

Sphingosine Kinase 1–Mediated Inhibition of Fas Death Signaling in Rheumatoid Arthritis B Lymphoblastoid Cells

Xiujun Pi, Shi-Yu Tan, Michael Hayes, Liqun Xiao, James A. Shayman, Song Ling, and Joseph Holoshitz

Objective. It is becoming increasingly apparent that B cells play an important role in the pathogenesis of rheumatoid arthritis (RA). Due to the scarcity of B cells in RA, it has been technically difficult to functionally characterize B cell apoptosis in this disease. As a necessary first step to identify candidate aberrations, we investigated Fas-mediated signaling events in immortalized peripheral blood B lymphoblastoid cell lines (LCLs) from patients with RA and controls.

Methods. Cell death was determined by the MTS assay, and apoptosis was detected by the TUNEL assay and DNA laddering. Proteolytic activation of caspase 3 was determined by immunoblotting, and its enzymatic activity was determined by a fluorometric technique. Messenger RNA (mRNA) expression was quantified by real-time polymerase chain reaction (PCR) analysis. The functional role of sphingosine kinase (SPHK) was determined by measuring its enzymatic activity, by quantifying the levels of its product, sphingosine 1-phosphate (S1P), and by investigating the ability of the SPHK inhibitor *N,N*-dimethylsphingosine and isozyme-specific small interfering RNA (siRNA) oligonucleotides to reverse signaling aberrations.

Results. LCLs from patients with RA displayed disease-specific Fas-mediated signal transduction impairment with consequent resistance to cell death. RA LCLs displayed high constitutive SPHK activity and

increased levels of S1P. Real-time PCR analysis showed higher SPHK-1 mRNA expression levels in RA patients compared with paired controls. Increased SPHK-1 (but not SPHK-2) mRNA levels were observed in synovial tissue from RA patients. Competitive inhibitors of SPHK reversed the resistance of RA LCLs to Fas-induced apoptosis. Additionally, resistance to Fas-mediated signaling was reversed by siRNA oligonucleotides specific for SPHK-1 but not by oligonucleotides specific for SPHK-2.

Conclusion. These findings demonstrate disease-specific resistance to Fas-mediated death signaling in patients with RA and implicate increased SPHK-1 activity as the cause of this aberration.

Rheumatoid arthritis (RA) is a systemic disease characterized by infiltration of inflammatory cells into the synovium and hyperplasia of the synovial lining cells that lead to destruction of adjacent cartilage and bone (for review, see ref. 1). It has been previously postulated that either aberrant apoptosis or migration of cells from the peripheral blood into the synovial compartment may be responsible for pathologic stockpiling of cells in the rheumatoid pannus (2–7).

Over the past several years, interest in the pathogenic role of B lymphocytes in RA has reemerged (for review, see ref. 8). In addition to the realization that antibodies do play important roles in experimental models of RA and in the human disease, there is increasing evidence that B lymphocytes accumulate and mature in the inflamed synovium, where they can form ectopic germinal centers (9–11) and activate T cells (12). Additionally, experimental treatments with anti-CD20 antibodies have shown promise (13) and further support the growing consensus that B lymphocytes play an important role in the pathogenesis of RA. The propensity of B lymphocytes to accumulate in the synovium has been attributed to the local trophic influence of resident

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Table 1. Clinical features of the patients with rheumatoid arthritis*

Patient/sex/ age, years	Disease duration	RF	Nodules	Erosions	Therapy	HLA-DRB1
1/M/62	23 years	+	+	+	Gold, MTX	0401/0404
2/M/46	8 years	+	+	+	AZA, gold, HCQ, MTX, SSZ	0401/0404
3/F/48	6 years	+	+	+	HCQ, MTX	0401/0404
4/F/42	6 months	-	-	-	NSAID	0401/11
5/M/44	11 years	+	+	-	MTX, prednisone	0401/0404
6/F/62	33 years	+	+	+	AZA, gold, HCQ, MTX, SSZ	0401/0401
7/M/54	10 years	+	+	+	Gold	0401/15
8/F/45	7 years	+	+	+	MTX, NSAID	0401/17
9/M/64	16 years	-	-	+	NSAID	0101/0701
10/F/53	5 months	+	-	-	NSAID	17/17
11/F/21	14 months	+	-	-	Gold, HCQ, NSAID	0101/0404
12/M/72	19 years	-	-	+	NSAID, prednisone	0401/14
13/M/52	4 months	+	-	+	NSAID, prednisone	0101/15
14/F/34	11 years	+	-	+	NA	0401/1201
15/F/53	9 years	+	-	+	NA	0401/1104
16/M/44	NA	+	+	+	NA	0101/0301
17/F/NA	NA	NA	NA	NA	NA	0101/1302
18/F/37	5 years	+	+	+	NA	0401/0401
19/F/36	6 years	+	+	+	NA	0404/1401
20/M/32	10 years	-	-	+	NA	0401/0701
21/F/62	13 years	+	+	+	NA	0401/0701
22/F/65	3 years	+	-	-	NA	0401/0401
23/M/58	6 months	+	-	-	NSAID	0405/11

* RF = rheumatoid factor; MTX = methotrexate; AZA = azathioprine; HCQ = hydroxychloroquine; SSZ = sulfasalazine; NSAID = nonsteroidal antiinflammatory drug; NA = not available.

synoviocytes (9,10). However, what the mechanisms governing their homing to the synovium are, and whether B cells are intrinsically resistant to programmed cell death, are questions that are presently unanswered.

There is ample evidence that B cell Fas-mediated cell death plays a key role in the maintenance of self tolerance, and that failure of this mechanism can lead to autoimmunity (for review, see ref. 14). However, due to the marked scarcity of peripheral B lymphocytes in RA (15,16), little is known about Fas-mediated cell death of B lymphocytes in this disease.

In this study, we investigated the efficiency of Fas-mediated death signaling in peripheral blood B lymphocytes. In an attempt to overcome the scarcity of peripheral B lymphocytes in RA, we used immortalized lymphoblastoid cell lines (LCLs). Our data indicate that LCLs from patients with RA are uniquely resistant to Fas-mediated cell death. The aberration is attributable to overactivity of sphingosine kinase 1 (SPHK-1), with resultant overproduction of sphingosine 1-phosphate (S1P), a sphingolipid previously shown to inhibit apoptosis and to regulate lymphoid migratory pathways (17,18).

