SPHINGOSINE KINASE 1 REGULATES THE EXPRESSION OF PROINFLAMMATORY CYTOKINES AND NITRIC OXIDE IN ACTIVATED MICROGLIA

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Summary

Microglia, the resident immune cells of the central nervous system (CNS), play a pivotal role in the pathway leading to various neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, prion diseases and HIV-dementia. Activation of microglial cells causes neurotoxicity through the release of a wide array of inflammatory mediators including proinflammatory cytokines, chemokines and reactive oxygen species. Microglial activation has been implicated as one of the causative factors for neuroinflammation in various neurodegenerative diseases. Therefore, suppression of microglia-mediated inflammation has been considered as an important therapeutic strategy for neurodegenerative diseases.

The sphingolipid metabolic pathway plays an important role in inflammation, cell proliferation, survival, chemotaxis, and immunity in peripheral macrophages. In this study, we demonstrate that sphingosine kinase1 (SphK1), a key enzyme of the sphingolipid metabolic pathway, and its receptors are expressed in the BV2 microglial cells and SphK1 alters the expression and production of proinflammatory cytokines and nitric oxide in microglia treated with lipopolysaccharide (LPS). LPS treatment increased the SphK1 mRNA and protein expression in microglia as revealed by the RT-PCR, Western blot and immunofluorescence. Suppression of SphK1 by its

inhibitor, N, N Dimethylsphingosine (DMS), or siRNA resulted in decreased mRNA expression of TNF-α, IL-1β, and iNOS and release of TNF-α and nitric oxide (NO) in LPS-activated microglia. However, addition of sphingosine 1 phosphate (S1P), a breakdown product of sphingolipid metabolism, restored the increased expression levels of TNF-α and IL-1β and production of TNF-α and NO in activated microglia exposed to DMS or transfected with SphK1 siRNA. Hence, to summarize, suppression of SphK1 in activated microglia inhibits the production of proinflammatory cytokines and NO, and the addition of S1P to microglia reverses the suppressive effects. Since the chronic proinflammatory cytokine production by microglia has been implicated in neuroinflammation, modulation of SphK1 and S1P in microglia could be looked upon as a future potential therapeutic method in the control of neuroinflammation in neurodegenerative diseases.

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Abbreviations

BBB – Blood brain barrier

bp- basepairs

CNS- Central nervous system

DAPI- 4'-6-Diamidino-2-phenylindole

DMEM- Dulbeco's modified eagles medium

DMS- N, N Dimethylsphingosine

ELISA- Enzyme-linked immunosorbent assay

FBS- Fetal bovine serum

HRP- Horseradish peroxidase

IF- Immuofluorescence

IFN-γ–Interferon γ

IL-1β - Interleukin 1β

IL-1R1- Interleukin-1 receptor type 1

iNOS- inducible nitric oxide synthetase

kD-kiloDalton

LPS- Lipopolysaccharide

NO- Nitric oxide

PBS- Phosphate buffered saline

RT-PCR- Reverse transcription polymerase chain reaction

SE- Standard error

SphK1, 2- Sphingosine kinase 1 and 2

S1P- Sphingosine-1-phosphate

TE- Trypsin-EDTA

TMB-Tetramethylbenzidine

TNF-α- Tumour necrosis factor α

TNFR1 or 2 – TNF-α receptor type 1or 2

FTY720- Fingolimod

Chapter 1: Introduction

1.1 The central nervous system

The central nervous system (CNS) consists of the brain and spinal cord. Microscopically, the brain has around 1-2 x 10^{11} neurons and many more glial cells (namely-oligodendrocytes, astrocytes, microglia and ependymal cells). The glial cells are capable of dividing mitotically throughout life in contrast to the neurons and are derived from the ectoderm with the exception of the microglia, which is of monocytic lineage. The glial cells are separated from the neurons in the CNS by extracellular fluid by about 10-20nm intercellular space, which comprises about 15-20% of the brain volume. The glial cells do not participate in generating action potentials and have no synapses. The glial cells are subdivided into macroglia and microglia. The macroglia consist of the astrocytes and oligodendrocytes (Noback, 2005).

1.2 Microglia: history and types

Microglial cells were first described by Franz Nissil (Nissil, 1899) as rod cells, whose function was considered to be similar to leukocytes. Ramon Y Cajal (Cajal, 1913) described microglial cells as the 'third element' of the CNS, which refers to a group of cells that are morphologically distinct from the first and second elements, namely neurons and astrocytes. Del Rio-Hortega (P. Río-Hortega, 1920) differentiated this third element into microglia

and oligodendrocytes and was also the first to describe the two types of microglia: amoeboid and ramified.

Microglia, comprising 10-20% of the total glial cell population of the CNS, are the resident macrophage cells within the entire neuroaxis and represent the primary immunocompetent cells that protect against invasions by various routes, be it infectious agents or tumours. True to their macrophage nature, they also remove cellular debris from within the CNS. Thus they act as vigilant guardians of the brain and spinal cord. Although similar to peripheral macrophages, they possess distinguishing electrophysiological and biochemical properties, which make microglia different from the macrophages (Squire, 2008).

Microglia contain lysosomes and vesicles characteristic of macrophages, a sparse endoplasmic reticulum and a few cytoskeletal fibers (Squire, 2008). They usually have small rod shaped somas from which numerous processes extend (Squire, 2008). Processes from different microglia rarely overlap or touch (Squire, 2008). They are found in the CNS within the parenchyma (parenchymal microglia), and in the circumventricular organs.

Microglia exist in different morphological and functional forms (Noback, 2005):

- Resting ramified microglia: They are known as the resident brain macrophages and found in the normal adult CNS. They have finely branched and ramified processes (Noback, 2005).

- Activated amoeboid or reactive nonphagocytic microglia: They are found in areas of secondary reaction as in nerve transection and in CNS inflammation and are capable of producing cytokines (Noback, 2005).
- Phagocytic microglia or reactive phagocytic microglia: They are found in areas of trauma, infection and neuronal degeneration (Noback, 2005).

1.3 Origins of microglia

Microglia are of myelomonocytic lineage and are derived from hemangioblastic mesoderm. They become part of the CNS parenchyma during early embryonic development around the time when neurulation is completed (Streit, 2001). The fetal macrophages (Takahashi, et al., 1989) are known to populate the developing neuroectoderm as early as the 8th embryonic day in rodents (Alliot, et al., 1999). These fetal macrophages are considered to be the earliest detectable microglial precursor cells. With the further development of the CNS in the embryo, the fetal macrophages change from their rounded shape to embryonic microglia, which have short processes. At the perinatal stage, the embryonic microglia change into amoeboid microglia and these cluster around the supraventricular corpus callosum (Hurley, et al., 1999, Ling and Wong, 1993). The amoeboid microglia persist in the corpus callosum for the first two postnatal weeks, migrate into the cerebral cortex and differentiate into fully ramified microglia. A few microglia may also be replaced by

perivascular (space around the medium and small sized cerebral vessels) cells which are mononuclear phagocytes, replaced continuously by bone marrow progenitors (Hickey and Kimura, 1988),

1.4 Functions of microglia

Microglia are involved in clearance of apoptotic cells (Polazzi and Contestabile, 2002) during brain remodeling in embryogenesis and also brain remodeling through their assistant role in synapse stripping and matrix reorganization (Harry and Kraft, 2008). They also participate in the induction of neuronal death in cerebellum during normal development (Marin-Teva, et al., 2004). In the adult brain, microglia are in intimate contact with neurons and serve important maintenance functions and are capable of responding to subtle changes in the microenvironment, (Alemany, et al., 2007, Davalos, et al., 2005, Kreutzberg, 1996, Nimmerjahn, et al., 2005, Raivich, 2005) These cells play a major role in phagocytosis and clearance of aberrant or excess proteins e.g. β amyloid (Harry and Kraft, 2008).

In the CNS injury, microglia actively monitor and control the extracellular environment, walling off areas of the CNS from non-CNS tissue, and remove degenerating and dysfunctional cells (Harry and Kraft, 2008). The activated microglia in response to CNS inflammation secrete pro-inflammatory cytokines such as TNF-α and IL-1β and serve as antigen presenting cells (Carson, et al., 1998, Carson and Sutcliffe, 1999, Frei and Fontana, 1997, Hickey and Kimura, 1988) Increases in TNF-α and IL-1β have

been observed prior to neuronal death (Harry, et al., 2008, Lefebvre d'Hellencourt and Harry, 2005, Matusevicius, et al., 1996) and recent studies suggest that the activation of pro-inflammatory factors such as TNF-α can participate in causation of neuronal death (Harry and Kraft, 2008, Harry, et al., 2008, Kaushal and Schlichter, 2008). In adddtion, microglia produce multiple secreted factors including pro- and anti-inflammatory cytokines, nitric oxide, reactive oxygen species (ROS), glutamate, and growth factors (Harry and Kraft, 2008). Microglia also express the glutamate transporter, GLT-1 and, thus, may provide a level of protection through the elimination of extracellular glutamate (Nakajima, et al., 2001). Microglia can facilitate the apoptosis and phagocytosis of infiltrating T cells through various signaling pathways leading to a subsequent down regulation of microglial immune activation (Magnus, et al., 2002)

Ageing leads to neurodegeneration which might not only be due to a loss of neuroprotective properties, but also the actual loss of microglia (Ma, et al., 2003). This loss of microglia in senescence appeared to be caused by increased intracellular accumulation of iron leading to intracellular oxidative damage (Streit, et al., 2008).

The mechanisms by which the myriad functions and actions of microglia take place need to be studied in order to understand and apply it in possible therapeutic modulations. Hence *in vitro* studies are conducted by activating microglia by various stimuli such as LPS, β-amyloid, and IFN-γ, thrombin and proinflammatory cytokines. LPS, which is an endotoxin, is one

of the components of the outer membrane of gram negative bacteria and is an activator of microglia. LPS has been shown to activate the microglia by crossing the blood-brain barrier (BBB) in areas of loss of structural integrity of the BBB. Such an activation of microglia leads to the expression of proinflammatory cytokines, chemokines and reactive oxygen species that modulate inflammation. The endogenous receptor CD14 on microglial cells is the target for the LPS (Rivest, 2003).

β-amyloid which are present in neurofibrillary tangles and senile plaque in the brain of Alzheimer's disease patients, are known to be surrounded by reactive microglia indicating its potential role in the disease process (Dheen, 2007). Microglia activated by β-amyloid have been known to express proinflammatory cytokines and chemokines such as IL-1β, IL-8, IL-10, IL-12, TNF-α etc (Dheen, 2007).

IFN-γ is another known activator of microglial cells and serves important functions in innate and adaptive immunity (Dheen, 2007). Microglia in murine models show significantly increased myelin phagocytosis, proteolytic enzyme secretion and oxidative stress in response to IFN-γ (Dheen, 2007).

1.5 Sphingolipids

Lipids account for approximately 10% of the weight of the wet brain and half the dry matter of the brain (Sastry, 1985). The complex lipids are of

two types- glycerolipids and sphingolipids. The sphingolipids contain the long chain amino alcohol, sphingosine. The sphingolipids are derived from ceramide, which occurs in large concentrations in the nervous tissue and they include sphingomyelins, cerebrosides, sulfatides and gangliosides (Sastry, 1985).

Sphingomyelin accounts for 4.2-12.5% of the phospholipid content of the brain in various species. The peripheral nerves and the white matter have a higher concentration of sphingomyelin which forms a major component of myelin membrane (Sastry, 1985).

The production and metabolism of sphingolipids occur via *de novo* synthesis and the salvage pathway. The endoplasmic reticulum is the site for the *de novo* synthesis of sphingolipids. Palmitoyl CoA and serine get condensed to form 3-ketosphinganine in the presence of the catalytic action of serine palmitoyl transferase. Next, the 3-ketosphinganine is then reduced by a NADH dependant reductase to produce dihydrosphingosine. Ceramide synthase then adds different lengths of acyl chains to produce dihydroceramide (Ogretmen and Hannun, 2004). This is subsequently desaturated via dihydroceramide desaturase to form ceramide. Ceramide is then phosphorylated by ceramide kinase to ceramide-1-phosphate which is a bioactive sphingolipid. After ceramide formation, the remaining reactions occur in the Golgi apparatus and result in the incorporation of ceramide into glycolipids and sphingomyelin. Sphingolipids can also be recycled and ceramide can be produced by the salvage pathway, in which

glucocerebrosidase and sphingomyelinase breakdown various membrane glycolipids and sphingolipids. Ceramidases remove acyl chain from ceramide substrates and form sphingosine. Sphingosine can be recycled back to ceramide *via* ceramide synthases or, sphingosine can be phosphorylated to sphingosine-1-phosphate (S1P) by sphingosine kinases (Hannun and Obeid, 2008, Olivera, et al., 1998). S1P is dephosphorylated by sphingosine-1-phosphate phosphatase to form sphingosine. The final step in the biosynthesis is the irreversible cleavage of S1P into ethanolamine phosphate and hexadecenal by S1P lyase (Snider, et al., 2010).

