A Role for Neutral Sphingomyelinase-mediated Ceramide Production in T Cell Receptor–induced Apoptosis and Mitogen-activated Protein Kinase–mediated Signal Transduction

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Summary

Studying apoptosis induced by T cell receptor (TCR) cross-linking in the T cell hybridoma, 3DO, we found both neutral sphingomyelinase activation and production of ceramide upon receptor engagement. Pharmacological inhibition of ceramide production by the fungal toxin, fumonisin B1, impaired TCR-induced interleukin (IL)-2 production and programmed cell death. Addition of either exogenous ceramide or bacterial sphingomyelinase reconstituted both responses. Moreover, specific inactivation of neutral sphingomyelinase by antisense RNA inhibited IL-2 production and mitogen-activated protein kinase activation after TCR triggering. These results suggest that ceramide production by activation of neutral sphingomyelinase is an essential component of the TCR signaling machinery.

Key words: neutral sphingomyelinase • ceramide • T cell receptor • signaling • activation • apoptosis

T cells recognize peptide fragments in the context of MHC molecules using the surface TCR complex. The TCR consists of two distinct groups of transmembrane proteins: a clonally unique and antigen-specific heterodimer and an associated set of invariant subunits, termed CD3, that are essential for TCR signal transduction (1). After stimulation of the TCR, the CD3 complex recruits cytoplasmic tyrosine kinases of the src (e.g., fyn and/or lck) and the syk (i.e., syk and/or ZAP-70) families. These interactions result in increased protein tyrosine phosphorylation and trigger numerous biochemical changes, such as elevation of cytosolic calcium, and protein kinase C (PKC)[1] and mitogen-activated protein (MAP) kinase activation (2, 3).

Integration of these changes will activate various transcription factors that regulate T cell differentiation as well as immune responses. Developing thymocytes bearing receptors with high affinity for self-antigens undergo negative selection (4–9) that is mediated by apoptosis (10–13). On the other hand, thymic T cells expressing TCRs that have low affinity for self-MHC molecules are selected to mature to the single positive stage and migrate to the peripheral lymphoid tissues (9, 14–16). In the periphery, T cell stimulation can initiate diverse molecular programs leading to distinct outcomes (activation, anergy, or death) depending on the differentiation state of the T lymphocyte (i.e., responsiveness to a second signal) and/or the context in which the antigenic peptide is presented (i.e., availability of a second signal). Peripheral T cells that have expanded in response to a foreign antigen are eliminated if the antigen stimulates a large fraction of T cells and persists for a prolonged time (17–21). This process, called activation-induced cell death (AICD), is essential to protect the organism against the deleterious effects of uncontrolled T cell expansion and the production of toxic levels of cytokines.

We have recently developed a functional system to identify cDNAs involved in TCR-induced programmed cell death (PCD; reference 22). DNA sequence analysis of one of the cDNAs identified with this system indicated that it could be involved in sphingolipid synthesis (our unpublished data). This suggested to us that the sphingolipid pathway might be implicated in the apoptotic process initi-

[1]*Abbreviations used in this paper:* aSMase, bSMase, and nSMase, acidic, bacterial, and neutral SMase; CM, ceramide; CoA, coenzyme A; CsA, cyclosporin A; EGFP, enhanced green fluorescent protein; Erk, extracellular signal regulatory kinase; FB1, fumonisin B1; MAP, mitogen-activated protein; PCD, programmed cell death; PKC, protein kinase C; PLC, phospholipase C; SM, sphingomyelin; SMase, sphingomyelinase.

ated by TCR triggering. Here we demonstrate that TCR cross-linking results in activation of neutral sphingomyelinase (nSMase) and production of ceramide (CM). Blockage of CM production by either the fungal toxin, fumonisin B1 (FB1), or nSMase antisense RNA impaired TCR-induced IL-2 production and PCD, suggesting that CM is an essential second messenger of the TCR.

Materials and Methods

Cell Lines, Abs, and Reagents. The T cell hybridomas 3DO, 2B4, and the human cell line Jurkat were grown in RPMI 1640, containing 2 mM glutamine, 25 nM β-mercaptoethanol, 10 mg/ml streptomycin, 10 mg/ml gentamicin. Anti-CD3ε was purified on a protein A–Sepharose column from culture supernatants of the 2C11 hybridoma cell line. FITC-conjugated 2C11, anti–mouse TCR-β H57, anti–human TCR HIT3a, anti–mouse Fas Jo2, and anti–hamster IgG Abs were purchased from PharMingen. Anti–mouse CD28 37.51 was diluted from ascites fluids (23). Anti–mouse CD45R B220 Ab conjugated to microbeads was from Miltenyi Biotech GmbH. Anti–mouse class II mAb, clone M5/114 (24), was biotinylated. FB1, CM, and bacterial SMase (bSMase) were purchased from Biomol, and daunorubicin and cyclosporin A (CsA) were from Sigma.

Splenic T Cell Purification. Splenic T cells from Balb/c mice were isolated by negative depletion of B cells and class II^+ cells. B lymphocytes and macrophages were coated with B220 conjugated to microbeads and biotinylated rat M5/114 Abs, followed by incubation with microbeads conjugated to streptavidin. The cells were passed through a MACS column in the MACS separator, and the flow through was recovered. The purity of the T cell preparation was 85–92% as determined by staining of the recovered cells with FITC-conjugated 2C11 Ab.

