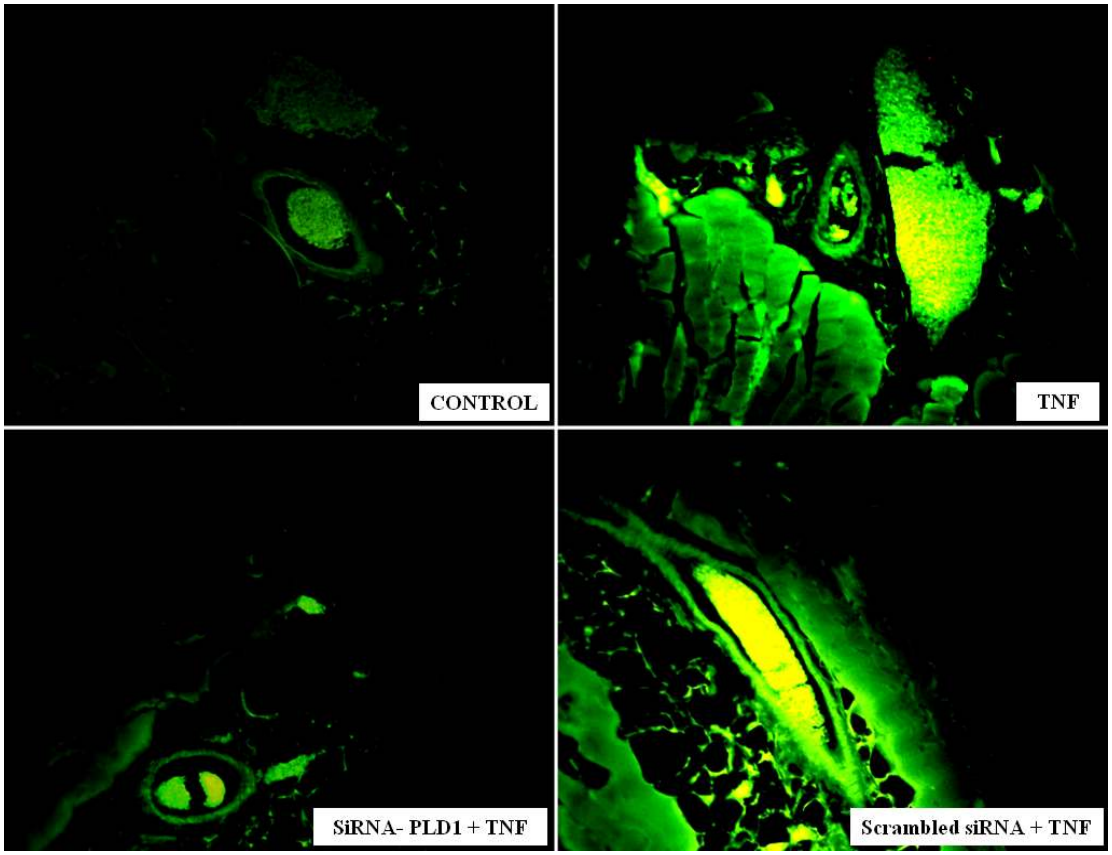


Figure 4.11: TNFα–induced ICAM1 expression in peritoneal tissues is dependent on PLD1 in BALB/c mice

The panels show TNFα (5 μg/mouse) induced (i.p) ICAM1 expression pattern using immunohistochemistry in the peritoneal tissues after 2 hours of TNFα administration. The panels indicate the peritoneal tissues from control mice (CONTROL); from mice injected with TNFα (TNF); from mice injected with siRNA-PLD1 prior to TNFα administration (siRNA-PLD1 + TNF) and from mice injected with scrambled siRNA prior to TNFα administration (scrambled siRNA + TNF). Results shown are representatives of multiple sections and fields from three different mice for each category.

4.3 Discussion

The PLD isoform expression profile in BALB/c mice has been reported in detail, including its differential expression in the various organs and tissues (Kim et al., 2007). However, the expression profiles in murine leukocytes were not reported and we have confirmed the presence of both the isoforms of PLD in murine peripheral blood leukocytes of this background. In the present study using a peritonitis model in mice, we have validated the function of PLD1 in the mediation of TNFα–induced inflammatory process *in vivo*. This is in concordance with our earlier *in vitro* finding that PLD1 mediates TNFα-triggered responses (Chapter 3). This study is the first study which validates the *in vivo* relevance of PLD isoform in the process of inflammatory responses. Increase in body temperature is a systemic response to infection or inflammation and it is known to be mediated by endogenous pyrogens like TNFα and IL-6 (Zanetti et al., 1992). It has also been reported that TNFα–induced pyrexia in mice was found to be mediated by IL-6 (Sundgren-Andersson et al., 1998). Our study shows that TNFα-induced pyrexia was inhibited when PLD1 was knocked down. We have also shown that PLD1 is necessary for TNFα-induced IL-6 production in both *in vitro* (Chapter 3) and *in vivo*. The inhibition of TNFα–induced increase in body temperature could be due to the decrease in the levels of IL-6. Therefore, we can suggest that PLD1 mediates TNFα–induced pyrexia by modulating the production of IL-6. The production of proinflammatory cytokines are one of the characteristic features of inflammation. Our study has vividly shown that PLD1 is essential in TNFα (i.p) induced systemic and local increase in the levels proinflammatory cytokine and/or chemokines which are important in the amplification of

the inflammatory process. This we suggest, based on the fact that the knock down of PLD1 *in vivo* prevented the TNFα-triggered increase in levels IL-6, MIP-1α and MIP-1β both in the peritoneal lavage and serum. IL-6, apart from modulating homeostatic functions like proliferation, differentiation, survival and apoptosis also plays a major role in the amplification of inflammatory responses (Kamimura et al., 2003; Kishimoto, 2006). Its level is reported to rapidly increase during inflammatory responses due to injury, infection and stress (Kamimura et al., 2003; Kishimoto, 2006). Increased or abnormal IL-6 levels are associated with a plethora of inflammatory conditions like inflammatory bowel disease, multiple myeloma, rheumatoid arthritis and some autoimmune diseases (Grisius et al., 1997; Hirano et al., 1988; Holtkamp et al., 1995; Kawano et al., 1988; Stuart et al., 1995; Wong et al., 2001). IL-6 level was also been found to be increased in C5a mediated peritonitis is mice in an earlier study (Vlasenko and Melendez, 2005). Decrease in the levels or effects of IL-6, a key amplifier of inflammatory process are being considered as an option to reduce inflammation associated morbidity. MIP-1α (Macrophage inflammatory protein -1 alpha) and MIP-1β (Macrophage inflammatory protein -1 beta) produced in response to inflammatory stimuli contribute to the process by modulating responses like chemotaxis, degranulation, phagocytosis and signaling mediator synthesis (Maurer and von Stebut, 2004). Dysregulation of MIP-1 has been associated with inflammatory disorders including arthritis (Maurer and von Stebut, 2004; Murdoch and Finn, 2000). Inhibiting PLD1 can reduce the concentrations of these cytokines. Thus, reducing the latter's adverse effect on the injured or inflamed tissue. The next important feature of an inflammatory response followed by the increase in the levels of proinflammatory cytokines is cellular adhesion

and cell migration into the inflamed tissue. Neutrophil migration and cellular chemotaxis have been reported to be mediated by PLD (Cui et al., 1997; Hayes et al., 1999; Lehman et al., 2006). We have shown that there has been an increase in the infiltration of neutrophils to the inflamed peritoneum as shown by the neutrophil counts in the peritoneal lavage and cellular migration by H and E staining in the inflammed region of the peritoneal tissue. This response was found to be mediated by PLD1. Cell adhesion is a stage which occurs prior to cellular migration and PLD has been implicated in the process of cell adhesion in phagocytes (Iyer et al., 2006). This is mediated by the cell adhesion molecules consisting of Integrins. Cell adhesion molecules also plays a role in the amplification of the inflammatory process by regulating and aiding in leukocyte migration (Granger and Kubes, 1994). Intercellular adhesion molecule -1 (ICAM-1) and vascular cell adhesion molecules (VCAM) expressed in immune cells and endothelial cells are known to be upregulated in inflammatory conditions (Koizumi et al., 1992; Staunton et al., 1989). CAMs are therefore being targeted in the treatment for inflammatory disorders. We have observed in this study that the increase in ICAM-1 and VCAM expression in the peritoneal tissue following TNFα administration is dependent on PLD1. In other words CAM expressions were downregulated when PLD1 was inhibited. A study reported that LPS induced cell adhesion is mediated by ICAM-1 and VCAM was regulated by ERK1/2 and NFκB but was not affected by p38 and JNK (Ogawa et al., 2003). This corroborates our earlier finding that PLD1 selectively mediates TNFα–induced responses including proinflammatory cytokines via ERK1/2 and NFκB (Sethu et al., 2008). Therefore, we can suggest PLD1 dampens the inflammatory process by inhibiting the amplifying responses by interfering with TNFα-triggered ERK1/2 and