Of all the products of sphingolipid synthesis, ceramide, sphingosine and S1P have been established in cell signaling roles. Ceramide has an important role in cellular stress responses such as cell cycle arrest, serum and nutrient deprivation, terminal differentiation, apoptosis and cell senescence (Hannun and Obeid, 2008). It has also been implicated in inflammation and skin homeostasis (Snider, et al., 2010). The action of ceramide on inflammation can be mediated by one of its phosphorylated products ceramide-1-phosphate, which activates phospholipase A2 (Nakamura, et al., 2006). In addition, ceramide-1-phosphate is required for the membrane translocation of phospholipaseA2 and downstream production of PGE2 (Lamour, et al., 2009)

Upon the degradation of ceramide, to sphingosine, S1P is rapidly formed via phosphorylation which then binds to G protein coupled receptors (S1P receptors). S1P has been implicated in myriad cell signaling pathways such as angiogenesis, cell migration and movement, cell survival and

proliferation, cellular architecture, cellular contacts and adhesions, heart development, vascular development, atherogenesis, acute lung injury and acute respiratory distress, tumourogenecity, metastasis, inflammation and immunity (Alemany, et al., 2007, Hait, et al., 2006).

1.6 Sphingosine kinases

1.6.1 Types and Location

Two isoforms of SphKs have been characterized so far: SphK1 and SphK2. In humans, SphK1 is located on chromosome 17 and SphK2 is located on chromosome 19 (Bryan, et al., 2008). SphK1 is present in the cytosol (Kohama, et al., 1998) and unlike Sphk1, the localization of SphK2 is cell type specific (Okada, et al., 2005, Sankala, et al., 2007). Both of the kinases phosphorylate erythro-sphingosine (Sphingosine), dihydrosphingosine and phytosphingosine, which are key sphingolipids (Melendez, 2008). In adult mouse, SphK1 is present abundantly in the spleen, heart, lung and brain, whereas SphK2 is expressed in the brain, kidney and the liver (Liu, et al., 2000). SphK1 translocates from the cytosol to the membrane periphery where it phosphorylates sphingosine into S1P. Sphk1 translocation to the plasma membrane has been shown to be facilitated by calcium and integrin binding protein1 (Jarman, et al., 2010). Another possible mechanism for this translocation is *via* TNF-α by the means of phospholipase D1 dependant mechanism in monocytes (Sethu, et al., 2008)

Many proteins affecting the activity of SphK1 have emerged, which include D-catenin/neural plakophilin- related armadillo repeat protein (Fujita, et al., 2004), aminoacyclase 1(Maceyka, et al., 2004), eukaryotic elongation factor 1A (Leclercq, et al., 2008), filamin A (Maceyka, et al., 2008), sphingosine kinase 1-interacting protein (Lacana, et al., 2002), and platelet endothelial adhesion molecule-1 (Fukuda, et al., 2004). Protein phosphatase 2A has been shown to deactivate SphK1 (Barr, et al., 2008) and cytosolic chaperonin containing TCP-1 has been shown to mediate proper folding of SphK1 (Zebol, et al., 2009)

Another mechanism of regulation of SphK1 is at the transcriptional level, where the SphK1 promoter was shown to be up regulated in response to LPS in RAW macrophages leading to possible protection from apoptosis (Hammad, et al., 2006). Hypoxia inducible factor 2α has also been shown to upregulate SphK1 expression selectively in glial cells, thereby leading to S1P secretion and enhancement of transcellular angiogenesis (Anelli, et al., 2008). Exposure of SphK1 to DNA damage, TNFα and proteolysis causes its downregulation (Taha, et al., 2004).

1.6.2 Activation of SphKs

The SphKs have been shown to be activated by various factors including (Bryan, et al., 2008): (a) Growth factors -platelet derived growth factor, epidermal growth factor, vascular endothelial growth factor, nerve growth factor, basic fibroblast growth factor, transforming growth factor β,

and insulin like growth factor-1; (b) Cytokines: TNF-α, interleukins; (c) Hormones: prolactin and estradiol; (d) Hypoxia, and (e) Histamine.

1.6.3 Functions of SphK1 and S1P

The cellular levels of sphingosine, ceramide and S1P and the activation/inactivation of SphK1 play major roles in myriad biological processes. S1P is known to be a modulator of cell proliferation, survival, apoptosis, migration, and Ca^{+2} hemostasis (Alemany, et al., 2007).

S1P can act intracellularly as a second messenger and extracellularly as a ligand for G-Protein coupled receptors coded by endothelial differentiation genes and are known as S1P receptors (S1P1, S1P2, S1P3, S1P4, S1P5) (Ozaki, et al., 2003, Rosen and Goetzl, 2005) thereby modulating cellular processes including proliferation, stimulation of adherent junctions, enhanced extracellular matrix assembly, formation of actin stress fibers and inhibition of apoptosis induced by ceramide or growth factor withdrawal (Alvarez, et al., 2007, Melendez, 2008) . The modulations of these functions have been studied in peripheral macrophages and other immune cells (Gude, et al., 2008, Hammad, et al., 2008, Melendez, 2008).

S1P1 signaling is known to be essential for embryonic blood vessel development (Liu, et al., 2000). S1P has also been shown to elicit egress of lymphocytes into the blood in an S1P1 dependant manner. S1P2 and S1P3 activate phospholipase-C and Rho and the knockout of both these receptors in mice decreases litter size and survival rates (Ishii, et al., 2002). Although not

studied extensively, S1P4 is known to be expressed in lymphocytes and is therefore involved in T-cell proliferation (Wang, et al., 2005). S1P5 is expressed in dendritic and natural killer cells (Walzer, et al., 2007). Activation of S1P receptors also takes place *via* growth factors such as platelet derived growth factor, which activates SphK1 and thus activating S1P receptors (Olivera and Spiegel, 1993).

The differences in the actions of the various S1P receptors are of important consequence in the CNS. Neurite extension and retraction are important in CNS development and is regulated by contrasting actions of Rho and Rac, which control the actin cytoskeleton (Li, et al., 2002). Upon activation of S1P1 by S1P, there is upregulation of Rac which is required for neurite outgrowth (Estrach, et al., 2002). In contrast, when S1P2 is activated by S1P, it upregulates Rho, which induces collapse of growth cones and inhibition of neurite outgrowth (Nakamura, et al., 2002). Also, glial cell line derived neurotrophic factor transactivates SphK/S1P signaling and induces neurite extension via S1P1 (Murakami, et al., 2007).

S1P1 is expressed prominently in cerebral cortical proliferative zone and in ventricular areas of mesenchephalon and coincides with the period of neurogenesis (McGiffert, et al., 2002). When neural stem cells are exposed to S1P, they differentiate into neurons and astrocytes (Harada, et al., 2004). Therefore S1P receptors and S1P play a very important role in neurogenesis.

Apoptosis in neural cells is essential for establishing functional neural populations and to eliminate defective neurons. Ceramide in low

concentrations maintains the immature hippocampal neurons and promotes cell death in ageing hippocampal neurons while in high concentrations ceramide causes cell death (Mitoma, et al., 1998). Hence S1P is required for the remodeling of the developing brain into the functional adult brain. S1P by itself can lead to the release of glutamate from the hippocampus (Kajimoto, et al., 2007). All these studies suggest that S1P may be linked to memory formation in the brain since the hippocampus is the site of memory and the remodeling of brain is the basis for functional connections between neurons essential for memory formation. In neurodegenerative diseases like Alzheimer's, the memory loss could therefore be attributed to the actions of S1P and the sphingolipid pathway.

S1P receptor expression is not the only determinant of S1P activity. S1P exists in high levels in plasma (Caligan, et al., 2000) and is found in low levels in tissues (Edsall, et al., 2000). This S1P gradient is important in the homing of immune cells to the site of inflammation since S1P levels are high in inflammatory conditions (Rivera, et al., 2008). Serum S1P levels are also higher than plasma levels (Yatomi, et al., 1997) due to the release of S1P from platelets during the blood clotting and in the presence of thrombin (Yatomi, et al., 1997). Red blood cells and vascular endothelial cells are also considered important sources of S1P in plasma (Jessup, 2008).

The actions of S1P are considered to be a consequence of intracellular production, export to extracellular space, and activation of S1P receptors. The SphK1 and S1P pathway is a complex one, with crossing over with G protein

coupled receptor and receptor tyrosine kinase pathways. Generally, S1P is considered important for growth and survival, whereas sphingosine and ceramide are associated with cell growth arrest and apoptosis (Ogretmen and Hannun, 2004). Therefore the balance between ceramide and sphingosine versus S1P could be the determinant of cell growth and survival (Spiegel and Milstien, 2003). SphK1 is the enzyme that leads to the production of S1P from sphingosine and ceramide, and is therefore critical in the balance between ceramide/sphingosine and S1P.

It has been found that SphK1 plays a role in activation of immune cells and also in chemotaxis and wound healing (Melendez, 2008). SphK1 has been reported to be involved in LPS and TNF-α mediated inflammatory processes (Hammad, et al., 2008, Melendez, 2008) in immune cells.

1.6.4 Clinical significances of sphingolipid pathways

The many functions and roles of the sphingolipid pathway are being studied with interest. The roles discovered range from that of metabolism, tumour formation, inflammation, signaling pathways, absorption and transport, to receptor function for viruses and bacteria (Duan and Nilsson, 2009).

Sphingolipid pathways have been implicated also in myriad other diseases such as asthma, inflammatory bowel disease, colon carcinogenesis, and rheumatoid arthritis. In asthma, SphK1 and S1P regulate many processes of the asthmatic attack. Mast cell degranulation and migration is dependant on

the transactivation of S1P2 receptor which regulates the antigen binding to its receptor (Jolly, et al., 2004). S1P induces airway smooth muscle contraction (Rosenfeldt, et al., 2003) and also influences eosinophil migration (Roviezzo, et al., 2004).

Rheumatoid arthritis is a chronic autoimmune disease involving inflammation of joints resulting in debilitating pain and deformities. The most recent therapeutic strategies target TNF-α. TNF-α activates SphK1 leading to production of S1P in rheumatoid arthritis patients. S1P causes proliferation and cytokine production in the synovial cells of the joints (Kitano, et al., 2006). Also, S1P is elevated in the synovium of these patients (Lai, et al., 2008). These findings suggest that S1P indeed plays an important role in rheumatoid arthritis.

Inflammatory bowel disease is characterized by inflammation of the intestines as the name suggests and malabsorption of nutrients. It has been treated with various modalities, immmunosuppression and steroids being the main modality. TNF-α has been implicated in the disease process also and since it is known to activate SphK1, the sphingolipid pathway is of importance in this disease process (Pettus, et al., 2003, Sethu, et al., 2008).

In the CNS, the sphingolipid pathway has been shown to play a key role in neuron specific functions such as regulation of neurotransmitter release and proliferation and survival of neurons and glia (Okada, et al., 2009).

S1P receptors play an important role in autoimmune diseases such as multiple sclerosis. This has come to light from studies conducted with the drug

fingolimod (FTY720). Multiple sclerosis is and autoimmune disease where T-cells migrate across BBB and attack myelin in the CNS. This leads to demyelination, axonal damage and the resultant clinical picture of multiple sclerosis which is that of loss of optimal function of the skeletal muscles due to nerve damage and also includes loss of vision. FTY720 is a pro-drug which is converted into an S1P mimetic by the action of SphK2, which leads to internalization and degradation of the S1P receptor and also its prolonged downregulation (Matloubian, et al., 2004). Therefore the signal given by S1P for the migration of immune cells is no longer present, and this in theory could help reduce the progression of multiple sclerosis. Indeed, the results of a phase 2 randomized double blind placebo controlled clinical trial evaluating FTY720 in the treatment of multiple sclerosis shows that the relapse rate of the treated group was significantly lower than the placebo group (Kappos, et al., 2006). Such clinically relevant results are very promising in the role of S1P in future treatment modalities.

