Anti-Inflammatory Effects of Sphingosine Kinase Modulation in Inflammatory 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 of cellular processes, including angiogenesis, proliferation, and apoptosis. Recently, interest in SIP has focused on two distinct cellular roles, namely its function as an intracellular second messenger, or extracellularly as a specific and high-affinity ligand for a family of G protein-coupled receptors previously known as the endothelial differentiation gene (EDG) family. To date, five SIP receptors in the endothelial differentiation gene family have been identified, including EDG-1, EDG-3, EDG-5, EDG-6, and EDG-8, now collectively known as SIP1-5 (2–4).

Sphingosine kinase (SphK) is a key enzyme in the sphingolipid metabolic pathway, responsible for phosphorylating sphingosine into sphingosine-1-phosphate (S1P). SphK/S1P play a critical role in angiogenesis, inflammation, and various pathological processes. Recently, SIP was identified as a receptor for S1P in many cells of hematopoetic lineage and in keratinocytes, macrophages, and dendritic cells. Moreover, by employing a specific antisense knock-down approach, SphK1 was increased in intracellular S1P level via SphK stimulation. We have previously shown that inhibition of SphK activity by N,N-dimethylsphingosine (DMS), a potent SphK inhibitor, leads to reduced Ca2+ mobilization, enzyme release, and chemotaxis, cytokine, and chemokine production in human neutrophils, monocytes, and macrophages (12, 13, 15). Moreover, by employing a specific antisense knock-down approach, SphK1 was found to be a critical regulator in TNF-α-mediated IL-1β and IL-6 proinflammatory responses in human monocytes (14).

Elevated levels of proinflammatory cytokine production characterize rheumatoid arthritis (RA) synovial inflammation (16, 17). In particular, within inflamed RA synovial membrane, the levels of proinflammatory cytokines (namely TNF-α, IL-1β, and IL-6) exceed those of anti-inflammatory agents (IL-1RA and IL-10), and this likely contributes directly to cartilage and bone erosion through promoting matrix metalloproteinase (MMP) production and dysregulated chondrocyte/osteoclast function (16–18). Moreover, successful therapeutic targeting of cytokines in RA, particularly TNF-α, had demonstrated their critical pathogenic importance (17–19). There are multiple pathways that drive cytokine production in RA synovium with
significant contribution from B cells, macrophages, mast cells, and fibroblasts (16, 17). T cell effector function may be via cytokine secretion (e.g., IL-17) or through cell contact-dependent cognate interactions with macrophages via ligand pairs such as LFA-1/ICAM-1 and CD40/CD154 (20–24). Recently, elevated SphK1, S1P, and S1P 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 prostaglandin E2 production (25, 26). In the present study, we investigated whether targeting SphK activity, either by DMS or specific blockade of SphK1 through a molecular approach, may possess any immunomodulatory, antiarthritic properties. We show that synovial fluid of RA patients exhibited higher levels of S1P than did those of osteoarthritis (OA) patients and in RA-derived human cells in which DMS suppressed cytokine and MMP-9 release by PBMCs and by monocytes following cell contact-dependent interaction with activated T lymphocytes. The potential clinical relevance of these observations is illustrated by the ability of DMS and SphK1 small interfering RNA (siRNA) to effectively suppress murine collagen-induced arthritis (CIA) both in vivo and in vitro via specific suppression of the pathologic proinflammatory and Th1 responses.