PATIENTS AND METHODS

Study subjects. A panel of LCLs from a total of 78 individuals (23 patients with RA, 8 with systemic lupus ery-

thematosus [SLE], 10 with juvenile RA [JRA], 3 with autoimmune thyroiditis, and 5 with insulin-dependent diabetes mellitus [IDDM], as well as 29 healthy controls) was used in this study. The RA group (58% women and 42% men, mean \pm SD age 49.2 ± 11.9 years) and the control group (61% women and 39% men, mean \pm SD age 45 ± 12.3 years) did not differ demographically. The salient clinical features of the RA group are shown in Table 1. A panel of LCLs from 10 RA-discordant monozygotic twin pairs (7 female pairs and 3 male pairs, mean \pm SD age 45.2 ± 10.7 years) was used in some experiments. Synovial tissues obtained during joint replacement surgery in 14 patients with RA and 9 patients with osteoarthritis (OA) were used to quantify synovial tissue SPHK messenger RNA (mRNA) expression.

Cells and culture conditions. LCLs were prepared from peripheral blood B cells, using a standard technique (19), and were cultured in supplemented RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Irvine Scientific, Santa Ana, CA). In all experiments, test and control cells were obtained from lines that had been maintained in identical tissue culture conditions, with identical cell density and viability.

Induction of apoptosis. Fas-mediated apoptosis was induced in LCLs by incubating 5×10^4 cells/well with 100 ng/ml of anti-Fas monoclonal antibody CH-11 (IgM; Medical and Biological Laboratories, Watertown, MA) in 96-well microplates. Control mouse IgM monoclonal antibody (TEPC-183; Sigma, St. Louis, MO) was used in equivalent concentrations. Cell death was determined at different time points, using a commercial MTS kit (Promega, Madison, WI). Microplate wells were read using an enzyme-linked immunosorbent assay plate reader at 490 nm.

Analysis of DNA fragmentation. Demonstration of DNA laddering on agarose gels was performed as previously described (20), with some modifications. Briefly, cells were lysed in Tris-EDTA (TE) buffer containing 0.2% Triton X-100, pH 8.0. Fragmented DNA was separated from intact chromatin by microfuging at 14,000 rpm at 4°C for 20 minutes. The resulting supernatant was treated with 1 mg/ml of proteinase K at 37°C overnight, then extracted with phenol-chloroform-isoamyl alcohol (at a ratio of 25:24:1) 3 times. DNA was precipitated by the addition of 3 volumes of absolute ethanol, in the presence of 0.3M sodium acetate, pH 5.2, and incubated overnight at -20°C and then pelleted by centrifugation at 14,000 rpm at 4°C for 20 minutes. The pellet was washed twice with 75% ethanol and dissolved in 30 μ l of TE buffer containing 10 μ g/ml of RNase overnight at 37°C. DNA samples were separated by electrophoresis on 1.8% agarose gels in the presence of ethidium bromide.

A TUNEL assay was performed as previously described (21). Cells were fixed in 1% buffered formaldehyde (pH 7.4) for 15 minutes on ice, washed in phosphate buffered saline (PBS), and stored at 4°C in 70% ethanol. Subsequently, cells were resuspended in 50 μ l of a solution containing 5 units of terminal deoxynucleotidyl transferase (Boehringer Mannheim, Indianapolis, IN), 2.5 mM COCl₂, 0.2M potassium cacodylate, 25 mM Tris HCl, 0.25 mg/ml bovine serum albumin (BSA), and 0.5 nM biotin-16-dUTP (Boehringer Mannheim). Cells were incubated in this solution at 37°C for 30 minutes, then rinsed in PBS and resuspended in 100 μ l of staining solution containing 2.5 μ g/ml fluoresceinated avidin, 4 \times concentrated saline sodium citrate buffer, 0.1% Triton X-100, and 5% (weight/volume) nonfat dry milk. Cells were incubated in this solution for 30 minutes at room temperature in the dark. Cells were then rinsed in PBS containing 0.1% Triton X-100 and resuspended in 1 ml of PBS containing 5 μ g/ml propidium iodide and 0.1% RNase (Sigma). Green (biotinylated dUTP) and red (DNA) fluorescence of individual cells were measured with an Epics Elite Flow Cytometer (Coulter, Hialeah, FL).

Caspase 3 activity. The assay for caspase 3 activity was based on a previously published method (22). Briefly, cells were lysed by agitation for 15 minutes in assay buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.4). The lysates were centrifuged at 16,000g for 10 minutes, and the supernatants were collected. The caspase 3 substrate Ac-DEVD-AMC (Axxora, San Diego, CA) was added at a final concentration of 50 μ M. The assay was performed in opaque black 96-well plates (OptiPlate-96 F; PerkinElmer, Boston, MA). The plates were read using a Fusion-Alpha HT instrument (365-nm excitation filter and 425-nm emission filter; PerkinElmer) for 3 hours.

Western blotting. Five million cells were washed and suspended in 100 μ l of lysis buffer (1% sodium dodecyl sulfate [SDS], 1 mM sodium vanadate, 10 mM Tris [pH 7.6]), and boiled for 5 minutes. After microcentrifugation at 14,000 rpm for 10 minutes, supernatants were recovered. The protein concentration of the lysates was determined with bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). For caspase 3 immunoblots, cell lysates (10–20 μ g of protein per lane) were separated on 15% SDS-polyacrylamide gels under reducing conditions, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), and probed with mouse

monoclonal anti-caspase 3 (clone 19, IgG2a; Signal Transduction Lab, Lexington, KY) followed by sheep F(ab')₂ fragment horseradish peroxidase-conjugated, anti-mouse immunoglobulin (Amersham, Piscataway, NJ). For poly(ADP-ribose) polymerase (PARP) immunoblots, cell lysates (10 μ g of protein per lane) were separated on 7.5% SDS-polyacrylamide gels, transferred to PVDF membranes, and probed with monoclonal mouse anti-human PARP antibody C2.10 (IgG1; Enzyme Systems Products, Dublin, CA). Antibody binding was detected with an enhanced chemiluminescence detection system (Amersham).