The part that the sphingolipids play in inflammation as discussed earlier, is particularly interesting, since many neurodegenerative conditions such as Alzheimer's disease find their origin or progression due to inflammation. Therefore, the sphingolipid pathway modulation may become a potential goldmine for future therapeutic methods in the treatment of neurodegenerative conditions.

1.7 Aims and hypothesis of this study

1.7.1 Aims of this study

Microglial activation in response to inflammatory stimuli is considered to be the hallmark in neurodegenerative diseases of the CNS. In the normal state, microglia act as scavengers of the CNS by removing damaged cells. But the chronic stimulation of microglia causes production of proinflammatory chemokines and cytokines such as TNF-α leading to further neuronal damage rather than just a scavenging action. Hence the modulation of microglia by various involved pathways is considered to be an important step in preventing neurodegenerative disease progression.

It has been reported that secretion of TNF-α, a proinflammatory cytokine is reduced with inhibition of SphK1 (Niwa, et al., 2000) which is present in abundance in the CNS as a major component of the lipid membranes. TNF-α secreted by activated microglia has been shown to be involved in neuroinflammation (Block and Hong, 2005, Dheen, et al., 2007). Hence, the modulation of TNF-α levels could form a potential therapeutic basis for the treatment of neuroinflammatory conditions. Due to the similarities between immune cells and microglia, it is therefore quite likely that SphK1 and S1P would play significant roles in microglial activation. The purpose of this study was therefore to understand the interactions between the sphingolipid pathway and activated microglia and also the effects on proinflammatory cytokine production by activated microglia of DMS, a

methylated derivative of sphingosine and a known inhibitor of SphK1. To address this,

- The presence of SphK1 and S1P receptors on microglia was confirmed
- The effect of activation of microglia on SphK1 was investigated
- The effects of suppression of SphK1 by SiRNA on the production of proinflammatory cytokines were determined.
- DMS, a methylated derivative of sphingosine and a known chemical inhibitor of SphK1 was used to confirm the effects on proinflammatory cytokines.
- S1P was exogenously administered to evaluate its pro/anti inflammatory effects on activated microglia.

1.7.2 Hypothesis

Modulation of SphK1 in resting and activated microglia regulates the expression and production of proinflammatory substances.

Chapter 2: Materials and Methods

2.1 Cell culture

BV2, a murine microglial cell line, which is a suitable model for *in vitro* study of microglia (Bocchini, et al., 1992) was used in this study.

2.1.1 Materials

Trypsin-EDTA (X0930, TE, Biowest France)

Fetal bovine serum (SV30160.03, FBS HyClone, Utah, USA)

Dulbecco's modified eagle's medium (D1152, DMEM, NUMI Sigma, USA)

Antibiotic antimycotic cocktail (A5955, Sigma, USA)

75cm^2 tissue culture flasks (NUNC, Denmark)

2.1.2 Procedure

The cells were grown in a 75cm^2 treated flask and washed with phosphate buffered saline solution (PBS) twice and then treated with Trypsin-EDTA in PBS for 3minutes at 37° C. The TE was inactivated by equal volume of 1x FBS. The solution was centrifuged at 1000rpm at 4° C for 5 min. The supernatant was discarded and the pellet was resuspended in 10 ml of DMEM containing 10 % FBS and 1 % antibiotic antimycotic cocktail (10 % medium). The cells were counted using a hematocytometer and approximately 2x10^{6}cells were plated into each flask containing 10 ml of 10 % FBS in DMEM and grown at 37° C and 5 % CO_2 in an incubator. The cells were subcultured every 2-3 days. For experiments, the BV2 cells were maintained

in DMEM without antibiotics or FBS for the required periods of treatment (Basic medium). For extraction of RNA and protein, $2x10^6$ cells were plated into cell culture dishes. For siRNA treatment and immunofluorescence, $2x10^5$cells were used per well in a 6 well plate or 24 well plate respectively.

2.2 Treatment of cell culture

2.2.1 Materials

LPS (L6529, Sigma, USA)

N, N Dimethylsphingosine -DMS (310500, Calbiochem, Germany)

Sphingosine-1-phosphate- S1P (S9666, Sigma, USA)

Fetal bovine serum (SV30160.03, FBS HyClone, Utah, USA)

2.2.2 Procedure

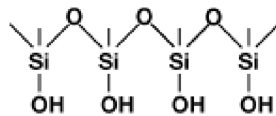
Cells were plated onto cell culture dishes and grown in 10 % DMEM/FBS with antibiotics overnight. The following day, medium was discarded and washed twice with PBS. The cells were grown in basic medium and treated with LPS (1 µg/ml), DMS (10 µM) and with S1P (10 nM) in various experimental combinations for different time points (30 min, 1 h, 3 h, 6 h) in the incubator. The control was taken as cells grown in basic medium for the same time periods. The medium was then discarded and washed twice with ice cold PBS and the cells and supernatant were used for extraction of RNA, protein, ELISA, immunofluorescence etc.

2.3 RNA extraction & Reverse transcription polymerase chain reaction (RT-PCR)

2.3.1 Principles

2.3.1.1 RNA extraction

The RNA extraction procedure combines the selective binding properties of a silica-based membrane with the speed of microspin technology. Nucleic acids, either DNA or RNA, are adsorbed onto the silica-gel membrane in the presence of chaotropic salts, which remove water from hydrated molecules in solution. Polysaccharides and proteins do not adsorb and are removed. A specialized high-salt buffer system allows upto 100 µg of RNA longer than 200 bases to bind to the silica membrane.



Structure of silica gel used for RNA extraction. (adapted from- http://www1.qiagen.com/resources/info/qiagen_purification_technologies_1.aspx#structure)

Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to a spin column, where the total RNA binds to the membrane and

contaminants are efficiently washed away. After a wash step, pure nucleic acids are eluted under low- or no-salt conditions in small volumes. High-quality RNA is then eluted in 30–100 µl water.

2.3.1.2 RT-PCR

Polymerase chain reaction (PCR) is a method that allows exponential amplification of short DNA sequences (usually 100 to 600 bases) within a longer double stranded DNA molecule. PCR entails the use of a pair of primers, each about 20 nucleotides in length that are complementary to a defined sequence on each of the two strands of the DNA. These primers are extended by a DNA polymerase so that a copy is made of the designated sequence. After making this copy, the same primers can be used again, not only to make another copy of the input DNA strand but also of the short copy made in the first round of synthesis. This leads to logarithmic amplification. Since it is necessary to raise the temperature to separate the two strands of the double strand DNA in each round of the amplification process, a major step forward was the discovery of a thermo-stable DNA polymerase (Taq polymerase) that was isolated from *Thermus aquaticus,* a bacterium that grows in hot pools; as a result it is not necessary to add new polymerase in every round of amplification.

Real time PCR or quantitative PCR is a variation of the standard PCR technique used to quantify DNA or messenger RNA (mRNA) in a sample.

Using sequence specific primers, the relative number of copies of a particular DNA or RNA sequence can be determined. The term relative is used since this technique tends to be used to compare relative copy numbers between tissues, organisms, or different genes relative to a specific housekeeping gene. The quantification arises by measuring the amount of amplified product at each stage during the PCR cycle. DNA/RNA from genes with higher copy numbers will appear after fewer melting, annealing, extension PCR cycles. Quantification of amplified product is obtained using fluorescent probes and specialized machines that measure fluorescence while performing temperature changes needed for the PCR cycles. SYBR green is a dye that binds to double stranded DNA but not to single-stranded DNA and is frequently used in real-time PCR reactions. When it is bound to double stranded DNA it fluoresces very brightly. During extension, increasing amount of dye binds to the newly formed double-stranded DNA, resulting in an increase in the fluorescence signal. Thus, the fluorescence measurement performed at the end of the extension step of every PCR cycle reflects the increasing amount of amplified DNA. After a few cycles, the fluorescent signal is first recorded as statistically significant above background signal. This point is described as threshold cycle (Ct), which occurs during the exponential phase of amplification (Gibson, et al., 1996).In addition, the specificity of the amplification and PCR product verification can be achieved by a melting curve of the PCR product (Ririe, et al., 1997).

After several (often about 40) rounds of amplification, the PCR product is analyzed on an agarose gel and is abundant enough to be detected with an ethidium bromide stain. In order to measure messenger RNA (mRNA), the method was extended using reverse transcriptase to convert mRNA into complementary DNA (cDNA), which was then amplified by PCR and, again analyzed by agarose gel electrophoresis.

Thus the steps involved in RT-PCR can be enumerated as follows:

1. mRNA is copied to cDNA by reverse transcriptase that has an endo H activity， using an oligo dT primer. This removes the mRNA allowing the second strand of DNA to be formed.
2. Denaturation ：cDNA is denatured at more than 90 degrees (~94 degrees) so that the two strands separate.
3. Annealing：The sample is cooled to 50 to 60 degrees and specific primers are annealed that are complementary to a site on each strand. The primers sites may be up to 600 bases apart but are often about 100 bases apart, especially when real-time PCR is used.
4. The temperature is raised to 72 degrees and the heat-stable Taq DNA polymerase extends the DNA from the primers.
5. After 30 to 40 rounds of synthesis of cDNA, the reaction products are analyzed by agarose gel electrophoresis. The gel is stained with ethidium bromide

Using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The data are represented as the fold change of target gene expression normalized to an endogenous reference gene, relative to a calibrator. For the treated samples,

evaluation of 2-ΔΔCt indicates the fold change of gene expression relative to the untreated control. For this method to be valid, the amplification efficiencies of the target and reference must be approximately equal.

2.3.2 Materials

Qiagen RNeasy mini kit (74106, Qiagen, Germany)

M-MLV Reverse transcriptase (M170A, Promega, Madison, USA)

Oligo (dT) 15 primer (c110A, Promega, Madison, USA)

dNTP mix (U1240, Promega, Madison, USA)

RNasin -RNase inhibitor (Promega, USA, Cat. No. N2111,)

LightCycler Fast Start DNA master plus SYBR Green 1 kit (03515885001, Roche Mannheim, Germany)

TAE buffer (Invitrogen, USA, Cat. No. 15558034)

100bp DNA step ladder (Promega, USA, Cat. No. G6951)

LightCycler instrument (Roche Molecular Biochemicals)

GeneGenius (Syngene, UK)

Spectrophotometer (Eppendorf, Germany)

2.3.3 Procedure

2.3.3.1 RNA extraction procedure from BV2 cells

Total RNA from BV2 microglial cells subjected to various treatments was extracted as per the instructions given by the Qiagen RNeasy mini kit.

Approximately, 1×10^6 cells were used to extract total RNA. Cells were lysed in 650 µl of RLT buffer (containing a highly denatured guanidine isothiocyanate which inactivates RNase) and then scraped from the flask with the scraper. The lysate was homogenized, then centrifuged for 30s at 14000g in a microfuge and the supernatant was mixed with 650 µl of 70 % ethanol to clear lysate. The sample was applied to an RNeasy mini spin column (silica-gel membrane, maximum binding capacity is 100 µg of RNA longer than 200 bases) and spun for 30 sec at 14000g and then flow-through was discarded. The RNA bound to the membrane was washed with buffer RW1 and RPE sequentially. High-quality RNA was then eluted in 20 µl of RNase free water. The concentration and purity of the extracted RNA was evaluated spectrophotometrically at 260 and 280 nm (Biophotometer, Eppendorf, Germany). The RNA samples were stored at -80° C until experiments.

2.3.3.2 Procedure for cDNA synthesis

Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) is an RNAdependent DNA polymerase that is used for first-strand synthesis of cDNA from RNA molecules. 2 µg of RNA was mixed with 2 µl of Oligo (dT) 15 and incubated at 70° C for 5 min. Each sample was then made to a final volume of 25 µl on ice with the following regents: 1 µl of M-MLV Reverse transcriptase, 5 µl of M-MLV RT 5x buffer ， 0.7 µl of RNasin, 0.5 µl. of dNTP mix and nuclease free water and incubated at 42° C for 60 min and 70°

C for 10 min. The cDNA thus obtained was then diluted 3 times in sterile water and stored at -20° C.