Culture and Stimulation Conditions. For TCR stimulation, 3DO and 2B4 cells were seeded on plates coated overnight at 4°C with 1 μg/ml of 2C11 Ab. For Fas stimulation, cells were preincubated for 30 min at room temperature with 1 μg/ml of anti–mouse Fas Ab Jo2, washed twice with medium, and seeded on plates coated overnight at 4°C with 5 μg/ml of anti–hamster IgG. Splenic T cells were stimulated using plates coated overnight with 1 μg/ml of H57 Ab. For costimulation, the anti–mouse CD28 37.51 Ab was added in solution at a 1:5,000 dilution from ascites fluids. Jurkat cells were cultured on plates coated overnight at 4°C with 5 μg/ml of anti–human TCR Ab HIT3a. FB1 was added to the cell cultures 30 min before stimulation. Daunorubicin was used at 10 μM. In the experiments shown in Fig. 4, exogenous CM (10 μM), nSMase (0.1 U/ml), and CsA (100 nM) were added to the cell cultures at the time of TCR stimulation.

Cell Death Determination, IL-2 Measurements, and Northern Blot Analysis. The percentage of cell death was assessed by measuring the DNA content of isolated nuclei stained with propidium iodide (25). IL-2 production was determined by ELISA (Genzyme). Expression of FasL and Nur77 mRNAs was quantitated by Northern blot analysis. Each lane was loaded with 20 μg of total RNA isolated from cells treated with 2C11 for the indicated time. The amount of mRNA loaded was normalized by using cyclophilin expression as an internal control.

CM Quantification. CM was quantified by the diacylglycerol kinase assay. After incubation with the indicated stimuli, cells were pelleted by centrifugation, washed twice with ice-cold PBS, and extracted with chloroform/methanol/1 N HCl (100:100:1, vol/vol/vol). The dried samples were resuspended in a 40-μl reaction mixture containing 5 mM of cardiolipin (Avanti Polar Lipids), 1 mM diethylenetriaminepentaacetic acid (DTPA; Sigma), 7.5% octyl-β-d-glucopyranoside (Calbiochem), 10 mM imidazole. After five cycles of freeze–thaw, the reaction was started by adding to the lipids suspension 100 μl of reaction buffer (100 mM imidazole-HCl, pH 6.6, 100 mM NaCl, 25 mM $MgCl_2$, and 2 mM EGTA), 20 μl of 20 mM dithiothreitol, 19 μl cold 10 mM ATP, 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; DuPont-NEN), and 10 μl *Escherichia coli* diacylglycerol kinase (Calbiochem). After 1 h at room temperature, the reaction was stopped by adding 0.5 ml of $CHCl_3/CH_3OH$/1 N HCl (100:100:1) and 85 μl of PBS, pH 7.4. 100 μl of the lower organic phase was resolved by thin-layer chromatography on silica gel 60 plates (Whatman) using a solvent system of chloroform/acetone/methanol/acetic acid/water (10:4:3:1) and visualized by autoradiography. Incorporated ^{32}P was quantified by scintillation counting. The level of CM was determined by comparison with a standard curve generated concomitantly with known amounts of CM (CM type III; Sigma).

CM Synthase Assay. CM synthase activity was determined as described previously (26). In brief, 75×10^6 cells were pelleted, washed once with cold PBS, and resuspended in 300 μl of homogenization buffer (25 mM Hepes, pH 7.4, 5 mM EGTA, 50 mM NaF, and 10 μg/ml each of leupeptin and soybean trypsin inhibitor). Cells were homogenized, and the lysates were centrifuged at 800 *g* for 5 min. The postnuclear supernatant was centrifuged at 250,000 *g* for 30 min. The microsomal membrane pellet was resuspended in 1 ml of homogenization buffer. Microsomal membrane protein (75 μg) was incubated in 1 ml reaction mixture containing 2 mM $MgCl_2$, 20 mM Hepes (pH 7.4), 20 μM defatted BSA (Sigma), varying concentrations (0.2–20 μM) of dihydrosphingosine (Biomol), 70 μM unlabeled palmitoyl-coenzyme A (palmitoyl-CoA; Sigma), and 3.6 μM (0.2 μCi) [1-^{14}C] palmitoyl-CoA (55 mCi/mmol; NEN Life Science Products). The reaction was started by addition of palmitoyl-CoA, incubated at 37°C for 1 h, and then stopped by extraction of lipids using 2 ml of chloroform/methanol (1:2). Lower phase was removed, concentrated, and applied to a silica gel 60 thin-layer chromatography plate. Dihydroceramide was resolved from free radiolabeled fatty acid using a solvent system of chloroform/methanol/3.5 N ammonium hydroxide (85:15:1), identified by iodine vapor staining based on comigration with CM standards, and quantified by liquid scintillation counting.

