-Supplemental Data-

Pseudomonas aeruginosa stimulates nuclear Sphingosine-1-phosphate generation and epigenetic regulation of lung inflammation

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Running Title: Nuclear SPHK2/S1P in Lung Inflammation

METHODS

Reagents and Antibodies. 10 X TBS (170-6435, BioRad Hercules, CA), 10X TAE Buffer (161-0743, BioRad, Hercules, CA), 10X Tris/Glycine/SDS Buffer (161-0772, BioRad, Hercules, CA), 10X Tris/Glycine Buffer(161-0771, BioRad, Hercules, CA), RIPA Buffer (R0278, Sigma-Aldrich, St. Louis, MO), 1X TE Buffer (51235, Lonza, Basel, Switzerland), PBS (21-040-CV, Corning, Corning, NY), Dimethyl Sulphoxide (DMSO), and Hybri-Max (D2650, Sigma-Aldrich, St. Louis, MO), Formalin Solution, neutral buffered, 10% (HT501128, Sigma-Aldrich, St. Louis, MO), ChIP Lysis Buffer (sc-45000, Santa Cruz Biotechnology, Dallas, TX), Phosphatase Inhibitor (524625-1SET, EMD Millipore, Billerica, MA), Protease Inhibitor (539134-1SET EMD Millipore, Billerica, MA), Novex Wedgewell 4-20% 10 well Tris-Glycine Gel (XP04200BOX, Thermo Fisher Scientific, Waltham, MA), Novex Wedgewell 10% 10 well Tris-Glycine Gel (XP00100BOX, Thermo Fisher Scientific, Waltham, MA), Novex Wedgewell 4-20% 15 well Tris-Glycine Gel (XP04205, Thermo Fisher Scientific, Waltham, MA), Novex Wedgewell 10% 15 well Tris-Glycine Gel (XP00105BOX, Thermo Fisher Scientific, Waltham, MA), 2-Mercaptoethanol (M3148, Sigma-Aldrich, St. Louis, MO), Laemelli 6X, Nonreducing Sample Buffer (BP-111NR, Boston BioProducts, Ashland, MA), Pierce BCA Protein Assay Kit (23225, Thermo Fisher Scientific, Waltham, MA), Pierce ECL Western Blotting Substrate (32106, Thermo Fisher Scientific, Waltham, MA), Tween 20 (BP337-100, Fisher Scientific, Waltham, MA), Restore PLUS Western Blot Stripping Buffer (46430, Thermo Fisher Scientific, Waltham, MA), Bovine Serum Albumin (A7906-100G, Sigma-Aldrich, St. Louis, MO), ECL Prime Western Blotting Detection Reagent (RPN2232, GE Healthcare Life Sciences, Pittsburgh, PA), Precision Plus Protein Dual Color (161-0374, BioRad, Hercules, CA), Goat Anti-Mouse IgG (170-6516, BioRad, Hercules, CA), Goat Anti-Rabbit IgG (170-6515, BioRad, Hercules, CA), HDAC2 (IP Preferred) (2545S, Cell Signaling Technology, Danvers, MA), Acetyl-Histone H4 (8647P, Cell Signaling Technology, Danvers, MA), Acetyl-Histone H3 (9649P, Cell Signaling Technology, Danvers, MA), Histone H3 (9715, Cell Signaling Technology, Danvers, MA), Histone H4 (2592S, Cell Signaling Technology, Danvers, MA), SphK2 (Thr578) (A8423, Assay BioTech, Fremont, CA), SphK1 (ab37980, Abcam, Cambridge, UK), Lamin B1 (ab16048, Abcam, Cambridge, UK), Actin (A15441, Abcam, Cambridge, UK), GAPDH (sc-25778, Santa Cruz Biotechnology, Dallas, TX), Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (A22188, Thermo Fisher Scientific, Waltham, MA), Mouse IL-6 ELISA (M6000B, R&D Systems, Minneapolis, MN), Mouse TNF-α Immunoassay (MTA00B M6000B, R&D Systems, Minneapolis, MN), HDAC Fluorometric Activity Assay Kit (10011563, Cayman Chemical, Ann Arbor, MI), Fetal Bovine Serum Heat Inactivated (12306C, Sigma-Aldrich, St. Louis, MO), DMEM (11995-065,