2.3.3.3 Procedure for real time polymerase chain reaction (RT-PCR)

RT-PCR was carried out using LightCycler Fast Start DNA master plus SYBR Green 1 kit as per the manufacturer's instructions. The oligonucleotide primer sequences used are tabulated below. The primer sequences used for the S1P receptors were derived from (Kimura, et al., 2008):

Table 1: Primer sequences used

	Sense	Antisense	Size (bp)
TNF-α	CGTCAGCCGATTTGCTATCT	CGGACTCCGCAAAGTCTAAG	205
IL1β	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG	229
iNOS	GCTTGTCTCTGGGTCCTCTG	CTCACTGGGACAGCACAGAA	217
β-actin	TCACCCACACTGTGCCCATCTACGA	GGATGCCACAGGATTCCATACCCA	314
SphK1	CTTCTGGGCTGCGGCTCTATTCTG	GGAAAGCAACCACGGGCACA	507
S1P1	5'ACTATATTCTCTTCTGCACCAC 3'	5'GCTTCGAGTCCTGACCCA 3'	80-120
S1P2	5'TGTCACTCTGTCCTTAACTC 3'	5'GGCCACTTGTCTCTCGAT 3'	
S1P3	5'CAACTTGGCTCTCTGCGACCT 3'	5'ACTGTTGGAGACAGACTGAACG 3'	
S1P4	5'CTCTACTCCAAGGGCTATGT 3'	5' TGGAGACTTCTGCCCATT 3'	
S1P5	5'GTGTGTGCCTTCATTGTG 3'	5'CAGGTCCGACAAAGTGAG 3'	

2.5 µl of cDNA was used per sample. Each sample was run with a corresponding internal control, β actin. After pre incubation at 95˚ C for 10 min, the PCR was performed as follows: 35-45 cycles of denaturation at 95˚ C for 5 sec, annealing at 60˚ C for 5 sec, and elongation at 72˚ C for PCR product size per 25 sec. The crossing points (the cycle number at which the Lightcycler detected the upstroke of the exponential phase of PCR product formation) were taken normalized with β actin for each sample. Statistical significance was estimated using Student's *t*-test and the fold change was calculated using the $2^{-\{\Delta\Delta Ct\}}$ method (Livak and Schmittgen, 2001).

2.4 Western immunoblot assay

2.4.1 Principles

Protein expression analysis is a very important tool of modern molecular biology. One key approach to explore protein expression is to analyze them by electrophoresis. SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis), was developed in the mid 1960s, and is widely used to separate proteins according to their net charge, size and shape. During electrophoresis, SDS serves to denature proteins by binding to hydrophobic regions of proteins and causes them to unfold into extended polypeptide chains, thus becoming dissociated from other proteins and freely soluble in the SDS solution (β-mercaptoethanol is also used to break the S-S bonds in proteins). The negatively charged SDS also wraps the proteins and

makes them negatively charged and thus the proteins move through the gel matrix towards the positive electrode .The movement is inversely proportional to the log of their molecular weight (William Wu, 2003).

Western Blotting is a procedure in which different types of proteins are separated by SDS-PAGE and then immobilized onto a solid support PVDF (polyvinlylidene difluoride) or nitrocellulose membrane. The protein of interest is then detected by incubating the membrane with a specific antibody probe. Thus a standard western blot involves 4 steps:

- separation of proteins by SDS-PAGE
- transfer of proteins onto a solid membrane
- incubation of the membrane with specific antibodies
- detection of hybridized signals

2.4.2 Materials

Protein extraction kit (78501, Pierce, IL, USA)

Protease inhibitor cocktail kit (78410, Halt^{TM}, IL, USA)

Protein assay kit (500-0007, Bio-Rad, California, USA)

Mouse anti β actin Abcam ab18061, Cambridge, USA,

Rabbit polyclonal SphK1 ABGENT AP7237c, San Diego, USA

Horseradish peroxide conjugated goat anti rabbit secondary antibody (7074, Cell Signaling Technology, Boston, MA, USA)

Enhanced chemiluminescence detection system (34095, Thermo scientific, Supersignal West Femto maximum Sensitivity Substrate)

Stripping buffer (0021059, Pierce, IL, USA)

Quantity One Software (Bio-Rad, version 4.4.1, California, USA).

Table 2: Reagents used for Western Blotting

10% resolving gel:	
H_2O	7.9ml
30% acrylamide mix	6.7ml
1.5 M Tris (pH 8.8)	5.0ml
10% SDS	0.2ml
10% ammonium persulfate	0.2ml
TEMED	0.008ml
5% stacking gel:	
H_2O	5.5ml
30% acrylamide mix	1.3ml
1.0 M Tris (pH 6.8)	1.0ml
2.0 10% SDS	0.08ml
10% ammonium persulfate	0.08ml
TEMED	0.008ml

1x SDS gel-loading buffer:	
50mM Tris.Cl (pH 6.8) 100mM dithiothreitol 2% SDS 0.1% bromophenol blue 10% glycerol	
Tris-glycine electrophoresis buffer:	
25mM Tris 250mM glycine 0.1% SDS	
Transfer buffer:	
25mM Tris 250mM glycine 20% Methanol	
1X TBS:	
Tris base NaCl H_2O up to 1 liter; adjusted to pH 7.6 with 2N HCl	2.42 g 0.8 g
1X TBST:	

1X TBS 1 liter 0.1% Tween 20	

2.4.3 Procedure

Protein extracts were made from BV2 cells subjected to different treatment conditions using protein extraction kit and protease inhibitor cocktail kit and were quantified using protein assay kit**.** 20 μg of each protein sample was separated on 10 % SDS-polyacrylamide gel and transferred to polyvinylidene difluoride transfer membrane. The membranes were blocked with 5 % non-fat dry milk for 1h and incubated with primary antibodies according to manufacturer's recommendations (1:10,000 for β actin; 1:500 for SphK1) overnight. The membranes were washed with Tris buffered saline with 0.1% Tween-20 (TBST) three times and incubated with horseradish peroxide-conjugated goat anti rabbit secondary antibody for 1h. The immunoblots were developed using enhanced chemiluminescence detection system. The membranes were stripped with stripping buffer and incubated with β actin as internal control and developed. The optical density of the bands was analyzed with Quantity One Software。

2.5 Immunofluorescence

2.5.1 Principles

The technique of immunofluorescence was first described by Coons (Coons, 1960). Fluorescence is defined, based upon the physical definitions of the properties of the matter, as the ability to emit light without noticeable delay when irradiated. Fluorescence from untreated materials is known as primary or natural or self or auto fluorescence. When fluorescence is generated using additional fluorescent substances, it is known as secondary fluorescence. In secondary fluorescence, the first step involves the treatment of unlabelled antibody with the antigen in the sample to be tested. This is followed by the addition of a second flourochrome conjugated antibody, which reacts with the unlabelled antibody. This complex is then visualized using high sensitivity fluorescent microscopes.

Cells are fixed to prevent the antibody from being bleached out during the experiment. Blocking is done to prevent nonspecific staining. The washing steps ensure removal of loosely bound and any unbound antibody (Wulf Storch, 2000).

2.5.2 Materials

Poly lysine (P4707, Sigma, USA)

Goat–anti-rabbit fluorochrome conjugated secondary antibody (AP132C, CY3 or AQ132F, FITC Chemicon, Temecula, CA, USA)

Rabbit polyclonal SphK1 antibody (Abgent AP7237c, San Diego, USA)

Rabbit TNF-α antibody (Cell Signaling Technology 3707, Boston, MA, USA)

FITC conjugated tomato (*lycopersicom esculentum*) Lectin (Sigma L0401, St. Louis, USA)

4', 6- diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes D1306, USA)

2.5.3 Procedure

Cover slips were sterilized and placed into 24 well plates. The cover slips were treated with poly lysine for 2 h at 4° C. BV2 cells were subcultured and grown in 10 % FBS medium overnight. The next day, the medium was discarded and the cells were washed with PBS twice. The cells were treated as per experimental requirements, then washed and fixed in 4 % paraformaldehyde for 30 min at 4° C. The cells were washed and blocked with normal goat serum at room temperature. The cells were then incubated with primary antibodies diluted in 5 % serum at recommended dilutions (1:100 for SphK1; 1:1000 for TNF-α) overnight at 4° C. The next day, the cells were washed 3 times for 10min each and incubated with the fluorochrome conjugated goat–anti-rabbit secondary antibody (1: 200, diluted in 5% Serum) at room temperature for 1h in the dark. Subsequently, the cells were counterstained with lectin (1:100), a commonly established marker for

microglia and incubated with DAPI (1:50,000) a nuclear marker, for 5 min at room temperature, washed, and mounted onto slides using fluorescent mounting medium. All steps were carried out in the dark. The slides were observed under confocal microscope (Olympus Fluoview 1000, Tokyo, Japan).

2.6 siRNA gene silencing

2.6.1 Principles

Recent advances in molecular biology have shown that gene expression can be effectively silenced in a highly specific manner through the addition of double stranded RNA (dsRNA) (Fire, et al., 1998, Hannon, 2002, Napoli, et al., 1990). Individual genes can be silenced by interfering with mRNA transcription. This is done *via* a small double-stranded RNA. An RNase III –like enzyme named DICER (Bernstein, et al., 2001) snips short interfering RNAs (siRNA) from longer double stranded RNAs made by either self-copying gene sequences, by replicating viruses, or by regulatory RNA sequences known as microRNAs. All the double stranded RNAs are cleaved by DICER enzyme into short siRNA pieces that can suppress gene expression. The short siRNA pieces unwind into single strand RNAs, which then combine with proteins to form into a multi-subunit protein complex called RISC [RNA-Induced Silencing Complex] in an ATP dependent step (Nykanen, et al., 2001). The RISC guides the siRNAs to the target RNA

sequence and then captures a native mRNA molecule that complements the short siRNA sequence. At some point the siRNA duplex unwinds, and it appears that the antisense strand remains bound to RISC and directs degradation of the complementary mRNA sequence by a combination of endo and exonucleases. If the pairing between native mRNA and siRNA piece is essentially perfect, the native mRNA is cut into unusable RNA fragments that cannot be translated. If however, the pairing is less than perfect then the RISC complex binds to the mRNA and blocks ribosome movement along the native mRNA also halting translation. The net effect is that no protein is made.

2.6.2 Materials

predesigned siRNA (Ambion, CA, USA)

OPTIMEM I reduced serum medium (31985, GIBCO, Invitrogen, USA)

Oligofectamine (12252-011, Invitrogen, USA)

siRNA *Silencer*® Select Negative Control siRNA (4390843, Ambion, CA, USA)

2.6.3 siRNA sequences

The SphK1 siRNA sequences used were:

Table 3: siRNA sequences

siRNA	Sense	Antisense
1.	GCAAGCAUAUGGAACUUGAtt	UCAAGUUCCAUAUGCUUGCcc
2.	GGUACGAGCAGGUGACUAtt	UUAGUCACCUGCUCGUACCca
3.	UGAUACUCACCGAACGGAAtt	UUCCGUUCGGUGAGUAUCAgt

2.6.4 Procedure for siRNA silencing of SphK1

The BV2 cells were subcultured and plated onto 6 well plates at a density of $1x10^5$ cells/ml. The total volume used per well was 2 ml. The cells were grown in DMEM with 10 % FBS without antibiotics for 24 h to achieve 30-50% confluency of the cells. 10 µl of predesigned siRNA (50nm) with 100 µl of OPTIMEM I reduced serum medium and 10 µl of Oligofectamine with 100 µl of OPTIMEM were incubated at room temperature for 10 min separately. 100 µl per sample of the Oligofectamine + OPTIMEM medium was mixed with the siRNA mix, and incubated for 20 min at room temperature. The cells were washed and 800µl of OPTIMEM and 200µl of the final siRNA mix was added to per well and incubated for 8.5 h. 1ml of 10 % FBS in DMEM without antibiotics was added per well and then incubated for 22 h. The transfected microglia were then treated with different combinations

(S1P, LPS, S1P+LPS) for the time points followed previously (30 min, 1 h, 3 h, 6 h). The transfection conditions were optimized to obtain a suppression efficiency of more than 70-80%. The suppression efficiency was calculated by comparison with cells treated with scrambled (control) siRNA *Silencer*® Select Negative Control siRNA.