Assay for Neutral and Acidic SMase. The cells were harvested and washed three times with ice-cold PBS, 100 μM Na_3VO_4 at 4°C. Cells were disrupted by five cycles of freezing–thawing (in methanol/dry ice) in 100 μl of lysis buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM EGTA, 1 mM sodium vanadate, 10 mM β-glycerol phosphate, 1 mM PMSF, 5 mM dithiothreitol, 20 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin. The lysate was centrifuged for 10 min at 1,000 *g* at 4°C, and the supernatant (post-nuclear homogenate) was centrifuged for 60 min at 100,000 *g* at 4°C. The resulting pellet (membrane fraction) was resuspended in 50 μl of lysis buffer. The membrane preparation was incubated for 30 min at 37°C with ^{14}C-SM (1,000,000 dpm, 10 nmol) in a mixed micelle assay containing 100 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and 0.1% Triton X-100 (final volume 100 μl). The reaction was stopped by adding 800 μl of $CHCl_3/CH_3COOH$ (2:1, vol/vol) and 250 μl of water. The radioactivity was determined by liquid scintillation counting. To determine the aSMase activity, membranes were prepared from cells using lysis buffer containing 20 mM

Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM EGTA, and 1 mM PMSF. The micelle assay used contained 100 mM sodium acetate, pH 5, and 0.1% Triton X-100.

SM Quantification. 3DO cells were grown for 48 h in the presence of 0.5 μCi/ml (80 Ci/mmol) [^{3}H]choline chloride. Post-labeling cells were washed with PBS, reseeded at 0.5 × 10^6 cells/ml in RPMI, and rested for 2–4 h. Cells were then subjected to a variety of treatments. After treatment, cells were harvested and cell pellets were resuspended in 3 ml of chloroform/methanol (1:2). Standard Bligh and Dyer extraction was used to recover lipids. Lipids dried under vacuum were resuspended in 50–100 μl of chloroform and spotted on thin-layer chromatography plates, and plates were developed in chloroform/methanol/acetic acid/water (50:30:8:5). Plates were sprayed with En^{3}Hance and exposed to film for 24–48 h. The labeled SM spots were scraped into scintillation fluid and counted in a scintillation counter.

Human nSMase Cloning and Transfection. Human nSMase cDNA was pulled out by PCR from a cDNA library derived from human fetal liver (Invitrogen) and cloned in pcDNA3.1 vector (Invitrogen). The library was screened using primers designed from the human nSMase sequence recently published (27).

Transient transfections in Jurkat T cells were performed by electroporating 50 μg of the indicated cDNAs together with 1 μg of En^{3}Hance Green Fluorescent Protein-N1 (EGFP; Clontech). The efficiency of transfections was monitored by analyzing the percentage of EGFP$^+$ cells by flow cytometry. EGFP$^+$ cells were sorted using a Becton Dickinson FACStar™.

Immunoprecipitation and Immunoblot. Cells were lysed in a buffer comprised of 60 mM Tris-HCl, pH 7.8, containing 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, and phosphatase and protease inhibitors as described previously (28). Post-nuclear fractions were precleared with protein A–trisacryl beads (Pierce) and subjected to immunoprecipitation with a mixed mAb preparation directed against phospholipase Cγ1 (PLCγ1; Upstate Biotechnology) bound to protein A/G–agarose beads (Pierce). Proteins were eluted with sample buffer, resolved by SDS-PAGE under reducing conditions, and transferred to nitrocellulose membranes (Hybond-C super; Amersham Pharmacia Biotech). Protein detection was via an antiphosphotyrosine primary Ab (4G10; Upstate Biotechnology) with a second Ab (rabbit anti–mouse IgG; Cappel) followed by ^{125}I–protein A (ICN Biomedicals). Immunoblots were stripped according to the membrane manufacturer's instructions and reprobed with other Abs. Immunoblots were scanned on a PhosphorImager (Molecular Dynamics) to produce the images shown, with no manipulation except for the adjustment of the exposure range. Densitometry was performed using ImageQuant™ software (Molecular Dynamics).

Results

FB1 Inhibits TCR-induced FasL Expression, Cell Death, and IL-2 Production. To test the hypothesis that the sphingolipid pathway might be implicated in the apoptotic process initiated by TCR triggering, we used an inhibitor of sphingolipid synthesis, FB1 (29). As shown in Fig. 1 A, this compound protected the T cell hybridoma 3DO from TCR-induced cell death.