Gibco, Gaithersburg, MD), L-Glutamine (25030-081, Gibco, Gaithersburg, MD), Penicillin/Streptomycin (15140122, Gibco, Gaithersburg, MD), 0.05% Trypsin (25300-054, Gibco, Gaithersburg, MD), QIAquick PCR Purification Kit (28104, Qiagen, Hilden, Germany), Ethidium Bromide (161-0433, BioRad, Hercules, CA), 6X DNA Loading Dye (R0611, Thermo Fisher Scientific, Waltham, MA), Quick-Load Purple 2-Log DNA Ladder (N0550G, New England BioLabs, Ipswich, MA), Formaldehyde (BP531-500, Thermo Fisher Scientific, Waltham, MA), Sodium Bicarbonate (7.5%) (25080-094, Thermo Fisher Scientific, Waltham, MA), 0.5 M EDTA (15575-038, Invitrogen, Carlsbad, CA), DEPC-Treated Water (G-3223-125, GeneMate BioExpress, Kaysville, UT), SDS 10% Solution (AM9822, Invitrogen, Carlsbad, CA), Sodium Chloride Solution (S6316, Sigma-Aldrich, St. Louis, MO), Molecular Biology Grade Water (46-000-CM, Corning, Corning, NY), RNAse A (EN0531, Thermo Fisher Scientific, Waltham, MA), Proteinase K (P8107S, New England BioLabs, Ipswich, MA), Micrococcal nuclease (MO247S, New England BioLabs, Ipswich, MA), Protein A/G Plus-Agarose (sc-2003, Santa Cruz Biotechnology, Dallas, TX), Dynabeads Protein G for Immunoprecipitation (10004D, Invitrogen, Carlsbad, CA), FastStart Universal SYBR Green Master (Rox) (19317900, Sigma-Aldrich, St. Louis, MO), Triton X (CAS 9002-93-1, Sigma-Aldrich, St. Louis, MO), Donkey-anti Rabbit IgG 568 (A10042, Thermo Fisher Scientific, Waltham, MA), Goat-Anti Mouse IgG 568 (A11031, Thermo Fisher Scientific, Waltham, MA), Alexa Fluor 488 (A21206, Thermo Fisher Scientific, Waltham, MA), DAPI (4', 6-diamidino-2-phenylindole) (D1306, Thermo Fisher Scientific, Waltham, MA), Alexafluor 647 (A21244, Thermo Fisher Scientific, Waltham, MA), ProLong Gold Antifade Reagent with DAPI (P36935, Thermo Fisher Scientific, Waltham, MA), Absolute Ethanol (BP2818-500, Fisher Scientific, Waltham, MA), Isopropanol (67-63-0, Sigma-Aldrich, St. Louis, MO), Chloroform (C606SK-1, Fisher Scientific, Waltham, MA), TRIzol Reagent (15596018, Life Technologies, Carlsbad, CA), GeneSilencer siRNA Transfection Reagent (T500750, Genlantis, San Diego, CA), Control siRNA (sc-37007, Santa Cruz Biotechnology, Dallas, TX), PKC δ siRNA (sc-36246, Santa Cruz Biotechnology, Dallas, TX), SphK2 siRNA (sc-39226, Santa Cruz Biotechnology, Dallas, TX), LE Agarose (E-3120-500, GeneMate BioExpress, Kaysville, UT), Luria Broth (L24040-500, Grainger, Lake Forest, IL), HEPES (15630-080, Gibco, Gaithersburg, MD), Dispase (04942078001, Roche Diagnostics, IN), DNase I (M0303S, New England Biolabs, USA), ACK Lysis Buffer (10-548E, Lonza, Basel, Switzerland), EpCAM Monoclonal Antibody (13-5791-82, eBioscience, San Diego, CA)