Materials and Methods

Human studies

Peripheral blood (PB) was collected from RA patients who fulfilled the American College of Rheumatology 1987 diagnostic criteria (27), following approval from the hospital’s institutional review board (Tan Tock Seng Hospital, Singapore), and written informed consent was obtained from all patients. PB T cell and monocyte subpopulations were prepared as described previously (28). Briefly, human Jurkat or RA patient-derived PB T cells were stimulated for 72 h with PHA (5 μg/ml)/PMA (10 nM, both Sigma-Aldrich) in RPMI 1640 with 2 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (all from Invitrogen), then fixed in 4% paraformaldehyde. Control nonactivated Jurkat and PB T cells and were also fixed for comparative purposes. Fixed Jurkat or PB T cells were then cocultured with the myelomonocytic U937 cells or autologous RA PB monocytes in the presence or absence of DMS (Cayman Chemical). DMS was prepared as a 10 mg/ml stock in ethanol and diluted to the appropriate concentration in RPMI 1640 before use as described previously (8, 9, 11–13). The antisense down-regulation of SphK1 was conducted as previously described (8, 12, 14). The sequences of the oligonucleotides were: 5′-CC GCAAGGATCCATAACCTC-3′ antisense for SphK1, and 5′-GGCTCC TTCTGACGGTCA-3′ as scrambled control (Qiagen). Supernatants were harvested after 48 h of coculture for cytokine estimation by ELISA. Synovial fluid was obtained from RA and OA patients and stored at −70°C until estimation of S1P by ELISA (Rheumatology Division, University of California, Los Angeles). Clinical details are as follows: for RA, mean age 49.1 ± 6.0 years (mean ± SEM), mean disease duration 72.6 ± 22.4 mm, 4 male/10 female; for OA, mean age 52.1 ± 4.9 years, mean disease duration 74.1 ± 25.2 mo, 3 male/5 female. At the time of synovial fluid aspiration all RA patients were receiving methotrexate only. The sequences of the oligonucleotides were: 5′-GCAAGGUCUCUGCAAGC-3′, antisense 3′-GAACUCGGACGC-5′; scramble control, sense 5′-GACUCUGAU-3′, antisense 5′-GGCAGGAUUAG-3′, as previously described (28). Male DBA/1 mice (22–25 g) were divided into two groups of 12 mice. For the SphK1 group, mice were treated with siRNA against SphK1 (5 μg/animal; equivalent to 200 μg/kg, in 0.2 ml final volume of PBS) i.p. on days 20–22, and then once every 2 days until day 36. Control mice received scramble siRNA at the same time points. The level of SphK1 siRNA knockdown is consistent with published murine studies (typically 4–8 μg/animal) where SphK2 protein expression remains unaffected (28, 49). The level of SphK1 inhibition was assessed by Western blot in spleen cells freshly isolated from mice treated with either SphK1 or scramble 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 (28, 49).

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, 2 mM HEPES buffer, and 10% heat-inactivated FCS (all from Invitrogen). Cells were stimulated with graded concentrations of CIA (50 μg/ml proved to be optimal, and therefore data for this are shown) or Con A (5 μg/ml) for 72 h, 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-well plates as described above for 96 h and measured by Alamar blue according to the manufacturer’s recommendations (Sorotec).

Serum collection

Using cardiac puncture, serum was collected at day 45. Blood was allowed to clot and was then centrifuged and aliquots of serum were stored at −70°C before cytokine, S1P, and anti-collagen Ab levels analyses by ELISA.

ELISA

All cytokines, S1P, and anti-collagen Ab levels were assayed by ELISA. Human IL-1β, IL-6, TNF-α, MCP-1 (all BD Biosciences), and MMP-9 (Amersham) assays were performed according to the manufacturers’ instructions. Detection limits were as follows: IL-1β, 0.05 ng/ml; TNF-α, 1 pg/ml; and MCP-1 all at 10 pg/ml; MMP-9 at 2 ng/ml. Similarly for murine ELISA, the Ab pairs for TNF-α, IFN-γ, IL-6, and IL-10 were obtained from BD Biosciences with the detection limits all at 10 pg/ml. S1P levels in synovial fluid of RA/OA patients and serum from DBA/1 mice were analyzed using a S1P competitive ELISA kit (Echelon Biosciences) according to the manufacturer’s instructions. Sensitivity of the assay was 30 nm. Anti-collagen II 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 χ² contingency analysis. Cytokine- and collagen-specific IgG levels were compared using Student’s t test.
Results

Detection of S1P in RA synovial fluid

We first compared the levels of S1P in synovial fluids collected from 14 RA patients and 8 patients suffering from OA, a degenerative joint disease. S1P levels were measured by a competitive ELISA, and up to 17.51 ± 4.23 μM (mean ± SD) of S1P was detected in RA synovial fluids, significantly higher than those observed in OA fluids (3.45 ± 0.85 μM, p < 0.05, Mann-Whitney U test). As the levels of S1P detected in synovial fluid of RA patients could be complicated by nonspecific binding of rheumatoid factor, RA synovial fluids (n = 10) were diluted 1/10 with delipidized pooled human serum, then spiked with 1 μM of external S1P and assayed by ELISA. The average recovery rate was 96% (range 89–107%), thus ruling out any potential interference by rheumatoid factor in our ELISA.

