Intracellular S1P is not essential for NFκB induction

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Background: Sphingosine kinases (Sphk) were proposed to be essential for inflammatory responses.

Results: Robust inflammatory responses were seen in macrophages that lack Sphk. However, intracellular sphingolipids and autophagic vesicles were induced.

Conclusions: Sphingosine kinases are not required for inflammation.

Significance: Attenuation of Sphk activity may not be critical for inflammation but could lead to altered sphingolipid levels and autophagy.

SUMMARY

Sphingosine kinases (Sphk), which catalyze the formation of sphingosine 1-phosphate (S1P) from sphingosine (Sph), have been implicated as essential intracellular messengers in inflammatory responses. Specifically, intracellular Sphk1-derived S1P was reported to be required for NFκB induction during inflammatory cytokine action. To examine the role of intracellular S1P in inflammatory response of innate immune cells, we derived murine macrophages that lack both Sphk1 and Sphk2 (mo Sphk dKO). Compared to WT counterparts, mo Sphk dKO cells showed marked suppression of intracellular S1P levels while sphingosine and ceramide levels were strongly upregulated. Cellular proliferation and apoptosis were similar in mo Sphk dKO cells compared to WT counterparts. Treatment of WT and Sphk dKO mo with inflammatory mediators TNFα or E. coli LPS resulted in similar NFκB activation and cytokine expression. Furthermore, LPS-induced inflammatory responses, mortality and thioglycollate-induced macrophage recruitment to the peritoneum were indistinguishable between mo Sphk dKO and littermate control mice. Interestingly, autophagic markers were constitutively induced in bone marrow-derived macrophages from Sphk dKO mice. Treatment with exogenous sphingosine further enhanced intracellular sphingolipid levels and autophagosomes. Inhibition of autophagy resulted in caspase-dependent cell death. Together, these data suggest that attenuation of Sphk activity, particularly Sphk2, leads to increased intracellular sphingolipids and autophagy in macrophages.

INTRODUCTION

Sphingosine 1-phosphate (S1P) is a biologically active lipid that regulates many physiological processes, such as lymphocyte trafficking and vascular development (1,2). S1P is generated by phosphorylation of free sphingosine (Sph) by two sphingosine kinases (Sphks) 1 and 2, which are highly conserved and ubiquitously expressed (3,4). Cellular levels of S1P are regulated not only through its biosynthesis but also degradation by S1P lyase (SPL) (5), S1P phosphatases (SPPs) (6), and intracellular lipid phosphate phosphatases (LPPs) (7). S1P exerts
biological functions mostly through five cell surface G protein–coupled receptors S1P₁–S1P₅. In addition, it has recently been proposed that intracellular S1P directly binds to several proteins and regulates their functions. For example, S1P was proposed to bind to and activate TRAF2, HDAC1 and 2 to block their activity and mitochondrial protein prohibitin to modulate respiration (8-10).

Since Sphk enzymes are required to produce S1P, mice that lack both sphingosine kinases do not contain detectable levels of S1P (11). Because such global Sphk double KO mice are embryonic lethal due to a vascular defect, it was suggested that S1P signaling via its receptors constitute an essential event in embryonic vascular development (11). However, single isofrom knockout, i.e., Sphk1⁻/⁻ or Sphk2⁻/⁻ mice are phenotypically normal (12), suggesting that Sphk1 and Sphk2 have redundant functions and can compensate for each other to fulfill essential functions. Indeed, Sphk1 mRNA and activity were induced and plasma S1P was elevated in Sphk2⁻/⁻ mice (13-16), suggesting that lack of Sphk2 leads to a compensatory upregulation of Sphk1 expression. The mechanism responsible for this compensation is not known.

Recently, it was proposed that Sphk enzymes are essential for inflammatory responses. For example, intracellular S1P produced from Sphk1 was proposed to bind to the TNFα receptor associated factor (TRAf)-2 and stimulate its E3 ubiquitin ligase activity as a key mechanism for NFkB signaling (9). Since TRAF-2 is a critical intermediate in the signal transduction of inflammatory cytokines (17-19), this work suggested an essential role of intracellular S1P in cytokine-induced inflammatory pathways. Other reports showed that inhibition of Sphk1 by its inhibitor and/or siRNA decreased expression of proinflammatory cytokines (20-22). On the other hand, studies that attenuated Sphk1 activity or expression by either pharmacological inhibitors or Sphk1 siRNA rendered macrophages sensitive to M. smegmatis infection (23), and deletion of Sphk1 exhibited disparate effects in mice against the inflammation and injury induced by LPS (24-26). In addition, we and others have reported that Sphk1⁻/⁻ and Sphk2⁻/⁻ mice do not exhibit attenuated inflammatory responses in several inflammatory models (25,27,28). Therefore, the role of Sphk enzymes in inflammation is unclear at present.