Quantitative real-time PCR: All Primers used for Real time PCR analysis were obtained from IDT, Coralville, IA. IL-6 Forward, 5'-CCAAGAGGTGAGTGCTTCCC-3', IL-6 Reverse Primer, 5'-

CTGTTGTTCAGACTC TCTCCCT-3', TNF-α Forward Primer, 5'-CCCTCACACTCAGATCATCTTCT-3', TNF-α Reverse Primer, 5'-GCTACGACGTGGGCTACAG-3', GAPDH Forward Primer, 5'-AGGTCGGTGTGAACGGATTTG-3', GAPDH Reverse Primer, 5'-TGTAGACCATGTAGTTGAGGTCA-3', ChIP DNA primers targeting the NFkB binding site in the proximal promoter region of mouse IL-6 gene: Forward Primer, 5'-CCCACCCTCCAACAAAGATT-3', Reverse Primer, 5'-GAATTGACTATCGTTCTTGGTG-3'

Mice models

All animal experiments were approved by the Institutional Animal Care and Use committee at University of Illinois at Chicago. *Sphk1* (*SphK1*$^{-/-}$), and *Sphk2* (*SphK2*$^{-/-}$) knockout mice were originally provided by Dr. Richard Proia, National Institutes of Health, Bethesda, MD under an MTA agreement. The knockout mice were backcrossed onto the C57BL/6 background for 8 generations. All *in vivo* experiments were carried out with age-matched (6-8 weeks) male and female mice. The mice were housed in the University of Illinois Animal Care Facility with free access to a standard laboratory chow diet in a half-day light cycle exposure and temperature controlled specified-pathogen free environment.

Preparation of *Pseudomonas aeruginosa* culture. The parent strain *Pseudomonas aeruginosa* (*PA* 103) was used for all the experiments. Preparation of the cultures and determination of colony-forming units (CFU) were carried out as described earlier [1]. The bacterial concentration was confirmed by plating out the diluted samples on sheep blood agar plates. For *in vitro* experiments, the bacterial preparations that were heat-inactivated at 60 ^{o}C for 20 min were used.

***Pseudomonas aeruginosa* infection of mouse lung.** Age (8-9 weeks)- and weight (20-22 g)-matched male and female WT, *Sphk1*$^{-/-}$, and *Sphk2*$^{-/-}$ mice were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) as per approved protocol and were administrated a single intratracheal infusion of sterile PBS or *PA*103 in PBS (1x10^{6} CFU/mouse). After 24 or 48 h of treatment, bronchoalveolar lavage (BAL) fluid was collected using 1 ml of sterile Hanks Balanced Salt Buffer, and differential cell counts were performed using cytospin as described[1]. Cytokines in the BAL fluids were measured using enzyme-linked immuno-absorbent assay, and H_2O_2 by Amplex Red Hydrogen Peroxide/Peroxidase kit (Invitrogen). Left lungs were removed and fixed with 10% formalin, and paraffin embedded 5μm thick sections were stained with hematoxylin and eosin for immunohistochemistry. Right lung was snap frozen in liquid nitrogen and stored at -80°C

for total lysates, Western blots and RNA. For survival studies, mice were administered 1 x 10^7 CFU/animal of *PA*103 and monitored at least 4 times daily for 4 days.

Isolation of alveolar Type II epithelial cells from mouse lung.