2.7 ELISA

2.7.1 Principles

The cytokine ELISA (Enzyme-Linked Immunosorbent Assay) is a specific and highly sensitive method for quantitative measurements of cytokines or other analytes in solutions. A specific monoclonal antibody against the protein of interest (cytokine) is coated on a microtiter plate. A second monoclonal antibody, used for detection, binds to a different epitope on the protein. The secondary antibody is labeled with biotin, which allows subsequent binding of a Streptavidin conjugated enzyme. Any unbound reagents are washed away. When the substrate/chromophore Tetramethylbenzidine (TMB) is added, a color reaction will develop that is proportional to the amount of protein bound. The objective is to allow development of a color reaction through enzymic catalysis. The reaction is allowed to progress for a defined period after which the reaction is stopped by the alteration in pH (addition of H_2SO_4) of the system, or addition of an inhibiting reactant. Finally, the color is quantified by the use of a

spectrophotometer reading at the appropriate wavelength for the color produced. The concentration of protein is determined by comparison with a standard curve with known concentrations of protein. The detection limits for cytokine ELISAs are commonly in the lower picogram/ml range.

2.7.2 Materials

Mouse TNF-α ELISA kit (eBioscience; Cat #88-7324)

2N H_2SO_4 (Sigma-Aldrich, Cat # 320501; MO, USA)

2.7.3 Procedure for TNF-α quantification by ELISA

ELISA was carried out as per the instructions provided in the kit. NUNC Maxisorp 96 well ELISA plate was coated with 100 µl/well of capture antibody in coating buffer. The plate was sealed and incubated overnight at 4° C. The wells were aspirated and washed 5 times with >250 µl/well Wash Buffer, for 1min each time. The concentrated assay_diluent was diluted with 4 parts of distilled water and blocking was done with 200 µl/well of 1X assay diluent per well and incubated at room temperature for 1 h. The wells were aspirated and washed. Recombinant TNF-α standard diluted in 1X assay diluent was added 100 µl/well of standard to the appropriate wells. 2-fold serial dilutions of the standards were done to make the standard curve. 100 µl/well of the medium from the samples was added to the appropriate wells. The plate was covered and sealed and incubated overnight at 4° C for maximal

sensitivity. The wells were aspirated and washed for a total of 5 washes. 100 µl/well of detection antibody diluted in 1X assay was added to each well. The plate was sealed and incubated at room temperature for 1 h. The wells were aspirated and washed. 100 µl/well of Avidin-HRP diluted in 1X assay diluent was added per well. The plate was sealed and incubated at room temperature for 30 minutes. The wells were aspirated and washed. 100 µl/well of Tetramethylbenzidine (TMB) Substrate Solution was added to each well. The plate was incubated at room temperature for 15 min. 50 µl of 2N H_2SO_4 stop solution was added to each well. The results were read using a microplate spectrophotometer at 450nm. The standard curve using TNF-α standards was used to calculate the TNF-α production of the samples.

2.8 Nitric Oxide Assay

2.8.1 Principles

Nitric oxide (NO) is oxidized to nitrite (NO2-) and nitrate (NO3-) in biological systems. Therefore, concentrations of these anions are used as a quantitative measure of NO production. Nitrate is converted into nitrite utilizing nitrate reductase. Further addition of Griess reagents convert nitrite into a deep purple azo compound. Measurement of the absorbance of the azo chromophore accurately determines the total nitric oxide production.

2.8.2 Materials

Nitric Oxide Colorimetric BioAssay Kit (Cat # N2577-01, USBiological, Massachusetts, USA)

2.8.3 Procedure

The NO production was quantified colorimetrically using the nitric oxide colorimetric bioassay kit. NUNC Maxisorp 96 well plate was coated with 200 µl/well of diluted assay buffer. 80 µl/well of the supernatant collected from the differentially treated (LPS, S1P, LPS+S1P and siRNA) cell cultures were added to each well. 10 µl/well of the reconstituted enzyme cofactor mixture was added. 10 µl/well of the reconstituted nitrate reductase mixture was added subsequently to each well. The plate was covered and incubated at room temperature for 1-4h. 50µl/well of Griess reagent R1 was added per well and 50µl of Griess reagent R2 was added to each well immediately. Colour was allowed to develop for 10 min at room temperature and the absorbance was read using a microplate spectrophotometer at 540nm. The standard curve using nitrate standards was used to calculate the NO production of the samples using the following formula:

[Nitrate+Nitrite](µM)=[(A540-Y-intercept)/(slope)x(200µl/sample volume(µl))]xDilution

Chapter 3: Results

3.1 SphK1 is expressed in microglial cells

The protein and mRNA expression of SphK1 was detected in BV2 microglial cells as revealed by the immunofluorescence (Fig. 1A-C) and PCR (Fig.1 D, E) analyses, respectively. The SphK1-positive cells were confirmed to be microglia by colocalization with lectin (Fig.1 A-C). Western blot analysis also revealed that SphK1 was expressed in the microglial cells (Fig.2G).

3.2 S1P receptors 1-5 are expressed in BV2 microglial cell line

S1P acts intracellularly as a second messenger and extracellularly via G-protein coupled receptors, $S1P_{1,2,3,4,5}$ (Ozaki, et al., 2003, Rosen and Goetzl, 2005). PCR analysis showed that S1P receptors, $S1P_{1,2,3,4,5}$ were expressed in BV2 cells (Fig.1E).

3.3 Expression of SphK1 is increased in activated microglia

SphK1 expression was increased in LPS-activated microglia. Immunofluorescence (IF) analysis showed the increase in SphK1 expression maximally at 1h after LPS treatment with a return to baseline levels at 6h post-treatment (Fig.2A-F). This result was confirmed by the Western blot analysis which showed that the expression of SphK1 in activated microglia increased

significantly by 40%, at 1h post-treatment. However, the increase declined to base level by 6h post-treatment (Fig.2G-H).

3.4 Suppression of SphK1 by DMS reduced the TNF-α production

Effects of suppression of SphK1 by DMS on TNF-α production in LPS activated BV2 microglia were studied by IF and RT-PCR. IF showed the increased expression of TNF-α in microglia treated with LPS and this increase was attenuated by the DMS treatment (Fig 3A). Moreover, treatment of microglia with DMS alone also resulted in reduction of TNF-α expression compared to that of untreated samples. The real time RT-PCR analysis showed that LPS treatment increased the TNF-α mRNA expression in microglia but, upon concomitant suppression of SphK1 with DMS in LPS activated microglia, the level of TNF-α mRNA expression was significantly reduced by 51% at 1h, 18% at 3h post treatment and was at par with LPS alone treatment at 6h (Fig.3B). Moreover, in microglia treated with DMS alone, TNF-α expression level appeared to be reduced or unaltered at different time points tested.

3.5 Exogenous administration of S1P in BV2 microglia increased the TNF-α production

The effects of administration of S1P on TNF-α expression in BV2 cells was studied by IF and RT-PCR. IF showed increased expression of TNF-

α in microglia upon treatment with LPS or S1P (Fig.4 A-L). When S1P was administered in LPS activated BV2 microglia, the TNF-α expression was markedly increased compared to LPS alone treated samples (Fig. 4 D, H, L). The real time RT-PCR analysis showed a significant increase in the expression of TNF-α in microglia treated with S1P and LPS for 1h, 3h and 6h (Fig.4M). Upon addition of S1P to LPS activated microglia, the TNF-α mRNA expression level was significantly increased by 126% at 1h post-treatment, compared to cells treated with S1P alone. ELISA further confirmed that the exogenous administration of S1P increased the TNF-α release in untreated and LPS-activated microglia. Moreover, the release of TNF-α was found to be reduced significantly in untreated microglia and LPS-activated microglia with the suppression of SphK1 by DMS (Fig.4 N).

3.6 Suppression of SphK1 by siRNA reduced TNF-α production in LPS activated microglia

Immunofluorescence analysis showed that SphK1 immunoreactivity was markedly reduced in BV2 microglia transfected with SphK1 specific siRNA ($SphK1^-$) compared to negative control cells (Fig 5 A, B). The siRNA transfection efficiency in BV2 microglia was found to be 80%, as revealed by real time RT-PCR (Fig.5C, D). Immunofluorescence analysis showed reduced expression of TNF-α in $SphK1^-$ microglia, compared to cells transfected with negative control (Fig.5.E, F). However, administration of S1P increased the expression of TNF-α in $SphK1^-$ cells treated with or without LPS (Fig 5 H, I).

Treatment of LPS alone was unable to increase the expression of TNF-α in SphK1$^-$ microglial cells (Fig.5G). The real time RT-PCR analysis showed that TNF-α mRNA expression was significantly silenced (80%) in SphK1$^-$ cells, compared to that in negative control (Fig.5J). LPS treatment was unable to increase TNFα expression in SphK1$^-$ cells compared to negative controls. However, treatment of S1P with or without LPS increased the expression of TNF-α by 20% in SphK1$^-$ cells, compared to that in negative control (Fig.5J). ELISA analysis confirmed that suppression of SphK1 with siRNA suppressed TNF-α production in untreated and LPS-treated microglia (Fig.5 K). However, exogenous administration of S1P with or without LPS increased TNF-α production in SphK1$^-$ cells (Fig.5 K).

3.7 Suppression of SphK1 by DMS reduced the mRNA expression level of IL-1β in BV2 microglia

Effect of SphK1 suppression by DMS on IL-1β mRNA expression in LPS-activated BV2 microglial cells was studied by the real time RT-PCR. IL-1β mRNA expression level was found to be reduced about 30% in microglia treated with DMS for 30min, compared to that of control (Fig.6B). LPS treatment increased IL-1β mRNA expression but, upon concomitant suppression of SphK1 with DMS in LPS activated microglial cells, the expression level of IL-1β was reduced significantly at 30min, 1h and 3h post-treatment (Fig.6B). However, the reduction was not significant after 6h post-treatment.

3.8 Exogenous administration of S1P in BV2 microglia increased the IL-1β mRNA expression

The real time RT-PCR analysis showed that the IL-1β mRNA expression was increased significantly in BV2 microglial cells treated with LPS for 30min, 1h, 3h and 6h, compared to that of control. This increase in the expression level of IL-1β was further augmented by the addition of S1P.The maximum increase in expression in cells treated with LPS and S1P was detected at 3h post-treatment (Fig.6C). Moreover, the significant increase of IL-1β mRNA expression level in microglia treated with S1P alone was detectable at 3h post-treatment.

3.9 Suppression of SphK1 (SphK1-) by siRNA reduced IL-1β mRNA expression in LPS- activated microglia

Real time RT-PCR analysis showed a significant decrease (40%) in the expression level of IL-1β in $SphK1^{-}$ microglia compared to that of negative control. LPS treatment was unable to increase IL-1β mRNA expression in $SphK1^{-}$ cells compared to negative controls. However, S1P treatment (for the same duration as that of LPS) with and without LPS increased the expression level of IL-1β significantly in $SphK1^{-}$ cells, in comparison to that of negative control (Fig.6D).

3.10 SphK1 regulates the iNOS mRNA expression in activated BV2 microglia

The real time RT-PCR analysis showed that LPS induced iNOS expression in BV2 cells at 6h post-treatment (Fig 7A, B). The LPS-induced increase in the iNOS expression in microglia was attenuated (20%) significantly by the concomitant suppression of SphK1 with DMS for 6h (Fig.7B). However, DMS was unable to alter the iNOS expression in activated BV2 cells significantly in early time points studied (data not shown).

LPS-induced iNOS mRNA expression in BV2 cells was further augmented significantly by the concomitant addition of S1P (Fig 7C).

3.11 Suppression of SphK1 ($SphK1^{-}$) by siRNA reduced the iNOS mRNA expression in LPS-activated microglia.

The real time RT-PCR analysis showed the significant suppression (82%) of iNOS expression in $SphK1^{-}$ microglia, compared with negative controls (Fig. 7D). In addition, treatment of $SphK1^{-}$cells with LPS, and S1P with or without LPS increased the iNOS expression compared to that observed in control $SphK1^{-}$cells (Fig.7D). However, this increase was not above that of negative control.

3.12 SphK1 regulates NO production in BV2 microglial cells

Nitric oxide assay showed the limited production of NO in $SphK1^-$ microglia treated with LPS and the increase in production of NO upon administration of SIP. In $SphK1^-$ cells treated with LPS, the NO levels remained at baseline control levels (Fig.7E). However, exogenous administration of S1P with or without LPS increased NO production in $SphK1^-$ cells significantly (Fig.7E).