NFκB activation. It is an accepted fact that proinflammatory cytokine production and phagocytic cells infiltration mediate and amplify the inflammatory process. Targeting these events will aid in the dampening of any aberrant inflammation. In this accord, our earlier *in vitro* study and the present *in vivo* study strongly showcase the potential for PLD1 as a novel therapeutic target in the management of TNFα associated inflammatory conditions.

CHAPTER 5

Sphingosine Kinase1 mediates TNFα–induced inflammatory response *in vivo*

5.1 Introduction

Over the recent past, Sphingolipids have emerged as key contributors in the cellular signaling process, in addition to their structural role in eukaryotic cell membranes. Sphingolipids influence signaling cascades by their potent bioactive metabolites like Ceramide and Sphingosine – 1 – phosphate (S1P) which regulates cellular homeostasis like cellular proliferation, differentiation and apoptosis (Melendez, 2008; Spiegel and Milstien, 1995; Spiegel and Milstien, 2002; Spiegel and Milstien, 2003). S1P mediates signaling as an intracellular second messenger and as an extracellular ligand to specific receptors (Spiegel and Milstien, 2002; Spiegel and Milstien, 2003). S1P is generated by the specific phosphorylation of Sphingosine by an enzyme called Sphingosine Kinase (SphK). Two mammalian highly conserved *SPHK* isoforms (*SPHK* 1 and *SPHK* 2) has been identified, cloned and characterized (Kohama et al., 1998; Liu et al., 2000; Melendez et al., 2000). The SphK isoforms are associated with distinct cellular functions due to their differing kinetic properties, tissue distribution and temporal expression patterns (Spiegel and Milstien, 2003).

Increase in SphK activity with S1P production and associated responses like intracellular calcium release, cytokine – chemokines production, chemotaxis, super-oxide

production, degranulation and increase in the expression of cell adhesion molecules following the activation of plasma membrane receptors like FcγR1 (Melendez et al., 1998b), FcεR1 (Melendez and Khaw, 2002), fMLP receptor (Alemany et al., 1999), C5aR (Ibrahim et al., 2004; Melendez and Ibrahim, 2004), TNFR (Xia et al., 1998; Zhi et al., 2006) and PDGF receptors (Olivera et al., 1999) in immune cells have been reported. SphK has also been strongly implicated in T cell activation and migration (Matloubian et al., 2004; Pappu et al., 2007). Based on these reports it is quite evident that SphK in response to inflammatory stimuli triggers proinflammatory cytokine production, cellular migration and activation of vascular endothelial cells, thereby, associating it with inflammatory responses. SphK and S1P have been associated with inflammatory conditions and autoimmune conditions including rheumatoid arthritis (Kappos et al., 2006; Kitano et al., 2006; Sekiguchi et al., 2008).

Inflammatory disorders have been associated with a potent pro-inflammatory cytokine, Tumor Necrosis Factor alpha (TNFα) (DeVries et al., 1999; Matsumoto and Kanmatsuse, 1999; Palladino et al., 2003; Plo et al., 2000; Tracey and Cerami, 1993; Tracey and Cerami, 1994). It is produced by a variety of cells including immune cells and functions both in an autocrine and paracine way through its receptors. TNFα elicits a wide spectrum of cellular responses like proliferation, differentiation, mediation of inflammation and regulation of immune responses (Chen and Goeddel, 2002; MacEwan, 2002a). Its immunomodulatory and inflammatory role is brought about by the production of various cytokines, activation of leukocytes and lymphocytes, enhancing the expression of adhesion molecules, initiating adherence of neutrophils and monocytes to the endothelium and promoting inflammatory cell migration (Baud and Karin, 2001;

Hehlgans and Mannel, 2002; MacEwan, 2002a; Tracey and Cerami, 1993; Tracey and Cerami, 1994). Dysregulation in TNFα production or signaling has been associated with inflammatory diseases such as multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus and inflammatory bowel diseases (Aggarwal, 2003; Chen and Goeddel, 2002; Tracey and Cerami, 1993; Tracey and Cerami, 1994). In an attempt to identify strategies to reduce TNFα associated morbidity in inflammatory conditions, TNFα blockade by decreasing its level and also by inhibiting its molecules involved in the TNFα-triggered signaling pathways such as PDE4, p38 MAP-kinase and NFκB inhibitors are currently being explored (Palladino et al., 2003). SphK has emerged as one of the potential targets in this regard to dampen TNFα associated inflammatory response as it is known to mediate TNFα-induced intracellular signaling and associated responses in immune cells (Xia et al., 1998). In our recent study we have reported that SphK1 is coupled to TNFα signaling events and associated inflammatory responses *in vitro* (Zhi et al., 2006). SphK modulation has been reported to manage inflammatory condition like rheumatoid arthritis (Lai et al., 2008b), asthma (Lai et al., 2008a) and C5a induced peritonitis (Pushparaj et al., 2008) in mice. However, the direct the role of SphK1 in TNFα-mediated responses is yet to be validated *in vivo*. In the present study, we investigated whether modulating SphK by specific blockade of SphK1 through RNA interference has an anti inflammatory role in TNFα–induced peritonitis in mice. Here, we have shown that an increase in the inflammatory mediators and responses following intra peritoneal administration of TNFα was suppressed in mice with specific knock down in

the expression of SphK1. This indicates the potential of SphK1 as a target in the management of TNFα associated inflammatory conditions.

5.2 Results

5.2.1 Knock down of mSphK1 *in vivo*

Isoform specific knock down of murine Sphingosine kinase1 (mSphK1) was established by intravenous administration of a siRNA against mSphK1. The dose and administration regimen to obtain a significant knock down of the gene product was based on our earlier experiments (Pushparaj et al., 2008). The details of the methodology are described in detail in Chapter 2 of this thesis. Figure 5.1 exhibits a significant decrease in the levels of mSphK1 in peripheral blood leukocytes. The isoform specific knock down of SphK1 with expression of mSphK2 unaffected using the same siRNA sequence was reported in our earlier study (Pushparaj et al., 2008). Therefore, this result indicates achievement of isoform specific knock down of SphK1 *in vivo*.

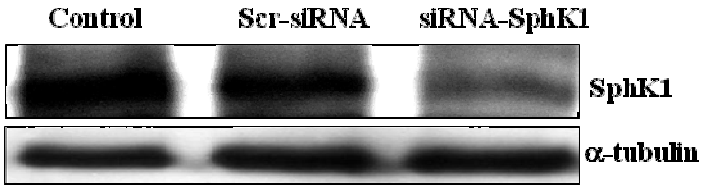


Figure 5.1: Specific knock down of SphK1 in BALB/c mice using siRNA
Western blot analysis shows the knockdown of murine Sphingosine kinase 1 (mSphK1) in BALB/c mice peripheral blood leukocytes, by the use of specific siRNA for SphK1 (4 μg/mouse) administered via intravenous tail vein injection regimens as described earlier. Lysates probed for SphK1 from peripheral blood leukocytes were obtained from, untreated mice which severed as control (Control); from mice injected with scrambled siRNA (Scr-siRNA) and from mice injected with siRNA-SphK1 (siRNA-SphK1). In addition, α-tubulin was probed for loading control. The immunoblot shown here is a representative of three different replicate experiments done. This was performed by obtaining peripheral blood leukocytes lysates from three different mice for each category.