Determination of SPHK enzymatic activity and S1P levels. To measure SPHK activity, cells were washed twice with cold PBS, centrifuged, and resuspended in 200 μ l of the buffer (20 mM Tris buffer, pH 7.4, containing 20% [volume/volume] glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM NaF, 10 μ g/ml leupeptin and aprotinin, and 1 mM PMSF). Cells were frozen and thawed 4 times by transferring from liquid nitrogen to a 37°C water bath. Cell lysates were centrifuged for 30 minutes at 14,000 rpm/minute. The buffer was mixed with supernatant that contained 70–200 μ g of protein to a final volume of 190 μ l. The reactions were started by the addition of 10 μ l of γ -³²P ATP (2 μ Ci, 20 mM) and sphingosine-BSA, 50 μ M final concentration, containing 100 mM MgCl₂. Samples were incubated for 30 minutes at 37°C, and reactions were terminated by the addition of 20 μ l of 1N HCl. Lipids were extracted by the addition of 0.8 ml of chloroform, methanol, and concentrated HCl (100:200:1 [v/v/v]). Phases were separated by the addition of 240 μ l of chloroform and 240 μ l of 2N KCl. The organic phase was evaporated under N₂ and resuspended in 50 μ l of chloroform-methanol mixture (at a ratio of 1:2). Lipids were resolved by thin-layer chromatography on Uniplate silica gel G (Analtech, Newark, DE) using 1-butanol, methanol, acetic acid, and water (80:20:10:20 [v/v/v/v]) as solvent. The radioactive spots corresponding to authentic S1P were located by standard S1P, which was visualized by charring with cupric sulfate-phosphoric acid spray. Radioactive SP-1P spots were quantified with a PhosphorImager (Molecular Dynamics, Fairfield, CT).

To determine S1P levels, 5 \times 10⁶ cells were washed twice with cold PBS, centrifuged, and resuspended in 500 μ l of buffer (138 mM NaCl, 3.3 mM NaH₂PO₄, 2.9 mM KCl, 1 mM MgCl₂, 1 mg/ml glucose, and 20 mM HEPES [pH 7.4]). Three milliliters of ice-cold chloroform-methanol (1:2 [v/v]) was added to the buffer, followed by thorough mixing and sonication for 30 minutes. Phases were separated by adding 2 ml of chloroform, 2 ml of 1M KCl, and 100 μ l 7N NH₄OH. The alkaline bottom phases were used for sphingosine quantification. The alkaline upper phases were transferred to new tubes, and 3 ml of chloroform and 200 μ l of concentrated HCl were added. The bottom chloroform phases formed under these new acidic conditions were evaporated under N₂ and used for S1P quantification, as previously described (23).

Extraction of total RNA and reverse transcription (RT). Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). Briefly, 1 \times 10⁷ LCL cells or 50–100 mg of dissected synovial tissue was placed in 600 μ l of lysis buffer containing 4M guanidinium salt and β -mercaptoethanol, and homogenized by using a microultrasonic cell disrupter. After the addition of 600 μ l of 70%

ethanol, the mixture was loaded onto an RNeasy spin column and centrifuged for 0.5 minutes at 8,000g. The column was washed with a buffer containing 70% ethanol and centrifuged twice. After treatment with DNase I, total RNA was collected with 50 μ l of diethyl pyrocarbonate--treated water and stored at -70°C . The concentration and purity of RNA were determined by absorbance at 260 nm and 280 nm. Complementary DNA (cDNA) was synthesized from 1 μ g total RNA by using MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA). Negative controls were prepared using all reagents except the RNA sample or without reverse transcriptase.

RT-polymerase chain reaction (RT-PCR). Exon-spanning primers specific for SPHK-1, SPHK-2, and β -actin were designed based on published cDNA sequences. The oligonucleotide sequences were as follows: for SPHK-1, forward 5'-TTGAACCATTATGCTGGCTATGAG-3', reverse 5'-CAGCAGGTTTCATGGGTGACA-3'; for SPHK-2, forward 5'-TTTGCCCTCACCTTACATC-3', reverse 5'-TTCAGAGATCATGGGAAGCA-3'; for β -actin, forward 5'-AGAAATCTGGCACCACACC-3', reverse 5'-AGAGGCGTACAGGGATAGCA-3'.

Reactions were carried out in PCR Master Mix buffer (Promega), containing 50 mM Tris HCl (pH 9), 3 mM MgCl_2 , 400 μM dNTPs, and 50 units *Taq* DNA polymerase. The reaction mixture contained 22.5 μ l PCR Master Mix, 2 μ l of primers at a concentration of 20 μM , 5.5 μ l cDNA, and 15 μ l H_2O . PCR incubations were conducted in a programmable thermal controller (ThermoHybaid, Philadelphia, PA). Each cycle included denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 60 seconds. The final extension was performed at 72°C for 5 minutes. Thirty cycles were performed for PCR tubes containing β -actin template, whereas 38 cycles were used for amplifying SPHK. The DNA was separated on a 1.5% agarose gel. Gels were stained for 20 minutes in Tris-borate-EDTA buffer containing 0.5 $\mu\text{g/ml}$ ethidium bromide, destained for 30 minutes, examined on an ultraviolet transilluminator, and photographed with a Bio-Rad gel scanner (Bio-Rad, Hercules, CA).

Real-time quantitative PCR. Reactions were carried out in $2\times$ PCR Master Mix (Applied Biosystems) in a total volume of 50 μ l containing 5 μ l (2 μ l for β -actin) of cDNA, 0.4 μM of each PCR primer, 0.2 μM of TaqMan probe with passive reference, and 25 μ l of PCR Master Mix. SPHK and β -actin genes were amplified in separate tubes in duplicate. The amplification parameters were 50°C for 2 minutes, 95°C for 10 minutes, and 50 cycles at 95°C for 15 seconds and at 60°C for 1 minute. The following exon-spanning primers and probes were used: for SPHK-1, forward 5'-TTGAACCATTATGCTGGCTATGAG-3', reverse 5'-CAGCAGGTTTCATGGGTGACA-3', probe 5'-FAM-ACGCTATTGCTGTGCCGCCG-TAMRA-3'; for SPHK-2, forward 5'-GACGGGCTGCTCCATGAG-3', reverse 5'-TTCACAGCTTCCTCCAGTCA-3', probe 5'-FAM-TGCTGAACGGGCTCCTAGATCGCC-TAMRA-3'; for β -actin, forward 5'-AGAAAATCTGGCACCACACC-3', reverse 5'-AGAGGCGTACAGGGATAGCA-3', probe 5'-FAM-ACC-GCGAGAAGATGACCCAGATC-TAMRA-3'.