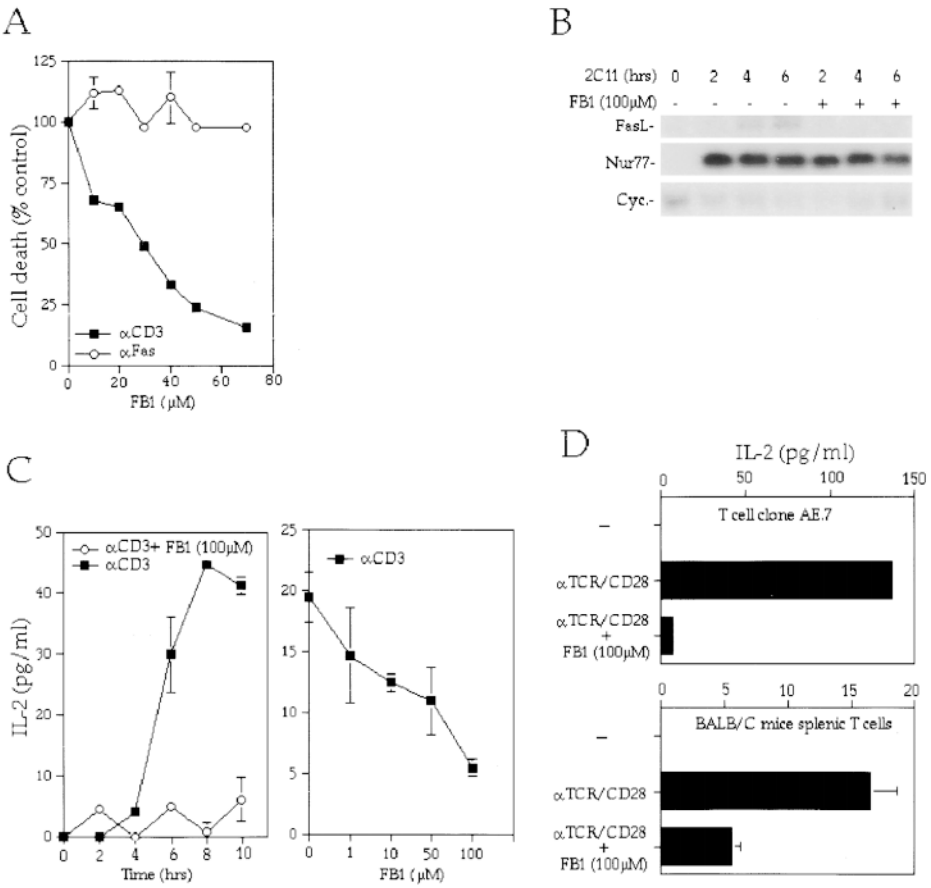

Figure 1. FB1 interferes with TCR signaling. (A) Pretreatment for 30 min of 3DO cells with FB1 inhibits TCR-induced apoptosis. The inhibitory effect is dose dependent. Fas-induced cell death was not affected by this toxin. Apoptosis was measured 8 h after TCR triggering and 4 h after Fas stimulation. (B) Induction of FasL expression, detectable by Northern blot analysis 4 h after TCR cross-linking, is blocked by FB1 (100 μM). Nur77 expression, also induced by TCR stimulation, is unaffected. Cyclophilin (Cyc.) mRNA expression was used as an internal standard control to allow normalization to the amount of mRNA loaded. (C) FB1 also reduces antigen receptor–triggered IL-2 production in 3DO cells. In the experiments shown in the lower panel, IL-2 secretion was measured 6 h after stimulation of 3DO cells with the 2C11 mAb, in the presence of the indicated concentrations of FB1. In the experiments shown in the upper panel, FB1 was used at a 100 μM concentration. (D) FB1 also reduces IL-2 production induced by stimulation of both antigen receptor (TCR) and the costimulatory receptor CD28 in AE.7 T cell clone and freshly isolated splenic T cell. Data points shown in A, C, and D represent the mean of three independent experiments ± SE.

Stimulation of the TCR on a T cell hybridoma induces FasL upregulation, and the engagement of Fas by FasL activates PCD (30–32). Considering that activation of the sphingolipid pathway has been described to occur after Fas triggering in several cellular systems (33, 34), we also tested the effect of FB1 on Fas-induced cell death. This toxin did not affect Fas-triggered apoptosis (Fig. 1 A), indicating that FB1 interfered with the TCR-induced death pathway upstream of Fas. A key event of TCR-induced apoptosis is upregulation of FasL. Our results raised the possibility that FB1 inhibited FasL induction after TCR triggering, which indeed turned out to be the case. FasL mRNA expression, detectable 4 h after TCR stimulation, was blocked by FB1 (Fig. 1 B). This inhibition was specific for FasL, since expression of Nur77 mRNA, another gene known to be induced upon receptor triggering (35, 36), was not affected (Fig. 1 B).

FB1 could either specifically interfere with PCD or compromise early events in TCR signaling. To differentiate between these two possibilities, we measured IL-2 levels in culture supernatants after TCR triggering. Pretreatment with FB1 inhibited IL-2 production in 3DO cells (Fig. 1 C), indicating that this compound interfered with signaling events that are common to the activation and cell death pathways. To determine if this observation was true for normal T cells, the clone A.E7 was tested as well as freshly isolated splenic T lymphocytes (Fig. 1 D). FB1 reduced production of IL-2 after simultaneous stimulation of either T cell population with anti-TCR and anti-CD28 Ab (Fig. 1 D).

TCR Triggering Induces nSMase Activation, SM Hydrolysis, and CM Production. Inhibition of IL-2 production and cell death by FB1, which is known to interfere with the de novo synthesis of CM by inhibiting CM synthase, suggested that CM may participate in early events in TCR signaling. Therefore, we tested whether TCR triggering induced production of CM. As shown in Fig. 2 A, intracellular CM concentration increased rapidly upon TCR stimulation.

