Supporting Online Material for

**SphK1 Regulates Proinflammatory Responses Associated with Endotoxin and Polymicrobial Sepsis**

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Materials and Methods

Patients The prospectively enrolled patients admitted and treated for sepsis in the Intensive Care Units of the University Hospital, Faculty of Medicine, of the Central University of Venezuela, from January 2008 to April 2009. All patients presented clinical and/or laboratory variables that fulfilled the criteria for, and were diagnosed with, aseptic systemic inflammatory response syndrome or polymicrobial sepsis, as defined in reference S1. Healthy male and female volunteers served as controls. The study was approved by the Human Subjects Institutional Committee of the Faculty of Medicine and University Hospital of the Central University of Venezuela, and written informed consent was obtained from patients and volunteers. All patients enrolled were evaluated according to the Acute Physiological and Chronic Health Evaluation (APACHE) II score, a disease classification system that predicts hospital mortality after intensive care unit (ICU) admission. Thirty patients were enrolled having an APACHE II score in the severe range (average: 27.43 ± 6.79 SD), the average age was 52.1 (age-range 17-84) and the male to female ratio was 50/50%. Controls consisted of 15 healthy volunteers, with an average age of 31.5 ± 6.2 SD (age-range 22-45), and a male to female ratio of 53.3/46.7%.

Human cells and bacteria Human peripheral blood mononuclear cells, neutrophils and CD14+ monocytes were purified from peripheral blood of healthy donors as described (S2). Human peritoneal leukocytes were obtained from peritoneal fluid of the above-described patients.
Cultures of *S. aureus*, *P. aeruginosa* and BCG were stopped in the logarithmic growth phase, washed twice in PBS and inactivated by incubating for 30 min at 80 °C.

**Flow cytometric and functional analysis of human cells** Purified monocytes and neutrophils were cultured in the absence or presence of inactivated bacteria (monocytes/neutrophils: bacterial ratios were about 1/10–1/100), LPS (100 ng ml-1), LTA (100 ng ml-1) and mycolic acid (10 g ml-1). All cells were incubated with PBS or 20% human serum for 1 h on ice to block Fc receptors. Cells were then fixed with formalin and permeabilized with saponin. After staining with either antibody 21C7 (mouse IgG1, anti-SphK1) or 1B7.11 (control mouse IgG1, anti- 2,4,6 TNP; American Type Culture Collection), followed by human-adsorbed phycoerythrin (PE)-conjugated goat anti-mouse IgG (Southern Biotechnology Associates), cells were analysed on a flow cytometer. Four-colour analysis of human peritoneal leukocytes was performed using anti-SphK1, CD15 (Immunotech), CD14 (Immunotech) and CD16 (Immunotech) monoclonal antibodies conjugated with allophycocyanin (APC), CyChrome, PE and fluorescein isothiocyanate (FITC), respectively.

**Polymerase Chain Reaction (PCR)**

RNA was extracted from the cells using the Qiagen RNeasy® midi kit (Research Biolabs, Singapore) following manufacturer’s instructions. The concentration and purity of the RNA were determined spectrophotometrically by measuring the absorbance at 260 nm and 280 nm using NANO drop machine (Biofrontier Technology). Reverse transcription of 1 µg of RNA/sample was performed with
Transcriptor first strand cDNA synthesis kit (Roche Diagnostics, Germany) using hexamer primers. Light Cycler® RNA Amplification Kit (Roche Diagnostics, Germany) was used for real time PCR.

For real time PCR, the concentration of target genes was determined using the comparative CT method and values were normalized to an internal GAPDH or β-actin control. All primers were run simultaneously, with GAPDH or β-actin in triplicates. Efficiency of all the primers was calculated and was included in data analysis using the Light Cycler® 480 software version 1.2.9.11.