DMS inhibits cell contact-induced cytokine production via cognate interactions

T cells from RA synovium or PB, particularly those activated by PHA/PMA, or cytokines like IL-15 or IL-18 are capable of driving macrophages to produce TNF-α in a cell contact-dependent manner (29, 30). We therefore investigated the ability of DMS, a potent SphK inhibitor, to influence the production of proinflammatory cytokines such as TNF-α by monocytes in response to activated T cells in a cell contact coculture system (22–24). Jurkat T cells were cultured with PHA and PMA, fixed with paraformaldehyde, and then cocultured with human U937 monocytic cells in the presence or absence of DMS. As expected, activated T cells induced substantial production of TNF-α, IL-6, IL-1β, and MCP-1 by U937 cells (Fig. 1A–D). Such cytokine synthesis was markedly reduced when the U937
cells were treated with DMS in a dose-dependent manner (Fig. 1A–D). Similar data were obtained when identical experiments were performed using PB T cells derived from RA patients and cocultured with autologous PB monocytes (Fig. 2).

It was important to determine whether similar effects could be achieved through direct inhibition of SphK. To this end, we performed parallel studies in which the ability of SphK1 antisense oligonucleotide to modify IL-6 and TNF-α production by U937 monocyes was evaluated. We observed that coculture-induced IL-6 and TNF-α release was significantly suppressed by SphK1 antisense oligonucleotide when compared with scrambled control (Fig. 1, B). No evidence of increased apoptosis was observed at the concentrations of DMS employed using annexin V/7-aminoactinomycin D staining by FACS analysis (annexin V+ U937 3.9 ± 1.7% vs 10 μM DMS 3.8 ± 1.7%, 5 μM DMS 3.5 ± 1.3%, 1 μM DMS 3.9 ± 1.8%; annexin V+ RA PB monocytes 6.8 ± 1.8% vs 10 μM DMS 7.6 ± 2.1%), indicating that reduced cell survival is unlikely to explain the observed inhibition. These results therefore demonstrate that SphK modulation may regulate proinflammatory cytokine synthesis in a system directly relevant to clinical arthritis.

**DMS inhibits cell contact-induced MMP-9 production**

Overproduction of MMP-9 has been observed in the synovial fluid of RA patients. MMP-9 from macrophages and neutrophils is thought to play a key role in the migration of these cells during inflammation in RA (31). To determine the role of SphK in cell contact-induced MMP-9 synthesis by monocytes, Jurkat T cells or RA PB T cells were stimulated, fixed, and then cocultured with U937 monocytic cells or autologous PB monocytes in the absence or presence of DMS as described above. Such coculture-induced monocyte MMP-9 release was significantly suppressed by DMS in a dose-dependent manner (Fig. 3A). Moreover, similar reduction was observed in experiments using PB T cells and monocytes derived from RA patients (Fig. 3B).

**FIGURE 3.** Effect of DMS on cell contact-induced MMP-9 production by monocytes. Jurkat T cells or PB T cells purified from RA patients (n = 5) were stimulated with PMA/PHA for 72 h, fixed in paraformaldehyde, and cocultured with U937 monocytic cells or autologous PB monocytes in the absence or presence of DMS for 48 h. DMS significantly suppressed MMP-9 release by U937 cells in a dose-dependent manner (A) or RA patients at 10 μM DMS (B). Data are means ± SEM of triplicate cultures and are representative of five similar experiments (A) or five RA patients (B). Ts indicates PMA/PMA activated T cells; Tc, medium control. *, p < 0.05 by Student’s t test.

**FIGURE 4.** DMS dose-dependently attenuated the progression of murine CIA. Collagen-primed DBA/1 mice were injected i.p. with 200 μg/kg DMS (n = 16 mice/group), 400 μg/kg DMS (n = 16 mice/group), or with PBS carrier control (n = 15 mice/group) from days 21 to 23 and then once every 2 days until day 45. Mice were monitored for disease progression as indicated by (A) incidence, (B) mean articular index, and (C) mean paw thickness (mm). DMS-treated mice developed significantly less severe disease in a dose-dependent manner compared with PBS carrier controls. Data are mean ± SEM. *, p < 0.05 by Mann-Whitney U test.