Alveolar type II (ATII) epithelial cells from 8-week-old WT mice were isolated as described[2]. Briefly, mice were anesthetized with intraperitoneal injection of ketamine, and euthanized. The trachea was exposed by dissection and a cannula was inserted and lungs were perfused with 1.5 ml of dispase II and tied immediately with a suture thread. Lungs were collected, incubated in 1 ml of dispase II for 45 min at 37°C, transferred to 100-mm petri-dishes containing 10 ml of DMEM medium plus 25 mM HEPES, 1% Pen/Strep, and 20 U/ml of DNase I and gently teased with forceps, until only connective tissue were visible. The cell suspension was then passed through a 70-micron mesh; cells were resuspended in DMEM media containing 25 mM HEPES, 10% FBS, and 1% Pen/Strep and incubated at 37°C for 2 h on IgG coated plates. Following incubation, non-adherent cells were centrifuged at 1500 rpm for 5 min and the supernatant discarded. One ml ACK lysis buffer was added to the pellet and incubated for 1 min, and DMEM media was added to neutralize the buffer. Cells were collected and centrifuged for 5 min at 1500 rpm, supernatant was discarded, and 1 ml of ACK lysis buffer was added again until no RBC's were present. When RBC's were no longer present, 4µl of anti-EpCAM biotin conjugated antibody was added and incubated at 37°C for 20 min in the dark. Following incubation, cells were washed in 1 ml of FACS buffer, then resuspended in 500µl of FACS buffer (1X PBS + 2% FBS) with 20µl streptavidin coated conjugated magnetic beads and incubated for 30 min. Cells were then washed in 1 ml of MACS buffer, twice, then resuspended in 200µl MACS buffer and in an IMAC apparatus, allowing cells to bind for 8 min. Bound fractions were resuspended in 1 mL of fresh MACS buffer and unbound fractions were removed. Cells were then resuspended in DMEM media containing 25 mM HEPES, 10% FBS, 1% Pen/Strep, and 10 µM/ml ROCK inhibitor and incubated in collagen (rat-tail), and incubated at 37°C. The medium was changed every 2 days, and the primary ATII cells were used within 7 days of isolation. The purity of the ATII epithelial cells was verified using flow cytometry for co-staining with Ep-CAM and cytokeratin and co-staining with Ep-CAM and SP-C.

MLE-12 cell culture

Mouse transformed Lung Epithelial cells (MLE-12) were cultured in Dulbecco's Modified Eagle Medium (DMEM) complete medium, 4.5 g/L glucose. L-glutamine and Sodium pyruvate (10%

FBS, 100 U/mL penicillin and streptomycin) at 37°C and 5 % CO_2 and were allowed to grow approximately 90 % confluence in 35-mm or 100-mm petri-dishes.

Exposure of lung epithelial cells to heat-inactivated *Pseudomonas aeruginosa*

For *PA* infection, cells were starved in medium with 2% FBS, without antibiotics, overnight before exposure to heat-inactivated *PA* at a multiplicity of infection (MOI) of 50 (1 x 10^6 CFU/ml) for 3 h. Cells were then harvested, cell lysates prepared, and immunoblotted to detect proteins. Control cells were treated with endotoxin-free sterile saline.

Isolation of nuclear fraction from epithelial cells

Nuclei from MLE-12 cell or primary HBEpCs (~90% confluence) were prepared by sucrose density gradient differential centrifugation. Briefly, nuclei were prepared from MLE-12 or HBEPC cells, grown in 100-mm dishes. After *PA* (1 x 10^6 CFU/ml) infection for 2 h, cells were washed three times with PBS, trypsinized, detached from the cell culture dishes and then centrifuged at 300 x g for 10 min. The cell pellets were washed again with PBS and resuspended in 5 ml buffer A (10 mM HEPES-KOH [pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT), and homogenized ten times using a Dounce homogenizer with a tight pestle. Cell homogenates were centrifuged at 200 x g for 5 min at 4°C and supernatant was collected and stored as cytoplasmic fraction. The pellet was resuspended in 3 ml of 0.25 M sucrose, 10 mM MgCl2, and layered over 3 ml 0.35 M sucrose, 0.5 mM $MgCl_2$, and centrifuged at 1,400 x g for 5 min at 4 °C. The nuclear pellet was collected, resuspended in RIPA buffer, sonicated for 10 seconds at setting 5, and centrifuged at 10,000 x g for 10 min at 4 °C and the supernatant collected as the nuclear fraction. The purity of the nuclear preparation was determined by western blotting and immunostaining for lamin B with anti0-lamin B antibody, a component of nuclear lamina.