*Quantitative real time PCR* - Primers for genes of interest were designed according to their cDNA sequences from PubMed. The uniqueness of the suitable pairs chosen was checked by “blast” (http://www.ncbi.nlm.nih.gov/blast/). All primers were purchased from Research BioLab (Singapore). SPHK1 (F: atgctggctatgagcaggtc; R: gtgcagagacagcaggttca); GAPDH (F: aegaccccttcattgacctcaa; R: gctggtgctggcatggactgtg)

**siRNA and Western Blots** For the siRNA studies, cells were incubated for 48 hr, with 1μg/ml of siRNAs: Validated siRNAs to silence human SphK1 (SPHK1, SI02660455), PKCδ (PRKCD, SI02660539), and a scrambled negative-control siRNA (SI03650318) were purchased from Qiagen. siRNAs were transfected as previously described (S3).

**Gel electrophoresis and Western blots.** 40 μg of protein from total cell lysates/samples were resolved in 10% polyacrylamide gels (SDS-PAGE) under denaturing conditions and then transferred to 0.45 μm nitrocellulose membranes as previously stated (S3). The blots were probed using polyclonal anti-SphK1 and anti-SphK2 antibodies (Exalpha Biologicals, MA, USA); anti-PKCδ, PKCε, and PKCα (Santa Cruz Biotechnology, USA); anti-α-tubulin (Upstate, VA, USA) was used as a
loading control. Bands were visualized using anti-IgG HRP-conjugated secondary antibodies, and the ECL Western Blotting Detection System (GE Healthcare, UK).

**SphK1-inhibitor** The SphK1-specific inhibitor was synthesized, purified and validated as recently described (S4).

**SphK activity assay** SphK activity from total cell extracts was analysed using a radiometric assay as previously described (S5).

**Measurement of S1P** Generation of S1P following receptor activation as previously described (S5, S6). Briefly, cells were preincubated overnight in medium containing [3H]serine (2 μCi/ml) to label cellular sphingolipids and free sphingosine pools. Following labeling, cells were stimulated, and the reactions were terminated at the specified times. Lipids were extracted and resolved by TLC and analysed relative to S1P standards. Bands corresponding to S1P were excised from the plate and counted by liquid scintillation spectrometry. Results were calculated as a percentage of the total radioactivity incorporated in the lipids.

**DAG Kinase activity assay** DAG kinase activity was measured as previously described (S6). Briefly, Diacyl glycerol-containing lipid extracts are reconstituted into mock membrane micelles and incubated with DAG kinase and [32P-γ]ATP. DAGs present in the sample will be phosphorylated to PtdOH. PtdOH is then resolved by TLC relative to standards and quantified by liquid scintillation.
**Ceramide Kinase activity assay** Ceramide kinase activity was measured in a similar protocol as that for the DAGkinase assay. Briefly, Ceramide-containing lipid extracts are reconstituted into mock membrane micelles and incubated with Ceramide kinase and [32P-γ]ATP. Ceramides present in the sample will be phosphorylated to Ceramide-Phosphate. Ceramide-Phosphate is then resolved by TLC relative to standards and quantified by liquid scintillation.

**NFκB Activity** NFκB was analyzed using the MercuryTM TransFactor-“Profiling Kit-Inflammation” (BD), following the manufacturer’s instructions. The enzymatic product was analyzed in a standard plate reader as previously described (S3).

**Cytokine production triggered by LPS or BLP in human macrophages** Cells (2 x 10^6) pretreated or not with siRNAs for 48h, or compound 5c for 30 min, were stimulated by the addition of LPS (100 ng/ml), or BLP (100 ng/ml), for 24 h. Following stimulation, the supernatants were collected and stored at -20°C until use. Levels of human TNF-α, IL-1β, IL-6, IFNα and IFNβ, in the culture supernatants were evaluated using ELISA (R&D Systems, Minneapolis, MN). HMGB1 levels were also measured using ELISA (Shino-Test: 326054329).

