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Antisense Knockdown of Sphingosine Kinase 1 in Human Macrophages Inhibits C5a Receptor-Dependent Signal Transduction, Ca$^{2+}$ Signals, Enzyme Release, Cytokine Production, and Chemotaxis$^1$

Alirio J. Melendez$^2$ and Farazeela Bte Mohd Ibrahim

The anaphylatoxin C5a is produced following the activation of the complement system and is associated with a variety of pathologies, including septic shock and adult respiratory distress syndrome, and with immune complex-dependent diseases such as rheumatoid arthritis. C5a has been shown to regulate inflammatory functions by interacting with its receptor, C5aR, which belong to the rhodopsin family of seven-transmembrane GPCRs. However, the intracellular signaling pathways triggered by C5aR on immune-effector cells are not well understood. In this report we present data showing that, in human monocyte-derived macrophages, C5aR uses the intracellular signaling molecule sphingosine kinase (SPHK)1 to trigger various physiological responses. Our data show that C5a rapidly stimulates the generation of sphingosine-1-phosphate, SPHK activity, and membrane translocation of SPHK1. Using an antisense oligonucleotide against SPHK1, we show that knockdown of SPHK1 abolishes the C5a-triggered intracellular Ca$^{2+}$ signals, degranulation, cytokine generation, and chemotaxis. Our study shows for the first time that SPHK1 not only plays a key role in the generation and release of proinflammatory mediators triggered by anaphylatoxins from human macrophages but is also involved in the process of immune cell motility, thus pointing out SPHK1 as a potential therapeutic target for the treatment of inflammatory and autoimmune diseases.

Materials and Methods

All chemicals and reagents unless stated otherwise were bought from Sigma-Aldrich (Singapore).

Isolation of human primary monocytes and differentiation to macrophages

Mononuclear cells were isolated from heparinized fasting venous blood by Ficoll-Hypaque centrifugation as previously described (27). The 20 ml of blood (anticoagulated with 10 U/ml heparin) was layered carefully on 15 ml of Ficoll-Hypaque gradient and centrifuged at 500 × g, without brakes, at room temperature for 30 min. The mixed mononuclear band was aspirated and the cells were washed three times in phenol red RPMI 1640 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine and suspended in a known volume. Leukocyte count was performed on a Coulter counter and then cells were plated (5 × 10⁶ cells) in six-well culture plates in RPMI 1640 medium. Incubation was conducted at 37°C for 2 h in 5% CO₂/95% air, after which nonadherent cells were removed by washing the wells twice with RPMI 1640, and the remaining adherent cells were grown in the culture medium supplemented with 10% FCS and 2 mM glutamine. The medium was replaced every 2–3 days. The cells were used after 8 days of culture. Cell viability, determined by trypan blue exclusion, was >94% in all experiments.

SPHK activity in whole macrophages and generation of S1P

SPHK activity in intact cells was measured by assaying the amount of intracellular S1P generation following receptor activation as previously described (25, 28). Briefly, cells were preincubated overnight in medium containing [3H]serine (2 µCi/ml) to label cellular sphingolipids and free sphingosine pools. Following labeling, 2 × 10⁶ cells/sample were stimulated by the addition of 5 nM C5a and warming to 37°C, and the reactions were terminated at the specified times. Lipids were extracted and analyzed by TLC on silica gel G60. Standard S1P was applied with the samples, and the lipids were visualized using iodine vapors. Bands corresponding to S1P were scraped and counted in a scintillation counter. Results were calculated as a percentage of the total radioactivity incorporated in the lipids.

SPHK activity in cell extracts

Human macrophages, 2 × 10⁶ cells/sample, were stimulated by the addition of 5 nM C5a. Following C5a stimulation the cells are lysed and cell extracts are assayed for SPHK activity. SPHK activity was measured as previously described (24, 29). Briefly, the system is based upon the SPHK catalyzed transfer of the γ-phosphate group of ATP (using a mixture of cold ATP and [γ-32P]ATP (1 µCi/sample)) to a specific substrate, the products were separated by TLC on silica gel G60 (Whatman, Maidstone, U.K.) and visualized by autoradiography. The radioactive spots corresponding to S1P were scraped and counted in a scintillation counter.

