Distinct Roles of Sphingosine Kinase 1 and 2 in Murine Collagen-Induced Arthritis

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Sphingolipids are sources of important signaling molecules in addition to their role as structural components of the eukaryotic cell membranes. In particular, sphingolipid metabolites such as ceramide and sphingosine-1-phosphate (SIP) have emerged as a new class of potent bioactive messengers involved in an array cellular processes, including angiogenesis, proliferation, and apoptosis (1, 2). To date, a total of five G protein-couple SIP receptors have been identified, designated S1P1–5 (3, 4). Sphingosine kinase (SphK) is a key enzyme in the sphingolipid metabolic pathway, responsible for phosphorylating sphingosine into SIP. Two mammalian SphKs, SphK1 and SphK2, have been identified and characterized (5–7). Stimulation of various plasma membrane receptors, such as the fMLP receptor (8), the C5a receptor (9), and TNF-α receptor (11), lead to rapid increase in intracellular SIP level via SphK activity. The pivotal role of SphK and SIP in inflammation has been widely established (2, 4).

We have previously shown that inhibition of SphK activity leads to reduced Ca2+ mobilization, enzyme release, chemotaxis, cytokine, and chemokine production in human neutrophils, monocytes, and macrophages (9–12). Moreover, SphK1 was found to be a critical regulator in TNF-α-mediated IL-1β and IL-6 proinflammatory responses in human monocytes (11). In RAW macrophages, TNF-α-induced PGE2 production can be inhibited by down-regulation of SphK1 via small interfering RNA (siRNA) inhibition (13). SphK1 expression is increased in human inflammatory bowel disease (IBD) colons. Importantly, in a murine model of IBD, SphK1-deficient mice are protected from disease manifestations such as weight loss, colon pathology, anemia, and leukocytosis, indicating the therapeutic potential of SphK1/SIP modulation in the treatment of inflammatory diseases (14). In contrast, SphK2 is required for the maintenance of Th cells homeostasis. CD4+ T cells from SphK2-deficient mice exhibit a hyperactivated phenotype with enhanced proliferative and Th1 cytokine-secreting capacities, and promote IBD in SCID mice. This is in part due to IL-2-induced abnormal accentuated STAT5 phosphorylation and is independent of S1P (15).

Rheumatoid arthritis (RA) is a chronic, destructive, autoimmune joint disease characterized by elevated levels of proinflammatory cytokine production (16, 17). In particular, within inflamed RA synovial membrane, the levels of proinflammatory cytokines exceed those of anti-inflammatory mediators. Moreover, successful therapeutic targeting of cytokines in RA, particularly TNF-α and IL-6, had demonstrated their critical pathogenic importance (16–18). Elevated SphK1, SIP, and SIP1 have been detected in RA synovium, and SIP signaling via SIP1 was found to promote synoviocyte proliferation, cytokine-induced COX-2 expression, and PGE2 production (19). Lymphoblastoid cell lines from patients with RA were shown to be resistant to Fas-mediated cell death, in
part due to over-activity of SphK1 (20). In RA primary fibroblast-like synovocytes (FLS), S1P1 was shown to be essential for the survival of FLS, and S1P1/S1P5 for FLS migration; activation of S1P1/S1P5 promotes release of proinflammatory cytokines including IL-6, IL-8, and TNF-α (21).

We have previously shown that synovial fluid of RA patients exhibited higher levels of S1P than those with osteoarthritis. Furthermore, N,N-dimethylphosphinosine (DMS), a potent SphK inhibitor, significantly reduced inflammatory mediator release by RA-derived peripheral blood monocytes following cell contact-dependent interaction with activated T cells (22). In a murine collagen-induced arthritis (CIA) model, i.p. administration of DMS significantly suppressed joint destruction and proinflammatory cytokine production in vivo and in vitro (22). In the present study, we investigated by targeting SphK activities, down-regulation of SphK1, or SphK2 via siRNA, may modulate the development of CIA. We found SphK1 down-regulation can effectively suppress synovial inflammation, joint erosion, and proinflammatory cytokine responses in vivo and in vitro whereas SphK2 knockdown resulted in enhanced disease severity. These data identify the distinct role of SphK1 and SphK2 in regulating the development of inflammatory arthritis.