Measurement of IL-6 and TNF-α

Media from MLE-12 cells and HBEpCs or BAL fluids from mouse lung were centrifuged at 10000 x g for 10 min at 4°C, and IL-6 and TNF-α levels in the supernatants were measured using a commercially available ELISA kit, according to the manufacturer's instruction (R&D).

Measurement of H_2O_2

BALF from mice were collected and centrifuged at 10,000 × g, for 20 min at 4°C, and H_2O_2 measurements were performed immediately using Amplex Red Hydrogen Peroxide/Peroxidase kit (Invitrogen), according to the manufacturer's instruction.

HDAC activity

HDAC activity was measured in cell nuclei isolated from MLE-12 cells or HBEpCs as outlined above using a commercially available kit according to the manufacturer's instruction (Cayman).

Transfection of HBEpCs with small interfering RNA

Depletion of endogenous SPHK1 and SPHK2 proteins in cells was carried out using gene-specific siRNA. Pre-designed siRNA of mouse and human *SphK1, SphK2*, or nonspecific/non-targeting siRNA, were used to transfect MLE-12 or HBEpCs. Each siRNA contained at least 3 different sequences targeting the mRNA of each gene. Prior to transfection, cells were starved in basal medium containing 2% FBS for 24 h. Next day, 50 nM scrambled, or gene specific siRNA complexes were prepared in Gene Silencer transfection reagent according to the manufacturer's recommendation and cells were transfected in serum-free media for 4 h and the media was replaced with fresh complete medium supplemented with 10% FBS and growth factors. After 72 h post-transfection cells were stimulated with vehicle or *PA* for 3 h. Knockdown of target protein was confirmed by Western blotting.

Immunoblotting

HBEpCs, MLE-12 or ATII cells (~90 % confluence) were stimulated with vehicle or 50 MOI of heat-inactivated *PA* 103 for 2 h, washed with PBS, and lysed with 100µl of RIPA buffer, with protease and phosphatase inhibitors. Lysates were then sonicated for 10 seconds at a setting of 3 in a probe sonicator and centrifuged at 10,000 x g for 10 min at 4°C. Supernatants were collected and protein concentration was measured using BCA protein assay kit. Samples for Western blot were prepared with 10-20µg protein and 6x Laemmli buffer, placed on a heat block at 100°C for 5 minutes. The cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% or 4-20% precast gel (Invitrogen) at 225 V for 40 min. Blots were transferred onto 0.22nm nitrocellulose membranes for 1.5 h at 70 volts. Membranes were rinsed with wash buffer (Tris buffer saline + Tween 20) for 5 minutes following transfer, then blocked in blocking buffer (Tris buffer saline + Tween 20 + 1% BSA) for 2 h, and were probed with the primary antibody of interest overnight at 4°C. The next day, membranes were washed with washing buffer 3 times, 10 min each, incubated with respective HRP-conjugated secondary antibody for 1 h, and washed with washing buffer 3 times, 10 min each. Protein bands were

detected using super signal luminol enhancer. Band intensities were quantified by densitometry using ImageJ software.