5.2.2 TNFα–induced acute peritonitis in mice is mediated by SphK1

The role of SphK1 in TNFα–induced acute peritonitis model was evaluated by investigating the effects of SphK1 knock down on TNFα (i.p) induced responses like, increase in body temperature, proinflammatory cytokine and chemokines production, cellular infiltration, migration and expression of cell adhesion molecules in the vascular endothelium.

5.2.2.1 Temperature response

Pyrexia or increase in body temperature is a classical systemic response associated with increase in TNFα levels. Therefore, in this study the role of SphK1 in whole body temperature changes in mice injected (intra-peritoneal) with TNFα was investigated. Figure 5.2 indicates the increase in body temperature by a couple of degree celcius following TNFα administration and inhibition in this increase in temperature by one degree celcius in mice pretreated with SphK1 siRNA. The body temperature changes in mice injected with scrambled siRNA was similar to that of the TNFα control mice (Figure 5.2). This indicates that SphK1 modulates this systemic response during an inflammatory reaction.

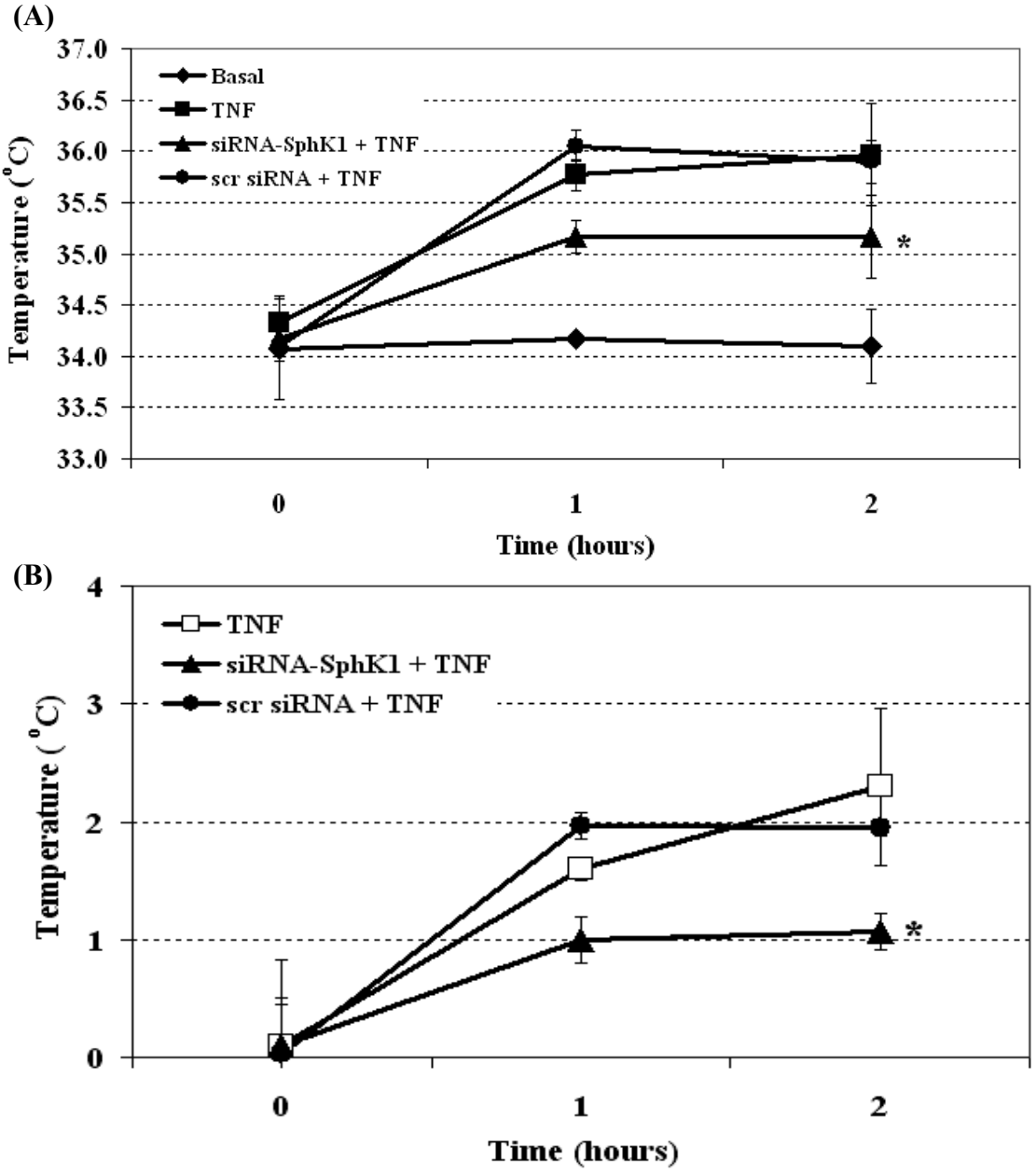


Figure 5.2: SphK1 knock down alters rectal temperature changes triggered by TNFα in BALB/c mice

The graphs indicate TNFα (5 μg/mouse) induced rectal temperature changes observed over the time points indicated. TNFα was administered via intra-peritoneal (i.p) injections. The above indicates, temperature pattern in control mice (♦); temperature changes after TNFα administration (■); temperature changes in mice injected with siRNA-SphK1 (▲) and scrambled siRNA (●) prior to TNFα administration. Graph A indicates changes in actual temperature and graph B indicates difference in temperature compared to that of the untreated controls. Results shown are the mean ± the standard deviation of measurements from three different mice for each category. (*) indicates Student's *t* test *p* value <0.05.

5.2.2.2 Production of proinflammatory cytokine and chemokines

Increase in the levels of proinflammatory cytokines and chemokines are important events in any inflammatory response. It is quite well known that one of the means by which TNFα brings about its amplification of inflammatory process is by stimulating the release and production of proinflammatory cytokines and chemokines. Therefore, we measured the levels of IL-6, MIP-1α and MIP-1β in the peritoneal lavage and in the serum of mice in all the categories. There was a significant increase in the levels of these inflammatory mediators both in the serum and peritoneal lavage of mice treated with TNFα compared to that of their untreated counterparts (Figure 5.3 – 5.5). However, there was an inhibition in the levels of the cytokine and chemokines produced in mice pretreated with siRNA- SphK1 prior to TNFα injection (Figure 5.3 – 5.5). This strongly suggests the role of SphK1 in the production of proinflammatory mediators to an inflammatory stimulus.