Materials and Methods

Induction and assessment of CIA

Male DBA/1 mice at 8–10 wk old were obtained from the Laboratory Animals Centre, National University of Singapore (NUS). Animal experiments were conducted according to the Institutional Guidelines for Animal Care and Use Committee, NUS. Male DBA/1 mice received 200 µg of bovine type II collagen (CII, Sigma-Aldrich) in Freund’s complete adjuvant (Difco) by intradermal injection (day 0). Collagen (200 µg in PBS) was given again on day 21 by i.p. injection. Mice were monitored for signs of arthritis for which severity scores were derived as follows: 0, normal; 1, erythema; 2, erythema plus swelling; 3, extension/loss function, and total score of sum of four limbs, giving a maximum score of 12 per mouse. Paw thickness was measured with a dial-caliper (Kroeplin). For histological assessment, mice were sacrificed and the hind limbs removed, fixed in 10% neutral-buffered formalin, and 5-µm sections were stained with H&E (Sigma-Aldrich). The quantification of arthritis was performed by a ‘treatment-blind’ observer and a score was assigned to each joint based on the degree of inflammation, synovial hyperplasia, and erosion. Each parameter was analyzed separately against a set of predefined sections graded 0 (normal), 1 (mild), 2 (moderate), and 3 (severe).

Treatment protocols

To investigate the role of SphK1 and SphK2 in murine CIA, siRNA sense and antisense strands were purchased from Qiagen with the following sequences: SphK1, sense +5′-GGCCAGAGCUCUGCCAGUC-3′, antisense +3′-AGACUGCAAGGUCUCCCUCC-5′; SphK2, sense +5′-GCCCUAAGCAGAAGGCAC-3′, antisense +3′-GUGCUGAUAUGUG UAGGCGGT5′; Scramble control, sense +5′-GACUACAGUGAACCUAGCU-3′. For both SphK1 and SphK2 groups, mice (22–25 g) were treated with siRNA against SphK1 or SphK2 (5 µg/animal; equivalent to 200 µg/kg, in 0.2 ml final volume of PBS) i.p. on days 22–24, and then once every 2 days until day 45. Control mice received scrambled siRNA at the same time points. The dose of siRNA chosen is consistent with our previous in vivo murine studies (typically 4–8 µg/animal) (23, 24). The level of SphK1 and SphK2 inhibition was assessed by Western blot in spleen cells freshly isolated from mice treated with either SphK1, SphK2, or scrambled control siRNA. Mice were sacrificed 24 h after the last treatment and cell lysates were separated by 10% SDS-PAGE, transferred onto membrane, and probed with anti-SphK1 or anti-SphK2 Abs as previously described (9, 23, 24).

Collagen-specific in vitro culture

Draining lymph nodes (popliteal and inguinal; four per mouse) were aseptically removed from the mice and passed through cell striainers (BD Biosciences) to prepare a single-cell suspension. Cells were cultured at 2 × 10^6 cells/ml in RPMI 1640 medium supplemented with 2 mM l-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES buffer, and 10% heat-inactivated FCS (all from Invitrogen). Cells were stimulated with graded concentrations of CII (50 µg/ml proved to be optimal and therefore data for this are shown) or Con A (5 µg/ml, Sigma-Aldrich) up to 96 h as indicated, and the supernatants from parallel triplicate cultures were stored at −70°C until analysis of cytokine concentrations by ELISA. Proliferation assays were performed in triplicate in a 96-wells plate as described above for 96 h and measured by Alamar Blue according to manufacturer’s recommendations (SerenTeck).