Immunoprecipitation

After appropriate treatments, cells were pelleted in ice-cold PBS, lysed in standard lysis buffer (Cell Signaling), and sonicated. Lysates were then centrifuged at 1,000 x g for 10 min at 4°C. Supernatants were collected and protein assayed using BCA protein assay kit. For immunoprecipitation (IP), equivalent amounts of protein (1 mg) from each sample were pre-cleared with control IgG conjugated to A/G agarose beads at 4°C for 1 h, centrifuged at 1,000 x g for 10 min at 4°C in a microfuge centrifuge. Supernatants were collected and incubated overnight with primary antibody conjugated to A/G agarose beads at 4°C, with rotation. After 18-24 h, the samples were centrifuged at 1,000 x g for 5 min at 4°C in a refrigerated microfuge centrifuge and the pellet containing the agarose beads were washed three times with lysis buffer. After brief centrifugation at 1,000 x g for 5 min, the beads were collected by removing supernatant buffer, and 40 µl of SDS sample buffer [100 mM Tris-HCl (pH 6.8), 4% SDS, 0.1% bromophenol blue, 20% glycerol, 200 mM DTT was added to the beads and boiled. Lysates were then subjected to SDS-PAGE followed by Western blotting. Proteins were detected by immunoblotting using appropriate primary antibodies, and HRP-conjugated anti-rabbit or anti-mouse secondary antibodies.

Lung tissue immunofluorescence and immunohistochemistry staining

Mouse lung was infused with O.C.T. compound containing 4% paraformaldehyde. The lung lobes were embedded in O.C.T. compound and frozen immediately on dry ice. Five-micrometer sections of mouse lung were fixed with paraformaldehyde (4%) and embedded in optimal cutting temperature blocking PBS buffer (5% BSA, 0.05% Triton X-100) for 1 h. Sections were incubated overnight with rabbit anti-SPC antibody, mouse anti-SphK2, or mouse anti-p-SPHK2 antibody. Sections were then washed in PBS containing 0.05% Tween 20 for 3 times (20 min/time), and then incubated with donkey anti- rabbit and rabbit anti-mouse secondary antibodies conjugated with Alexa Fluor 568 and Alexa Fluor 488 for immunofluorescent staining, or biotinylated secondary antibodies for immunohistochemistry staining for 1 h and then washed with PBS for 3 times (20 min/time). Nuclei were stained with DAPI, followed by rinsing and mounting in Vectashield mounting medium (Vecta Laboratories). At least 20 Images were obtained with Nikon fluorescence microscopy from different area of the slide. Intensity of p-SPHK2 staining was quantified by Image J software. In brief, single channel for p-SPHK2 images was selected, a

common threshold was set for all the images; intensity of p-SPHK2 was obtained by the measure function of the software. For immunohistochemistry quantification, p-SPHK2 or SPHK2 signal was selected by the function of color threshold, settings were consistent for all images analyzed. Selected p-SPHK2 areas were inverted and the intensity of selected area was measured by the software.

Immunofluorescence microscopy

MLE-12 or primary ATII cells grown on chamber slides were grown to ~90% confluence and starved in 2% FBS overnight. Cells were then treated with *PA*103 for 3 h, fixed with 3.7% formaldehyde, permeabilized with 0.25% Triton X-100, and incubated with blocking buffer for one 1 h (PBS + Tween 20 + 2% BSA). Cells were then incubated overnight with appropriate primary antibody (1:300). Cells were washed thrice with PBS and incubated with secondary antibodies (1:200 dilution) in PBS-T containing 2% BSA. Glass cover slips were mounted on the chamber slides with mounting fluid containing DAPI to stain nuclei. Cells were examined using an Olympus Fluoview 1000 and Nikon Eclipse TE2000-S immunofluorescence microscope and Hamamatsu digital camera with 63X oil immersion object and MetaVue software. Analysis was done using FIJI software. To quantify fluorescence intensity of nuclear SPHK2, a single in focus plane was acquired, and an outline was drawn around the DAPI stained nuclei region and the fluorescence intensity value for SPHK2 were measured in the outlined area, normalized and plotted relative to vehicle control. Nuclear localization was analyzed by Pearson's correlation coefficient (PCC).