In order to examine in the issue of intracellular S1P as a critical mediator of cytokine-induced inflammatory responses in innate immune cells, we investigated murine macrophages that lack both Sphk isoenzymes. Our results suggest that intracellular S1P is not required for macrophages to respond to TNF-α or LPS. Rather, Sphk2 is involved in an intracellular metabolic network that maintains sphingolipid homeostasis, which when perturbed, leads to accumulation of sphingolipid metabolites and compensatory autophagy.

**EXPERIMENTAL PROCEDURES**

**Animals** - We obtained C57BL/6 mice and LysM-Cre mice, which expresses the Cre recombinase driven by the lysozyme M promoter (29) from the Jackson Laboratory. Sphk1floxt/lox, Sphk2floxt/lox, Sphk1⁻/⁻ and Sphk2⁻/⁻ mice were described previously (11,30). By crossbreeding, myeloid-specific Sphk1/Sphk2 knockout mice (Sphk1floxt/lox Sphk2⁻/⁻ LysM-cre⁺ or Sphk1floxt/lox Sphk2floxt/lox LysM-cre⁺) were generated. All mice were >8 wk old when used for the described experiments. Comparisons were done to cre-littermate controls. All studies were performed under animal protocols approved by the IACUC of Weill Cornell Medical College, the University of California, San Francisco or the French Ministry of Agriculture.

**Reagents and Cell Culture** - C2-ceramide, Sph and S1P were purchased from Avanti Polar Lipids. Doxorubicin (Dox), ammonium chloride (NH₄Cl) and 3-methyladenine (3-MA) were purchased from Sigma. Bone marrow-derived macrophages (BMDM) were isolated as described previously (31). The cells were cultured in DMEM containing 10% FBS and 20% L-cell conditioned media as a source of macrophage colony-stimulating factor (M-CSF). Macrophages were obtained as a homogeneous population of adherent cells after 5-7 days of culture. Thiglycolate-elicted peritoneal macrophages were isolated from mice 4 days after injection of 2 ml of sterile thiglycolate (Sigma). The cells were incubated at 37°C in a humidified 5% CO₂ atmosphere.

**Western blot analysis** - Cells were washed with ice-cold phosphate-buffered saline and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.25% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 1 mM Na orthovanadate, and 1 x protease inhibitor cocktail). Protein concentrations of supernatants were determined by BCA protein assay kit (Pierce Chemical Co.). Equal amounts of protein were separated on a 10% SDS-PAGE and blotted onto a nitrocellulose membrane.
performed using the following antibodies: p-p65, p65, p-JNK, p-p38 (Cell Signaling); Sphk1, COX-2 (Cayman Chemical); Sphk2 and β-Actin (Abcam). Blots were developed with the Western blot development kit from GE Healthcare.

Cell viability assay- The survival of macrophages under experiments was evaluated by the MTT dye reduction method. After each incubation time, the cells were incubated with yellow MTT dye [3-(4,5-dimethylthiazol-2-yl)-2, diphenyl-tetrazolium bromide (Sigma). The formazone crystals were dissolved in DMSO and OD was measured at 570 nm in a spectrometer Spectra max 250 (Bio-Rad).

RNA isolation and qRT-PCR analysis- Total RNA was extracted from treated cells using RNA Stat-60 (Tel-Test Inc) according to the manufacturer's instructions. Reverse transcription was carried out using First Strand cDNA synthesis kit for reverse transcription-PCR (avian myeloblastosis virus) (Roche Applied Science) using random primers. Real time PCR was performed on a 7500 real time PCR system (Applied Biosystems) using Fast SYBR® Green Master Mix (Applied Biosystems), relative RNA levels were calculated using the ΔΔCT method (31). Primer sets for qRT-PCR were Sphk1 (5'-AGGTGGTGATGGGCTAATG-3', 5'-TGCTCGTACCCAGATAGTG-3'), Sphk2 (5'-TGGTGCAATCTCCTGAA-3', 5'-CCAGACACAGTGACAATGCC-3'), IL-1 (5'-TTCTTTGGTTATGGCTTGG-3', 5'-TTCTTTGGTTATGGCTTGG-3'), IL-6 (5'-CAGGGAGGAGCTTCACAG-3', 5'-TCCACGATTTCGCCAGAAAC-3'), TNF-α (5'-CAGTTGCTTGTTGCTACG-3', 5'-CATCGATGAGCTATGACGT-3'), Gapdh (5'-AGAACATCATCTCCTGTGATCC-3', 5'-CAGATTGGGGGTAGGAACAC-3').