Serum collection

Using cardiac puncture, serum was collected on day 27 before onset of clinical arthritis and at day 46. Blood was allowed to clot, then centrifuged and aliquots of serum stored at −70°C before cytokines, S1P, and anti-collagen Ab levels analysis by ELISA.

ELISA

Murine TNF-α, IFN-γ, IL-6, and IL-10 (BD Biosciences) in serum and culture supernatants were assayed by ELISA using paired Abs according to the manufacturer’s instructions. The lower limit of detection for TNF-α, IFN-γ, IL-6, and IL-10 was 10 pg/ml. S1P levels in serum from DMSA/1 mice were analyzed using a S1P competitive ELISA kit (Echelon Bio-sciences) according to the manufacturer’s instructions. Sensitivity of the assay was 30 nM. Anti-CII Ab titers of individual sera were detected with biotin-conjugated anti-mouse IgG1 or IgG2a, followed by conjugated avidin peroxidase (all BD Biosciences) and developed with tetramethylbenzidine substrate (Sigma-Aldrich).

Statistical analysis

Clinical and histological scores were analyzed with the nonparametric Mann-Whitney U test. Differences between cumulative incidences at a given time point were analyzed by the chi-square contingency analysis. Cytokine, S1P, and collagen-specific IgG1 levels were compared using the Student’s t test.

Results

SphK1, but not SphK2 down-regulation, suppressed development of CIA

We first investigated the effects of SphK1 and SphK2 down-regulation on the development of CIA in DBA/1 mice, a surrogate model of human RA. DBA/1 mice were immunized with CIA/CFIA as described in Materials and Methods. Mice began to show clinical signs of arthritis on day 30 after immunization. DMSA/1 mice were given i.p. 200 µg/kg of siRNA against either SphK1, or SphK2, or scrambled siRNA as control on day 20–22, and then once every 2 days until day 45. The severity of arthritis was measured by sequential estimation of the articular index and was compared for involved animals within each group. SphK1 siRNA significantly suppressed the incidence and severity of development of CIA when compared with control mice that received scrambled siRNA (p < 0.05, Fig. 1, A–C) whereas SphK2 siRNA failed to suppressed the severity of developing CIA (Fig. 1), suggesting such inhibition is dependent of SphK1 rather than SphK2. Furthermore, mice that received SphK2 siRNA developed severe inflammatory, polyarticular disease by day 33 as indicated by mean articular index (Fig. 1B) whereas the incidence of arthritis was comparable to mice that received scrambled control.

The siRNA dose chosen is consistent with our previous in vivo murine studies (typically 4–8 µg/animal) where we observed SphK1 protein expression in lysates of PBMC, spleen, lung, and liver was effectively down-regulated by administration of SphK1 siRNA (23, 24). To determine the specificity and levels of SphK1 and SphK2 protein reduction, spleen cells were isolated from mice (n = 5) 24 h after the last SphK1 or SphK2 siRNA treatment as described above, and we performed Western blot analysis to study SphK1/2 modulation. Fig. 2, A–C shows SphK1 protein expression in the spleen lysates was effectively down-regulated by SphK1 siRNA treatment, whereas SphK2 remains unaffected, suggesting the reduction of CIA disease activity was specific and is dependent of SphK1 rather than SphK2. Similarly, protein levels of SphK2...
but not SphK1 was effectively reduced in SphK2 siRNA recipients. We observed both SphK1 and SphK2 expression was reduced but not SphK1 was effectively reduced in SphK2 siRNA recipients (Fig. 2D), suggesting that systemic inflammatory responses were modified.