Phospholipase C (PLC) activity

Human macrophages, 2 × 10⁶ cells/sample, were stimulated by the addition of 5 nM C5a. Following stimulation, inositol 1,4,5-triphosphate (IP₃) was generated and visualized by autoradiography. The radioactive spots corresponding to IP₃ were scraped and counted in a scintillation counter.

Cytosolic calcium measurement

Cytosolic calcium was measured as previously described (24). Briefly, 2 × 10⁶ cells/ml were loaded with 1 µg/ml fura 2-AM (Molecular Probes, Leiden, The Netherlands) in PBS, 1.5 mM Ca²⁺ and 1% BSA. After removal of excess reagents by dilution and centrifugation, the cells were resuspended in 1.5 mM Ca²⁺ supplemented PBS and warmed to 37°C in the cuvette. A total of 2 × 10⁶ cells/sample were stimulated by the addition of 5 nM C5a. Fluorescence was measured at 340 and 380 nm and the background-corrected 340:380 ratio was calibrated as previously described (24).

Protein kinase C (PKC) activity

PKC enzyme activity was measured as previously stated (30, 31), using the Biotrak Protein Kinase C enzyme assay kit (Amersham Biosciences). Briefly, the system is based upon the PKC catalyzed transfer of the γ-phosphate group of ATP (using a mixture of cold ATP and [γ-32P]ATP; 1 µCi/sample) to a peptide substrate specific for PKC. A total of 2 × 10⁶ cells/sample were stimulated by the addition of 5 nM C5a for 15 min. Following stimulation, PKC assays were conducted. Results are expressed as phosphorylation rate per picomolar of protein per minute.

Degranulation and enzyme release

β-Hexosaminidase is released from macrophages. Degranulation was measured using a previously described colorimetric assay to assess the release of β-hexosaminidase (24). Briefly, 2 × 10⁶ cells/sample were stimulated by the addition of 5 nM C5a for 30 min. Following stimulation, 50 µl of the sample supernatant was incubated with 200 µl of 1 mM p-nitrophenyl N-acetyl-β-D-glucosaminide for 1 h at 37°C. The total β-hexosaminidase concentration was determined by a 1/1 extraction of the remaining buffer and cells with 1% Triton X-100; a 50 µl aliquot was removed and analyzed as described. The enzyme concentration was determined by measuring the OD at 400 nm.

Cytokine detection

A total of 2 × 10⁵ cells/sample, pretreated or not with antisense oligos, were stimulated by the addition of 5 nM C5a for 24 h. Following stimulation, the supernatants were collected and stored at −20°C until use. TNF-α, IL-6, and IL-8 levels in the supernatants were measured by ELISA and detected using an enzyme-linked immunosorbent assay kit (Amersham Biosciences). The supernatants were diluted to a concentration of 5 nM C5a and the optical density of the supernatants was measured at 450 nm.
were evaluated using ELISA (R&D Systems, Minneapolis, MN) following the manufacturer’s instructions.

Chemotaxis assay
Chemotaxis was assayed using the Chemicon QCM Chemotaxis 3 μm 96-well Cell Migration Assay kit (catalogue no. ECM 515; Temecula, CA) following manufacturers instructions. A total of 2 \times 10^5 cells were used per sample and the concentration of C5a used for the migration assays was 5 nM.

RT-PCR analysis
mRNA from BMMC was isolated using the Qiagen midi kit for mRNA extraction. Specific forward (5’-TGAACCCGCGCGAAGGGC-3’) and reverse (5’-GGTCAGGCCGGCATCCAG-3’) primers were designed for the human SPHK1 to yield a 570 bp fragment, and specific forward (5’-CCATGGCGAG TTTGGCTC-3’) and reverse (5’-CATCCC CGGCAGTGCCAGTCCTCGG-3’) primers were designed for the human SPHK2 to yield a 356 bp fragment.