Intracellular sphingolipid analysis - Intracellular sphingolipid levels of ceramides (Cer), Sph and S1P were analyzed by the Lipidomics Analytical Core at the Medical University of South Carolina using LC-MS/MS methods (32).

Lysosomal staining with Dextran - For lysosome staining, BMDM were plated on Poly-D-lysine coated glass coverslip bottom dishes and incubated for 16 h with 2.2 mg/ml dextran conjugated to Rhodamine (70,000 mol. Wt.; Invitrogen) in complete growth media. Cells were washed thoroughly in complete media and then incubated in media with or without 20 mM sphingosine for 4 h. Live cells were imaged in medium 2 and 0.2% (w/v) glucose on a Zeiss LSM510 laser scanning confocal microscope using a 63x, 1.4 numerical aperture plan Apochromat objective. Cell temperature was maintained at 37°C with a heated stage and objective heater (33).

Flow cytometry- BMDM were harvested after 7 days of culture and then incubated on ice for 30 min with the indicated antibodies, washed, and processed using an LSR II flow cytometer (BD Biosciences). The antibodies used were PE anti-CD11b (BioLegend), and FITC-F4/80 (eBioscience). Stained cells were subsequently washed and analyzed using an LSR II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (version 8; Tree Star, Inc.).

Immunofluorescence - Cells grown on glass coverslips were washed with PBS, fixed in 2% paraformaldehyde solution for 15 min at room temperature, and permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. Immunofluorescence analysis was performed using anti LC3B antibody (Cell Signaling) and Alexa Fluor 594-conjugated secondary antibody (Invitrogen). Confocal laser-scanning microscopy analysis was performed using a Fluoview FV10i system (Olympus).

Endotoxemia - Female littermates 8 weeks or older were injected intraperitoneally (i.p.) with freshly prepared LPS (30mg/kg; E-coli 0111:B4; Sigma-Aldrich) in normal saline. Survival and righting reflexes were monitored every 12 hours for 5 days, after which all surviving mice were sacrificed. Experimental animals without righting reflex at time of monitoring were considered moribund, euthanized and included as non-survivors in survival curves generated by the Kaplan and Meier method using Graphpad Prism software.

Plasma cytokines - Blood from the retro-orbital venous plexus was collected with EDTA coated glass capillaries into EDTA tubes 8 hours after i.p. injection of 30 mg/kg LPS. Plasma was removed after blood centrifugation at 500 g for 10 minutes. Plasma levels of interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), interferon-γ (IFN-γ), tumor necrosis factor α (TNFα), and interleukin-12p70 (IL-12p70) were determined using the BD cytometric bead array (CBA, BD Biosciences) Mouse Inflammation kit according to the manufacturer’s instructions.
RESULTS

Sphks are not essential for myeloid cell survival, proliferation or differentiation- To explore the role of sphingosine kinase isoenzymes in macrophages, Sphk1flo×flo Sphk2−/− mice (Sphk2−/−) were used as the source of Sphk2−/− macrophages, Sphk1flo×flo Sphk2−/− mice were crossed with lysozyme-Cre mice to generate the myeloid Sphk1 and Sphk2 double knockout mice (MΦ Sphk dKO), which were the source of Sphk1 and Sphk2 null macrophages. The deletion efficiency of Sphk1 gene in isolated BMDM from MΦ Sphk dKO mice was almost complete as determined by PCR of genomic DNA (Figure 1A). RNA analysis by quantitative RT-PCR showed that both Sphk1 and Sphk2 transcripts were downregulated >90% in respective knockout BMDM cells (Figure 1B). Immunoblot analysis of BMDM cell extracts showed complete loss of expression of Sphk2 and both Sphk1 / Sphk2 in Sphk2−/− and MΦ Sphk dKO cells (Figure 1C).