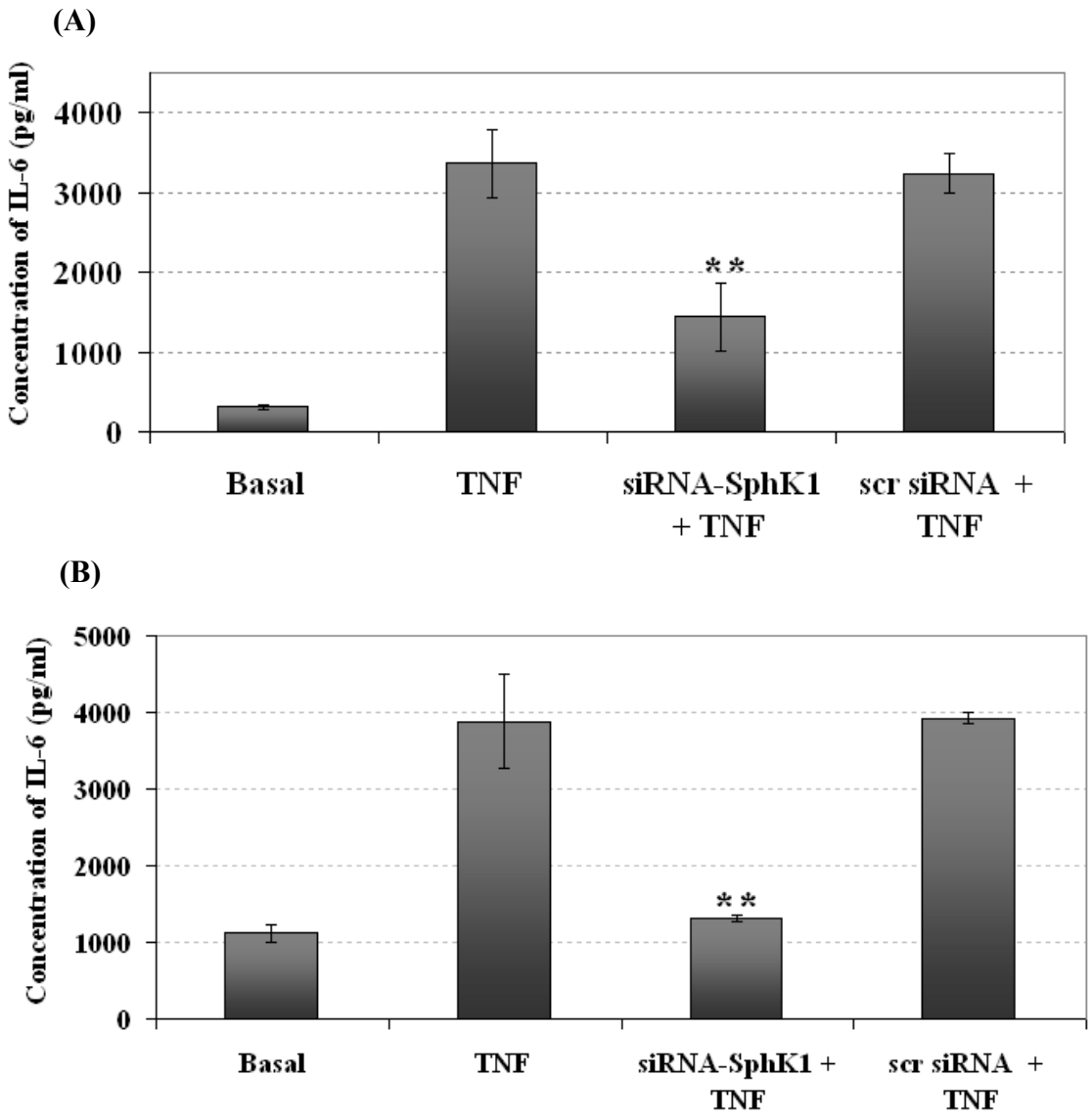
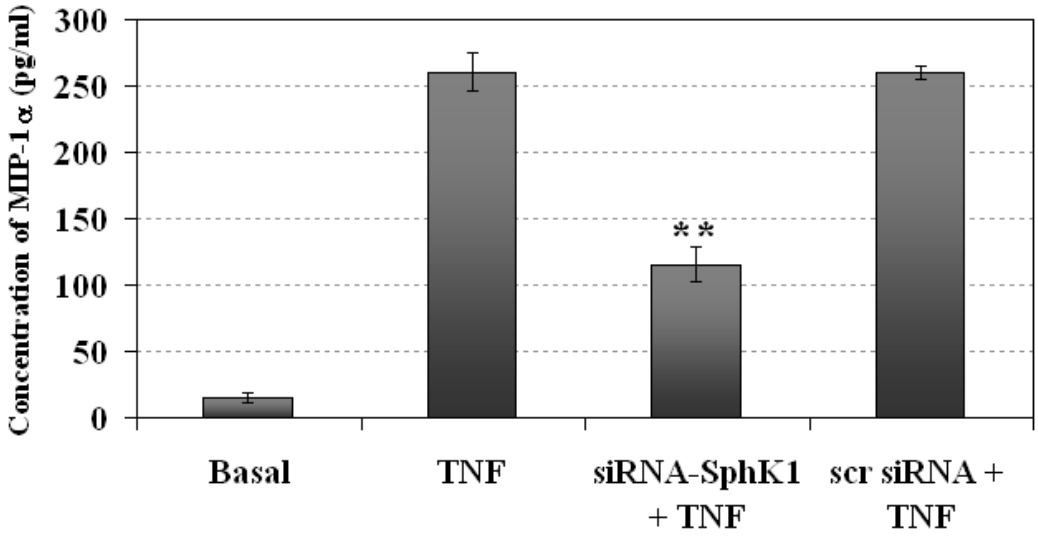


Figure 5.3: TNFα–induced IL-6 release is dependent on SphK1 in BALB/c mice

The graphs indicate the TNFα (5 μg/mouse) induced (i.p) IL-6 release in 2 hours in peritoneal lavage (A) and in serum (B). IL-6 levels in control mice (Basal); in mice injected with TNFα (TNF); in mice injected with siRNA-SphK1 prior to TNFα administration (siRNA-SphK1 + TNF) and in mice injected with scrambled siRNA prior to TNFα administration (scr siRNA + TNF). Results shown are the mean ± the standard deviation of triplicate measurements obtained from samples collected from three different mice for each category. (* *) indicates Student's *t* test *p* value <0.01.

(A)



(B)

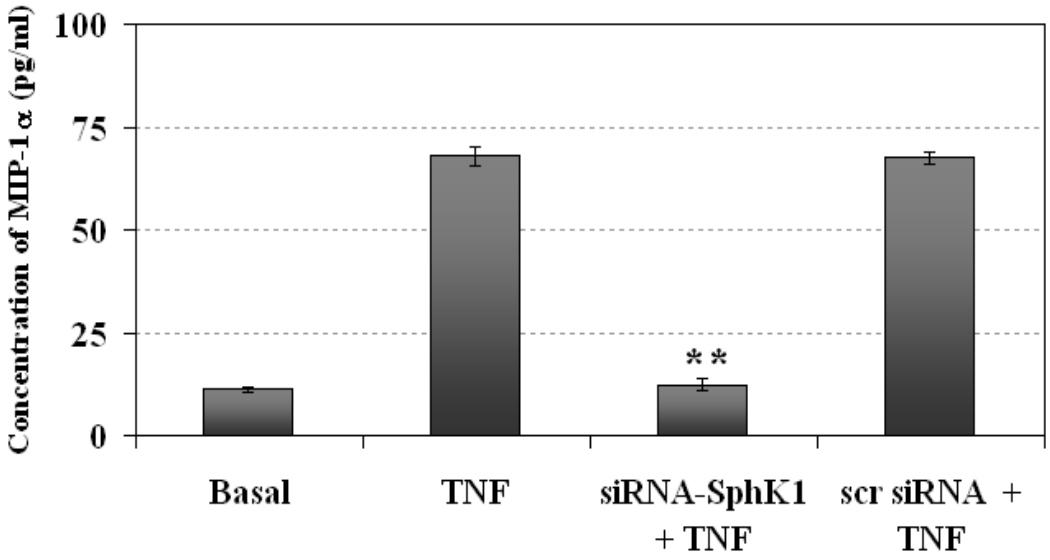


Figure 5.4: TNFα–induced MIP-1α release is dependent on SphK1 in BALB/c mice

The graphs indicate the TNFα (5 μg/mouse) induced (i.p) MIP-1α release in 2 hours in peritoneal lavage (A) and in serum (B). MIP-1α levels in control mice (Basal); in mice injected with TNFα (TNF); in mice injected with siRNA-SphK1 prior to TNFα administration (siRNA-SphK1 + TNF) and in mice injected with scrambled siRNA prior to TNFα administration (scr siRNA + TNF). Results shown are the mean ± the standard deviation of triplicate measurements obtained from samples collected from three different mice for each category. (∗∗) indicates Student's *t* test *p* value <0.01.