**Treatment with DMS inhibits the development of murine CIA**

We next investigated the effect of DMS on the development of CIA in DBA/1 mice, a surrogate model of human RA. DBA/1 mice were immunized with CII/CFA as described in Materials and Methods. Mice began to show clinical signs of arthritis on day 27 after immunization. Mice were injected i.p. daily with 200 μg/kg DMS, 400 μg/kg DMS, or with PBS carrier control from days 21 to 23 and then once every 2 days until day 45. DMS dose-dependently suppressed the incident, mean articular index, and mean paw thickness of developing CIA (Fig. 4). To determine whether DMS administration modified articular destruction, we evaluated cartilage and bone integrity histologically. Adjacent cartilage and bone erosion, synovial hyperplasia, and inflammatory infiltration into the joint compartment were clearly evident in the PBS carrier controls (Fig. 5). Each of these parameters was markedly suppressed in mice that received 400 μg/kg DMS (Fig. 5). Taken together, these data clearly indicate that DMS potently suppressed the development of CIA and that such activity can prevent progression of articular damage.

**Effect of DMS on serum cytokines, S1P, and anti-collagen Ab production in vivo**

A potential mechanism by which SphK modulation via DMS could suppress CIA pathology is by blocking proinflammatory cytokine...
release in vivo. To compare the extent of such modulation, serum cytokine concentrations from arthritic mice were measured by ELISA at the end of the treatment period (day 45). High concentrations of IL-6, TNF-α, and IFN-γ were detected in PBS carrier controls. These were present at significantly \( p < 0.05 \) reduced levels in mice treated with 400 \( \mu g/kg \) of DMS (Fig. 6, A–C). Moreover, reduction in serum IL-6 levels (Fig. 6A), a surrogate marker for suppression of the acute phase response, suggested that systemic inflammatory responses were modified. It was important to determine the level of S1P modulation achieved by DMS at either 200 or 400 \( \mu g/kg \), and serum S1P levels were assessed by ELISA. A significant reduction in serum S1P levels was observed in a dose-dependent manner in DMS recipients (Fig. 6D), suggesting that S1P responses were indeed modified by the presence of DMS. Finally, we sought evidence for anti-collagen Ab production. CII-specific IgG1 and IgG2a levels were also analyzed by ELISA and found to be reduced in mice treated with 400 \( \mu g/kg \) DMS (Fig. 6, E and F).

**FIGURE 5.** DMS treatment of mice with CIA resulted in reduced joint pathology. H&E sections prepared from hind paws (\( n = 6 \)/group) obtained at day 45 were scored for the presence of cartilage/bone erosion, synovial hyperplasia, and inflammatory infiltration as described in Material and Methods. The pathological changes differed significantly between the DMS (400 \( \mu g/kg \))-treated mice and the PBS recipients. \( *, p < 0.05 \) vs PBS control group by Mann-Whitney \( U \) test; data are means ± SEM.

**FIGURE 6.** Serum proinflammatory cytokines, S1P, and serum anti-collagen IgG2a and IgG1 titer in DMS-treated mice. DBA/1 mice treated with DMS or PBS carrier control were sacrificed on day 45, and serum was collected from five mice in each group. Levels of IL-6 (A), TNF-α (B), IFN-γ (C), and S1P (D) were determined by ELISA of individual samples. Anti-collagen IgG2a (E) and IgG1 (F) Ab levels were measured and expressed as mean absorbance (OD\(_{630}\)) ± SEM of individual serum measurements. Data are means ± SEM. \( *, p < 0.05 \) by Mann-Whitney \( U \) test.

**FIGURE 7.** Reduced in vitro collagen-specific responses in mice treated with DMS. Draining lymph node cells (\( n = 5 \)/mice/group) were harvested from mice on day 45 as described in Material and Methods and cultured with CII (50 \( \mu g/ml \)) 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. T cell proliferation was assayed by Alamar blue at 96 h (A). Significant suppression of IFN-γ (B), TNF-α (C), and IL-6 (D) production was observed in lymph node cultures removed from DMS (400 \( \mu g/kg \))-treated mice compared with PBS carrier controls. CII-induced production of IL-10 was significantly increased in DMS (400 \( \mu g/kg \)) recipients (E). Data are means ± SD of triplicate cultures. \( *, p < 0.05 \) by Student’s \( t \) test.