BMDM from WT, Sphk2−/− and MΦ Sphk dKO mice were analyzed for the levels of S1P, sphingosine and ceramides by LC/MS/MS. S1P levels were markedly diminished in both Sphk2−/− and MΦ Sphk dKO cells, suggesting that the activity of Sphk2 is important in the basal production of S1P (Figure 2A). Interestingly, sphingosine levels were significantly elevated in Sphk2−/− and MΦ Sphk dKO. This suggests that lack of Sphk isoenzymes leads to the accumulation of the substrate sphingosine. Furthermore, ceramide levels were also elevated (Figure 2B). Intracellular ceramide and sphingosine levels were at least two and one order of magnitude higher than S1P, respectively. These data suggest that lack of Sphk isoenzymes leads to metabolic “pileup” of sphingolipids.

Sphk2−/− and MΦ Sphk dKO mice were grossly indistinguishable from WT mice and no obvious abnormality was detected. To examine whether the loss of Sphk isoenzymes affects BMDM differentiation, macrophage makers (CD11b and F4/80) were analyzed by flow cytometry in BMDM. More than 98% cells expressed both of these macrophage markers (Figure 2C), suggesting that lack of Sphk isoenzymes does not impair differentiation of hematopoietic stem cells into macrophages. Moreover, kinetics of cell proliferation were similar among BMDM derived from WT, Sphk2−/−, and MΦ Sphk dKO mice (Figure 2D), suggesting that Sphk isoenzymes are not required for myeloid cell survival or proliferation in vitro.

Sphk1 and 2 are not necessary for macrophage inflammatory responses in vitro- Intracellular levels of sphingoid bases (Sph, dihydro-sphingosine), sphingoid base-1-phosphates (S1P, dihydro-S1P) and ceramide molecular species were quantified in BMDM after TNF-α treatment. Surprisingly, TNF-α treatment (4 h) did not result in significant alterations in intracellular sphingoid bases or the phosphorylated derivatives in BMDM isolated from Sphk2−/−, MΦ Sphk dKO and WT mice (Figure 3A).

Inflammatory responses in macrophages are regulated by key signal transduction intermediates, such as NFκB and stress activated protein kinase (SAPK) members, p38 and JNK. To examine the role of Sphk isoenzymes and intracellular S1P in macrophage inflammatory signal transduction, we examined the activation of NFκB p65, p38 SAPK and JNK phosphorylation. Since BMDM showed strong activation of pp65 and pp38 after 15 min treatment of TNF-α and 30 min treatment of LPS (data not shown), we treated BMDM with TNF-α for 30 min and LPS for 15 min. BMDM from Sphk2−/−, MΦ Sphk dKO and WT mice showed equivalent activation of pp65, pJNK and pp38 after treatment with LPS or TNFα (Figure 3B). Further, cyclooxygenase-2 (COX-2), a key downstream inflammatory gene was also induced to a similar extent in BMDM from Sphk2−/−, MΦ Sphk dKO and WT mice 24 hours after LPS treatment (Figure 3C). Similar findings were seen in thioglycollate-elicited peritoneal macrophages (data not shown).

We also treated BMDM with LPS for 2 and 6 hours, and mRNAs involved in the inflammatory response were detected by qPCR. The results indicate that IL-1, IL-6, and TNF-α mRNA were induced by LPS treatment, but no significant differences were observed between BMDM from Sphk2−/−, MΦ Sphk dKO and WT mice (Figure 3D).

Macrophage Sphk1 and 2 are not necessary for inflammatory responses in vivo- Next, to investigate the role of myeloid Sphk isoenzymes in inflammatory responses in vivo, we employed LPS to model the systemic inflammatory response characteristic of septic shock. We first observed that knocking out Sphk1 in all cells did not protect mice from LPS induced mortality (Figure 4A). To exclude compensation by Sphk2,
myeloid cell specific Sphk1/ Sphk2 double knock-out mice (Sphk1^flox/flox^; Sphk2^cre/cre^) were challenged with 30 mg/kg LPS. No significant difference was observed between MΦ Sphk dKO and littermate controls (Sphk1^flox/flox^; Sphk2^cre/cre^) in the plasma levels of inflammatory cytokines TNFα, INF-γ, MCP-1, IL-6, IL-12 and IL-12 or mortality (Figure 4B, C). We employed the thioglycolate-induced peritonitis model to explore whether macrophage recruitment is altered in Sphk2^−/− and MΦ Sphk dKO mice in response to inflammatory insult. The mice were injected with 3% thioglycolate and peritoneal macrophages were isolated by lavage 4 days later. Recruited peritoneal macrophage numbers were similar in WT, Sphk2^−/−, and MΦ Sphk dKO mice (Figure 4D). These results suggest that macrophage Sphk isoenzymes are not necessary for inflammatory responses in vivo.