PKC activity

MLE-12 cells grown to ~90% confluence in 100-mm cell culture dishes were treated with heat killed *PA*103 for different time points and lysed with RIPA buffer. Cell lysates were then sonicated and centrifuged at 10,000 x g for 10 minutes and PKC activity was measured with a kit according to the manufacturer’s instruction Kit (Enzo BioSceinces).

IL-6 gene expression

Total RNA was isolated from cells that were treated with vehicle and *PA*103 for 3 h using TriZOL reagent (Life Technologies), according to the manufacturer’s protocol. cDNA was generated using random primers and Real time PCR was done using Mouse IL-6 primers on the iCycler (BioRad).

ChIP Assay

MLE-12 cells grown to ~90% confluence on 100-mm dishes were treated with heat-inactivated *PA*103 for 3 h. Formaldehyde was added directly to the cell culture medium to a final concentration of 1% and incubated for 9 min. Glycine was then added to a final concentration of 125 mM and dishes were incubated in room temperature for 5 min, washed with PBS, cells were collected in a 15 ml tube, centrifuged at 800 x g for 5 min, and pellet was collected. The pellet was resuspended in ChIP lysis buffer (Santa Cruz) with protease and phosphatase inhibitors, and incubated at 4°C with rotation for 10 min. Samples were centrifuged at 2000 x g for 5 min and 1 mL Micrococcus nuclease (MNase) digestion buffer containing $CaCl_2$ was added to re-suspend the pellet, and samples were incubated at 37°C for 10 min. EDTA was added to a final concentration of 5 mM and samples were sonicated 3 times, 12 sec each, at a power of 6. Lysates were centrifuged at 10,000 x g for 10 min. Fifty microliter of chromatin was removed for analysis and the remaining supernatant was stored in -80°C. For chromatin analysis, 100µl of nuclease-free water, 6µl of 5M NaCl, and 2µl RNAse A was added to the 50µl sample and incubated at 37°C for 30 min. Following incubation, 2µl Proteinase K was added and samples were incubated at 65°C for 2 h. DNA was purified using Qiagen QiA Quick PCR purification kit; protein concentration was measured with a nano-drop and amount of digested DNA was viewed by loading 10µl of sample on a 1.2% agarose gel with 100bp DNA marker. Ten micrograms of digested chromatin were used for each IP. Fifty microliter of Protein A/G agarose beads were added to the digested DNA and samples were incubated for 1 h at 4°C with rotation. Samples were centrifuged at 4,000 x g for 5 min and the supernatant was transferred to a new tube. Ten microliters of supernatant were kept aside for input fraction. Chromatin DNA was immunoprecipitated using 5µg Acetylated Histone H3K9 antibody, 2µg of negative control (IgG), and 2µg of positive control ($H_3K_4Me_3$) for 4 h at 4°C with rotation. Fifty microliters of Protein A or G magnetic beads were added, and samples were incubated for 2 h at 4°C with rotation. Magnetic beads were pelleted using Dyna mag^{-2} Magnet stand (Invitrogen) and supernatant discarded. Magnetic pellets were washed with low salt wash buffer, high salt wash buffer, LiCl wash buffer, and 1 X TE buffer, twice. Two hundred and fifty microliters of Elution buffer was added to input fractions and allowed to incubate at room temperature for 30 min with rotation. Samples were then incubated overnight at 65°C. Samples were treated with 2µl RNAse for 30 min at 37°C. After incubation, 2µl Proteinase K was added and incubated for 1 h at 55°C. Magnetic beads were pelleted and supernatant was collected; DNA was isolated using Qiagen QiA Quick PCR purification kit. Real time PCR was performed using specific primers designed to amplify NF-kB binding site in IL-6 proximal promoter region.