CM is produced by two distinct routes: de novo synthesis and SM hydrolysis by SMases (37). The de novo synthesis of CM via stimulation of CM synthase has been implicated in the apoptotic pathway (38). Since FB1 has been shown to inhibit this enzyme (38, 29), we first tested whether CM production upon TCR triggering resulted from activation of CM synthase. As shown in Fig. 2 B, CM synthase activity was not increased upon TCR stimulation. Next, we analyzed whether TCR engagement induced activation of SMases, enzymes responsible for CM generation induced by stimulation of several membrane receptors, including nerve growth factor receptor (NGFR), TNFR, and Fas (39). SMases are known to exist in at least two

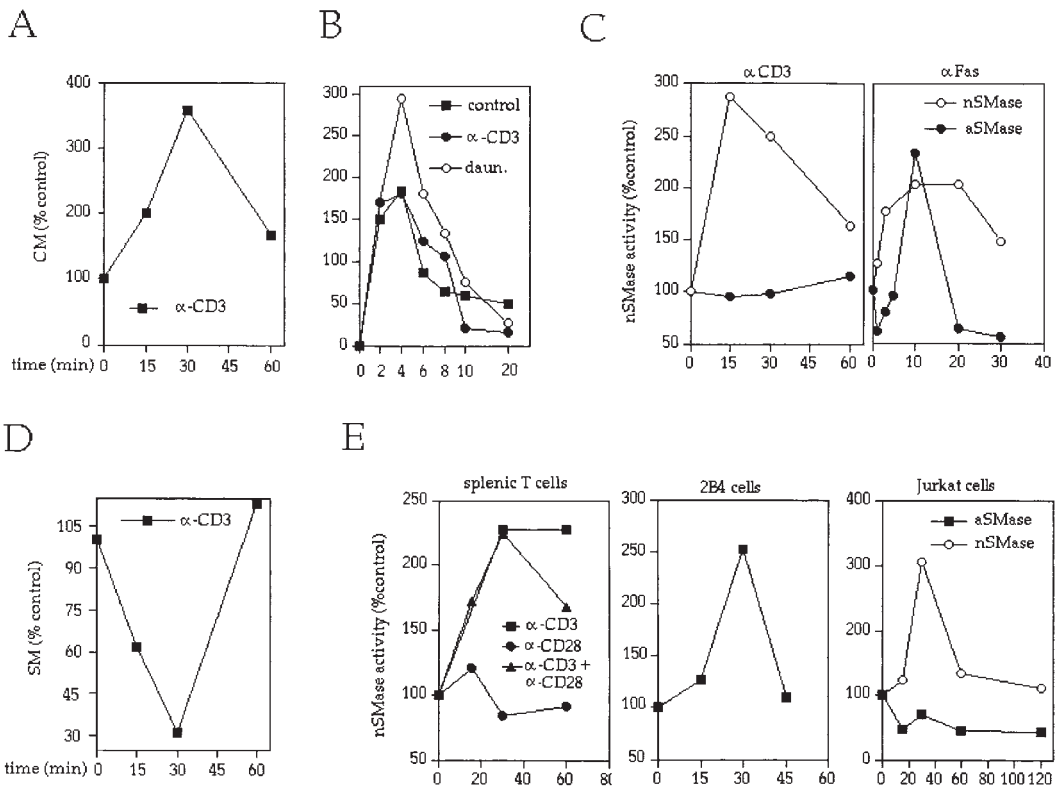

Figure 2. TCR stimulation induces nSMase activation, hydrolysis of SM, and increased intracellular levels of CM. (A) TCR triggering induces CM production. (B) The activity of CM synthase, which is enhanced by daunorubicin (daun.), is not affected by TCR stimulation. (C) TCR engagement specifically activates nSMase but not aSMase (C, left) and induces hydrolysis of SM (D). Stimulation of Fas activates both SMases (C, right). (E) TCR cross-linking induces nSMase activation also in splenic T cells from Balb/c mouse (left), murine T cell hybridoma 2B4 (middle), and human T cell clone Jurkat (right) cells. Stimulation of purified splenic T cells with an anti-CD28 Ab did not affect nSMase activity (left). Data presented are representative of at least three independent experiments.

forms, a Mg^{2+}-dependent membrane-bound form with a neutral pH optimum (nSMase) and a lysosomal acidic form (aSMase) (37). Triggering of the TCR resulted in activation of nSMase (Fig. 2 C). This activation was selective since the aSMase, which is readily stimulated upon Fas triggering, was not induced by the TCR (Fig. 2 C). The time course of nSMase activation paralleled CM production as well as hydrolysis of the nSMase substrate, SM (Fig. 2, A and D). Thus, the rapid increase in intracellular CM upon TCR stimulation depends on activation of nSMase.

To determine whether nSMase activation was a general and specific consequence of TCR engagement, we analyzed nSMase activity after triggering of the TCR and CD28 molecules in other T cell types. Purified splenic mouse T cells (Fig. 2 E, left), mouse T cell hybridoma 2B4 (Fig. 2 E, middle), and human T cell clone Jurkat (Fig. 2 E, right), all showed activation of nSMase in response to stimulation of the TCR. An isotype-matched Ab specific for the costimulatory molecule, CD28, did not activate nSMase or alter the TCR-dependent induction of this enzyme in purified splenic T cells (Fig. 2 E). Thus, nSMase is specifically activated by the TCR in all T cell types analyzed.

FB1 Blocks TCR-induced nSMase Activation and CM Production. Although FB1 is considered to be an inhibitor of CM synthase (38, 29), our data suggest that the toxin interferes with the TCR-dependent nSMase activation. Therefore, we measured nSMase activity, and CM and SM levels after TCR triggering. We found that FB1 inhibits activation of this enzyme and, consequently, SM hydrolysis and CM production (Fig. 3, A–C). In contrast, Fas-induced aSMase activation was not effected by FB1 (data not shown). Thus, FB1 inhibits TCR-induced CM production by interfering, either directly or indirectly, with nSMase activation.