Western blots
Unless stated other ways, 40 μg of lysate for each sample was resolved on 12% polyacrylamide gels (SDS-PAGE) under denaturing conditions and then transferred to 0.45-μm nitrocellulose membranes. After blocking overnight at 4°C with 5% nonfat milk in TBS, 0.1% Tween 20 and washing, the membranes were incubated with the relevant Abs for 4 h at room temperature. The membranes were washed extensively in TBS/0.1% Tween 20 (washing buffer). The blots were probed using specific, anti-SPHK1 polyclonal (made in house as previously described (21)), and a monoclonal anti-Arf1 (Santa Cruz Biotechnologies, Santa Cruz, CA), primary Abs. Bands were visualized using anti-rabbit HRP-conjugated, or anti-mouse HRP-conjugated, secondary Abs, and the ECL Western Blotting Detection System (Amersham Biosciences).
**Fluorescent microscopy**

After stimulation with 5 nM C5a, cells were fixed in 4% paraformaldehyde and deposited on microscope slides in a cytosin centrifuge, then permeabilized for 5 min in 0.1% Triton X-100 in PBS. Fluorescent labeling was performed using an anti-SHPK1 polyclonal Ab made by us as previously described (21). Staining was analyzed by fluorescence microscopy using a Leica DM IRB microscope, and images captured using a Leica DC 300F camera (Leica Microsystems, Bannockburn, IL).

**Antisense knockdown of SPHK 1**

The antisense down-regulation of SPHK1 was conducted as previously described (24). Antisense oligonucleotides were purchased from Oswell DNA Services (Southampton, U.K.); 20-mers were synthesized, capped at either end by the phosphorothioate linkages (first two and last two linkages), and corresponded to the reverse complement of the first 20 coding nucleotides for SPHK1, and a scrambled oligo for control. The sequences of the oligonucleotides were: 5'-CCCGCAGGATCCATAACCTC-3' antisense for SPHK1; 5'-CTGTTGAAGAAGAGGACGT-3' scrambled antisense for control. Cells were cultured in the presence of antisense for 48 h.

**Results**

**C5a stimulates SPHK activity**

We first investigated whether the C5aR would generate S1P and activate SPHK activity in the human macrophages. For this model, formation of [3H]S1P from [3H]sphingosine was first determined. Activation of the C5aR induced a rapid and transient increase in [3H]S1P generation (Fig. 1A), reaching its maximal [3H]S1P generation at 2 min. Parallel to the generation of S1P, direct measurement of SPHK activity showed that the enzyme is activated following C5aR engagement (Fig. 1B).

**SHPK1 is the enzyme activated by C5a antisense knockdown of SPHK1**

Two human SPHKs have recently been cloned and characterized, namely SPHK1 (32) and SPHK2 (33). First, the mRNA presence of specific SPHK isozymes present in the cells was examined; only the messenger for SPHK1 was found by RT-PCR in the human macrophages, and SKNMC were used as control (Fig. 2A). Fluorescent microscopy confirmed the presence of SPHK1, and showed that in resting cells SPHK1 is primarily cytosolic, but after C5aR engagement it rapidly translocates to the cell periphery (Fig. 2B, top).

We pretreated the cells with an antisense oligonucleotides specific for SPHK1, and found that the antisense knocks down SPHK1 levels by ~80% as shown by Western blot analysis (Fig. 2C), whereas an antisense control (scrambled oligo) had no effect on the SPHK1 levels (Fig. 2C).

In cells pretreated with the antisense oligo against SPHK1, C5a-stimulated formation of S1P was substantially inhibited (Fig. 2D). Direct measurement of SPHK activity was also inhibited in cells pretreated with the antisense against SPHK1 (Fig. 2E). However, pretreatment of the cells with a scrambled antisense oligonucleotide had no effect on S1P generation (Fig. 2D), nor on SPHK enzyme activity (Fig. 2E). These data show that the C5aR is capable of stimulating SPHK activity and S1P generation by specifically activating SPHK1.

**Role of SPHK1 on C5a-triggered Ca^{2+} signals**

Previous studies have demonstrated that SPHK mediates Ca^{2+} signals for several plasma membrane receptors (20–23), and because our results showed that C5a triggers SPHK activity in human macrophages (Figs. 1 and 2), we decided to investigate the role of SPHK1 in the C5a-triggered Ca^{2+} mobilization in these cells. C5a stimulation rapidly and transiently triggered calcium release from internal stores (Fig. 3A). However, in cells pretreated with the antisense against SPHK1, these Ca^{2+} signals were inhibited (Fig. 3A). Pretreatment of the cells with the scrambled antisense oligonucleotide had no effect on the C5a-triggered Ca^{2+} signals (Fig. 3A).