SPHK activity in nuclear fraction

The nuclear fractions (40 µg protein) were subjected to sphingosine kinase activity assay in 10 mM Hepes buffer (pH 7.4) containing 10 mM $MgCl_{2,}$ and 1 mM DTT in the absence or presence of 1µM sphingosine (Sph), dihydro Sph or FTY720 in the presence of 0.1% fatty acid BSA and 10 µM [γ-^{32}P] ATP (Specific activity 10,000 dpm/pmol) in a final volume of 100 µl for 30 min at 37^{0}C. The reaction was terminated by the addition of 0.8 ml of 1 N HCl followed by 1 ml of methanol and 1 ml chloroform to extract the lipids. The lower chloroform layer was subjected to thin-layer chromatography, and auto-radiography and radioactivity associated in S1P, DH S1P or FTY720-P was quantified by scintillation counting as described under METHODS. Values are means $\pm$ SEM of three independent experiments and expressed as pmoles of product formed/mg protein/min.

Lipid Extraction and quantification of sphingoid bases by LC-MS/MS

Lipids from cells or subcellular fractions were extracted by a modified Bligh and Dyer procedure [3] with the use of 2% formic acid for phase separation mostly as described [4]. D7-S1P (30 pmol), D7-16:0-ceramide (*N*-16:0-D7-sphingosine, 60 pmol) and D7-sphingosine (30 pmol) were employed as internal standards and were added during the initial step of lipid extraction. The extracted lipids were dissolved in methanol/chloroform (4:1, v/v), and aliquots were taken to determine total phospholipid content[5]. Samples were concentrated under a stream of nitrogen, re-dissolved in methanol, transferred to auto sampler vials, and subjected to sphingolipid LC-MS/MS analysis. All standards were from Avanti Polar Lipids (Alabaster, AL).

Analyses of sphingoid base-1-phosphates, ceramides, and sphingoid bases were performed by electrospray ionization tandem mass spectrometry (ESI-LC/MS/MS). The instrumentation employed was Sciex 6500 QTRAP hybrid triple quadrupole linear ion-trap mass spectrometer (AB Sciex, Redwood City, CA) equipped with an Ion Drive Turbo V ion spray ionization source interfaced with a Shimadzu Nexera X2 UHPLC system. All lipid molecules and their derivatives were separated using Ascentis Express RP-Amide 2.7 µm 2.1 x 50 mm column and gradient elution from methanol:water:formic acid (65:35:0.5, 5 mM ammonium formate) to methanol:chloroform:water:formic acid (90:10:0.5:0.5, 5 mM ammonium formate). S1P and DHS1P were analyzed as *bis*-acetylated derivatives with D7-S1P as the internal standard employing negative ion ESI and MRM analysis basically as described[6]. Ceramides and sphingoid bases were analyzed with D7-16:0-ceramide and D7-Sph as internal standards using positive ion ESI and MRM analysis basically as described[2]. To facilitate ceramide analysis, total lipids were

hydrolyzed using a methylamine reagent[7] for 2 h at 55ºC. Reagents were evaporated with nitrogen stream; the residual non-saponified lipids were dissolved in 0.2 ml of methanol and subjected to the LC-MS/MS analysis of ceramides. Standard curves were created for all measured analytes by mixing a fixed amount of the internal standard with variable amounts of corresponding analysts (Sph, DHSph, S1P, DHS1P, and variable ceramide species N-acylated with 16:0-24:1 fatty acids, all from Avanti Polar Lipids). The linearity and the correlation coefficients of the standard curves were obtained via linear regression analysis. Quantitation of ceramide molecules for which there are no standards available was performed with the best closest approximation to the available standards.

Statistical Analysis.

Data are expressed as mean ± SE from three independent experiments in triplicates unless otherwise indicated. Analysis of variance and student -Newman-Keuls test was used to compare means of two or more treatment groups. $P< 0.05$ was considered statistically significant. Survival analysis was performed using Kaplan Meier Survival analysis using Graphpad Prism 7. A-log rank test p-value <0.05 was deemed significant. Statistical tests were performed using Graphpad Prism version 7.0, Graphpad Software, La Jolla California, USA.

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