The Inhibitory Effect of FB1 on TCR-induced IL-2 Production and Apoptosis Is Reverted by Exogenous CM. Altogether, these findings indicate that FB1 impairs TCR signaling by inhibiting CM production. However, this toxin could also inhibit other pathways that are essential for the TCR to cause IL-2 production, FasL upregulation, and, hence, cell death. To distinguish between these two possibilities, we used bSMase or a cell-permeable CM analogue to reconstitute intracellular CM levels. Both treatments reversed the inhibitory effect of FB1 and restored TCR-induced IL-2 production (Fig. 4 A). However, neither compound reconstituted IL-2 production when TCR signaling was inhibited by CsA, an immunosuppressant that blocks dephosphorylation and nuclear translocation of the transcription factor nuclear factor of activated T cells (NFAT)-c1 (40). Furthermore, C2 CM restored TCR-induced PCD only when signaling was blocked by FB1 and not by CsA (Fig. 4 B).

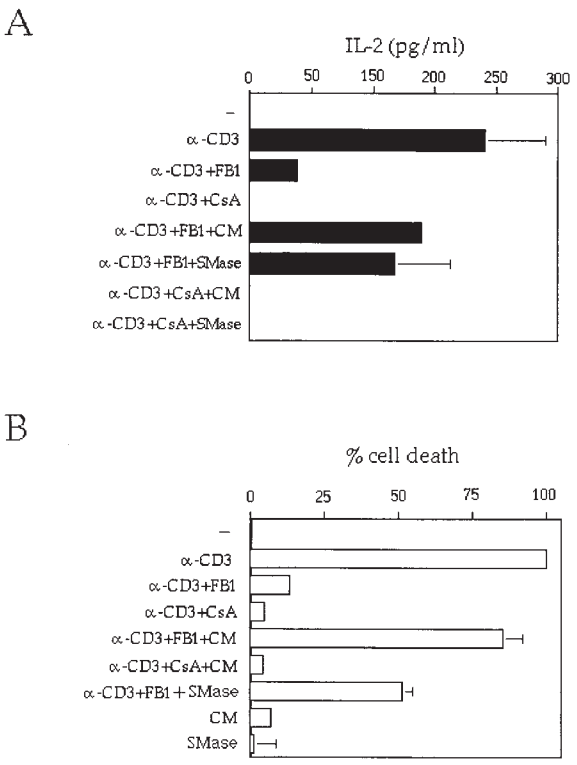

Figure 4. Reconstitution of IL-2 production and cell death by exogenous CM or bacterial SMase. 3DO cells were stimulated with plate-bound anti-CD3 Ab with or without FB1 preincubation. At the time of TCR stimulation, exogenous CM (10 μM), bSMase (0.1 U/ml), and CsA (100 nM) were added as indicated. Supernatants were analyzed for IL-2 production by ELISA 12 h after stimulation (A). In a separate experiment, cells were tested for apoptosis 8 h after stimulation (B). Data points represent the mean of three independent experiments ± SE.

Inactivation of nSMase by Antisense RNA Blocks TCR-induced IL-2 Production. To directly test the involvement of nSMase in TCR signaling, we cloned by PCR the cDNA coding for the recently identified and cloned human nSMase

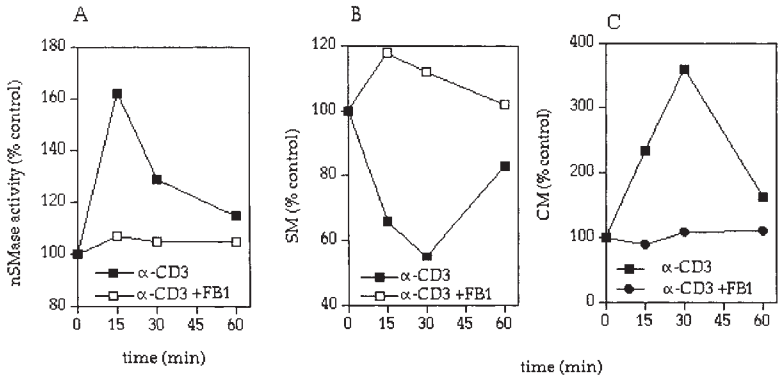

Figure 3. FB1 interferes with the TCR-induced nSMase activation. Preincubation of 3DO cells with FB1 (100 μM) blocks TCR-induced activation of nSMase (A). As a result, SM hydrolysis (B) and CM production (C) are also inhibited. Data shown are representative of five independent experiments.

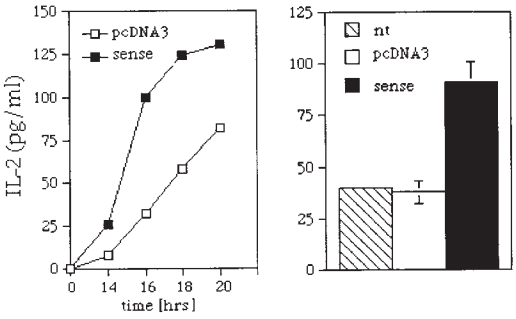

Figure 5. Overexpression of nSMase enhances TCR-induced IL-2 secretion. Jurkat T cells transfected with nSMase cloned in sense (sense) and stimulated with an anti-CD3ε Ab produce significantly higher levels of IL-2 compared with mock-transfected (pcDNA3) or nontransfected (nt) controls. The data presented in the right panel represent three separate transfections, and error bars indicate SEM. IL-2 levels were measured 12 h after transfection.