Sphk2 is critical for intracellular sphingoid base homeostasis in BMDM As shown above, Sphk2^−/− and MΦ Sphk dKO BMDM contained higher levels of sphingosine and ceramides than WT BMDM. When treated with exogenous sphingosine, further increases in intracellular sphingosine and ceramides were observed in MΦ Sphk dKO BMDM (Figure 5). In particular, C16 ceramide was greatly enhanced (Figure 2B). This was also observed, albeit to a slightly lesser extent in Sphk2^−/− BMDM. S1P was increased in Sphk2^−/− BMDM, which was expected since these cells express Sphk1. These data suggest that lack of Sphk isoenzymes leads to intracellular accumulation of sphingosine and ceramide in BMDM.

Treatment of WT BMDM with exogenous sphingosine led to strong induction of mRNA and protein for Sphk2 (Figure 6). This was also seen in HEK293 cells. In contrast, Sphk1 was not induced. These data suggest that accumulation of intracellular sphingosine leads to the compensatory increase in Sphk2 expression and the formation of S1P.

Sphk2 expression, cellular autophagy and cell death
Intracellular accumulation of sphingosine and ceramides could lead to alteration in membrane lipid composition of subcellular organelles. Disturbances in membrane lipid composition cause compensatory autophagy, a process by which organelles are destroyed intracellularly (34). Indeed, autophagic vacuoles, as determined by LC3B staining were enhanced in Sphk2^−/− and MΦ Sphk dKO BMDM (Figure 7A). Treatment with exogenous sphingosine further enhanced the accumulation of autophagic vacuoles, especially in MΦ Sphk dKO BMDM. Similarly, treatment with NH4Cl, which is known to increase the accumulation of autophagic vacuoles, also enhanced the number of LC3^+ vesicles in Sphk2^−/− and MΦ Sphk dKO BMDM. Immunoblot analysis using LC3B antibodies demonstrated the presence of active form of LC3B (LC3B-II) in Sphk2^−/− and MΦ Sphk dKO BMDM which was further increased by exogenous sphingosine and NH4Cl treatment (Figure 7B). Also, lysosome morphology was determined by the rhodamine dextran uptake assay (Figure 7C). Under control conditions, normal lysosome morphology was observed in the WT, Sphk2^−/− and MΦ Sphk dKO BMDM. WT BMDM lysosomes were unaffected following loading the cells with 5 μM sphingosine, while a small fraction of cells in the Sphk2^−/− and MΦ Sphk dKO BMDM contained slightly enlarged lysosomes. Upon loading cells with 20 μM sphingosine, BMDM begin to display enlarged lysosomes. Few macrophages with enlarged lysosomes were observed in the WT BMDM, while in Sphk2^−/− and MΦ Sphk dKO BMDM the majority of the cells exhibited enlarged lysosomes. The most severe phenotype was observed in the MΦ Sphk dKO BMDM. These data suggest that extremely high intracellular sphingosine levels promoted lysosomal abnormalities that parallel the accumulation of autophagosomes.

Exogenous sphingosine treatment led to increased cell death in Sphk2^−/− and MΦ Sphk dKO BMDM compared to WT BMDM (Figure 8A). In contrast, treatment with exogenous ceramide or S1P did not result in preferential toxicity of Sphk2^−/− and MΦ Sphk dKO BMDM (Figure 8C, D). Similarly, treatment of BMDM with the cytotoxic agent Doxorubicin, which induced caspase-dependent cell death, also did not induce preferential death of Sphk2^−/− and MΦ Sphk dKO BMDM (Figure 8B). These data suggest that accumulation of intracellular sphingosine leads to cell death due to excessive accumulation of sphingoid bases in Sphk2^−/− and MΦ Sphk dKO cells.

In WT BMDM, treatment with 3-methyadenine (3-MA), which inhibits autophagy by attenuation of PI-3-kinase (35,36), as well as NH4Cl, which inhibits lysosomal pH and prevents the fusion of autophagosomes to lysosomes, induced cell death in the presence of exogenous sphingosine (Figure 8C). Such treatments strongly induced activation of caspase-3 and -8 (Figure 8D). These data suggest that cellular autophagic pathways are cytoprotective in BMDM to compensate for intracellular sphingosine accumulation.