**DMS reduced in vitro collagen-specific proinflammatory immune responses**

We next investigated the immunological mechanisms by which SphK modulation suppresses articular inflammation. CIA is associated with a proinflammatory immune response, rendering it an excellent model to explore the effect of DMS upon functional T cell response in vivo. CIA-specific immune responses were examined in vitro in pooled draining lymph node cells obtained at day 45. Cells from DMS-treated mice produced significantly less CII-induced proliferation, IFN-γ, TNF-α, and IL-6 compared with cells from control animals (Fig. 7A–D), whereas antiinflammatory cytokine IL-10 synthesis was enhanced (Fig. 7E). Immune modulation by DMS in vivo was Ag-specific since Con A-induced production of IFN-γ, TNF-α, IL-6, and IL-10 in parallel cultures was not affected (data not shown).

**SphK1 siRNA attenuated the development of murine CIA**

To verify that the inhibitory effects of DMS from the above observations were mediated by direct SphK inhibition rather than
Table I. SphK1 siRNA attenuated the progression of murine CIAa

| Time (Day 36) | Scrambled siRNA (n = 12) | SphK1 siRNA (n = 12) |
|--------------|--------------------------|---------------------|
| Incidence (%) | 75%                      | 33.3%*              |
| Mean articular index | 5.86 ± 1.20               | 2.50 ± 0.64*        |
| Arthritic paws | 2.87 ± 0.34               | 0.50 ± 0.25*        |

a Collagen-primed DBA/1 mice were injected i.p. with scrambled or SphK1 siRNA (both at 5 μg/animal; equivalent to 200 μg/kg, n = 12 mice/group) from day 20 to 22 and then once every 2 days until day 36 as described in Materials and Methods. The siRNA dose chosen is consistent with our previous in vivo murine studies (typically 4–8 μg/animal) where we observed that SphK1 protein expression in lysates of PBMC, spleen, lung, and liver was effectively suppressed by administration of SphK1 siRNA (28, 49). To determine the levels of SphK1 protein inhibition, spleen cells were isolated from mice (n = 5) 24 h after the last treatment of SphK1 siRNA as described above, and they were found to be significant reduced when compared with mice that received scrambled control siRNA, while its isoenzyme, SphK2, remains unaffected (data not shown). The severity of arthritis was measured by sequential estimation of the articular index and was compared for involved animals within each group. In agreement with the previous DMS results, SphK1 siRNA significantly suppressed the incidence and severity of development of CIA when compared with control mice that received scrambled siRNA (p < 0.05, Table I), suggesting that such inhibition is dependent of SphK1 rather than SphK2. Therefore, these results clearly demonstrate that SphK modulation, either in the form of DMS administration or targeting SphK1 via siRNA, can directly inhibit proinflammatory cytokine synthesis and progression of arthritis.

Discussion

There is currently considerable interest in the potential of immunomodulatory therapies in the treatment of inflammatory diseases, particularly those targeting cytokine expression. Recently, SphK and SIP were implicated in various autoimmune conditions such as RA (25, 26), primary Sjögren’s syndrome (33), and multiple sclerosis (34). The pivotal role of SphK and SIP in inflammation has been widely established and was summarized in a recent review (2). SphK/SIP have pleiotropic effects on a variety of leukocytes such as T and B cells (28, 35, 36), macrophages and monocytes (16, 37), and neutrophils (13, 15). Moreover, studies have shown that SphK/SIP participate in several inflammatory responses such as leukocyte chemotaxis (12, 13), and cytokine production and blockade of SphK activity suppresses such responses (12–15, 28). Our data indicate that levels of SIP in the synovial fluid of RA patients were significantly higher than those of osteoarthritis patients. Moreover, we have shown SphK modulation either through DMS or SphK1 antisense oligonucleotide significantly reduced the levels of proinflammatory mediator synthesis in our cell contact assays. In a murine CIA model, i.p. administration of DMS significantly inhibited disease severity and reduced articular inflammation and joint destruction, as well as proinflammatory responses both in vivo and in vitro. Similarly, inhibition of SphK1, one of the two SphK isoenzymes, attenuated the development of CIA in vivo.