Emission fluorescence from individual PCR tubes at each cycle was monitored using an iCycler (Bio-Rad). Data were collected, consisting of the threshold cycle values corresponding to the PCR cycle number at which fluorescence was

detectable above an arbitrary threshold, based on baseline data within cycles 3–15. The threshold was determined to ensure that the threshold cycle values were obtained in the exponential phase of PCR, during which no rate-limiting components are expected. To construct a standard curve, cDNA was synthesized from total RNA extracted from cell culture and then diluted in 5 steps, 2–32 times. A standard curve of threshold cycle values against the log value of cDNA was generated for both SPHK and β -actin. The PCR product was checked on 1% agarose gels to ensure there was no amplification from genomic DNA. The relative concentrations of SPHK and β -actin were obtained from the standard curve, and the ratios of SPHK normalized against β -actin were calculated.

Inhibition of SPHK gene expression by small interfering RNA (siRNA). SPHK-1 and SPHK-2 gene expression in LCLs was knocked down by using specific siRNA oligonucleotides. The siRNA for the SPHK-1 gene was a 21-nucleotide siRNA directed at a target sequence 70 nucleotides downstream of the start codon (5'-AAGGGCAAGCC-TTGCAGCTC-3'), based on a published report (24). The siRNA for the SPHK-2 gene was a 21-nucleotide siRNA directed at a target sequence 645 nucleotides downstream of the start codon (5'-AACCTCATCCAGACAGAACGA-3'), which was designed using Qiagen siRNA software.

Cells were transfected with either the 21-nucleotide duplexes for SPHK (2 μg siRNA/sample) for gene silencing or a 21-nucleotide scrambled siRNA as negative control (Qiagen), or an enhanced green fluorescent protein vector (Amaya Biosystems, Gaithersburg, MD) for monitoring the transfection rates using nucleofection technology (Amaya Biosystems). Briefly, 10^7 cells were resuspended in a 100- μ l solution from the Nucleofector Kit T (Amaya Biosystems), following the manufacturer's guidelines for cell line transfection, using the provided cuvette and nucleofected with a Nucleofector apparatus. After being electroporated, the cells were immediately transferred into a culture flask containing culture medium prewarmed to 37°C . The cells were then cultured for 96 hours before analyzing the gene-silencing effects. The mRNA levels were measured by quantitative real-time PCR analysis.

RESULTS

As shown in Figure 1A, LCLs from normal controls died efficiently after exposure to the agonist anti-Fas antibody CH-11. In contrast, LCLs from patients with RA showed markedly reduced cell death. Disease susceptibility in RA is closely associated with HLA-DRB1 alleles encoding a shared epitope (SE) in the third hypervariable region of the HLA-DR β chain (25). Of note, we did not find any relationship between the presence of either 1 or 2 SE-encoding DRB1 alleles and resistance to Fas-mediated cell death in the RA group. Similarly, no resistance to Fas-mediated cell death was observed in healthy control subjects expressing either 1 or 2 SE alleles.

In order to more accurately assess the relative contribution of genetic versus nongenetic factors, we