**IKK-co-immunoprecipitation and Western Blot analysis** Cells (3 x 10^6) pretreated or not with compound 5c for 30 min, were stimulated by the addition of LPS (100 ng/ml) for 10 minutes. Following LPS stimulation, macrophages were lysed in Digitonin-containing buffer. Macrophagelysates (100 μg), were incubated with an anti-IKKα antibody (ProSci, Inc, CA, USA)- conjugated to immobilized Protein-A, for 2hr in a tumbler and at room temperature.
Following this, immunoprecipitated proteins were eluted, and the eluted proteins were separated by SDS-PAGE, followed by Western blotting into nitrocellulose membranes, as previously described \((S3)\). The membranes were probed with antiphosphoserine/threonine antibody (Abcam, Cambridge, UK); and for antibodies recognising IKKβ, IKKα, IKKγ, (ProSci, Inc, CA, USA); IkBβ (Abcam, Cambridge, UK); IkBα (Epitomics, CA, USA); p65, (Bethyl Laboratories, TX, USA); and PKCδ (Santa Cruz, Biotechnology, USA) primary antibodies. Bands were visualized using anti-IgG HRP-conjugated secondary antibodies, and the ECL Western Blotting Detection System (GE Healthcare, UK).

**PKC Activity** Following LPS stimulation (for the times described in the figure), PKC enzyme activity was measured using the Biotrak Protein Kinase C enzyme assay system (GE Healthcare, UK), as previously described \((S3)\).

**In vitro PKCδ activation** The activity of the human-recombinant PKCδ protein (Abcam, Cambridge, UK), was analysed *in vitro* substituting the phospholipid co-factors, Biotrak Protein Kinase C enzyme assay system (GE Healthcare, UK), for Sphingosine-triton-X100 micelles, in the presence or absence of recombinant SphK1 (BPS Biosciences, San Diego, CA, USA), or in the presence of Sphingosine-1-phosphate-triton-X100 micelles.

**Serum cytokines** Levels of mouse TNF-α, IL-1β, IL-6, MCP-1, IFNα and IFNβ in serum were measured using ELISA (R&D Systems, Minneapolis, MN). HMGB1 levels were also measured using ELISA (Shino-Test: 326054329).
Mice Wild-type C57/BL6 mice were bred in the National University of Singapore (NUS), SPHK1-/- and SPHK2-/- mice were generated as previously described (S7) and bred in the University of Glasgow (GU). All mice were maintained on a genetic background of C57/BL6. The study was carried out according to the NUS and GU guidelines for animal experimentation, and following approved protocols and project license. A standard pellet diet (Glen Forrest, WA, Australia) and water was given ad libitum. Animals were maintained under a constant 12-h light and dark cycle and an environmental temperature of 21-23° C. For all studies adult male mice were used.

LPS-induced endotoxemia Mice (8–10 weeks, 19–22 g) were randomly grouped (10 mice per group) and injected i.p. with a lethal dose of LPS (24 mg/kg in a final volume of 100 μl) (Sigma), in a blinded fashion. We administered 4 μg of siRNA per mouse, as previously described17, for three consecutive days prior to the LPS administration by i.v. injection. Our SphK1-specific inhibitor (5c) was administered i.v., at the doses shown in the figures, 30 min before LPS administration. Viability was monitored every 12 hours for 7 days.

Cecal ligation and puncture We performed CLP as described (S8). Briefly, we anesthetized mice (10/group) with ketamine (75 mg/kg, intramuscular injection) and xylazine (20 mg/kg). An abdominal midline incision was then performed and the cecum was isolated. We ligated the cecum 5 mm from the cecal tip, away from the ileocecal valve, and the ligated cecal stump was then perforated by two “through and through” punctures (22-gauge needle). We placed the cecum back into its normal intra-abdominal position and closed the abdomen with a running suture of 5-0 prolene. For survival studies, mice were observed for one week. For analysis of
peritonitis, mice were sacrificed at the indicated times, and their peritoneal cavities were washed with 2 ml of ice-cold PBS, 0.2% BSA. The recovered peritoneal lavage fluid was analyzed for different cell infiltrates and the level of cytokines was measured. We administered 4 μg of siRNA per mouse, as previously described (S7), for three consecutive days prior to the CLP procedure by i.v. injection, and for three days after the CLP. Our SphK1-specific inhibitor (5c) was administered i.v., at the doses shown in the figures, 30 min before CLP, and every day for three days after the CLP. Viability was monitored every 12 hours for 7 days.