The pathways that drive cytokine release in RA synovium remain unclear, but T cells play a significant role by direct release of IL-17 or via cell contact with synovium macrophages (17–19, 21, 29, 30). DMS was effective in suppressing T cell-monocyte interactions, an important pathway driving proinflammatory cytokine production in the synovium (29, 30). We have demonstrated in a series of cell contact experiments employing either Jurkat/U937 cells or PB T cells and autologous monocytes derived from RA patients that DMS significantly reduced cell contact-induced proinflammatory TNF-α, IL-1β and IL-6, and chemokine MCP-1 synthesis in a dose-dependent manner. More importantly, MMP-9, a collagenase that plays a direct role in cartilage degradation and subsequent bone erosion in RA (31), was significantly inhibited by the presence of DMS, which may potently block MMP-9 synthesis either by directly interfering with monocyte-T cell interaction (39) or indirectly by suppressing TNF-α and IL-1β (40).

CIA represents an ideal opportunity to explore the diverse inflammatory effects on SphK modulation in an inducible, autoimmune model. CIA has served as a translational model in which the role of inflammatory cytokines in RA, particularly TNF-α, IL-1β, and IL-6, were defined (16, 20). Our data indicate that DMS administration into CIA mice resulted in significantly reduced joint pathology and subsequent proinflammatory responses. Activation of various plasma membrane receptors, such as the FHLR receptor (11), the C5a receptor (12, 13), and TNF-α receptor (14) (receptors that are of direct relevance in RA), leads to rapid increase in intracellular S1P levels via SphK stimulation. S1P can promote neutrophils, monocytes, and lymphocyte activation and migration (12–15, 41–44). Our data suggest that SphK modulation via DMS may possess inhibitory action on inflammatory cell infiltration into the joint, subsequent synovial hyperplasia, and erosion as revealed by histological examination. Such suppressive action of DMS on leukocyte migration is consistent with previous studies showing that SphK plays a role in chemotaxis of human peripheral blood neutrophils and macrophages (12, 13, 15). Inhibition of SphK may have a direct effect on cellular migratory machinery such as calcium mobilization and expression of adhesion molecules, including VCAM-1 and E-selectin (45). Finally, reduced SIP production after DMS treatment may also be directly responsible for the reduced cell infiltration, as SIP has been shown to act as a chemoattractant signal (35, 36).

We show that blockade of SphK activity with DMS significantly reduced the levels of CII-specific IgGa and IgG2a Abs in the serum of arthritic mice. Our data indicate that DMS has an inhibitory action on the levels of circulating SIP in serum of treated mice in a dose-dependent manner as compared with carrier controls. Interestingly, B cell lines derived from RA patients are uniquely resistant to Fas-mediated apoptosis, in part due to overactivity of SphK and overproduction of SIP, which can inhibit apoptosis and regulate lymphoid migratory pathways (26). Additionally, FTY720-phosphate, which binds to SIP receptors, caused the rapid disappearance of peritoneal B cells by inhibiting their emigration from parathympic lymph nodes, as well as reducing peritoneal B cell-derived intestinal secretory IgA production (46). This suggests that SIP may play an important role in regulating B cell survival, trafficking, and Ab production.

It is important to establish that the inhibitory effects of DMS in the above observations were mediated by direct SphK inhibition rather than by nonspecific off-target side effects (32), and to determine the efficacy of SphK1 in murine arthritis using a
highly specific siRNA (28). Our data showed that i.p. administration of SphK1 siRNA significantly reduced both incidence and disease severity in the development of murine CIA. Recently, it was suggested that SphK1 knockout mice developed CIA with normal incidence and severity (47). It is possible that mice lacking the SphK1 gene during embryonic development may be adapted to not rely on this pathway for inflammatory responses postnatally. There are a number of precedents including the initial Src kinases, Syk, and Zap-70 knockout mice (48). Thus, blockade of SphK in normal animals may lead to modulation of inflammatory responses. To this end we have recently validated that SphK plays a critical role in vivo in a model of acute inflammation (49).

In conclusion, we demonstrate in this study that synovial fluid of RA patients exhibited higher levels of SIP than did their non-inflammatory OA counterparts and in RA-derived human cells in which DMS suppressed cytokine and MMP-9 release by PBMCs following cell contact-dependent interaction with activated T lymphocytes. While testing such hypotheses reliably in preclinical studies remains challenging, the potential clinical relevance of the above observations is illustrated by the ability of SphK modulation either through DMS or SphK1 siRNA to effectively suppress murine CIA both in vivo and in vitro via specific down-regulation of the pathologic proinflammatory and Th1 responses. Therefore, it is conceivable that SphK modulation may play a beneficial role in the treatment of inflammatory diseases such as RA.

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Disclosures

The authors have no financial conflicts of interest.

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