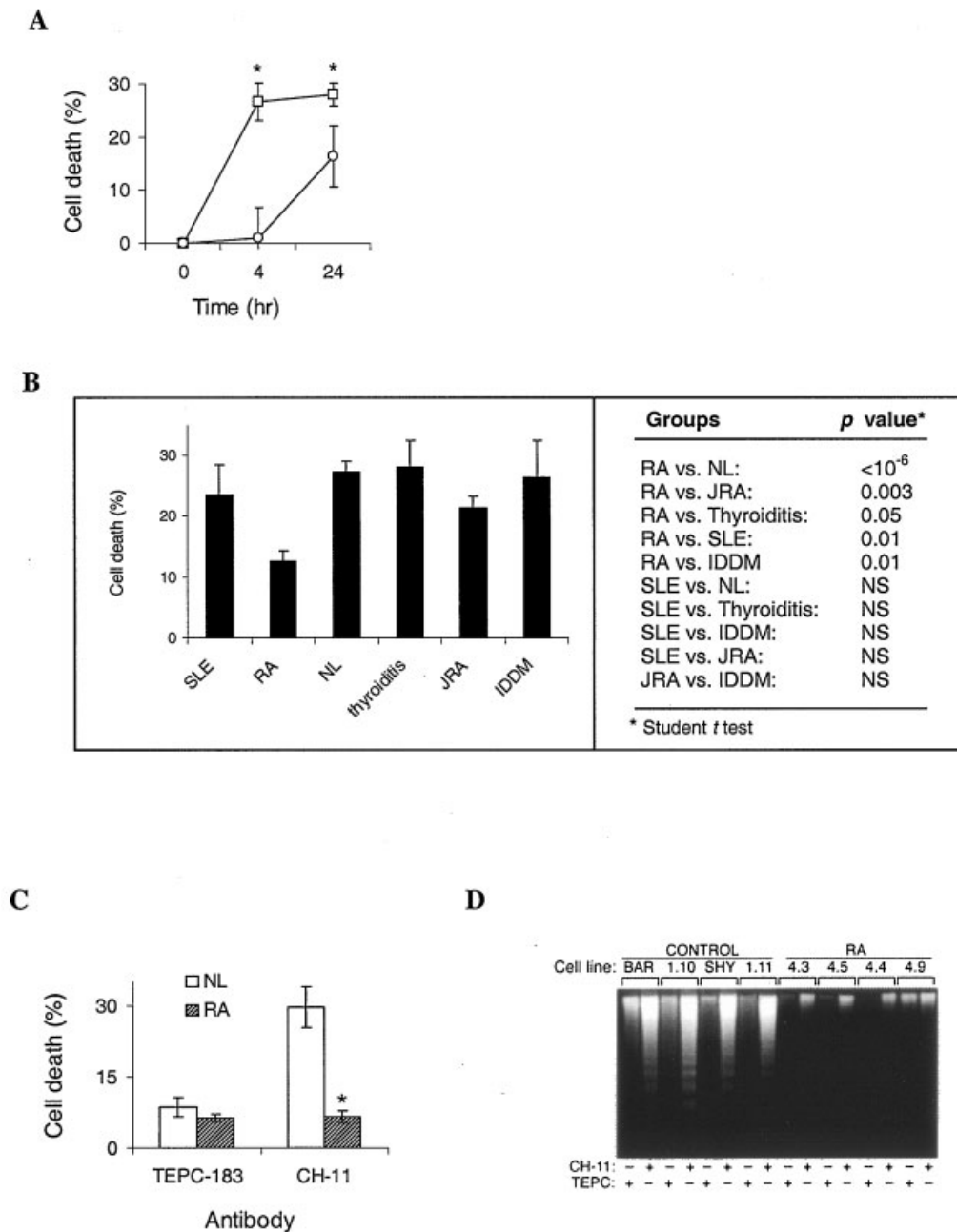


Figure 1. **A**, Resistance of rheumatoid arthritis (RA) lymphoblastoid cell lines (LCLs) to Fas-mediated cell death. LCLs from 15 healthy controls (□) and 15 patients with RA (○) were incubated with anti-Fas antibody. The percentage of cell death was determined at different time points thereafter, using the MTS method. **B**, Comparison of cell death in RA versus other autoimmune diseases. Fas-mediated cell death was determined in LCLs from 8 patients with systemic lupus erythematosus (SLE), 10 patients with juvenile RA (JRA), 5 patients with insulin-dependent diabetes mellitus (IDDM), 3 patients with autoimmune thyroiditis, 15 patients with RA, and 15 normal (NL) controls. The percentage of cell death 20 hours after anti-Fas antibody treatment was determined by the MTS method. NS = not significant. **C**, Determination of anti-Fas antibody-mediated DNA fragmentation by the TUNEL assay in LCLs from 6 normal controls and 5 patients with RA. Values are the mean \pm SD. **D**, Determination of anti-Fas antibody-mediated DNA fragmentation in LCLs from 4 normal controls and 4 patients with RA by agarose gel electrophoresis. Cells were treated with either anti-Fas antibody (CH-11) or an isotype-matched control antibody (TEPC-183), as described in Patients and Methods. * = $P < 0.05$.

Table 2. Susceptibility to Fas-mediated cell death in RA-discordant monozygotic twins*

Twin pair	HLA-DRB1	Fas-mediated cell death, %	
		Healthy twin†	RA twin‡
1	0401/1201	33	18
2	0401/1104	41	19
3	0101/0301	21	13
4	0101/1302	26	9
5	0401/0401	29	11
6	0404/1401	31	14
7	0401/0701	27	0
8	0401/0701	23	9
9	0401/0401	13	22
10	0405/11	50	14

* RA = rheumatoid arthritis.

† Mean \pm SD 29.4 \pm 3.3%.‡ Mean \pm SD 12.9 \pm 2.0%; $P = 0.0006$ versus healthy twin.

conducted experiments on a panel of 20 LCLs derived from 10 RA-discordant monozygotic twin pairs (Table 2). Fas-mediated cell death was significantly lower in the RA twin group compared with that in the healthy twin group (mean \pm SD 12.9 \pm 2.0% and 29.4 \pm 3.3%, respectively; $P = 0.0006$). In all but 1 pair of twins, resistance to Fas-mediated cell death was seen in the RA twin but not in the healthy twin ($P < 0.005$). Thus, we concluded that resistance to Fas-mediated cell death in RA represents a disease-related defect rather than a genetically determined defect.

Resistance to Fas-mediated cell death was observed in 22 of the 23 RA patients studied, independent of their age, sex, disease duration, the presence of rheumatoid factor, erosions, or rheumatoid nodules, and irrespective of their drug treatment (Table 1). The resistance was not observed in other autoimmune diseases (Figure 1B). For example, the mean \pm SD percentage of cell death induced by anti-Fas antibodies in LCLs from patients with JRA (21.5 \pm 2.1%), SLE (23.5 \pm 5.0%), IDDM (26.3 \pm 8.2), and autoimmune thyroiditis (28.2 \pm 4.4%) was significantly higher than that in the RA group (12.9 \pm 2.0%) ($P = 0.003$, $P = 0.01$, $P = 0.01$, and $P = 0.05$, respectively). No statistically significant differences were found between Fas-mediated cell death in the healthy group and any of the control autoimmunity groups, or among the different autoimmunity groups (Figure 1B).

Resistance of RA LCLs to Fas-induced cell death was attributable to failed apoptosis, as demonstrated by DNA fragmentation data. In control LCLs, Fas ligation triggered DNA fragmentation as determined by the TUNEL assay (Figure 1C) and by analysis of DNA

laddering by agarose gel electrophoresis (Figure 1D). In contrast, RA LCLs did not show evidence of DNA fragmentation. RA LCLs were not innately resistant to programmed cell death, because they displayed some, albeit delayed, Fas-mediated caspase 3 activation (Figure 2A). Additionally, LCLs from patients with RA and controls were equally sensitive to apoptosis following treatment with staurosporine (data not shown).

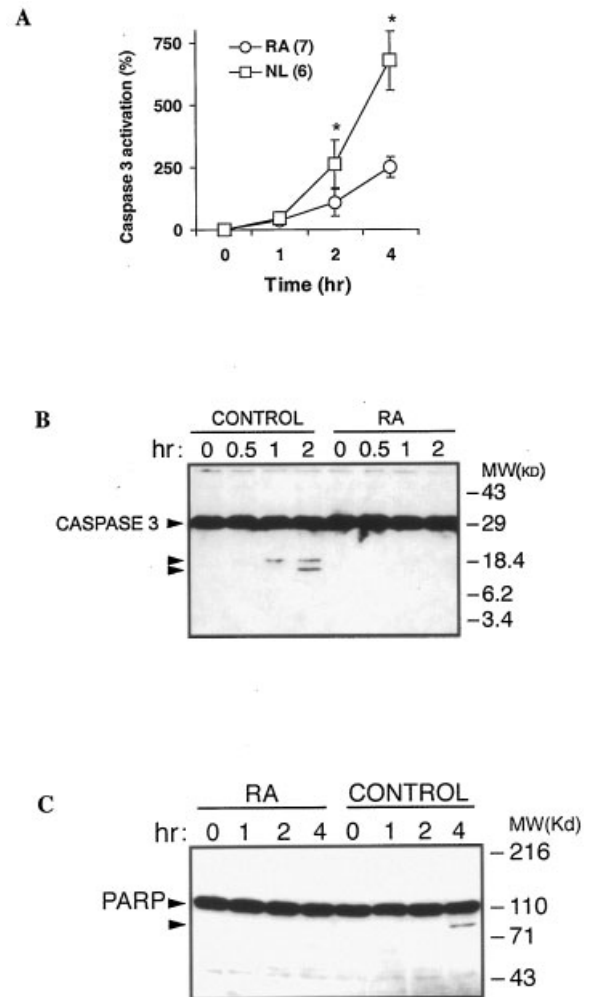


Figure 2. Fas-mediated signaling in RA LCLs. **A**, LCLs from 7 patients with RA and 6 normal controls were incubated with the anti-Fas antibody CH-11 over time, and caspase 3 activation was determined. Results represent the increase in caspase 3 activity above baseline levels. Values are the mean \pm SD. **B** and **C**, Western blot analyses with either anti-caspase 3 or anti-poly(ADP-ribose) polymerase (anti-PARP) were performed on LCLs at different time points following activation with the anti-Fas antibody CH-11. Representative LCLs from 1 patient with RA and 1 healthy control are shown. Similar patterns were obtained in all 4 pairs of RA and normal cell lines tested (results not shown). * = $P < 0.02$. See Figure 1 for other definitions.