(27). Northern blot analyses showed that nSMase is expressed in all human lymphoid tissues and cell lines tested, including spleen, lymph node, thymus, peripheral blood lymphocytes, bone marrow, fetal liver, and Jurkat T cells (data not shown). The nSMase cDNA was then cloned into the mammalian expression vector pcDNA3.1, in both sense and antisense orientation. Jurkat T cells were transfected with either sense or antisense constructs together with a plasmid coding for EGFP. The efficiency of transfection, determined by measuring the percentage of $EGFP^+$ cells, was between 60 and 70% (data not shown). Consistent with our hypothesis, overexpression of nSMase by transfection of the sense construct resulted in a significant increase in IL-2 production upon antigen receptor triggering (Fig. 5). In contrast, expression of the antisense construct reduced IL-2 production by ~50% compared with mock-transfected cells (Fig. 6 A, left). To accurately measure the inhibitory effect of the antisense vector and to determine whether expression of the nSMase antisense RNA efficiently depleted the cellular pool of nSMase protein, we isolated transfected cells by sorting $EGFP^+$ cells. Sorted cells were then assayed for TCR-induced IL-2 (Fig. 6 A, right) and CM production (Fig. 6 B). In this experimental setting, antisense nSMase almost completely blocked both IL-2 and CM production. Taken together, these results demonstrate that CM is an essential second messenger for the TCR to adequately signal for IL-2 production and PCD.

nSMase Is Required for TCR-induced MAP Kinase Activation. In an attempt to localize where in the TCR signaling pathway CM production plays its most important role, we studied the effect of nSMase inhibition on the activation of known biochemical signals that are induced upon TCR stimulation. A critical early event in TCR signal transduction is the induction of protein tyrosine kinase activity with ensuing phosphorylation of multiple substrates (41). However, inhibition of nSMase activity by transient transfection with an antisense vector had no effect on the overall level of protein tyrosine phosphorylation or the pattern of phosphorylated proteins (Fig. 7 A, top). To confirm this finding, we analyzed directly the phosphorylation status of PLCγ1, one of the targets of tyrosine kinases during TCR stimulation (42). No detectable inhibition of TCR-induced PLCγ1 tyrosine phosphorylation was observed in cells transfected with antisense nSMase (Fig. 7 A, bottom panels). Taken together, these data suggest that CM production is not required for the early events leading to the activation of this pathway.

TCR ligation activates the guanine nucleotide regulatory protein, Ras (43). GTP-bound Ras triggers a cascade of events that culminate in the phosphorylation and consequent activation of the MAP kinases, extracellular signal regulatory kinase (Erk)1 and Erk2. Inhibition of nSMase CM synthesis resulted in diminished MAP kinase activation (Fig. 7 B, top panels), suggesting that this pathway may be regulated by CM production. Pharmacological activation of PKC resulting from treatment of T lymphocytes with the phorbol ester, PMA, activates MAP kinase in a Ras-dependent manner. This effect of PMA was not affected by blockade of CM synthesis (Fig. 7 B, bottom panels). Together with the observation that early tyrosine kinase activation is insensitive to inhibition of nSMase activity, these data suggest that CM plays a role in MAP kinase activation upstream of Ras or on a parallel pathway.

Discussion

In this study, we show that ligation of the TCR induces production of CM, a molecule that acts as a second messenger in TCR signal transduction. This increase in intracellular CM results from specific activation of nSMase and SM hydrolysis.

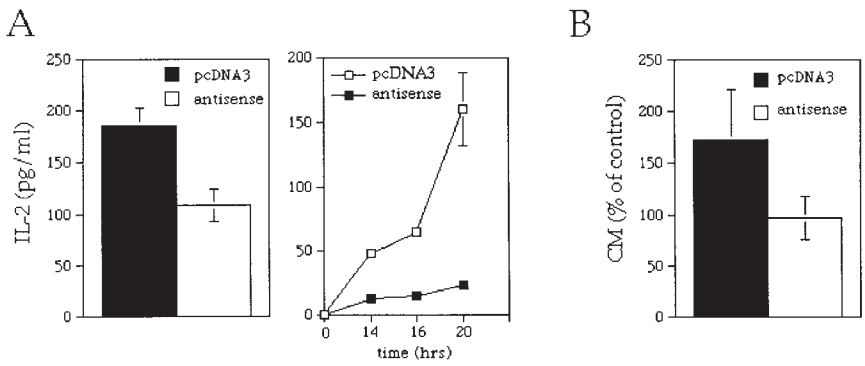

Figure 6. Overexpression of antisense nSMase inhibits TCR-induced CM production and IL-2 secretion. (A) Jurkat T cells, transfected with antisense nSMase expression plasmid (antisense) or empty vector (pcDNA3), were tested for IL-2 production before (left; measurements taken 20 h after TCR stimulation) and after (right) sorting of $EGFP^+$ cells. Sorted cells were also assayed for CM production 45 min after TCR stimulation (B). Data points represent the mean of three independent experiments ± SE.