**Effect of SphK siRNA treatment on serum cytokines**

Based on recent data from CD4+ T cells of SphK2 deficient mice, it was suggested that SphK2 may play an important role in regulating T cell responses and is independent of S1P (15). Therefore, SphK1/2 siRNA down-regulation may modify CIA pathology by altering proinflammatory cytokine profile in vivo. We studied the effect of siRNA treatment before the onset of clinical arthritis on day 27. Serum cytokines including IL-6, IL-10, TNF-α, and IFN-γ were all below our ELISA detection limits (< 10 pg/ml, data not shown), presumably as day 27 precedes the acute phase of CIA where anti-CII responses are still under development. Furthermore, no evidence of significant changes in serum S1P levels was observed during early stage of siRNA treatment (naive mice 2.0 μM ± 0.3, scrambled control 2.5 μM ± 0.4, SphK1 2.2 μM ± 0.4, SphK2 2.7 μM ± 0.6, mean ± SEM, n = 5 mice/group). We next investigated serum cytokine concentrations from arthritic mice with established disease at the end of the treatment period (day 46). High levels of IL-6, TNF-α, and IFN-γ were detected in scrambled controls and SphK2 siRNA recipients. These were present at significantly (p < 0.05) reduced levels in mice treated with SphK1 siRNA (Fig. 4, A–C). Moreover, serum IL-10 levels were significantly elevated in SphK1 siRNA recipients (Fig. 4D), suggesting that systemic inflammatory responses were modified.
SphK1-siRNA suppresses collagen-specific proinflammatory immune responses in vitro

We next investigated mechanisms whereby such effects were achieved. CIA is associated with a proinflammatory immune response, rendering it an excellent model to explore the effect of SphK1/2 down-regulation upon functional T cell response in vivo. CII-specific immune responses were examined in vitro in pooled draining lymph nodes obtained at day 27 before onset of disease. Cells from SphK1 siRNA-treated mice produced significantly less CII-induced IFN-γ, TNF-α, and IL-6 compared with cells from scrambled control animals (Fig. 5, A–C) whereas significantly increased in CII-induced production of IFN-γ and TNF-α was noted in SphK2 siRNA recipients (Fig. 5, A and B). Neither IL-10 production or proliferation was modified (Fig. 5, D and E). Next, CII-specific immune responses were examined in vitro in pooled draining lymph nodes obtained at day 46 from mice with

![Figure 3](#)

**FIGURE 3.** SphK1 siRNA administration significantly reduced articular inflammation and destruction. H&E sections prepared from hind paws (n = 6/group) obtained at day 46 were scored for the presence of cartilage/bone erosion, synovial hyperplasia, and inflammatory infiltration. SphK1 siRNA-treated mice developed significantly lower pathological changes than mice that received either SphK2 or scrambled siRNA (A). A representative H&E section from each group of mice is shown. SphK1 siRNA-treated mice showed reduced histological evidence of destruction (B). Profound cartilage and bone erosion, hyperplasia, and cellular infiltration were observed in the SphK2 (C) and scrambled siRNA-treated mice (D). Data are means ± SEM. *, p < 0.05 by Mann-Whitney U test.

![Figure 4](#)

**FIGURE 4.** Serum proinflammatory cytokines in siRNA-treated mice. Arthritic DBA/1 mice treated with i.p. 200 μg/kg SphK1, SphK2, or scrambled control siRNA were sacrificed on day 46, and serum was collected from five mice in each group. Levels of IL-6 (A), TNF-α (B), IFN-γ (C), and IL-10 (D) were determined by ELISA of individual samples. Data are means ± SEM. *, p < 0.05 by Student’s t test.

![Figure 5](#)