Chapter 4: Discussion

Microglial activation is considered the hallmark of neuroinflammation. It is well established that activation of microglia in various neurodegenerative diseases and by exposing the cells to LPS, β-amyloid, thrombin, and IFN-γ experimentally enhances the release of large amounts of proinflammatory cytokines such as TNF-α and IL-1β and reactive oxygen intermediates such as ROS and NO, contributing to neuroinflammation and neurodegeneration (Block and Hong, 2005, Combs, et al., 2001, Dheen, et al., 2007, McCoy and Tansey, 2008, Vilhardt, 2005). Hence, determination of various mechanisms controlling microglial activation is believed to be an important step towards the suppression of neuroinflammation.

Sphingolipids have been regarded as structural components of cell membranes until recently. Now it is well established that sphingomyelin hydrolysis produces ceramide generation, which can be then converted into ceramide-1-phosphate, sphingosine and sphingosine-1-phosphate. SphK1, one of the enzymes involved in the sphingolipid metabolic pathway, is known to play a role in the activation of immune cells (Melendez, 2008), and is involved in TNF-α mediated inflammatory processes in immune cells (Hammad, et al., 2008, Melendez, 2008). It has been reported that decreased levels of proinflammatory cytokines such as TNF-α and IL-1β are associated with inhibition of SphK1 (Maines, et al., 2008, Niwa, et al., 2000). These cytokines in CNS are largely produced by microglial cells in response to LPS, and are controlled by glucocorticoids (Allan and Rothwell, 2001, Nadeau and Rivest,

2002, Streit, et al., 1999). The chronic microglial reactivity and uncontrolled production of TNF-α and IL-1β are the direct causes of the neurodegeneration (Nadeau and Rivest, 2003, Streit, et al., 1999).

Sphingolipids form a part of the phospholipids content in the CNS. High levels of sphingolipids are found in peripheral nerves and the white matter since it is a constituent of myelin sheaths (Sastry, 1985). The present results have shown that microglia, the resident immune cells of the CNS, express SphK1. Since the presence of SphK1 was established in microglia, the next step was to find out which receptors of S1P were expressed in microglia since S1P would exert some of its many known actions *via* these receptors. The experiments showed that microglia express all the known five S1P receptors- S1P1, S1P2, S1P3, S1P4, S1P5. S1P1 activation is required for embryonic blood vessel development (Liu, et al., 2000). S1P2 and S1P3 play important roles in the number and survival of embryos in mice and hence are required for successful pregnancies (Ishii, et al., 2002). S1P4 is involved in T-cell proliferation (Wang, et al., 2005). S1P5 is expressed in dendritic and natural killer cells (Walzer, et al., 2007). Since S1P receptor activation leads to such varied and diverse effects, studies in the future should establish which receptors are involved in the neurodegenerative pathway to give a better understanding of the mechanisms involved in microglial activation.

The study has further revealed that SphK1 expression is upregulated in LPS-activated microglia. Various other studies have shown similar upregulation of SphK1 in response to inflammatory stimuli. SphK1 promoter

was shown to be upregulated in response to LPS in RAW macrophages (Hammad, et al., 2006). Hypoxia inducible factor 2α has also been shown to upregulate SphK1 expression selectively in glial cells. Such upregulation of SphK1 in inflammatory conditions indicate that this maybe a proinflammatory mechanism and it leads to the generation of S1P. Hence the proinflammatory role of the sphingolipid pathway maybe attributed to S1P and the activation of specific S1P receptors since the S1P receptors seem to have conflicting roles as shown by other studies on neurons. S1P1 activation by S1P leads to Rac upregulation leading to neurite outgrowth (Estrach, et al., 2002). In contrast, when S1P2 is activated by S1P, it upregulates Rho which induces collapse of growth cones and inhibition of neurite outgrowth (Nakamura, et al., 2002), Therefore it is possible that the proinflammatory activity of sphingolipid pathway in microglia may be a result of the activation of a specific S1P receptor. Hence as mentioned earlier, it is essential in future studies to find out which S1P receptor is responsible for the proinflammatory effects.

The study also shows concomitant increase in the release of TNF-α and IL-1β by activated microglia and therefore it is plausible that this effect is mediated *via* sphingolipid pathway, or that TNF-α and IL-1β activate the SphK1. Therefore we needed to confirm which came first in the sequence of events. For this, SphK1 would need to be inhibited first and then the microglia should be activated. The findings of this study show that the suppression of SphK1 activity in activated microglia by pretreatment of cells

with its inhibitor, DMS or transfection of cells with its siRNA, inhibited the expression levels of TNF-α, IL-1β and iNOS and release of TNF-α and NO. In addition, the BV2 microglial cells in the present study and purified microglia from primary cultures have been shown to express all or some of the five S1P receptors (Tham, et al., 2003). The results obtained clearly demonstrate that the sphingosine kinase signaling pathway is involved in inflammatory response of activated microglia in an autocrine/paracrine signaling fashion.

The activated microglia in response to CNS inflammation secrete pro-inflammatory cytokines such as TNF-α and IL-1β (Carson, et al., 1998, Carson and Sutcliffe, 1999, Frei and Fontana, 1997, Hickey and Kimura, 1988). It is well known that pro-inflammatory factors such as TNF-α can participate in causation of neuronal death (Harry and Kraft, 2008, Harry, et al., 2008, Kaushal and Schlichter, 2008). TNF signaling has been shown to have many functions such as regulation of BBB permeability, febrile responses, glutamatergic transmission and synaptic plasticity and scaling (McCoy and Tansey, 2008). Elevated levels of TNF-α are present in large number of neurological diseases such as ischemia, traumatic brain injury, multiple sclerosis, Alzheimer's disease and Parkinsosn's disease. Therefore modulation of TNF-α levels in neuroinflammatory conditions is an important method to delay the progression of these diseases. Activated microglia have also been known to express proinflammatory cytokines and chemokines such as IL-1β,

IL-8, IL-10, IL-12, etc (Dheen, 2007). This further suggests that S1P acts as an upstream factor via its receptors which induces the production of proinflammatory cytokines and neurotoxic substances such as NO in activated microglia.

The results also show that the exogenous addition of S1P could not restore the expression of cytokines completely in LPS activated SphK1- microglial cells although it enhanced the cytokine expression levels in untransfected LPS-treated BV2 cells. Since many of the biological responses of S1P are mediated *via* transactivation of S1P receptors (Anliker and Chun, 2004, Spiegel and Milstien, 2003), the present results suggest that the exogenous addition of S1P in SphK1-microglial culture was not sufficient to induce the transactivation of S1P receptors and their downstream signaling pathways. Moreover, the elevated NO release in LPS-activated SphK1- microglial cells in response to exogenous S1P did not correlate with mRNA expression level of iNOS, which is responsible for the rapid production of NO. It is suggested that this discrepancy could be attributed to the differential NO production which may be regulated at the level of protein translation.

The finding that the sphingosine signaling pathway is upregulated in activated microglia has important implications not only in inflammatory responses of microglial cells, but for other physiological and pathological processes regulated by S1P in neurons as it is a pleiotropic lipid mediator that regulates many different biological responses, including growth, survival, differentiation, cytoskeleton rearrangements, angiogenesis, vascular

maturation, and lymphocyte trafficking (Anliker and Chun, 2004, Olivera and Rivera, 2005, Olivera, et al., 2006, Rosen and Goetzl, 2005, Saba and Hla, 2004, Spiegel and Milstien, 2003).

The sphingosine signaling pathway has been implicated in neurodegenerative diseases such as Alzheimer's disease. Although the pathogenesis of Alzheimer's disease is not fully clear yet, it is understood that accumulation of β-amyloid causes neuronal degeneration and microglia activation. Studies in human neurons, oligodendrocytes and brain sections also provide direct evidence that β-amyloid causes sphingolipid pathway activation and ceramide accumulation (Okada, et al., 2009). S1P plays a role in neuronal excitability since S1P2 null mice have spontaneous seizures and increase in excitatory currents (Okada, et al., 2009). These studies taken together with the results of this study suggest that the sphingolipid pathway in an important aspect in microglial and neuronal responses to inflammation in neurodegenerative diseases.

4.1 Conclusion and scope for future study

Neurodegenerative conditions such as Alzheimer's disease and Parkinson's disease cause immeasurable loss of treasured memories and debilitating physical symptoms. Hence they captured wide attention and the need to find out possible cures or preventive strategies have gained urgency due to the increase in the lifespan of mankind, leading to an increase in the ageing population.

Microglial activation has been implicated as one of the key mechanisms in neurodegenerative conditions such as Alzheimer's disease and Parkinson's disease. Hence control and modulation of microglial activation is considered a viable means to achieve cure or control of these conditions. Therefore, the comprehensive effects of microglial modulation need to be studied to avoid any adverse effects. Recently the accumulation of the Aβ has been shown to cause neuronal degeneration in AD brains through abnormal sphingolipid metabolism (Okada, et al., 2009). It is well established that Aβ in AD brain activates microglia, which release large amounts of proinflammatory cytokines, and reactive oxygen intermediates, contributing to the neuroinflammation and neurodegeneration (Dheen, et al., 2005, Dheen, et al., 2007). It is possible that this inflammatory response of microglia in the AD brain is mediated *via* S1P signaling pathway.

The experimental results show that suppression of SphK1 activity thereby reducing S1P, in activated microglia leads to suppression of pro inflammatory cytokines and neurotoxic factors. This may be considered as a possible future therapeutic mode for the control of production of factors that contribute to neuroinflammation. However, the effects of suppression of SphK1 in microglia need to be carefully evaluated, as SphK1 is a known modulator of myriad other functions, not only in the CNS, but also other organ systems, and may cause undesirable side effects, if all the comprehensive effects are not taken into consideration. Hence, further studies need to be done

in order to evaluate the clinical efficacy of SphK1 suppression in neuroinflammatory conditions

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Figure Plates and Legends

Fig.1 (A-C) Immunofluorescence shows the expression of SphK1 (red) in the cytoplasm of BV2 cells, double labeled with lectin (green). (D, E) RT-PCR analysis of cDNA derived from BV2 cells shows the expression of SphK1 (507bp) and S1P receptors, S1P1,2,3,4,5 (80-120bp). Scale bar, 50µm.

Fig. 1 (A – E)

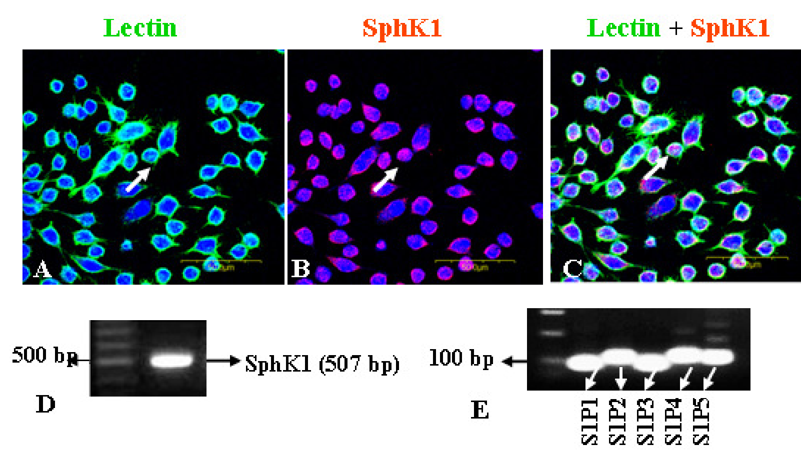


Fig.2 (A-F) Immunofluorescence images showing changes in SphK1 expression in microglia after treatment with LPS compared to untreated cells at 30min, 1 and 6h. The SphK1 expression peaks at 1h post treatment with LPS, but it returns to baseline levels at 6h. Western blot analysis (G, H) shows that the SphK1 protein expression increases after LPS treatment, and it reaches its maximum at 1h after treatment. The data represent the mean±SE of at least three independent experiments. Control vs LPS treated; $*p<0.05$. Scale bar (A), 50µm.