Resistance to Fas-mediated apoptosis in RA LCLs could not be attributed to reduced Fas receptor density, because similar levels of Fas expression were detected by flow cytometry on LCLs from normal individuals and patients with RA. Additionally, LCLs from controls and patients with RA expressed similar levels of CD30 and CD40 surface proteins (data not shown).

The findings described above suggest that an intracellular mechanism may be responsible for the observed resistance to apoptosis. Examination of the Fas pathway in RA LCLs revealed impaired signaling. Figure 2A compares caspase 3 activation following Fas ligation in LCLs from patients with RA and normal controls. As can be seen, although RA cells displayed some activation, it was markedly delayed compared with that in normal cells ($P < 0.02$). Impaired Fas-mediated signaling could also be demonstrated by the absence of signature proteolytic cleavage products of caspase 3 (Figure 2B) and poly (ADP-ribose) polymerase (PARP) following Fas ligation (Figure 2C). Thus, the resistance of RA lymphocytes to Fas-mediated apoptosis was associated with impaired signal transduction.

The finding of diminished and delayed caspase 3 activation in RA cells suggests that the Fas pathway is intrinsically intact but may be subjected to inhibition by an extrinsic regulatory mechanism. It has been previously proposed that the efficiency of Fas-mediated cell death signaling may be regulated by SPHK via its antiapoptotic product, S1P (26). We therefore assessed the role of SPHK. As can be seen in Figure 3A, RA cells displayed markedly increased SPHK enzyme activity compared with control cells ($P < 0.02$). The increased SPHK enzymatic activity in RA LCLs was associated, as expected, with increased intracellular levels of S1P (Figure 3B).

We next investigated whether constitutively active SPHK plays a role in the observed resistance of RA LCLs to Fas-mediated apoptosis. To that end, we first used a pharmacologic approach. Preincubation of cells with the SPHK competitive inhibitor *N,N*-dimethylsphingosine reversed the resistance of RA LCLs to Fas-mediated apoptosis to near-normal levels (Figure 3C). A similar effect was observed with another SPHK-specific inhibitor, DL-threo-dihydrosphingosine (data not shown).

The 2 known SPHK isoenzymes are SPHK-1 and SPHK-2. Transcripts for both isozymes are expressed in LCLs (Figure 4A). To quantify SPHK mRNA expression levels, we performed real-time PCR analyses on LCLs from 10 pairs of RA-discordant monozygotic twins. In 7 of the 10 twin pairs, SPHK-1 mRNA levels

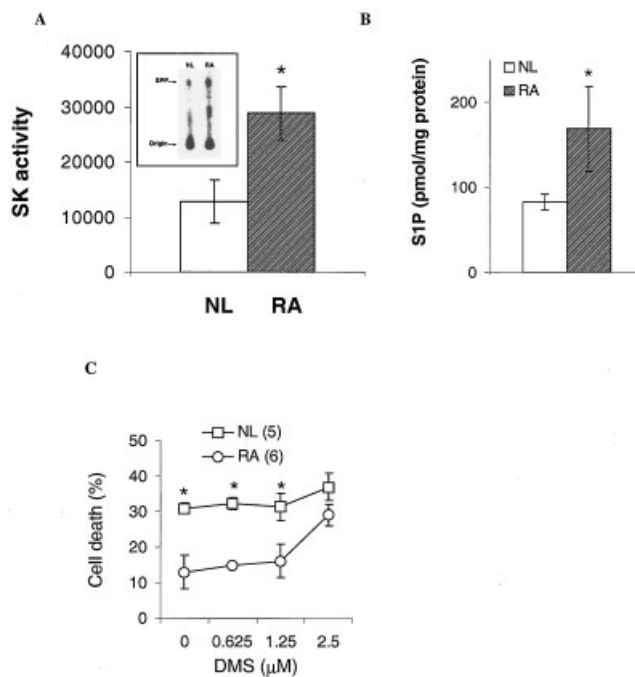


Figure 3. Role of sphingosine kinase (SPHK; SK) in resistance to Fas-mediated apoptosis. **A**, Comparison of constitutive SPHK activity in lymphoblastoid cell lines (LCLs) from 9 patients with rheumatoid arthritis (RA) and 11 normal (NL) controls. Boxed image shows a representative PhosphorImager display of the SPHK activity assay in 1 patient with RA (right) and her healthy twin sister (left). **B**, Constitutive levels of sphingosine 1-phosphate (S1P) in LCLs from 8 normal controls and 5 patients with RA. **C**, Reversal of RA LCL resistance to Fas-mediated apoptosis by *N,N*-dimethylsphingosine (DMS). Fas-mediated apoptosis was determined in LCLs from 6 patients with RA and 5 healthy controls, using the MTS method. Values are the mean \pm SD. * = $P < 0.02$.

were higher in the RA twin compared with the healthy twin. In contrast, SPHK-2 mRNA levels were clearly higher in the RA twin in only 2 of the 10 pairs (Figure 4B). The mean \pm SD SPHK-1 mRNA expression levels were 6.31 ± 1.58 -fold higher in the RA twin cells compared with their healthy cotwins ($P = 0.008$ by Mann-Whitney U test).