**FIGURE 5.** Down-regulation of SphK1 decreases in vitro CII-specific proinflammatory cytokine production. Draining lymph node cells (n = 5 mice/group) were harvested from mice on day 27 before onset of disease and cultured with CII (50 μg/ml) for up to 96 h. Cytokine concentrations in the culture supernatant (72 h for IL-6 and TNF-α; 96 h for IL-10 and IFN-γ) were determined by ELISA. Significant suppression of IFN-γ (A), TNF-α (B), and IL-6 (C) production was observed in lymph node cultures removed from SphK1 siRNA-treated mice compared with scrambled controls whereas IFN-γ and TNF-α was enhanced in SphK2 siRNA recipients (D). T cell proliferation was assayed by Alamar Blue at 96 h (E). Data are mean ± SD of triplicate cultures. *, p < 0.05 by Student’s t test.
established disease. Cells from SphK1 siRNA-treated mice produced significantly less spontaneous and CII-induced IFN-γ (Fig. 6A). SphK1 siRNA treatment also significantly reduced the levels of TNF-α, IL-1β, IL-6, MCP-1, and MMP-9 synthesis in cell-contact assays. Administration of DMS reduced clinically detectable inflammatory arthritis in DBA/1 mice (22). The present report now compares the role of SphK1 with that of SphK2, and documents the distinct role of these two isoenzymes in regulating inflammatory arthritis. Our data indicate that SphK1 can promote CIA, as down-regulation of its expression through siRNA significantly suppressed the development and severity of CIA, as well as proinflammatory responses in vivo and in vitro in CIA-immunized mice. In contrast, down-regulation of SphK2 via siRNA therapy resulted in exacerbation of disease and inflammatory changes.

Anti-collagen Ab production in vivo

Finally, we sought evidence for anti-collagen Ab production. On day 27, CIA-specific IgG1 and IgG2a levels were analyzed by ELSIA and found to be low and similar in all three groups of mice (data not shown). However, by day 46, CIA-specific IgG2a Abs, which are typically produced during a Th1 response, were found to be reduced in SphK1 siRNA-treated mice compared with scrambled controls and SphK2 siRNA-recipients (Fig. 7A). CIA-specific IgG1 levels were not significantly different (Fig. 7B).

Discussion

Although a critical role of the interaction between SphK and S1P in immune cell function has been identified (2, 4), the precise contribution of SphK1 and SphK2 to the development of autoimmune diseases such as RA is as yet poorly understood. We recently detected that synovial fluid of RA patients displayed higher levels of S1P than those with osteoarthritis (22). SphK modulation either through DMS, or SphK1 antisense oligonucleotide significantly reduced the levels of TNF-α, IL-1β, IL-6, MCP-1, and MMP-9 synthesis in cell-contact assays. Administration of DMS reduced clinically detectable inflammatory arthritis in DBA/1 mice (22). The present report now compares the role of SphK1 with that of SphK2, and documents the distinct role of these two isoenzymes in regulating inflammatory arthritis. Our data indicate that SphK1 can promote CIA, as down-regulation of its expression through siRNA significantly suppressed the development and severity of CIA, as well as proinflammatory responses in vivo and in vitro in CIA-immunized mice. In contrast, down-regulation of SphK2 via siRNA therapy resulted in exacerbation of disease and inflammatory changes.

SphK/S1P exert pleiotropic effects on a variety of leukocyte subsets such as the T and B cells, macrophages, monocytes, and neutrophils (2, 4). Moreover, studies have shown that SphK/S1P participates in several inflammatory responses, such as leukocyte chemotaxis, and cytokine production and blockade of SphK activity suppresses such responses (9–13, 22–24). SphK1 and S1P were implicated in various autoimmune conditions such as RA (19–21), primary Sjögren’s (25), and IBD (14). Our data show that IL-6, TNF-α, and IFN-γ were reduced in the SphK1-siRNA treated mice in vivo and in vitro. Elevated SphK1, S1P, and S1P₁ levels have been detected in RA synovium, and S1P signaling via S1P₁ was found to promote synoviocyte proliferation, inflammatory cytokine-induced COX-2 expression, and PGE₂ production (19, 20). In human RA primary FLS, S1P₁ was shown to be the essential signal for survival; S1P₂/S1P₃ stimulated FLS migration whereas activation of S1P₂/S1P₃ enhanced IL-6 and IL-8 secretion. In addition, TNF-α was shown to promote FLS S1P₁ expression and S1P₁-mediated IL-8 secretion (21). Thus, the above pathways are likely to contribute to the immunomodulatory effects of SphK1 in inflammatory arthritis.