Fig. 2 (A – G)

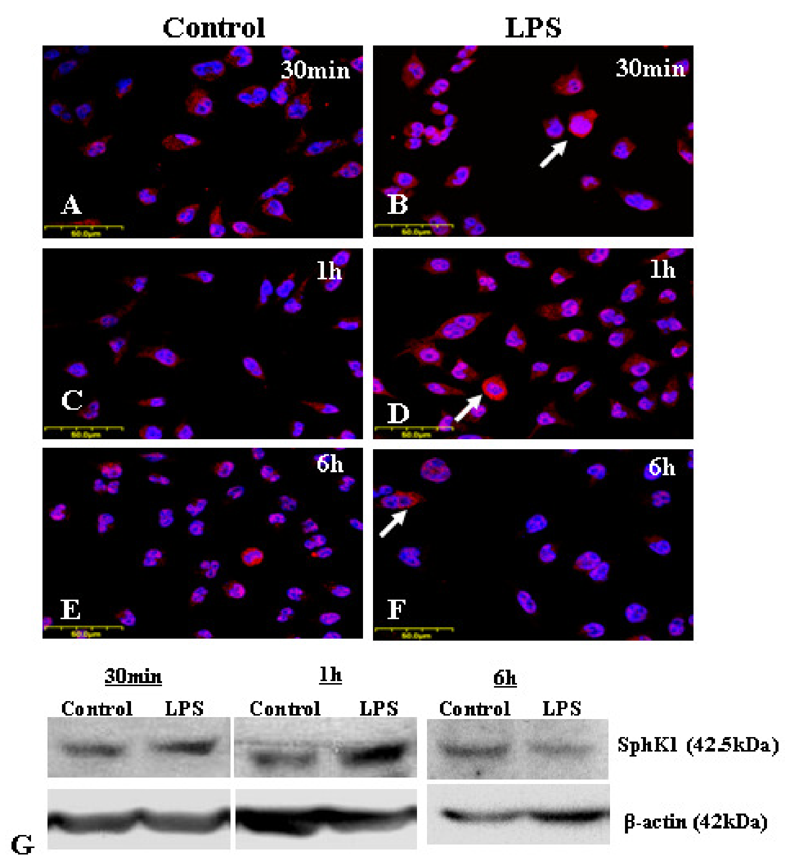


Fig. 2H

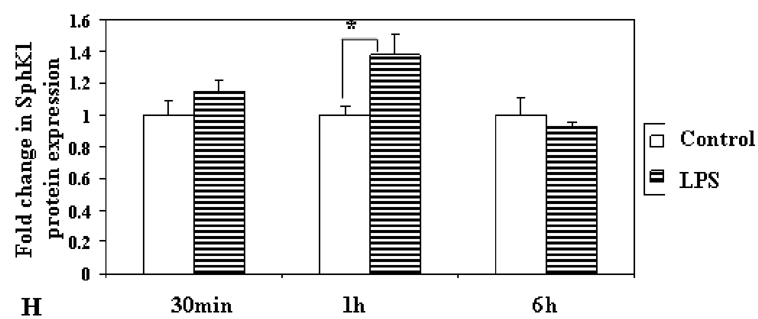


Fig.3 (A-L) Immunofluorescence images show that the TNF-α expression (green) is reduced with SphK1 suppression by DMS in LPS-treated BV2 cells at all time points (30min, 1h, 6h) when compared to cells treated with LPS alone. Note that LPS treatment gradually increases the TNF-α expression which peaks maximum at 6h in BV2 cells. (M, N) Real time RT-PCR analysis shows that TNF-α mRNA expression level is significantly increased in BV2 cells treated with LPS for 30min, 1, 3, and 6h. The maximum increase is found 3h after the treatment. Suppression of SphK1 with DMS in LPS-treated BV2 cells results in a significant reduction in TNF-α mRNA expression compared with controls at all time points studied. The data represent the mean±SE of at least three independent experiments. The PCR product (205bp) for TNF-α is shown (M). Control vs DMS-treated; LPS-treated vs LPS+DMS-treated; *$p<0.05$. Scale bar (A-L), 50μm.

Fig. 3 (A –M)

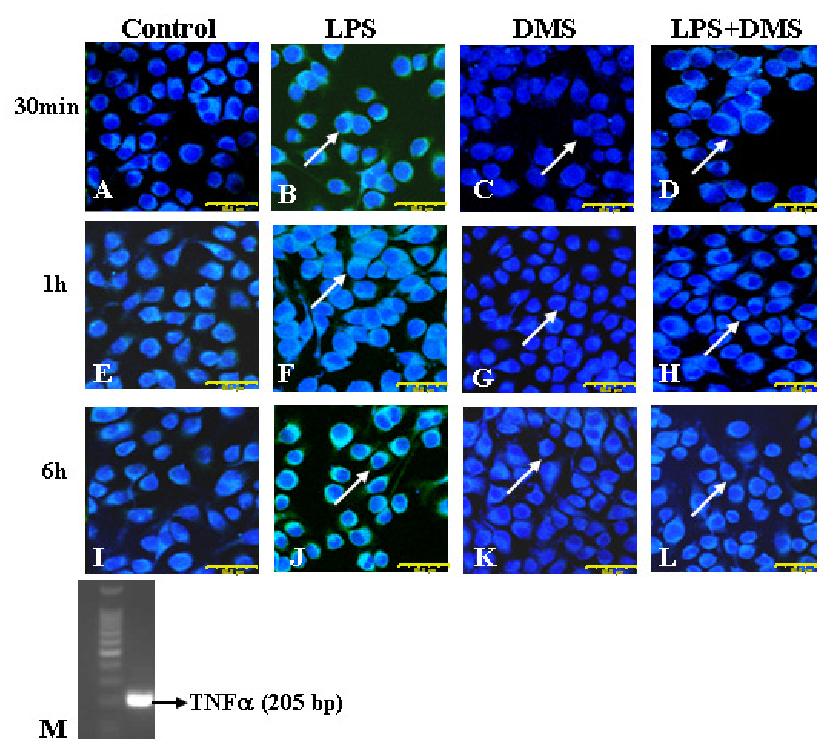


Fig. 3N

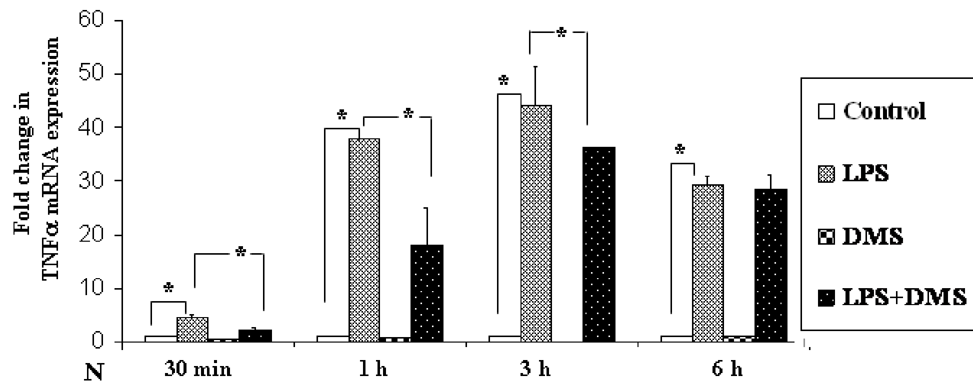


Fig.4 S1P treatment increases TNF-α levels in BV2 cells. (A-L) Immunofluorescence images showing increased expression of TNF-α upon treatment with S1P. The TNF-α expression is increased noticeably when BV2 cells are treated with S1P or LPS alone and S1P together with LPS. (M) RT-PCR analysis shows the increased mRNA expression level of TNF-α in BV2 cells treated with S1P or LPS or S1P+LPS at all time points studied (30min, 1h, 3h, 6h), compared with untreated controls. (N) ELISA further confirms that the TNF-α release is increased in BV2 cells treated with S1P alone and S1P together with LPS and is reduced in cells treated with LPS+DMS. The data shown are the mean±SE of at least three independent experiments. Control vs S1P-treated; LPS-treated vs LPS+S1P-treated; $^*p<0.05$. Scale bar (A), 50μm.

Fig. 4 (A – L)

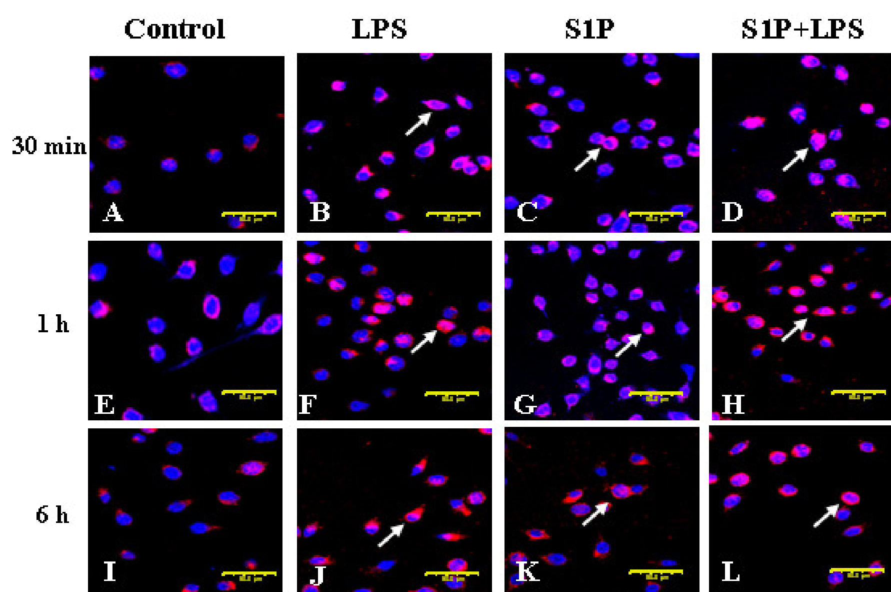


Fig. 4M

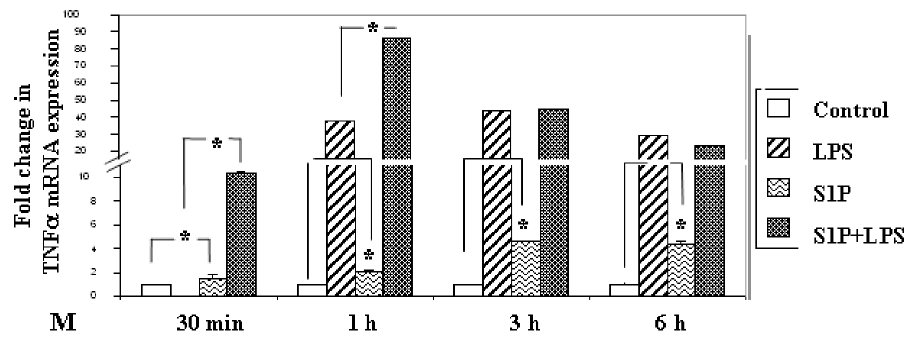
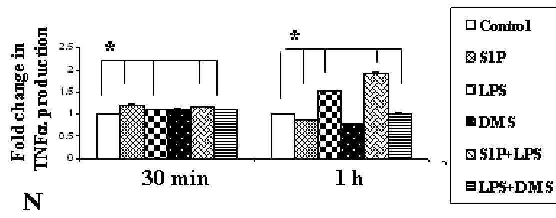


Fig. 4N



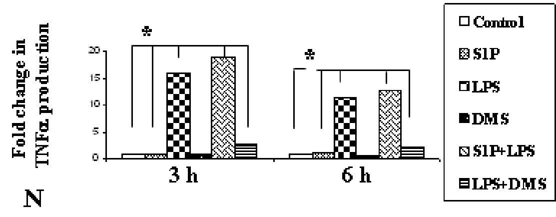


Fig.5 Suppression of SphK1 (SphK1-) with siRNA reduces TNF-α production in BV2 microglial cells. (A, B) Immunofluorescence images show that SphK1 immunoreactivity is markedly reduced in SphK1- cells compared to negative control cells. RT-PCR analysis shows that the efficiency of siRNA suppression of SphK1 is about 80% compared to negative controls (C). Crossing point of PCR cycles is also shown (D). (E-I) Immunofluorescence images showing reduced expression of TNF-α in SphK1- cells. The TNF-α expression is reduced in SphK1- cells (F) but is increased with S1P administration (H). LPS treatment is unable to increase the expression of TNF-α in SphK1- cells (G). However, the TNF-α expression is increased, when S1P is concomitantly administered with LPS in SphK1- cells (I). RT-PCR (J) and ELISA (K) analyses shows that TNF-α mRNA expression and production are decreased in SphK1- cells respectively, compared with negative controls. LPS treatment is unable to increase TNF-α mRNA expression and release in SphK1- cells. S1P treatment of SphK1- cells increases the production of TNF-α Upon treatment of SphK1- cells with S1P alone or S1P+LPS, the level of TNF-α mRNA expression level and release is moderately increased above base negative control level (J), although there is no significant increase in its release (K). The data represent the mean±SE of at least three independent experiments. Control vs treated; **$p<0.005$ Scale bar (A,B), 50µm.