The inhibitory action of antisense on Ca^{2+} signals was not due to inhibition of PLC stimulation. Preincubation of cells with the antisense against SPHK1 neither altered basal nor inhibited the C5a-triggered generation of IP_{3} (Fig. 3B).

**Role of SPHK1 on C5a-triggered physiological responses**

Following the role of SPHK1 on triggering Ca^{2+} signals, we investigated the role of SPHK1 in other well-characterized functional responses of macrophages following cellular activation by C5a, namely PKC activity, degranulation, cytokine release, and chemotaxis.

**FIGURE 3.** C5a-triggered cytosolic Ca^{2+} signals are inhibited by N,N-dimethylsphingosine showing a role for SPHK. A, Cytosolic Ca^{2+} triggered by C5a stimulation in the monocyte-derived macrophages, time course control (Cells untreated); cytosolic Ca^{2+} triggered by C5a in cells pretreated with the SPHK1 antisense (Cells+a.s.SPHK1); and cytosolic Ca^{2+} triggered by C5a in cells pretreated with the scrambled antisense (Cells+a.s.scrambled) are shown. Results are representative of three separate experiments. B, C5a-triggered PLC activity is not inhibited by the SPHK1 antisense. Basal IP_{3} generation in untreated macrophages (Cells basal), IP_{3} generation after C5a stimulation in the untreated macrophages (Cells+C5a), IP_{3} generation after C5a stimulation in macrophages pretreated with the SPHK1 antisense (Cells+C5a+a.s.SPHK1), and IP_{3} generation after C5a stimulation in macrophages pretreated with the scrambled antisense (Cells+C5a+a.s.scrambled) are shown. Results are the mean ± SD of triplicate measurements and of three separate experiments.
**C5a-triggered PKC activity.** PKC activity is important for many cellular responses, and most receptor trigger PKC activity. C5a triggered a fast increase of PKC enzyme activity in the macrophages (Fig. 4). Pretreatment with the antisense oligo against SPHK1 did not affect the PKC activity triggered by C5a, however, pretreatment with the PKC inhibitor, bisindolylmaleimide (BisI), indeed inhibited the PKC activity triggered by C5a (Fig. 4). This shows that SPHK1 does not play a role on the C5a-triggered activation of PKCs.

**C5a-triggered degranulation.** Enzyme release (degranulation) is an important effector function of phagocytic cells and has been shown to require Ca2+ signals. Therefore we investigated whether SPHK1 plays any role on the C5a-triggered degranulation. In contrast to PLC and PKC activities, C5a-stimulated degranulation was strongly inhibited in cells pretreated with the SPHK1 antisense oligo. C5a very rapidly triggered the release of β-hexosaminidase from the human macrophages (Fig. 5). Pretreatment of cells with the antisense oligo against SPHK1, substantially inhibited the C5a triggered enzyme release, whereas pretreatment with the scrambled antisense oligonucleotide had no effect on degranulation (Fig. 5). These results suggest a role for SPHK1 in regulating C5a-triggered migration.

**C5a-triggered chemotaxis.** One of the most important functions of anaphylatoxins is their role as chemoattractants, therefore, we decided to find whether SPHK1 plays any role in chemotaxis. C5a (5 nM) triggered a strong chemotactic response on the human macrophages (Fig. 6). However, pretreatment of cells with the antisense SPHK1 oligo substantially inhibited the C5a-triggered chemotaxis, whereas pretreatment of the cells with the scrambled antisense oligonucleotides did not alter the response (Fig. 6). This suggests a specific role for SPHK1 in the C5a-triggered macrophage migration.

