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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

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. The Journal of Immunology, 2004, 173: 1596–1603.

Activation of the complement cascade plays a key role in host defense. However, anaphylatoxins produced following the activation of the complement system are associated with a variety of pathologies, including septic shock and adult respiratory distress syndrome, and with immune complex-dependent diseases such as rheumatoid arthritis (1–3). Anaphylatoxins are generated by activation-induced cleavage of the third and fifth components of complement, C3a and C5a, respectively (4). C3a and C5a aremediators of proinflammatory and immunoregulatory activities (4). C5a is a 74 aa peptide shown to regulate inflammatory functions by interacting with its receptor, C5aR, which belongs to the rhodopsin family of seven-transmembrane G-protein-coupled receptors (GPCRs) (5, 6). Anaphylatoxin receptors are present on myeloid and nonmyeloid leukocyte populations, including granulocytes, and monocytes and macrophages (6, 8), lymphocytes (9, 10), and dendritic cells (DCs) (11, 12). One of the main functions of anaphylatoxins is the recruitment of leukocytes to the sites of infection, inflammation, and trauma. It is well recorded that C5a is a potent chemoattractant for all C5aR-expressing cell types (13–15), including the responses of macrophages to C5a (14, 15). However, the intracellular signaling cascades triggered by anaphylatoxins in human immune-effector cells are poorly understood. The aim of our study was to dissect the intracellular signaling pathways triggered by C5aR in human macrophages to better understand the C5a triggered responses in human macrophages. Recently, it has become clear that sphingolipids are sources of important signaling molecules. Particularly, the sphingolipid metabolites, ceramide and sphingosine-1-phosphate (S1P), have emerged as a new class of potent bioactive molecules, implicated in a variety of cellular processes such as cell differentiation, apoptosis, and proliferation (16–19). Interest in S1P focused recently on two distinct cellular actions of this lipid, namely its function as an extracellular ligand activating specific GPCRs and its role as an intracellular second messenger (20). Several findings enforced the notion of S1P as an important intracellular second messenger. First, activation of various plasma membrane receptors, such as the platelet-derived growth factor receptor (21, 22), the FcεRI and FcγRI Ag receptors (23–25), as well as the fMLP receptor (26), was found to rapidly increase intracellular S1P production through the stimulation of the SPHK. Second, inhibition of SPHK stimulation strongly reduced or even prevented cellular events triggered by these receptors, such as receptor-stimulated DNA synthesis, Ca\(^{2+}\) mobilization, and vesicular trafficking (21–26).

To our best knowledge this is the first study showing that C5a uses the intracellular signaling molecule SPHK1 as a key player in the physiological responses triggered by C5a in human macrophages. In this study we show that C5aR activation rapidly stimulates the production of S1P and SPHK activity. Moreover, knocking down the expression levels of SPHK1, with a specific antisense oligonucleotide, a number of the macrophage responses to C5a are inhibited, such as the C5a-stimulated intracellular Ca\(^{2+}\) signals, degranulation, cytokine production as well as cell migration. Thus, our data suggest that SPHK1 is indeed a key player in the proinflammatory responses triggered by human monocyte-derived macrophages stimulated by C5a.
Materials and Methods

Isolation of human primary monocytes and differentiation to macrophages

Mononuclear cells were isolated from heparinized fasting venous blood by Ficoll-Paque centrifugation as previously described (27). The 20 ml of blood (anticoagulated with 10 U/ml heparin) was layered carefully on 15 ml of Ficoll-Paque 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 (5–7 × 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 excised from the plate and counted by liquid scintillation spectrometry. 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 radioactivity 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, unlabelled 4,5-triphosphate (IP₃) was measured as previously described (24), using the BIOTRAK TRK 1000 kit (Amersham Biosciences, Buckinghamshire, U.K.).

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 system (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 × 10⁶ 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 at the indicated time points and stored at −20°C until use. TNF-α, IL-6, and IL-8 levels in the supernatants were measured as previously described (24), using the BIOTRAK TRK 1000 kit (Amersham Biosciences, Buckinghamshire, U.K.).
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 manufacturer’s instructions. A total of 2 × 10⁵ 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'-TGAACCGCGGCAAGGGC-3') and reverse (5'-TGTAGCCGGCCTACCCGC-3') primers were designed for the human SPHK1 to yield a 570 bp fragment, and specific forward (5'-CATGTTGCTCCT-3') and reverse (5'-CATCCC CGGGCAGTTG CAGTCCTCGG-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).