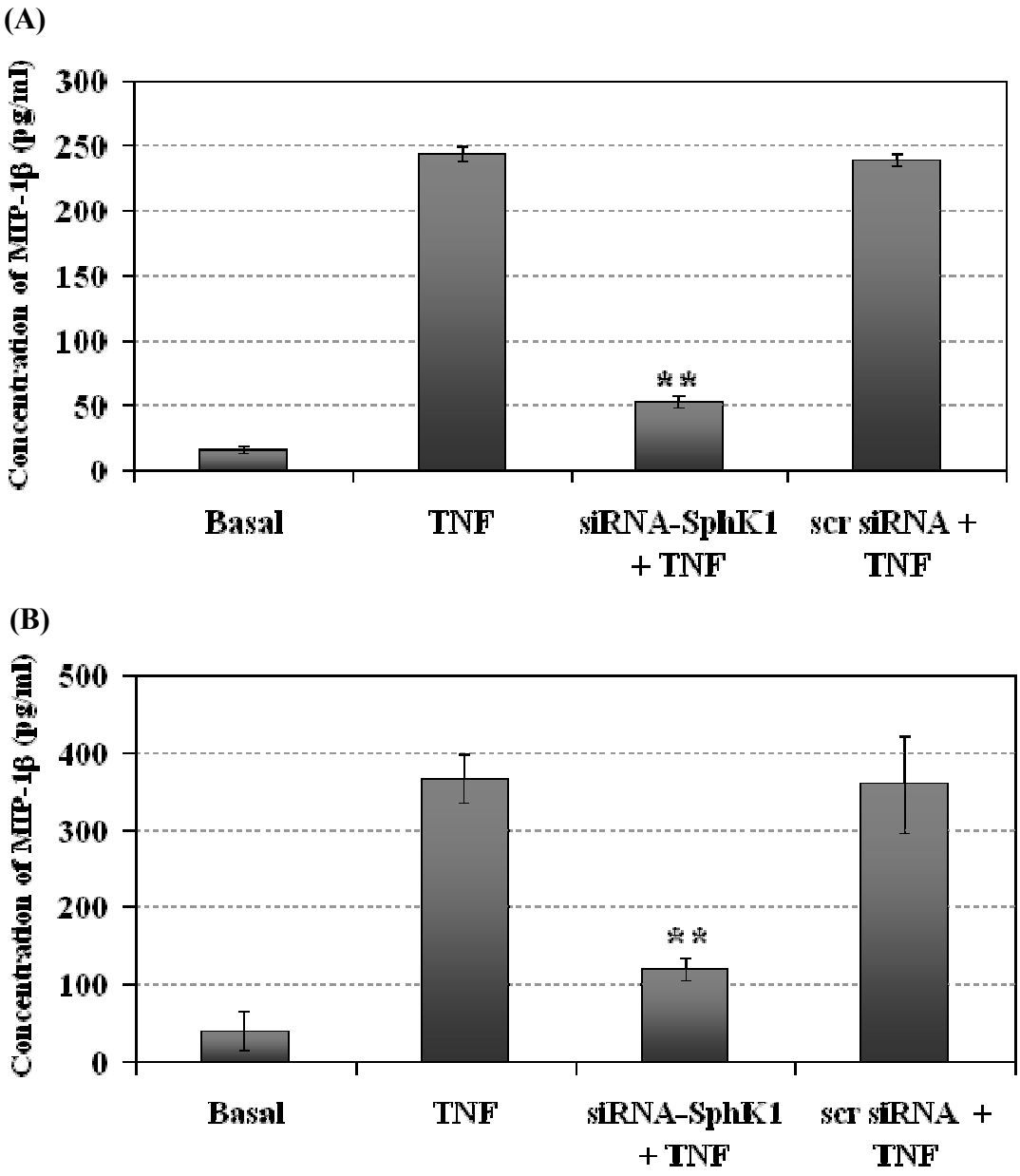


Figure 5.5: TNFα-induced MIP-1β release is dependent on SphK1 in BALB/c mice

The graphs indicate the TNFα (5 μg/mouse) induced (i.p) MIP-1β release in 2 hours in peritoneal lavage (A) and in serum (B). MIP-1β levels in control mice (Basal); in mice injected with TNFα (TNF); in mice injected with siRNA-SphK1 prior to TNFα administration (siRNA-SphK1 + TNF) and in mice injected with scrambled siRNA prior to TNFα administration (scr siRNA + TNF). Results shown are the mean ± the standard deviation of triplicate measurements obtained from samples collected from three different mice for each category. (**) indicates Student's *t* test *p* value <0.01.

5.2.2.3 Cellular infiltration/migration

Increase in vascular permeability and subsequent infiltration of immune cells to the inflammatory region or the site of injury is one of the early events in any inflammatory process. Neutrophils are the first of the immune cells to reach the site of inflammation. Therefore, we investigated the number of neutrophils in the peritoneal lavage at the end of two hours. Figure 5.6 shows a significant increase in the number of neutrophils in the peritoneal lavage of mice injected with TNFα when compared to that of the untreated controls. This indicates the influx of neutrophils into the site of inflammation, the peritoneal cavity. However, the observed increase in neutrophils in the lavage was significantly reduced in mice pretreated with SphK1 siRNA prior to TNFα administration (Figure 5.6).

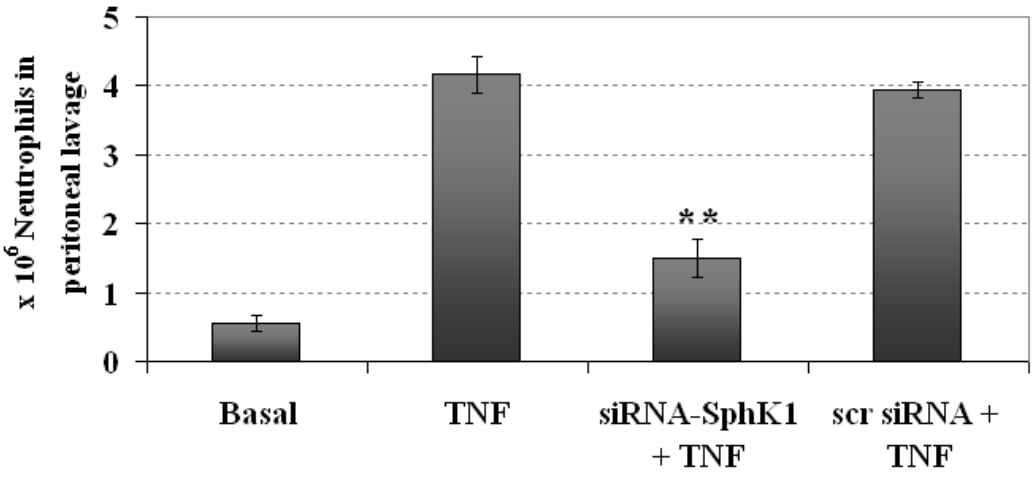


Figure 5.6: Neutrophils infiltration into the peritoneal lavage induced by TNFα is dependent on SphK1 in BALB/c mice

The graphs indicate the TNFα (5 μg/mouse) induced (i.p) neutrophil infiltration in 1.5ml of peritoneal lavage after 2 hours of TNFα administration. Number of neutrophils, in peritoneal lavage of control mice (Basal); in the peritoneal lavage of mice injected with TNFα (TNF); in the peritoneal lavage of mice injected with siRNA-SphK1 prior to TNFα administration (siRNA-SphK1 + TNF) and in the peritoneal lavage of mice injected with scrambled siRNA prior to TNFα administration (scr siRNA + TNF). Results shown are the mean ± the standard deviation of triplicate measurements of lavage samples collected from three different mice for each category. (**) indicates Student's *t* test *p* value <0.01.

We further evaluated the role of SphK1 in cellular migration patterns at the site inflammation induced by TNFα using H and E staining of the inflamed peritoneal tissues. There was increase in the cellular infiltration in the peritoneal tissues of mice treated with TNFα intra-peritoneal injection (Figure 5.7). The peritoneal tissues obtained from mice with SphK1 knock down prior to TNFα injection exhibited a marked decrease in the cellular infiltration pattern observed earlier (Figure 5.7).