**C5a-triggered cytokine production.** Another important role for anaphylatoxins is to promote the release of proinflammatory cytokines and chemokines. In this model we show that C5a stimulates TNF-α and IL-6 and IL-8 release from the human macrophages (Fig. 7). However, in cells pretreated with the antisense SPHK1 oligo, C5a-triggered release of TNF-α (Fig. 7A), IL-6 (Fig. 7B), and IL-8 (Fig. 7C) was inhibited. In contrast pretreatment with the scrambled antisense had no inhibitory effect on cytokine production, suggesting that SPHK1 mediates the C5a-triggered cytokine production on human macrophages.

Taking these results together, our data indicates that SPHK1 plays a key role on the C5a-triggered proinflammatory responses of human macrophages.

**Discussion**

Activation of C5aR triggers a number of rapid proinflammatory responses, including up-regulation of cell adhesion molecules, cytokine and enzyme release, and leukocyte migration (34, 35). Recently, C5a has been shown to have an immunoregulatory role able to stimulate mediators of both acute and chronic inflammation (36–39). The significance of C5a in several inflammatory diseases is demonstrated by the fact that agents that blocked the action of C5a also suppressed inflammation in several animal models (40–44). Most of these studies used blocking Abs raised against C5a (41, 43) or recombinant proteins that are receptor antagonists or analogues of C5a (40, 44). However, there are many problems associated with the use of such proteins to treat human patients. Immunogenicity is a problem and proteins are expensive to manufacture, very susceptible to degradation by proteases in serum or the gastrointestinal track, and generally display poor pharmacokinetic properties. More recently attempts have been made to make smaller molecules that are more stable, cheaper to make, have better bioavailability, and are more attractive as drug candidates for treating human diseases mediated by C5a (45, 46). However, very little is known about the intracellular signaling pathways activated by C5a in immune-effector cells, such as human macrophages.
SIP has been shown to play an important role in immune cell activation including cytoskeletal changes and degranulation. We report for the first time that SPHK1 is rapidly activated by C5a in the human macrophages. Our data shows that SPHK1 is involved in the C5aR-mediated Ca\(^{2+}\) mobilization in human macrophages. Furthermore, knocking down SPHK1 levels, with the specific SPHK1 antisense oligonucleotides, substantially inhibited the C5a-triggered calcium release from internal stores, in a manner that was independent of the PLC/IP3 pathway because the anti-sense did not have any effect on the C5a-triggered IP3 generation.

To further investigate the physiological roles of the SPHK1 in the C5a signaling pathways, we studied the effects of the antisense knockdown of SPHK1 on PKC enzyme activity, degranulation, cytokine production, and chemotaxis. Many physiological responses triggered in phagocytic cells are dependent on the activation of PKC isoforms. Treatment of the macrophages with the antisense oligonucleotides against SPHK1, which caused a marked inhibition on the C5a-induced intracellular Ca\(^{2+}\) concentration signals, had no effect on the C5a-triggered stimulation of protein kinase activity, whereas the PKC inhibitor BisI did indeed inhibit PKC activation. These data suggest that SPHK is not involved in the signaling pathway to PKC activation. However, there is a tight correlation between increase of intracellular calcium and degranulation (23, 24, 26). Pretreatment of human macrophages with the antisense against SPHK1 strongly inhibited the C5a-triggered release of \(\beta\)-hexosaminidase from the macrophages, indicating the requirement for SPHK1 in this response.

It is well established that C5a can trigger proinflammatory cytokines and chemokines, such as TNF-\(\alpha\), IL-6, and IL-8 production (36–39). These molecules share many activities, including the ability to induce fever and shock syndrome in animal models (47). In this report we show that C5a triggered the generation of TNF-\(\alpha\), IL-6, and IL-8 to different levels. Moreover, pretreatment of cells with antisense against SPHK1 substantially inhibited the production of TNF-\(\alpha\), IL-6, and IL-8 triggered by C5a. Inflammatory responses are characterized by the accumulation of macrophages at an inflammatory site. Where local inflammation is triggered by infection, trauma, or immune-complex deposition, C5a is likely to be an important chemokine. C5a has been shown to trigger chemotaxis in cell suspensions (48), and C5a-triggered cell migration has been used as a sensitive test for measuring the activation of the cell’s internal motile apparatus (49). In the present study we show that C5a is capable of triggering macrophage migration, and that pretreatment of cells with the antisense oligonucleotides against SPHK1 strongly inhibited the C5a-induced chemotaxis.