FIGURE 2. SPHK1 expression, subcellular localization, and antisense (a.s.) knockdown in the human monocyte-derived macrophages. A, RT-PCR analysis of mRNA expression levels for both SPHK1 and SPHK2 in the human monocyte-derived macrophages (Macrophage), and in a positive control for both isoforms, the human neuronal cell line SKNMC. Results are representative of at least three separate experiments. B, Fluorescent microscopy of cells immune-stained for SPHK1. Resting cells (Macrophage resting) and cells after stimulation with C5a for 2 min (Macrophage + C5a) are shown. Results are representative of three separate experiments. C, Western blot analysis of SPHK1 expression before and after antisense treatment are represented by SPHK1 expression levels in untreated monocyte-derived macrophages (Macrophage control), SPHK1 expression levels in cells pretreated for 48 h with the antisense against SPHK1 (Macrophage a.s. SPHK1), and SPHK1 expression levels in cells pretreated for 48 h with the scrambled antisense (Macrophage a.s.scrambled). The blot was simultaneously probed with an anti-Arf1 Ab to show equal loading for all the lanes. Results are representative of three separate experiments. D, Effect of the SPHK1 antisense on S1P generation following C5a stimulation. Cells were labeled with [3H]serine. Basal S1P generation was 1.2 pmols S1P/2 × 10⁶ cells. S1P generation in untreated macrophages following C5a stimulation (Macrophage + C5a), S1P generation after C5a stimulation in macrophages pretreated with the SPHK1 antisense (Macrophage + C5a + a.s.SPHK1), and S1P generation after C5a stimulation in macrophages pretreated with the scrambled antisense (Macrophage + C5a + a.s.scrambled) are shown. Results are the mean ± SD of triplicate measurements and from three separate experiments. E, Effects of the SPHK1 antisense on the C5a stimulated SPHK activity in the macrophages. SPHK activity from untreated macrophages following C5a stimulation (Macrophage + C5a), SPHK activity following C5a stimulation from macrophages pretreated with the SPHK1 antisense (Macrophage + C5a + a.s.SPHK1), and SPHK activity following C5a stimulation from macrophages pretreated with the scrambled antisense (Macrophage + C5a + a.s.scrambled) are shown. Results are the mean ± SD of triplicate measurements and from three separate experiments.
**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-SPHK1 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′-CTGGTGGAAAGAGGAGGCAGT-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]phospho increases 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).

**SPHK1 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 oligonucleotide 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 Ca2+ signals**

Previous studies have demonstrated that SPHK mediates Ca2+ 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 Ca2+ 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 Ca2+ signals were inhibited (Fig. 3A). Pretreatment of the cells with the scrambled antisense oligonucleotide had no effect on the C5a-triggered Ca2+ signals (Fig. 3A).

The inhibitory action of antisense on Ca2+ 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 IP3 (Fig. 3B).

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

Following the role of SPHK1 on triggering Ca2+ 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.

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**FIGURE 3.** C5a-triggered cytosolic Ca2+ signals are inhibited by N,N-dimethylphosphinoine showing a role for SPHK. A, Cytosolic Ca2+ triggered by C5a stimulation in the monocyte-derived macrophages, time course control (Cells untreated); cytosolic Ca2+ triggered by C5a in cells pretreated with the SPHK1 antisense (Cells +a.s.SPHK1); and cytosolic Ca2+ 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 IP3 generation in untreated macrophages (Cells basal), IP3 generation after C5a stimulation in the untreated macrophages (Cells+C5a), IP3 generation after C5a stimulation in macrophages pretreated with the SPHK1 antisense (Cells+C5a+a.s.SPHK1), and IP3 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 (Bis-I), 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 Ca^{2+} 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 degranulation.

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 IL6 and IL8 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.
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.

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 antisense 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 Bisl 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 β-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-α, 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-α, IL-6, and IL-8 to different levels. Moreover, pretreatment of cells with antisense against SPHK1 substantially inhibited the production of TNF-α, 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

FIGURE 7. TNF-α, IL-6, and IL-8 release triggered by C5a is inhibited in macrophages pretreated with the SPHK1 antisense (a.s.). A, Basal TNF-α release from untreated cells (Basal); TNF-α release from untreated cells after C5a stimulation (C5a control); TNF-α release after C5a stimulation in cells pretreated with the SPHK1 antisense (C5a + a.s.SPHK1); and TNF-α release after C5a stimulation in cells pretreated with the scrambled antisense (C5a + a.s.scrambled). 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.scrambled). 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.scrambled) 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 of sphingosine to S1P, in mast cells, regulates the activity of mast cells (54). Thus, mast cell activation triggered by the high-affinity IgE receptor (FceRI) activates the SPHK, which changes the balance of sphingosine toward S1P, and these two lipids have opposite effects. Although sphingosine inhibits the activation of members of the MAPK family, stress-activated protein kinase and AP-1, S1P activates these molecules, thereby inducing the proinflammatory action of mast cells, including the expression of TNF-α and IL-5 genes products (54). Moreover, it has been shown that FceRI activates SPHK-dependent Ca2+ mobilization in mast cells (23, 24), as well as S1P-dependent mast cell degranulation (24).

Phagocytic cell infiltration and proinflammatory cytokine production are universal components of a wide range of disease states including immune complex-mediated conditions such as nephritis (50), arthritis (51), and acute graft rejection (52). Agents that can inhibit phagocyte infiltration and/or the production of cytokines such as TNF-α and IL-6 may have wide therapeutic applications in the prevention and treatment of these and other disease states. The present study indicates that knocking down the levels of SPHK1, with an antisense oligonucleotide, very effectively inhibits the cytokine production and chemotactic response triggered by the anaphylatoxin C5a in human macrophages. Our findings also indicate new pathways in the intracellular signaling cascades triggered by anaphylatoxins, and point out the role of novel molecules such as SPHK1, in immune-inflammatory processes, aiding the validation of novel potential “druggable targets” for the development of new therapeutics for inflammatory diseases.

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References


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.