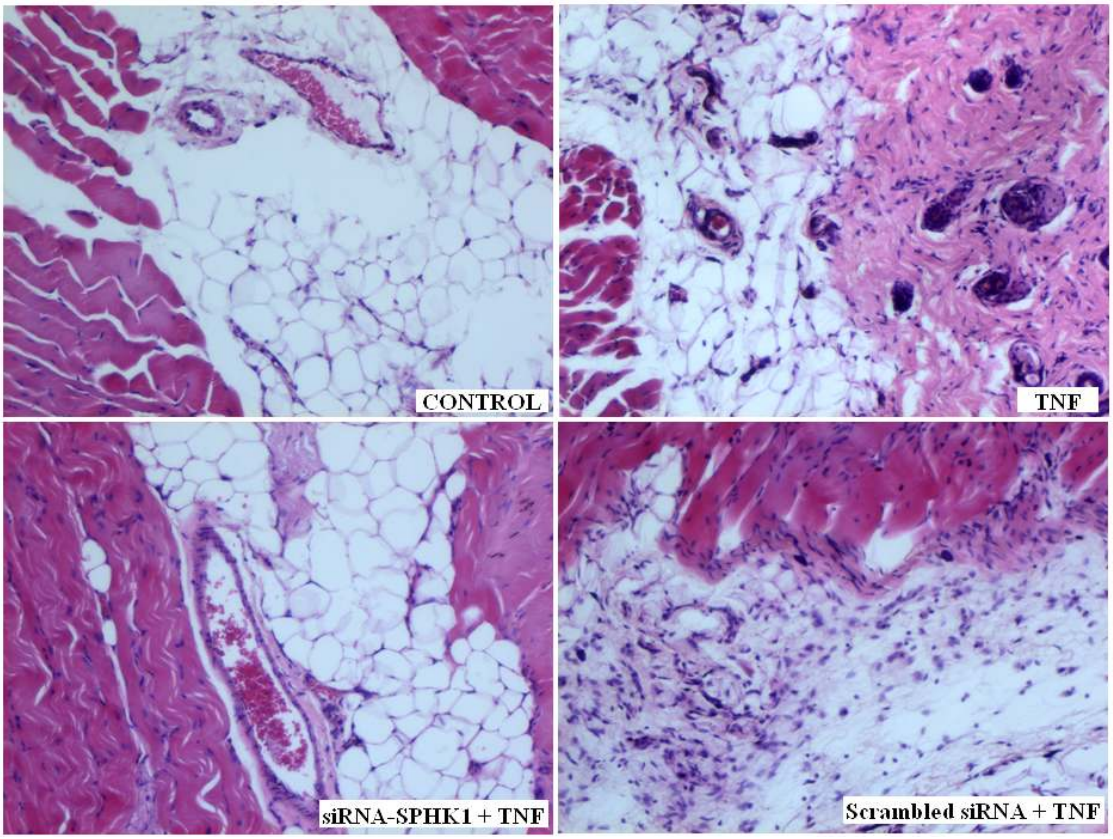


Figure 5.7: Cellular infiltration pattern in peritoneal tissue induced by TNFα is dependent on SphK1 in BALB/c mice
The panels show TNFα (5 μg/mouse) induced (i.p) cellular infiltration pattern using H and E staining in the peritoneal tissues after 2 hours of TNFα administration. The panels indicate the peritoneal tissues from control mice (CONTROL); from mice injected with TNFα only (TNF); from mice injected with siRNA-SphK1 prior to TNFα administration (siRNA-SPHK1 + TNF) and from mice injected with scrambled siRNA prior to TNFα administration (scrambled siRNA + TNF). Results shown are representatives of multiple sections and fields from three different mice for each category.

5.2.2.4 Expression of cell adhesion molecules

Cell adhesion molecules on both the vascular endothelium and the leukocytes play an important role in facilitating the cellular migration and infiltration of immune cells to the site of inflammation. Therefore, the expressions of ICAM1 and VCAM on the vascular endothelium following TNFα-induced inflammation were investigated using immunohistochemistry (IHC) in the peritoneal tissues of mice in our study model. TNFα administration induced an increase in the expression of VCAM and ICAM1 (Figure 5.8 and 5.9 respectively) on the vascular endothelial cells in the peritoneal tissues obtained. We have also reported that in the peritoneal tissues obtained from mice injected with siRNA - Sphk1 prior to TNFα administration, there was decrease in the expression of these cell adhesion molecules (Figure 5.8 and 5.9). These set of results clearly indicate the influence of SphK1 in cellular infiltration and migration in an inflammatory response.

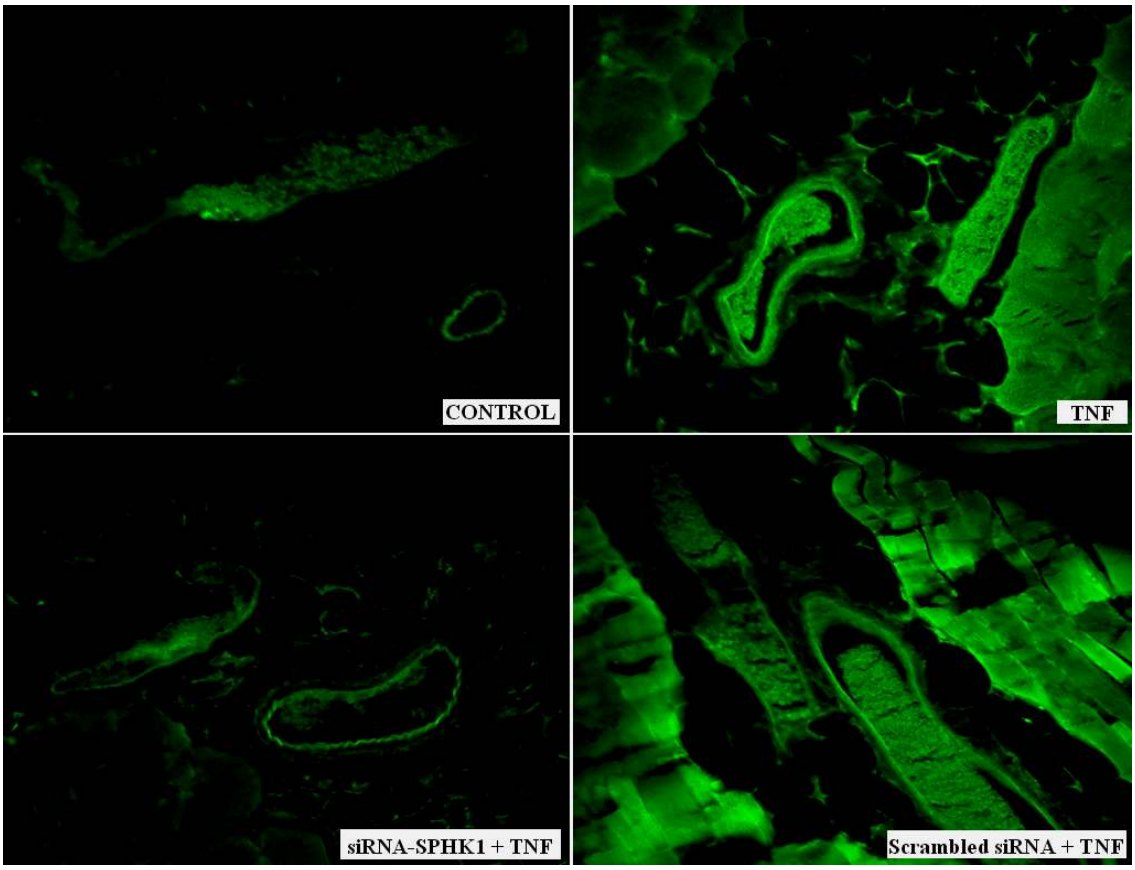


Figure 5.8: TNFα-induced VCAM expression in peritoneal tissues is dependent on SphK1 in BALB/c mice

The panels show TNFα (5 μg/mouse) induced (i.p) VCAM expression pattern using immunohistochemistry in the peritoneal tissues after 2 hours of TNFα administration. The panels indicate the peritoneal tissues from control mice (CONTROL); from mice injected with TNFα only (TNF); from mice injected with siRNA-SphK1 prior to TNFα administration (siRNA-SPHK1 + TNF) and from mice injected with scrambled siRNA prior to TNFα administration (scrambled siRNA + TNF). Results shown are representatives of multiple sections and fields from three different mice for each category.