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DISCUSSION

In this study, we addressed the issue of whether intracellular S1P produced by the Sphk isoenzymes is critical for inflammatory cells from the innate immune system, namely, bone marrow-derived macrophages. During inflammatory reactions, bone marrow-derived monocytes are recruited into tissues in response to chemokine signals and differentiate into inflammatory macrophages. Such cells produce and respond to immune stimuli such as cytokines and are essential for tissue inflammation and resolution (37,38).

The function of intracellular S1P is controversial. Prior to the cloning and characterization of the G protein-coupled receptors for S1P (39,40), S1P was considered to be a “second messenger” (41-43), in an analogous fashion to the well-characterized intracellular signaling molecules such as diacylglycerol and cAMP. However, it is now clear that many of the biological effects of S1P, for example, regulation of lymphocyte egress, endothelial cell barrier function, angiogenesis, fibroblast proliferation and survival require the action of the G protein-coupled S1P receptors (1,2,44). Indeed, specialized transporters for S1P, such as Spns2 mediate the export of S1P and maintain the vascular S1P gradient in vertebrates (45,46). In addition to the extracellular signaling mode, S1P and dihydroS1P are utilized as intermediates in critical intracellular lipid metabolic pathways. For example, during de novo sphingolipid synthesis or metabolic breakdown of exogenously-derived sphingolipids, metabolism of phosphorylated sphingoid bases by the S1P lyase enzyme is important in the downstream utilization of fatty acyl CoA and phosphoethanolamine into complex phospholipid synthesis (47,48). In contrast to well characterized role of S1P, i.e., extracellular ligand for GPCRs and a metabolic intermediate, the physiological relevance of S1P as a classical second messenger that activate intracellular transducer systems to modulate cellular responses is not as well established. Recent studies proposed specific intracellular targets of S1P, for example, TRAP2 and HDAC1 and 2 (8,9).

In this study, we developed a novel system to examine the intracellular role of S1P in macrophages. Since Sphk1 and 2 carry out redundant functions of producing S1P, and can compensate for each other in the absence of one isofrom, we developed a model in which both isoenzymes are lacking in macrophages. In MΦ Sphk dKO BMDM, S1P levels are markedly attenuated while sphingosine and ceramide levels were upregulated, suggesting metabolic pileup. Indeed, low intracellular levels of S1P and higher levels of sphingosine and ceramide, which are at least one and two orders of magnitude higher, respectively, suggests the high catalytic turnover of Sphk enzymes under basal conditions. Interestingly, macrophage proliferation, differentiation and survival were not affected by lack of Sphk enzymes. Thus, lack of S1P or enhanced sphingosine or ceramide in and of itself is not sufficient to alter cell proliferation and/or death.

Importantly, stimulation of BMDM or elicited peritoneal macrophages from MΦ Sphk dKO mice did not exhibit any defect in TNFα and LPS-induced inflammatory responses. In particular, the NFκB pathway was activated to a similar extent, suggesting that intracellular S1P is not necessary for the activation of this critical inflammatory signaling pathway. Moreover, Sphk1−/− and MΦ Sphk dKO did not protect from LPS induced systemic inflammation and death. This is particularly important since several reports have concluded that Sphk1 is an important pro-inflammatory enzyme necessary for TNF-α and LPS-induced NFκB activation (9,49) and LPS induced inflammation through Sphk1/ S1P3 signaling (26). Indeed, Sphk inhibitors were proposed as important novel therapeutics in the treatment of chronic inflammatory disease and sepsis (26,50,51). Our work using genetic models (27) questions the validity of this hypothesis. It is possible that the use of sphingosine kinase inhibitors with poor specificity as well as siRNAs, which can have off-target effects, contributed to these hypotheses. It is also worth noting that several reports dealing with sphingosine kinases and inflammation have been retracted from the literature (49,52-54). These factors, together with many reports (25,27,28), including the data shown in this report, suggest that intracellular S1P activation of NFκB and inflammatory responses is not a major, physiologically relevant pathway in inflammation.