Our data indicate that SphK1 down-regulation via siRNA might suppress synovitis as revealed by histological examination. Several mechanisms can be put forward to explain such observation. The suppressive action on leukocyte migration is consistent with previous studies showing that SphK1 play a role in chemotaxis of human peripheral blood neutrophils and macrophages (9, 10). Inhibition of SphK1 may have a direct effect of cellular migratory machinery such as calcium mobilization and expression of adhesion molecules including ICAM-1, VCAM-1, and E-selectin (9, 21).
In addition, significant reduction of serum S1P levels in SphK1 siRNA-treated mice may also be a contributing factor for the inhibition in synovitis, as S1P has been shown to act as a chemotactic signal (21, 27, 28). It is of interest that serum S1P levels were also lowered, but not as extensively, in SphK2-siRNA recipients.

Down-regulation of SphK1 significantly reduced serum levels of CII-specific IgG2a. The effect on Ab production may play a role in SphK1 siRNA-mediated amelioration of CIA as B cell-mediated pathology is of critical importance in RA (29). B cell lines derived from RA patients are uniquely resistant to Fas-mediated apoptosis, in part due to over-activity of SphK1, and over production of S1P, which can inhibit apoptosis and regulate lymphoid migratory pathways (20). In mice, FTY720-phosphate, which binds to S1P receptors, caused the rapid disappearance of peritoneal B cells and reduced intestinal secretory IgA production. This suggests S1P may play an important role in regulating B cell survival, trafficking, and Ab production (30).

SphK1 was found to be a critical regulator in TNF-α-mediated IL-1β and IL-6 proinflammatory responses in human monocytes (11). In RAW macrophages, TNF-α-induced PGE2 production can be inhibited by down-regulation of SphK1 via siRNA knockdown (13). SphK1 expression is increased in human IBd colons, and SphK1-deficient mice are protected from IBD pathology (14). These are in contrast to data generated from models of thioglycollate-induced peritonitis and CIA, where SphK1 knockout mice exhibit normal acute and chronic inflammatory responses (31). It is possible that mice lacking the SphK1 gene during embryonic development may be adapted not to rely on this pathway for inflammatory responses postnatally. This is supported by the fact that both SphK1 and SphK2 single knockout mice develop and reproduce normally, whereas complete elimination of S1P in the double knockout is embryonically lethal (32). This observation suggests that each isoenzyme of SphK may at least partially compensate for the lack of the other. Thus, blockade of SphK in normal animals may lead to modification of inflammatory responses, and we have recently validated that SphK1 indeed plays a critical role in regulating acute inflammation (23).

In contrast, the role of SphK2 in inflammation remains poorly defined. Our data suggest that down-regulation of SphK2 resulted in increased disease activity and proinflammatory responses. We have found that at day 27 before the development of arthritis, SphK2-siRNA treatment can potentially polarize in vitro T cell anti-CD4 response into a proinflammatory manner and is unlikely associated with changes in the level of serum S1P. Others have demonstrated similar findings; T cells from SphK2 knockout mice exhibit hyperactivated phenotype with enhanced proliferative and Th1 cytokine-secreting capacities, and promote IBd pathology in SCID mice, suggesting SphK2 may play an important role in maintaining T cell homeostasis (15). Hence, down-regulation of SphK2 could potentially alter Th1/2 cell balance and disease outcome. Future studies will be needed to address this possibility.

In conclusion, we have demonstrated the distinct role of SphK1 and SphK2 in regulating inflammatory arthritis. Down-regulation of SphK1 via siRNA significantly attenuated the development of CIA and proinflammatory responses when administered prophylactically. In contrast, SphK2 modulation can promote CIA-induced inflammatory arthritis. The present study clearly shows that such anti- and proinflammatory potential of SphK1/2 modulation may alter the outcome in RA synovitis and in other autoimmune diseases, such as IBd in which similar pathways are implicated; defining the functional dichotomy between the two isoenzymes is thus paramount. These findings raise the possibility that drugs that specifically target SphK1 activity may play a beneficial role in the treatment of inflammatory diseases such as RA.

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Disclosures

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