SPHK activity also plays a potential role in the physiological responses triggered on other immune-effector cells. Activated neutrophils generate a broad and a vigorous set of alterations in gene expression shaping the inflammatory response (50). It has been proposed that SPHK regulates neutrophil priming to provide an

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**FIGURE 6.** C5a-induced chemotaxis is inhibited in macrophages pretreated with the SPHK1 antisense (a.s.). Migratory number of cells shown include basal migration of untreated cells (Basal control); migration of cells pretreated with the SPHK1 antisense (Basal + a.s.SPHK1); migration of untreated cells toward the C5a containing chamber (C5a control); migration toward the C5a containing chamber of cells pretreated with the SPHK1 antisense (C5a + a.s.SPHK1); and migration toward the C5a containing chamber of cells pretreated with the scrambled antisense (C5a + a.s.scrambled). Results are the mean ± the SD of triplicate measurements and of three separate experiments.

**FIGURE 7.** TNF-\(\alpha\), IL-6, and IL-8 release triggered by C5a is inhibited in macrophages pretreated with the SPHK1 antisense (a.s.). **A.** Basal TNF-\(\alpha\) release from untreated cells (Basal); TNF-\(\alpha\) release from untreated cells after C5a stimulation (C5a control); TNF-\(\alpha\) release after C5a stimulation in cells pretreated with the SPHK1 antisense (C5a + a.s.SPHK1); and TNF-\(\alpha\) release after C5a stimulation in cells pretreated with the scrambled antisense (C5a + a.s.SPHK1) are shown. Results are the mean ± SD of triplicate measurements and of three separate experiments. **B.** Basal IL-6 release from untreated cells (Basal); IL-6 release from untreated cells after C5a stimulation (C5a control); IL-6 release after C5a stimulation in cells pretreated with the SPHK1 antisense (C5a + a.s.SPHK1); and IL-6 release after C5a stimulation in cells pretreated with the scrambled antisense (C5a + a.s.SPHK1) are shown. Results are the mean ± SD of triplicate measurements and of three separate experiments. **C.** Basal IL-8 release from untreated cells (Basal); IL-8 release from untreated cells after C5a stimulation (C5a control); IL-8 release after C5a stimulation in cells pretreated with the SPHK1 antisense (C5a + a.s.SPHK1); and IL-8 release after C5a stimulation in cells pretreated with the scrambled antisense (C5a + a.s.SPHK1) are shown. Results are the mean ± SD of triplicate measurements and of three separate experiments.
essential defense against infections (51), and in neutrophil inflammatory responses (26, 51). The recruitment of circulating neutrophils to damaged tissue plays a critical role in a number of physiological and pathophysiological events: circulating neutrophils attach to the activated endothelial cells in blood vessels through various adhesion molecules and then migrate through the endothelial cell layer into the injured sites; and SPHK activity has also been shown to modulate the expression of vascular cell adhesion molecules by endothelial cell (52), essential for the recruitment of leukocytes during the inflammatory response. Furthermore, it has been shown that TNF-α and IMLP-stimulated superoxide production can be regulated by SIP and inhibited by the SPHK inhibitor DL-threo-dihydrosphingosine (53).

Another important role for SPHK activity has been proposed for mast cell activation. It has been suggested that the differential ratio sphingosine to SIP, in mast cells, regulates the activity of mast cells (54). Thus, mast cell activation triggered by the high-affinity mast cell activation. It has been suggested that the differential ratio the proinflammatory action of mast cells, including the expression lipids have opposite effects. Although sphingosine inhibits the ac-

References

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Acknowledgment

role of SPHK in C5a signaling


Letter of Retraction


An investigation by the National University of Singapore concluded that Fig. 2A and 2C display irregular backgrounds that suggest the bands were improperly inserted. The investigation also concluded that Fig. 2A was duplicated in Zhi et al., Journal of Cellular Physiology, 2006, 208: 109–115, which has been retracted by Dr. Gary S. Stein, the Editor-in-Chief of that journal.

The Investigative Committee of the National University of Singapore concluded that Dr. Melendez committed serious scientific misconduct. The committee found no evidence indicating that other coauthors were involved in the scientific misconduct.