Although we find no role for sphingosine kinases in inflammatory responses, our results suggest a novel homeostatic role for sphingosine kinase in macrophages. We find that BMDM that lack Sphk2 or both Sphk isoenzymes show exaggerated autophagic vesicles. Addition of exogenous sphingosine further enhanced the autophagic response. Accumulation of autophagosomes may result from abnormal levels of sphingosine inducing abnormal lysosomes (55) and/or increased ceramide levels inducing enhanced autophagy (56). Indeed, enlarged lysosomes were more common in MΦ Sphk dKO BMDM treated with
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exogenous sphingosine. Interestingly, exogenous sphingosine treatment also induced apoptosis of BMDM. This suggests that accumulation of sphingolipid metabolites such as ceramides that modulate membrane domains trigger a compensatory autophagy response, which promotes cell survival. Indeed, inhibition of autophagy leads to caspase-dependent apoptosis. Previous work pointed out the importance of sphingolipids in the regulation of autophagy (56-58). Indeed, manipulation of sphingolipid metabolic enzymes modulates autophagy in various systems (34,59-63). We speculate that pharmacologic inhibition of Sphk isoenzymes could lead to intracellular sphingolipid accumulation and compensatory autophagic reponses in cells with high flux in the sphingolipid metabolic pathways.

We also found that enhanced sphingolipid accumulation due to Sphk dKO led to the induction of Sphk2 expression. Indeed, exogenous sphingosine treatment induced Sphk2 protein and mRNA. These data suggest that cells upregulate Sphk2 in response to exogenous sphingosine so that the sphingoid base may be metabolized to the phosphorylated metabolite, which can be secreted out of the cell or further metabolized by the S1P lyase. Specific mechanisms involved, i.e., how sphingolipid metabolites induce expression of Sphk2 transcription and/or protein expression is not known and needs to be further investigated.

In conclusion, our studies indicate that intracellular S1P generated by Sphk isoenzymes in macrophages is not needed for inflammatory responses. In contrast, Sphk isoenzymes maybe involved in a metabolic pathway that maintains membrane homeostasis. If Sphk levels are attenuated, enhanced sphingolipid levels could lead to enhanced Sphk2 expression and compensatory autophagy. These data are consistent with an important metabolic role for Sphk2 in cellular membrane homeostasis.

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FIGURES LEGENDS

Figure 1. Characterization of Sphk dKO macrophages. Efficiency of Sphk1 or Sphk2 deletion in isolated BMDM was determined by PCR of genomic DNA (A), quantitative RT-PCR of mRNA from BMDM (B) and immunoblot analysis of BMDM extracts (C). Data are representative of at least three independent experiments. (N/S-no signal detected).

Figure 2. Sphk isoenzymes are not essential for myeloid cell survival or differentiation. (A) Intracellular sphingoid bases and ceramide level in untreated BMDM were measured by LC-MS/MS. *p < 0.05, **p < 0.01 (compared with the WT group). N=6 per group. (S1P- sphingosine 1-phosphate; sph- sphingosine; ceramide – total ceramide molecular species). (B) The intracellular ceramide species in BMDM were measured by LC-MS/MS. BMDM were treated without or with 10 ng/ml TNF-α or 20 μM Sph for 4 hours. N=4-6 per group. Data represent mean ± SEM. (C) Flow cytometric detection of CD11b and F4/80 expression on BMDM after 7 days culture with 20% L929 conditional medium. Note that Sphk2 or mo Sphk dKO cells show similar level of differentiation (D) Cell proliferation of BMDM from 3 to 6 days after isolation as measured by the MTT assay. Data are representative of at least three independent experiments.

Figure 3. Sphk isoenzymes are not necessary for inflammatory responses. (A) Intracellular sphingoid bases and total ceramides in TNF-α treated BMDM were quantified by LC-MS/MS. *p < 0.05, **p < 0.01 (compared with the WT group). N=4 per group. Data represent mean ± SEM. (B) Immunodetection of total p65 and phosphorylated forms of p65, p38 and JNK in whole cell extracts of BMDM. Cells were either stimulated or not with 10 ng/ml TNF-α for 15 min or 100 ng/ml LPS for 30 min. (C) Immunodetection of COX-2 in whole cell extracts of BMDM stimulated or not with 100 ng/ml LPS for 24 hours. (D) qRT-PCR detection of TNF-α, IL-1 and IL-6 mRNA in BMDM cultured with 100 ng/ml LPS for 0, 2 and 6 hours. Bar graphs present data as fold changes to untreated (0 hour) cultures. (N=9) Data represent mean ± SEM.