For the therapeutic study, 2 mg/kg of 5c per mouse was administered i.v. at the indicated times following the CLP procedure. Co-Amoxiclav alone, 30mg/kg, the standard amount used in the clinic, or in combination with 5c, was injected every 12h after the first injection for 3 days. Co-Amoxiclav is the British approved name, for the combination of Amoxicillin trihydrate (a β-lactam antibiotic), with Potassium Clavulanate (a β-lactamase inhibitor). This combination results in an antibiotic with an increased spectrum of action and increased efficacy against β-lactamase-producing Amoxicillin-resistant bacteria.

**Histology** For morphological investigation, the lungs and liver of mice, 24h following the surgical-sham and CLP procedures, were carefully dissected out and immersed in 10% formalin fixative for 1 day. The specimens were then dehydrated through an ascending series of ethanol and cleared in toluene, before being embedded in paraffin. The tissue blocks were cut at 4 μm thickness by means of a Leica Rotary Microtome (Model 2165). Paraffin sections were mounted on albuminized glass slides by floating
and flattening the sections in a water bath at 45°C. The mounted sections were drained until dry and kept in an incubator at 30°C. Paraffin sections were first dewaxed in two changes of xylene and then passed through a descending series of alcohol and finally washed in deionized water, before staining with Hematoxylin and Eosin for 1-2 minutes at room temperature. The sections were rinsed three times in deionized water and then dehydrated quickly through an ascending series of ethanol and passed through xylene before being mounted with Permount. These were viewed with an Olympus microscope (Model BX 51) using 10X, 40X and 100X uplan Apo lenses.

**Bacteria count** Blood was collected (by cardiac puncture) from mice 24h after CLP. After serial dilutions of blood, 5 ml of each dilution was plated on blood agar plates. Bacteria were counted after incubation at 37°C for 24 h and calculated as CFU per whole peritoneal lavage or blood.