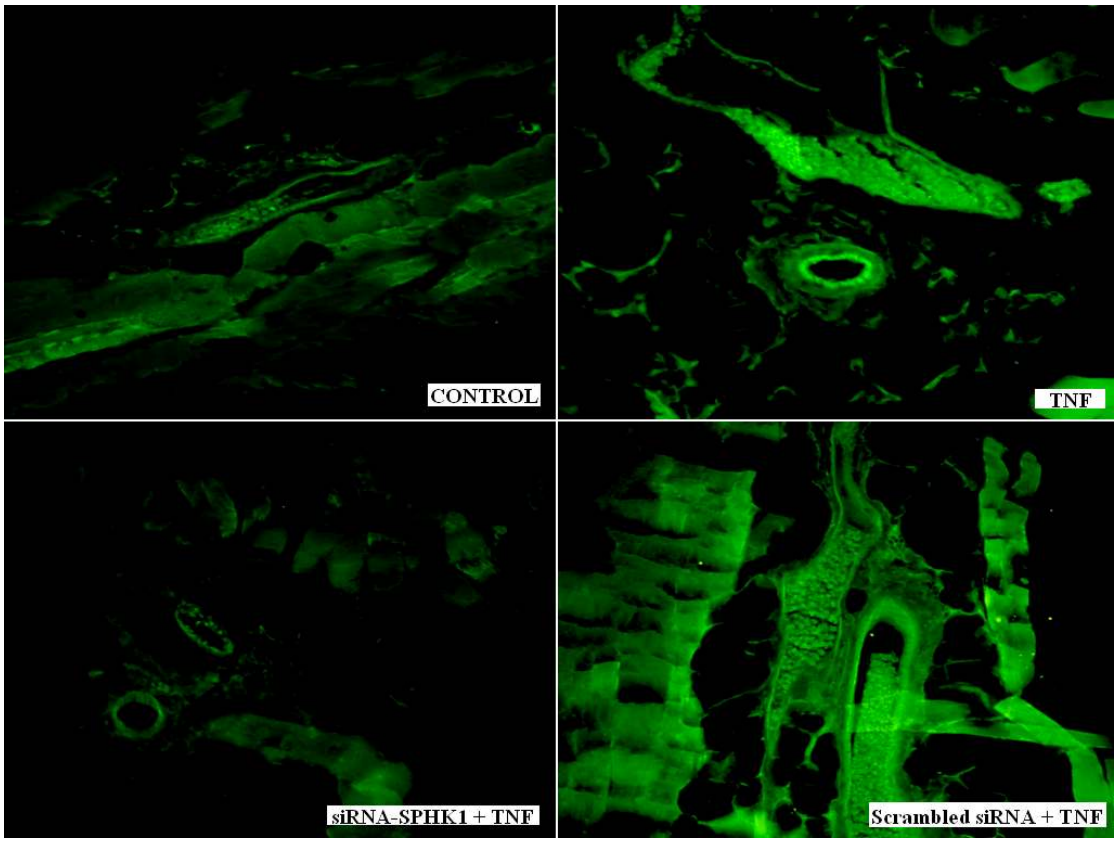


Figure 5.9: TNFα–induced ICAM1 expression in peritoneal tissues is dependent on SphK1 in BALB/c mice

The panels show TNFα (5 μg/mouse) induced (i.p) ICAM1 expression pattern using immunohistochemistry in the peritoneal tissues after 2 hours of TNFα administration. The panels indicate the peritoneal tissues from control mice (CONTROL); from mice injected with TNFα only (TNF); from mice injected with siRNA-SphK1 prior to TNFα administration (siRNA-SPHK1 + TNF) and from mice injected with scrambled siRNA prior to TNFα administration (scrambled siRNA + TNF). Results shown are representatives of multiple sections and fields from three different mice for each category.

5.3 Discussion

SphK and its metabolite S1P have been implicated in a wide range of physiological and pathological responses, including those associated with the immune system and inflammatory responses (Hla, 2004; Hla and Maciag, 1990; Kappos et al., 2006; Kitano et al., 2006; Melendez, 2008; Sekiguchi et al., 2008). The pivotal role of SphK and S1P in inflammation has been widely established and was elegantly summarized in a recent review (Melendez, 2008). Aberrant inflammatory responses are mediated by leukocytes. Therefore, modulation in their signaling and responses holds the key in dampening inflammation. Extensive studies have implicated the role of SphK in intracellular signaling and responses in leukocytes such as, neutrophils (Ibrahim et al., 2004; Niwa et al., 2000; Vlasenko and Melendez, 2005), mononuclear cells like monocytes, macrophages (Melendez et al., 1998b; Melendez and Ibrahim, 2004; Zhi et al., 2006), mast cells (Choi et al., 1996; Melendez and Khaw, 2002) and T lymphocytes (Lai et al., 2008a; Matloubian et al., 2004; Pappu et al., 2007). Infiltration of phagocytic cells and production of proinflammatory cytokines are characteristic features of any inflammatory disorder. Targeting or interfering with these events will aid in the dampening of aberrant inflammation.

Studies have also shown that blockade of SphK reduced the severity of inflammatory response by inhibiting the production of proinflammatory cytokines (Ibrahim et al., 2004; Lai et al., 2008a; Lai et al., 2008b; Melendez et al., 1998b; Melendez and Ibrahim, 2004; Zhi et al., 2006) and chemotaxis (Ibrahim et al., 2004; Melendez and Ibrahim, 2004). The role of SphK in inflammation and immune response *in vivo* has been reported with SphK inhibitors (Lee et al., 2004a; Vlasenko and

Melendez, 2005). Though isoform specific role of SphK has been reported to be associated with immune and inflammatory response, the selection of isoforms to specific responses remains rather unclear. SphK1 has been selectively associated with immune responses in FcγR1, FcεR1, C5a, TNFα associated signaling and responses (Ibrahim et al., 2004; Melendez et al., 1998b; Zhi et al., 2006). However, a recent report has indicated a role of SphK2 in mast cell activation (Olivera et al., 2007). In addition, it is currently reported that SphK2 specifically plays a role in the phosphorylation of FTY720 (Allende et al., 2004; Zemann et al., 2006), a sphingosine analogue currently in the clinical trials for the management of renal transplantation and multiple sclerosis (Kieseier et al., 2007; Tedesco-Silva et al., 2005). Both SphK1 and SphK2 have been reported to be involved in allergic responses in knock out models (Olivera et al., 2007). Other *in vivo* studies that used RNA interference method have proposed a role of SphK1 modulation to manage inflammatory condition like rheumatoid arthritis (Lai et al., 2008b) and asthma (Lai et al., 2008a) are in agreement with our current *in vivo* study. However, a study has reported an interesting but contradictory findings that SphK1 and/or SphK2 were found to be dispensable in both chronic and acute inflammatory responses in knock out mice models (Michaud et al., 2006; Zemann et al., 2007). This could be due to the action of a potential compensatory mechanism initiated in response to the absence of either SphK isoform during embryonic development, which may not be the case when it comes to transient *in vivo* knock down studies using short interfering RNA. However, more studies are necessary to understand and confirm the isoform specific roles of SphK in inflammatory reactions and other immune responses *in vivo*.