Figure 4. Myeloid Sphk is not critical for inflammatory responses in vivo. Survival curves of Sphk1−/− and Sphk1+/+ mice (A), Sphk1+/- Sphk2 flox/flox, LysM-Cre+ and Sphk1+/- Sphk2 flox/flox, LysM-Cre litter mate (B) mice after the injection of LPS (30 mg/kg i.p.). The number of mice in each group is indicated. (C) Plasma levels of cytokines 8 hours after the injection of LPS (30 mg/kg i.p.). The number of mice in each group is indicated. (D) Total macrophage numbers in peritoneal lavage samples were quantified 4 days after injection of 2 ml 3% thioglycolate. N=3; Data represent mean ± SD.

Figure 5. Accumulation of intracellular ceramide and Sph induced by exogenous sphingosine treatment. The intracellular levels of S1P (A), Sph (B) and total ceramides (C) in BMDM treated with 20 μM Sph for 4 hours were measured by LC-MS/MS. *p < 0.05 (compared with the WT group). N=4 per group. Data represent mean ± SEM.

Figure 6. Sphk2 is induced by exogenous Sph treatment. Immunodetection of Sphk1 or Sphk2 protein in C57BL/6 BMDM (A and B) and 293T cells (C). Cells were exposed to various concentrations of Sph for 4 hours or exposed to 20 μM Sph for various hours as indicated. On the right is the densitometric quantification of Sphk1/β-actin or Sphk2/β-actin. (D) qRT-PCR detection of Sphk1 and Sphk2 mRNA in C57BL/6 BMDM cultured with 20 μM Sph for 4 hours. *p < 0.05. Data (mean ± SEM) are representative of at least three independent experiments. N=3.

Figure 7. Autophagosomes are induced in macrophages that lack Sphk isoenzymes. (A) Immunostaining of LC3B in BMDM after 4 hours treatment of 5 μM Sph or 20 mM NH₄Cl.
Intracellular S1P is not essential for NFκB induction

(Scale bar=10 µm)  (B) Immunodetection of LC3B in BMDM after treated with various compounds as indicated for 4 hours. Data are representative of at least three independent experiments. (C) lysosomes were visualized with Dextran-rhodamine. Scale bar=10 µm.

**Figure 8. Spkk deleted BMDM are more sensitive to exogenous sphingosine-induced cell death.** BMDM were exposed to various concentrations of Sph (A), Dox (B) Ceramide (C) and S1P (D) for 24 hours as indicated. The viability of the cells was measured by MTT assay as described in the Methods section. **p < 0.01 (compared with the WT group). N=3; data are representative of at least three independent experiments.**

(E) WT BMDM were exposed to various compounds (20 µM Sph, 20 mM 3-MA, 20 mM NH₄Cl) as indicated for 24 hours and the viability of the cells was measured by MTT assay. *p < 0.05, **p < 0.01. N=3; data are representative of at least three independent experiments.  

(F) Immunodetection of cleaved caspase-3 and caspase-8 in BMDM after treated with various compounds (20 µM Sph, 20 mM 3-MA, 20 mM NH₄Cl) as indicated for 4 hours. N=3; data are representative of at least three independent experiments.
Fig 4

A

![Graph A](image1)

B

![Graph B](image2)

C

![Graph C](image3)

D

![Graph D](image4)
Fig 7

A

| Treatment                 | LC3B | WT                     | Sphk1^-/- Sphk2^-/- | Sphk2^-/- |
|---------------------------|------|------------------------|---------------------|-----------|
| NT                        | DAPI | [Image]                | [Image]             | [Image]   |
| 5 uM Sph                   | DAPI | [Image]                | [Image]             | [Image]   |
| 20 mM NH4Cl                | DAPI | [Image]                | [Image]             | [Image]   |

B

| Treatment | LC3B II | WT | Sphk1^-/- Sphk2^-/- | Sphk2^-/- |
|-----------|---------|----|--------------------|-----------|
| NT, Sph   | [Image] | [Image] | [Image] | [Image] |
| 10 uM Sph | [Image] | [Image] | [Image] | [Image] |
| 20 uM Sph | [Image] | [Image] | [Image] | [Image] |

C

| Treatment | Dextran rhodamine | WT | Sphk1^-/- Sphk2^-/- | Sphk2^-/- |
|-----------|-------------------|----|--------------------|-----------|
| UNT       | [Image]           | [Image] | [Image] | [Image] |
| 5 uM Sph  | [Image]           | [Image] | [Image] | [Image] |
| 20 uM Sph | [Image]           | [Image] | [Image] | [Image] |
Sphingosine kinases are not required for inflammatory responses in macrophages
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Hyuek Jong Lee’s name was misspelled. The correct spelling is shown above in the author line.