**Statistical analysis** The statistical significance among different experimental groups was analyzed by the unpaired Student’s *t* test.
Supplementary Figure 1. Bacterial products increase human SphK1 expression in vitro. (A) Flow cytometric analysis of intracellular SphK1 expression after incubation of human macrophages and neutrophils with the indicated activators, and untreated control cells. Isotype control indicates staining of cells with an isotype control antibody. (B) Western Blot analysis of SphK1 expression after incubation of human macrophages and with the indicated activators, and untreated control cells (Basal). α-tubulin indicates equal loading control. Data shown is representative of multiple patients’ cell-samples. (C) Quantitative Real-time PCR of SPHK1-mRNA expression after incubation of human macrophages with the indicated activators. Data shown as means ± SD from multiple patients (n=10). Student’s t test P values (**P< 0.01).
Supplementary Figure 2. SphK activity (A) in human macrophages stimulated with BLP, in cells pretreated with vehicle control (PBS), siRNA-SphK1, siRNA-Scramb., or the SphK1-inhibitor 5c. (B) NFκB activity in human macrophages stimulated as in (A). (C) Cytokine and HMGB1 production in untreated-nonstimulated human macrophages (Basal), or in human macrophages stimulated as in (A). (D) BLP-mediated cytokines and HMGB1 release by macrophages from septic patients after 16 hours under the indicated stimulation conditions. Basal indicates untreated-unstimulated cells, PBS was used as a vehicle control for 5c, the cytokines and HMGB1 levels were measured by ELISA. (E) NF-κB activity in macrophages from septic patients was measured by p65 binding to specific-DNA templates in an ELISA format. Data shown as means ± SD (n=4). Student’s t test p values (*P < 0.05 and **P< 0.01) compared with BLP -induced control macrophages (BLP PBS).
Supplementary Figure 3. Compound 5c does not inhibit DAGK, CerK, SphK2 or PKC-delta. The kinase assays were performed in the presence increasing amounts of compound 5c; Sphingosine Kinase 1 (SphK1 +5c); Sphingosine Kinase 2 (SphK2 +5c); Diacylglycerol Kinase (DAGK +5c); Ceramide-kinase (CERK +5c); Protein Kinase C-delta (PKC +5c). Data shown as means ± SD of triplicate measurements per point and of three separate experiments.
Supplementary Figure 3. LPS and BLP triggered S1P generation in human macrophages. (A) LPS-mediated cytosolic S1P production (top panel), and in the supernatant (bottom panel), in cells pretreated with control-vehicle (LPS+PBS), in cells pretreated with the siRNA for SphK1 (LPS+siRNA-SphK1), in cells pretreated with the scrambled siRNA (LPS+siRNA-Scramb.). (B) BLP-mediated cytosolic S1P production (top panel), and in the supernatant (bottom panel), in cells pretreated with control-vehicle (BLP+PBS), in cells pretreated with the siRNA for SphK1 (BLP+siRNA-SphK1), in cells pretreated with the scrambled siRNA (BLP+siRNA-Scramb.). Data shown as means ± SD from multiple patients (n=10). Student’s t test p values (**)p<0.01) compared with LPS or BLP -induced control macrophages (LPS+PBS or BLP+PBS).
Supplementary Figure 5. Western blots of: (A) PKCδ, PKCe and PKCa expression in macrophages before (Control PBS), and after treatment with siRNA against PKCd (siRNA-PKCd) or a scrambled control (siRMNA-Scramb.). Data shown are representative of 10 patients’ samples. (B) of immunoprecipitates from macrophages stimulated for 10 minutes under the indicated conditions. Blots were probed for phosphorylated-serine/threonine, IKKβ, IKKα, IKKγ, IκBβ, IκBα, p65 and PKCδ. (C) SphK1 expression in the liver, lungs and PBMCs of: 1 mice pretreated with vehicle, 2 mice pretreated with the siRNA-SphK1, 3 mice pretreated with the scrambled-siRNA. Blots were probed for SphK1 expression and for α-tubulin as loading control.
Supplementary Figure 6. Dose response effects of the SphK1 inhibitor on LPS induced lethality. Survival curves for LPS-induced lethality in mice pretreated with the stated amounts of 5c.

Supplementary Figure 7. Survival curves for CLP-induced lethality in SPHK1−/− or SPHK2−/− mice, in the presence or absence of pre-treatment with 2mg/kg of 5c.
Supplementary Figure 8. Bacteria in the blood measured 24h after CLP in: untreated mice (CLP), in mice pretreated with Co-Amoxiclav for 2h (CLP +Co-Am 2h), in mice pretreated with Co-Amoxiclav for 6h (CLP +Co-Am 6h), in mice pretreated with Co-Amoxiclav for 8h (CLP +Co-Am 8h), in mice pretreated with Co-Amoxiclav for 12h (CLP +Co-Am 12h), and for the negative control mice were Sham-operated (Sham). Data points correspond to 10 mice per treatment group. Student’s t test p values (*p<0.05 and **p<0.01) compared with CLP-induced control (CLP).
Table S1. Clinical Data of patients with sepsis

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<td>Average age and range</td>
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Data for healthy volunteers

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<td>Male/female ratio</td>
<td>% 53.3/46.7</td>
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Table S1. Top part of the table depicts the main characteristics of the recruited patients, including: age, sex, and APACHE II score, and bacteriological data. Bottom part of the table depicts the demographic information for the healthy volunteers.

References