In the present study, we have validated the function of SphK1 in the mediation of TNFα-induced inflammatory process *in vivo* using a TNFα induced peritonitis model in mice. This is in concordance with our earlier *in vitro* finding that SphK1 mediates TNFα triggered responses (Zhi et al., 2006). One of the systemic responses to infection or inflammation is pyrexia or an increase in body temperature. Endogenous pyrogens like TNFα and IL-6 are known to mediate this systemic response (Zanetti et al., 1992) and TNFα-induced pyrexia was found to be mediated by IL-6 in mice (Sundgren-Andersson et al., 1998). This study exhibited that a decrease in the expression of SphK1 inhibited this response. The reduction in TNFα–induced pyrexia could be due to the decrease in the levels of IL-6 as we have also shown that TNFα–induced IL-6 production is SphK1 dependent both *in vitro* (Zhi et al., 2006) and *in vivo* in this study. It can be suggested that SphK1 regulates TNFα-induced body temperature changes by modulating IL-6 levels. Increases in the levels of proinflammatory cytokines are one of the classical features in an inflammatory response. We have clearly shown in this study that TNFα-induced systemic and local rise in the levels proinflammatory cytokine and chemokines which are important in the amplification of the inflammatory process are mediated by SphK1. This is based on the results that TNFα-induced increase in levels of IL-6, MIP-1α and MIP-1β in the serum and peritoneal lavage was reduced with the decrease in the expression of SphK1 *in vivo*. IL-6 plays a role in the regulation of homeostatic functions like proliferation, differentiation, survival, apoptosis and in the amplification of inflammatory responses (Kamimura et al., 2003; Kishimoto, 2006). There is a rapid increases in its levels during inflammatory responses due to injury, infection or stress

(Kamimura et al., 2003; Kishimoto, 2006). Increased or abnormal IL-6 levels are associated with a inflammatory conditions like inflammatory bowel disease, multiple myeloma, rheumatoid arthritis and some autoimmune diseases (Grisius et al., 1997; Hirano et al., 1988; Holtkamp et al., 1995; Kawano et al., 1988; Stuart et al., 1995; Wong et al., 2001). An increase in the level of IL-6 in C5a induced peritonitis in mice in an earlier study was also found to be mediated SphK (Vlasenko and Melendez, 2005). Reductions in the levels or effects of IL-6 are being considered as an option to reduce inflammation associated morbidity. MIP-1α (Macrophage inflammatory protein -1 alpha) and MIP-1β (Macrophage inflammatory protein -1 beta) are produced in response to inflammatory stimuli. They contribute to the inflammatory process by modulating chemotaxis, degranulation and phagocytosis and signaling mediator synthesis (Maurer and von Stebut, 2004). Dysregulation of MIP-1 has been associated with inflammatory disorders including arthritis (Maurer and von Stebut, 2004; Murdoch and Finn, 2000). Inhibiting SphK1 can reduce the levels of these chemokines; thereby reducing it's adverse effect on the affected tissues. Neutrophils and monocyte migration or chemotaxis in response to inflammatory stimuli have been reported to be mediated by SphK (Ibrahim et al., 2004; Vlasenko and Melendez, 2005). Here, the results show an increase in the infiltration of neutrophils to the inflamed peritoneum as seen by the neutrophils counts in the peritoneal lavage and leukocyte infiltration by H and E staining of the peritoneal tissue was dependent on SphK1. Cellular adhesion to the vascular wall is an important feature assisting cellular migration into the site of inflammation. This is mediated by the cell adhesion molecules (CAMs) consisting of Integrins. Cell adhesion molecules also play a role in the amplification of inflammatory processes by regulating and aiding in

leukocyte migration (Granger and Kubes, 1994). Intercellular adhesion molecule -1 (ICAM-1) and vascular cell adhesion molecules (VCAM) expressed in immune cells and endothelial cells are known to be upregulated in inflammatory conditions (Koizumi et al., 1992; Staunton et al., 1989). CAMs are therefore being targeted in the treatment for inflammatory disorders. It has been observed in this study that the increase in ICAM-1 and VCAM expression in the peritoneal tissue following TNFα administration is dependent on SphK1. Studies have reported that LPS or TNFα–induced cell adhesion molecules expressions are regulated by ERK1/2 and NFκB (Ogawa et al., 2003; Xia et al., 1998). An earlier finding adds more specifics by reporting that SphK1 is the isoform that selectively mediates TNFα–induced responses including proinflammatory cytokine and cell adhesion molecule expression via NFκB (Zhi et al., 2006). Therefore, based on all the reports it can be proposed that SphK1 dampens the inflammatory process by inhibiting the amplifying responses by interfering with TNFα-triggered intracellular calcium release and NFκB activation. The present study along with an earlier *in vitro* study (Zhi et al., 2006) and other related reports clearly indicates that SphK1 is associated with the TNFα induced systemic response, proinflammatory mediators production and cellular infiltration *in vivo*. Any agent which can bring about specific inhibition of SphK1 is bound to possess the potential as a therapeutic agent in the management of the inflammatory conditions.

CHAPTER 6

6. Conclusions

Aberrant inflammatory responses have always been a major etiological factor for the onset and progression of a range of immune mediated inflammatory disorders. Since, TNFα was found to be strongly associated with inflammatory conditions, therapeutic management of such inflammatory disorders primarily targeted the production and action of TNFα. However, this strategy had certain adverse effects like increase in susceptibility to infections and resistant to therapy in certain patients. Therefore, the search for the alternative approaches resulted in targeting the signaling mediators which are involved in the amplification of inflammatory signals and resulting responses. For example, targeting signaling mediators like MAPKs and transcription factors has been shown to be advantageous. In this study, we have investigated the role of two important lipid based signaling mediators (PLD and SphK) in TNFα-mediated intracellular signaling and associated effector responses.

Early reports have clearly proven the pivotal roles of PLD and SphK in immune cell signaling and responses. Observation from the current *in vitro* studies in both monocytic cell line and primary monocytes have revealed that PLD mediates key TNFα–induced signaling events including calcium release, MAPKs and NFκB activation. The resulting inflammatory responses like pro-inflammatory cytokines (IL-6 and IL-1β) and chemotaxis were also found to be PLD dependent. PLD was associated with selective activation of ERK1/2 by mediating the translocation of Raf-1 to the cell

membrane following TNFα stimulation. However, activation of p38 kinase was not found to be dependent on PLD. Although, the activation of the transcription factor NFκB, well known to be associated with inflammatory responses, was shown to be dependent on PLD. In particular, this study has shown that the translocation of NFκB subunits into the nucleus, indicative of its activation was inhibited when the PLD activity was blocked. This study also exhibited that PLD was necessary for the degradation of IκBα which is highly relevant for facilitating the translocation of the NFκB subunits from the cytosol into the nucleus. Interestingly, an *in vitro* study has reported the role of SphK1 in TNFα signaling and responses in monocytes. Here, in this study we showed that PLD was upstream of SphK. Collectively, therefore it can be suggested that PLD mediates its signaling through SphK.

It was observed in this study that TNFα–induced the selective translocation of PLD1 but not PLD2 from the cytosol to the cell membrane. Our antisense based experiments, following up on this observation designed to determine the isoform specific role of PLD in TNFα signaling and responses, showcased the coupling of PLD1 to the signaling events and responses. Having explored the role of PLD1 in TNFα responses in a relevant cell line and a primary cell model, we furthered this study by investigating the role of PLD1 *in vivo*. siRNA based *in vivo* experiments revealed that TNFα-induced peritonitis in BALB/c mice was found to be mediated by PLD1 complementing our *in vitro* studies.

Earlier work from our team and part of the present investigations reported here has revealed the SphK1 also plays a vital role in the propagation of TNFα–induced

signaling and inflammatory responses. Therefore, experiments were planned to investigate the role of SphK1 *in vivo* using RNA interference. SphK1 was also found to influence TNFα-induced peritonitis in BALB/c mice.

Based on the results obtained in these studies we can propose that interfering with the expression and functions of specific isoforms of PLD or SphK can dampen aberrant inflammation. Therefore, it can be suggested that these lipid signaling mediators can be potential therapeutic targets in the management of immune mediated inflammatory disorders. Furthermore, improved or high therapeutic efficacy can be obtained either by only targeting the appropriate signaling mediators associated with the condition or by targeting these proinflammatory signaling mediators in combination with the current anti-TNFα treatment regimens. The combination strategy should be used appropriately based on the molecular pathogenesis of specific inflammatory conditions to counter any undesired effects triggered by the conventional therapy. The combination of conventional therapy with the inhibition of signaling mediators could be advantageous by possibly reducing the dose of the agents used or by sensitizing the affected individuals to conventional or current anti-TNF therapeutic strategies in the management of inflammatory disorders. However, we strongly suggest that more detailed and multifaceted *in vitro* and *in vivo* work are warranted to facilitate the development and translation of these potential targeting strategies to bed side therapeutic realities.

CHAPTER 7

7. References

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