Fig. 5A – 5I

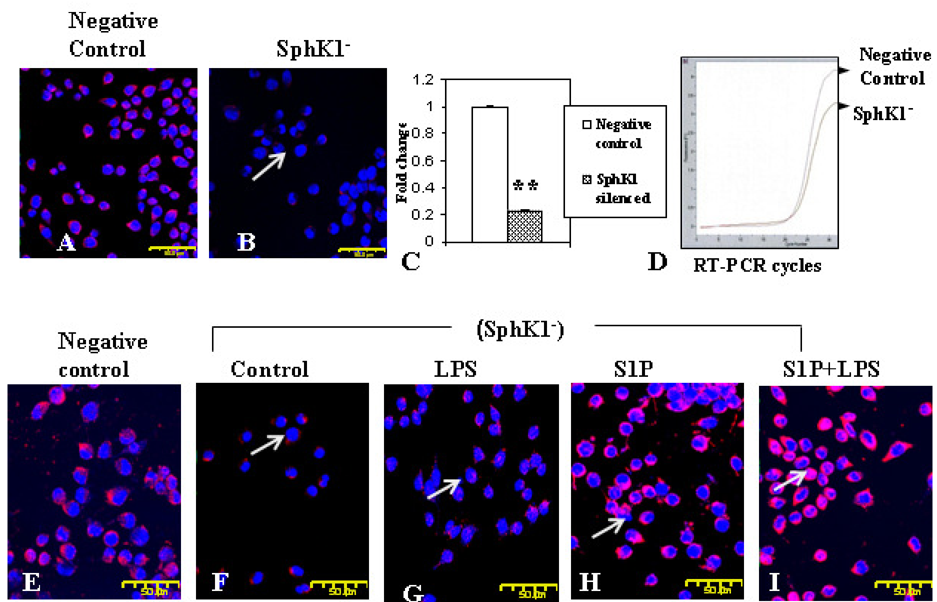
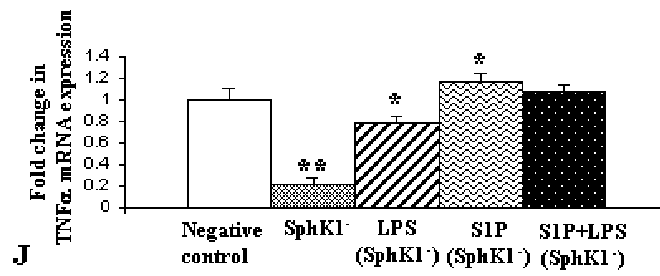


Fig. 5J – 5K



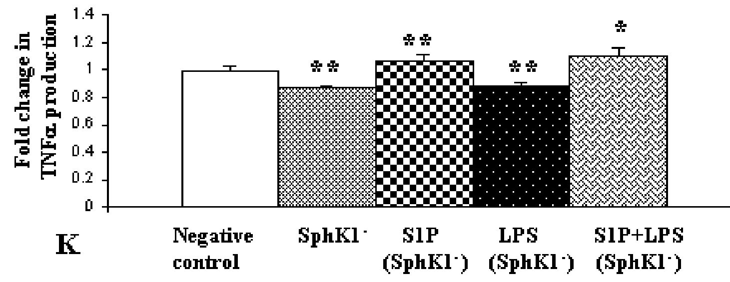


Fig.6 RT-PCR analysis showing time-course plot of IL-1β mRNA expression in BV2 cells. (A) IL-1β (229bp) is amplified in BV2 microglial cells. (B) LPS treatment increases IL-1β expression at all time points studied but upon concomitant suppression of SphK1 with DMS in LPS activated cells, the IL1β expression is markedly reduced until 3h post treatment with a return to baseline levels at 6h. (C) IL-1β mRNA expression level is further increased significantly in LPS-treated cells upon addition of S1P, compared to cells treated with LPS alone. (D) RT-PCR analysis showing the suppression of IL-1β mRNA expression in SphK1- cells. Suppression of SphK1 with siRNA results in a reduction in IL-1β mRNA expression compared with negative controls. LPS treatment is unable to increase IL-1β mRNA expression in SphK1- cells compared to negative controls. S1P treatment with or without LPS significantly increases the expression of IL-1β in SphK1- cells. The data represent the mean±SE of at least three independent experiments. Control vs treated; $^*p<0.005$

Fig. 6A – 6B

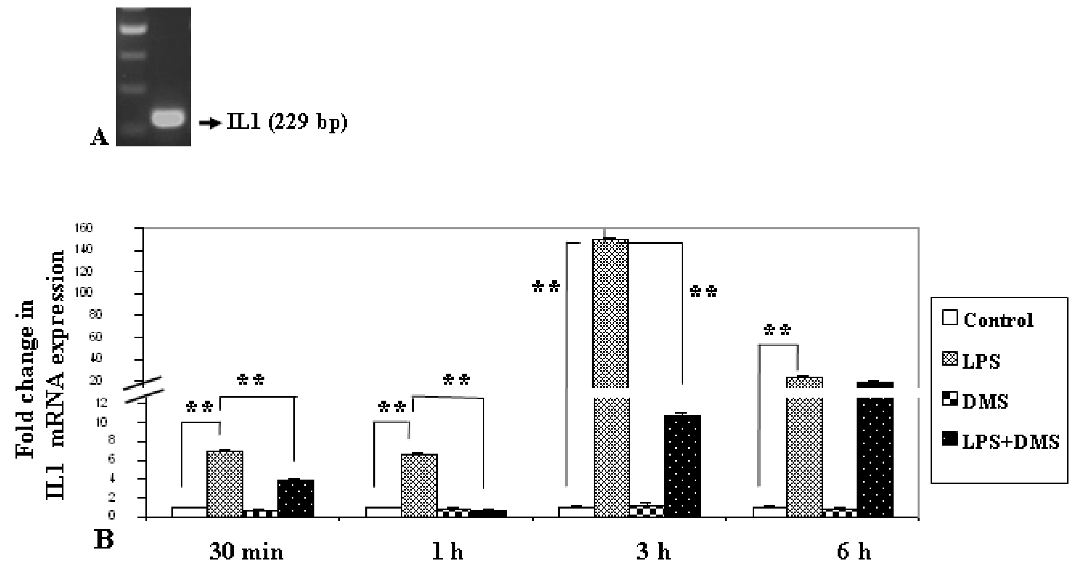


Fig. 6C

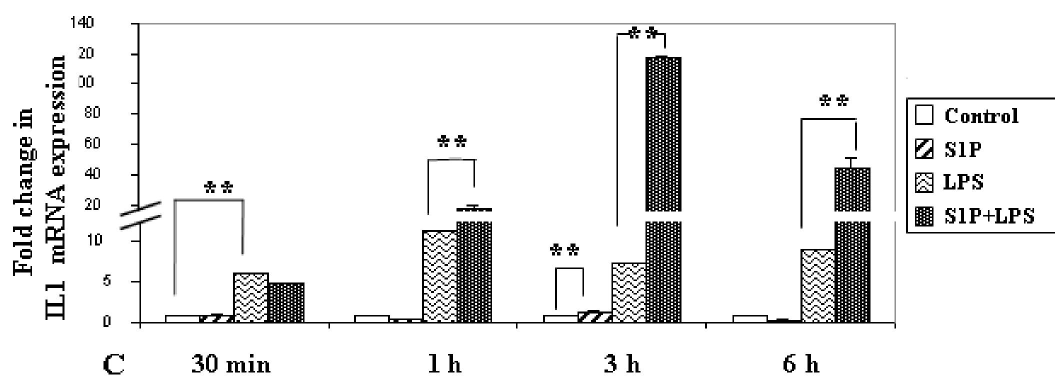


Fig. 6D

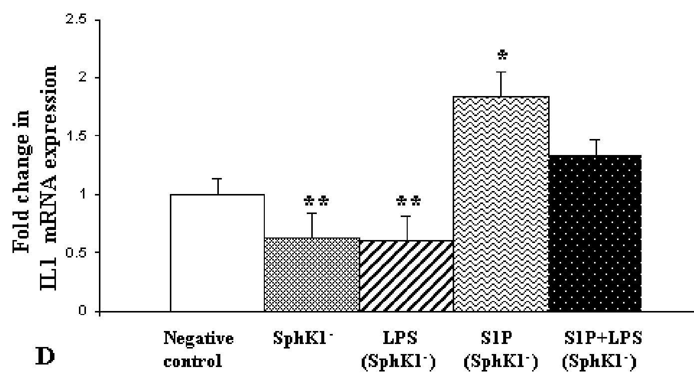


Fig.7 (A) RT PCR analysis showing suppression of SphK1 by DMS reduces iNOS mRNA expression in BV2 cells at different time points. (A) 217bp of iNOS PCR product is amplified. (B) LPS treatment increases iNOS expression in BV2 cells at 6h compared to control, but upon concomitant suppression of SphK1 with DMS in LPS activated cells, iNOS expression level is reduced significantly compared to cells treated with LPS alone. (C) RT-PCR analysis further shows that LPS-induced increase in iNOS mRNA expression in activated microglia is further augmented by the addition of S1P, compared to cells treated with LPS alone. (D) RT-PCR analysis shows that suppression of SphK1 with siRNA (SphK1-) decreases iNOS mRNA expression in BV2 cells, compared with negative controls. Treatment of SphK1-cells with LPS, and S1P with or without LPS increases the iNOS expression compared to that observed in control SphK1-cells (D). However, this increase was not above that of negative control. (E) Nitric oxide assay shows that in SphK1- cells, the NO production is limited compared to negative control. In SphK1- cells treated with LPS, the NO levels remains at baseline control levels. However, exogenous administration of S1P with or without LPS increases NO production significantly in SphK1- cells. The data represent the mean±SE of at least three independent experiments. Control/negative control vs treated samples; *p<0.005.

Fig. 7A – 7B

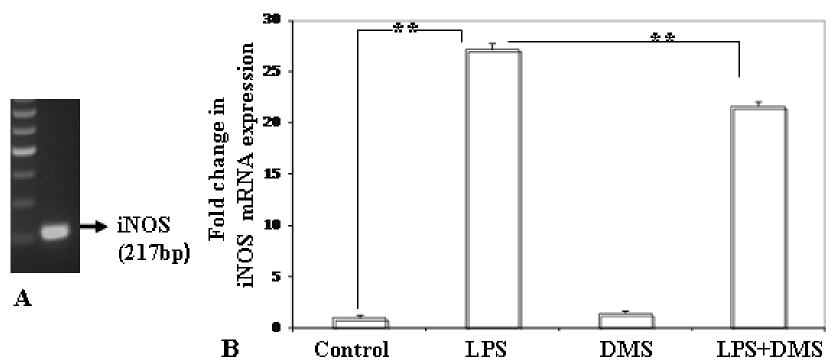


Fig. 7C

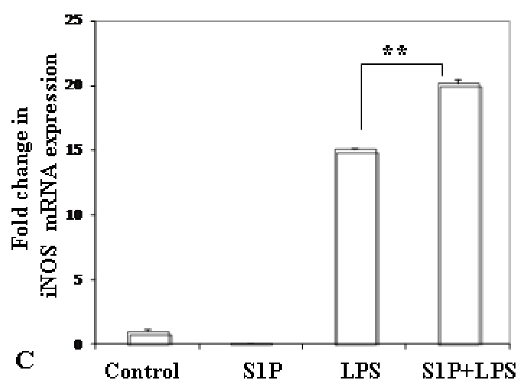


Fig. 7D

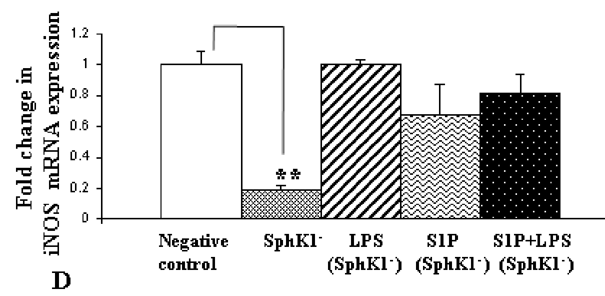


Fig. 7E