To investigate whether SPHK is responsible for aberrant Fas signaling, RA LCLs showing increased SPHK-1 and SPHK-2 transcripts and resistance to Fas signaling were transfected with siRNA oligonucleotides designed for either SPHK-1 or SPHK-2. Transfection with these siRNA oligonucleotides resulted in 52% and 57% suppression of SPHK-1 and SPHK-2 mRNA expression, respectively, as determined by real-time PCR (data not shown). As shown in Figure 4C, LCLs transfected with SPHK-1 siRNA showed markedly enhanced

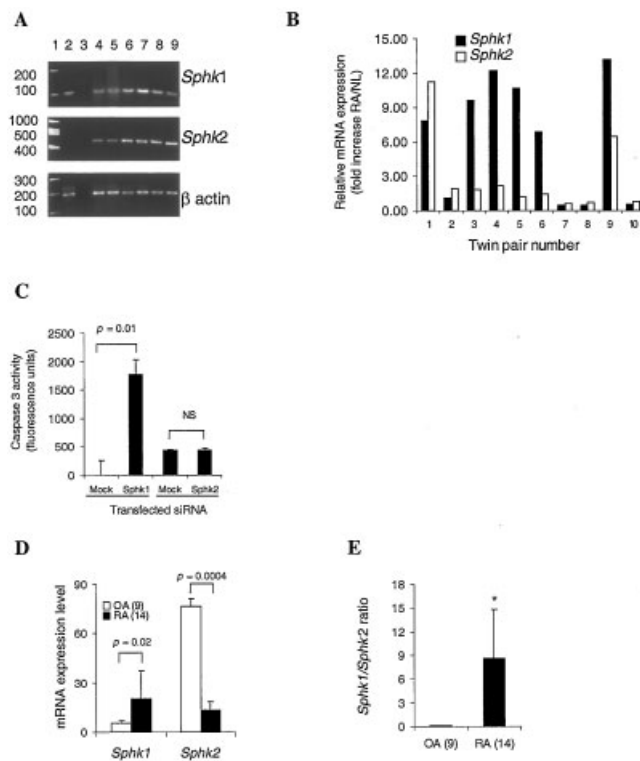


Figure 4. Identification of the sphingosine kinase (SPHK) isozyme. **A**, Reverse transcription–polymerase chain reaction (RT-PCR) analysis of SPHK-1 and SPHK-2. Lane 1, Molecular size markers; lane 2, positive control (human lung); lane 3, negative control (RNA without RT); lanes 4–9, lymphoblastoid cell lines (LCLs) from healthy twins (lanes 4, 6, and 8) and their respective cotwins with rheumatoid arthritis (RA) (lanes 5, 7, and 9). **B**, Real-time PCR analysis of *Sphk1* and *Sphk2* expression in 10 twin pairs. SPHK abundance was normalized to the β -actin expression level for each LCL. The data shown represent the ratio of SPHK abundance in the RA twin line relative to its healthy cotwin line. Values >1.0 indicate higher, and values <1.0 indicate lower SPHK mRNA expression in the RA twin relative to the healthy cotwin. **C**, Reversal of the Fas-mediated signaling aberration with SPHK-specific small interfering RNA (siRNA) oligonucleotides. LCLs from patients with RA were transfected with siRNA oligonucleotides specific for *Sphk1* or *Sphk2*. Control cultures were subjected to mock transfection. After 96 hours in growth culture, LCLs were incubated overnight with anti-Fas antibodies, and caspase 3 activity was determined. **D**, Real-time PCR analysis of *Sphk1* and *Sphk2* mRNA expression levels in synovial tissue from 14 patients with RA and 9 patients with osteoarthritis (OA). **E**, Ratio between *Sphk1* and *Sphk2* in synovial tissue from 14 patients with RA and 9 patients with OA. Values are the mean and SD. NS = not significant. * = $P = 0.00008$.

Fas-mediated caspase 3 activity ($P = 0.01$). In contrast, transfection with an SPHK-2–specific siRNA oligonucleotide had no effect (Figure 4C). Thus, we concluded that SPHK-1 overexpression is responsible for the observed resistance to Fas-mediated signaling.

Finally, in order to assess the relevance of these findings to RA, we used real-time PCR to measure SPHK-1 and SPHK-2 transcript levels in synovial tissues from 14 patients with RA and 9 patients with OA (Figures 4D and E). A striking difference between the 2 diseases was observed. In RA, SPHK-1 was more abundantly expressed than SPHK-2 ($P = 0.02$), while the opposite relationship was found in OA ($P = 0.0004$). The ratio of the SPHK-1 to SPHK-2 transcript expression level was 86.6-fold higher in RA than in OA (mean 8.66 versus 0.1; $P = 0.00008$ by Mann-Whitney U test).

DISCUSSION

The findings reported here demonstrate an impairment in Fas-mediated cell death signaling in RA LCLs and strongly implicate overactivity as a candidate mechanism. The characterization of biochemical mechanisms involved in cell death signaling requires an abundant supply of large numbers of homogeneous cells, which often is not feasible in human research. This technical caveat has slowed research into the mechanisms of B lymphocyte apoptosis in RA, due to the scarcity of peripheral B cells in this disease (15,16). In order to overcome this obstacle, we used immortalized B cell lines in this study. Test and control B cell lines were maintained under identical tissue culture conditions and expressed identical phenotypes. Therefore, as opposed to data obtained with primary cells, the aberrations observed here represent a genuine qualitative defect rather than a reflection of cell frequency–dependent phenomena. Additionally, by using RA-discordant monozygotic twin pairs, the possibility that genetic or demographic disparities are responsible for the differences between patients and controls has been effectively excluded.

The use of LCLs has been previously proven to be instrumental in uncovering important pathogenic mechanisms in other diseases, such as hypertension, glycogen and lipid storage diseases, chronic granulomatous disease, hematologic disorders, and solid tumors (27–33). One illustrative example is essential hypertension. Although LCLs have no relevance to blood vessel physiology, their use enabled the discovery of a G-protein signaling aberration in hypertension (27). Analogously, the relevance of LCLs to pannus pathology may be uncertain; however, the data presented here clearly demonstrate that those cells express a disease-specific signaling aberration and thus provide an insight into a potential pathogenic mechanism and a strong ra-

tionale for future exploration of the role of that aberration in RA.

Although genetic factors are known to play a role in RA susceptibility, the concordance rate of this disease in monozygotic twins has been estimated to be $\leq 15\%$ (34). The surprisingly low concordance rate suggests that nongenetic events may be necessary to trigger disease onset in genetically susceptible individuals. In this study, we demonstrated that resistance to Fas-mediated apoptosis is a disease-related, rather than a genetically determined, phenomenon. No association with particular HLA-DRB1 alleles could be found in unrelated subjects, indicating that the defect is not merely a consequence of the presence of the SE in RA. Furthermore, experiments with disease-discordant monozygotic twins indicate that the aberration is not determined genetically. These findings and the fact that the aberration is disease-specific strongly suggest that the signaling impairment observed here is directly relevant to the pathogenesis of RA.

Aberrations in Fas-mediated cell death signaling in RA have been previously demonstrated in T cells and in synoviocytes (for review, see ref. 3), while involvement of the Fas pathway in RA B cells is basically unknown. In murine autoimmune models, Fas signaling aberrations in B cells have been shown to play a major role in autoantibody production (for review, see ref. 35). Mice in which Fas expression is diminished (*lpr/lpr*) or in which its signaling is inefficient (*lpr^{cg}/lpr^{cg}*) develop autoantibodies. Although both T and B cells are affected in those genetic models, B cells contribute an autonomous effect to the autoimmune phenotype, independent of the T cell effect (36,37), suggesting that the Fas pathway plays a role in the maintenance of B cell tolerance, and that Fas-resistant B cells play a key role in autoimmunity. Similar to those models, the data presented here implicate Fas pathway impairment in RA B lymphocytes. Unlike the defects observed in those experimental models, however, our data indicate that in RA the Fas pathway is intrinsically intact but is inhibited by an extrinsic regulatory mechanism. Of note, inhibition of an otherwise intact Fas pathway has been shown to lead to autoimmunity in heterozygous *pten*^{+/-} mutant mice (38). Thus, impairment of Fas-mediated death signaling, either at the receptor-ligand level or further downstream, can lead to a similar end result of autoimmunity.

It is worth mentioning that cell accumulation in RA has a predilection for the joints. Therefore, the resistance of peripheral blood-derived cell lines observed here suggests that circulating lymphocytes pos-

sess latent resistance to Fas-mediated cell death. Upon activation, those cells may preferentially home to the joints and accumulate there due to the unique synovial microenvironment, which allows resident synoviocytes and homing lymphocytes to best exercise their abnormal survival tendencies in a codependent manner (9–12). Recent studies have identified BAFF as an important factor in synovial B cell survival (39) and autoimmunity (40).

Our data directly implicate SPHK overactivity as the underlying mechanism of impaired Fas-mediated death signaling in RA. We demonstrate here that RA LCLs display increased constitutive SPHK enzymatic activity, and that the majority of RA LCLs, as well as RA synovial tissues, have increased mRNA expression of SPHK-1. It should be cautioned, however, that due to the heterogeneous cellular composition of synovial tissues, the increased SPHK-1 transcripts reported here cannot be attributed exclusively to B cells. Further studies, using advanced methodologies such as in situ hybridization or laser capture microdissection, will be required to resolve this question.

Blocking SPHK activity with competitive inhibitors or knocking down SPHK-1 mRNA expression restored Fas-mediated death signaling. These findings are consistent with the current understanding of SPHK function (41–44). Enhanced SPHK activity promotes cell survival in many cell systems, including lymphocytes, in which SPHK has been shown to inhibit ceramide- and Fas-induced cell death (41). Consistent with our data, of the 2 known isozymes, SPHK-1 has been consistently shown to be a potent inhibitor of apoptosis, while SPHK-2 lacks that capability and has actually been reported to suppress cell growth and enhance apoptosis in certain cell systems (42,43). Importantly, SPHK has been previously implicated in inflammatory arthritis, because the locus containing *Sphk1* (at the telomeric region of chromosome 10) has been identified as an arthritis susceptibility region in rats (44).

It is worth noting that SPHK-1 overactivity and resistance to Fas-mediated death could be found in LCLs long after their isolation. This finding indicates that the observed aberration represents a genuine intrinsic defect rather than a transient response to cytokines, growth factors, medications, or other influences by the rheumatoid milieu. These findings suggest a lasting imprint of B cells derived from patients with RA as compared with controls. Among the candidate explanations that need to be considered are the following: different EBV infection rates between RA and control B cells (45), or an effect of preexistent triggers of B cell survival that may have led to lasting functional repro-

gramming in these cells. A third explanation for this nongenetic functional aberration involves epigenetic mechanisms. In recent years, there has been growing interest in the role of posttranscriptional events and epigenetic mechanisms in disease pathogenesis. Because SPHK-1 gene expression is epigenetically modified by a differentially methylated region at the 5' end of an adjacent GpC island (46), it is tempting to speculate that this mechanism might be involved in the aberration described in the current report.

The biologic effects of SPHK are attributable to its product, S1P. This sphingolipid metabolite displays both intracellular and extracellular activities and has been shown to play a role in diverse processes that may be relevant to RA pathogenesis, including angiogenesis, cell migration, cell growth, and cell differentiation (for review, see ref. 26). Of particular relevance to this study, S1P has been previously shown to suppress cell death induced by Fas ligand (47). The mechanism of the S1P-mediated antiapoptotic effect is incompletely understood. Although the relative role of S1P as an intracellular second messenger versus an extracellular paracrine or autocrine agonist is a matter of ongoing debate, its potency as an inhibitor of Fas-mediated death signaling is well documented. At nanomolar concentrations, S1P triggers a cell survival pathway, which involves activation of ERK, NF- κ B, and phosphoinositide 3-kinase (PI 3-kinase), as well as inhibition of JNK, p38, Bax, and caspase 3 (for review, see ref. 48). Our own data indicate that S1P survival signaling in LCLs involves activation of PI 3-kinase, inhibition of JNK, and suppression of executioner caspases via a G_i protein-coupled receptor mechanism (Tan S-Y, et al: unpublished observations).

S1P exerts potent effects that regulate the immune system through receptor-mediated survival signaling, chemotaxis, proliferation, and cytokine production (for review, see ref. 17). One of the most notable immune effects of S1P is its rapid receptor-mediated regulation of immune cell migration (49). S1P is now considered central in the physiologic immune response, and aberrations in its function could likely lead to immunopathologic processes and disease. In this context, it is noteworthy that a phosphoryl metabolite of the sphingosine analog FTY720, a promising new immunosuppressive prodrug (50), has been shown to prevent experimental autoimmunity models, including inflammatory arthritis in rats, presumably by inhibiting lymphocyte egress from the lymph node to the periphery (51).

In conclusion, we report a heretofore unknown

disease-associated dysregulation of SPHK, which inhibits Fas-mediated cell death signaling in RA. The reversibility of the aberration suggests that further characterization of the molecular mechanism(s) involved could help in identifying targets for therapeutic intervention in RA.

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