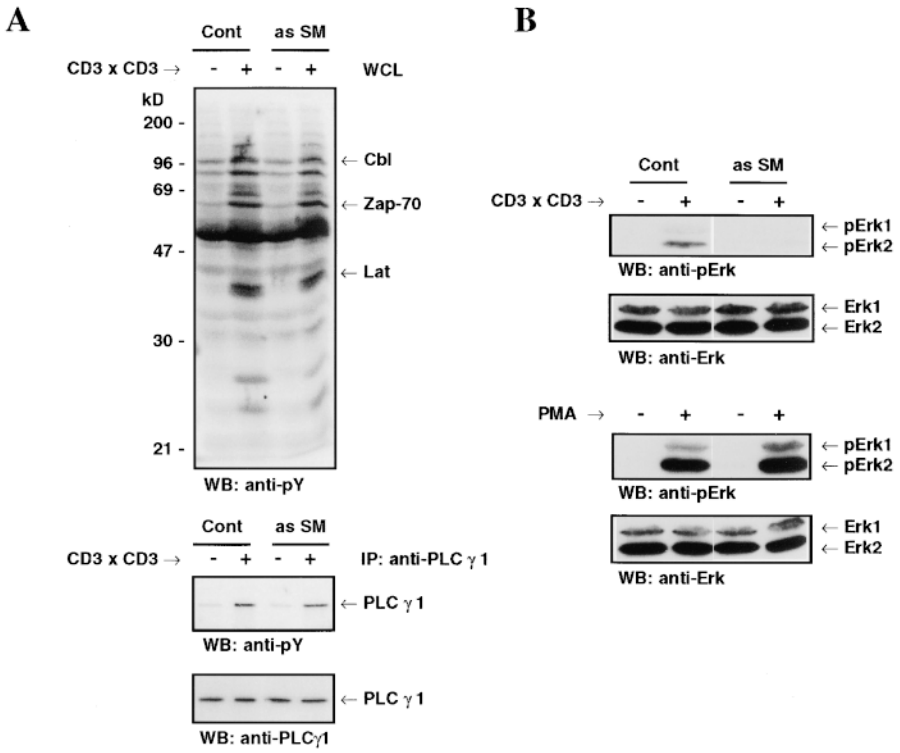

Figure 7. CM production is required for MAP kinase activation. (A) Jurkat cells, transfected with either empty vector (Cont) or antisense nSMase (as SM), were treated with medium alone or an anti-TCR Ab (CD3 × CD3) for 2 min at 37°C and immediately lysed. Total protein tyrosine phosphorylation was measured by Western blot (WB) analysis of whole-cell lysates (WCL) with an antiphosphotyrosine Ab (anti-pY; top). Three bands, attributable to LAT, Cbl, and ZAP-70 based on their electrophoretic mobility and the use of Abs against known tyrosine kinase substrates (data not shown), are indicated. Tyrosine phosphorylation of PLCγ1 was directly assayed by probing PLCγ1 immunoprecipitates (IP) with the antiphosphotyrosine Ab (middle). Equivalent amounts of PLCγ1 were immunoprecipitated in each experimental sample, as shown by stripping and reprobing the blot with an anti-PLCγ1 Ab (bottom). (B) Transfected cells were stimulated with either an anti-TCR Ab (CD3 × CD3) for 5 min or PMA for 10 min. Whole-cell lysates were first probed with an Ab specific for phosphorylated Erk1 and Erk2 (anti-pErk). After stripping, membranes were reprobed with an anti-Erk Ab to verify that equivalent amounts of MAP kinases were loaded in each lane.

Recently, an important role for molecules of sphingoid nature in the modulation of cell response to different extracellular signals has been uncovered. CM has been shown to act as a key molecule in a new signal transduction pathway (3). This phospholipid has been implicated in signaling through several membrane receptors, including nerve growth factor receptor (NGFR), TNFR, and Fas. Exogenous CM analogues have been shown to regulate processes such as PCD (33), IL-2 production (44), and FasL expression (45). Some downstream targets of this molecule have also been identified. The action of a 97-kD plasma membrane–bound serine/threonine protein kinase, CM-activated protein kinase (CAPK), is enhanced by elevation of cellular CM. The CM-activated protein phosphatase (CAPP), which is a member of the protein phosphatase 2A class of serine/threonine protein phosphates, is also a target of CM. CM may also stimulate the guanine nucleotide exchange factor, Vav, a putative activator of Ras-like guanine nucleotide regulatory proteins regulating cytoskeletal assembly in hematopoietic cells. Finally, PKCζ, an atypical PKC that is insensitive to phorbol esters and diacylglycerol, may also be a direct target for CM (37, 46).

An early critical event in TCR signaling is the phosphorylation of CD3/ζ–ζ chains, docking sites for the T cell–specific tyrosine kinase, ZAP-70. Activation of ZAP-70 kinase, which is regulated via tyrosine phosphorylation, is essential for many of the early events in TCR signaling. One of the targets of tyrosine kinases during TCR stimulation is PLCγ1 (42), whose enzymatic activity is enhanced by tyrosine phosphorylation (47). PLCγ1 catalyzes the formation of inositol (1,4,5)-trisphosphate and 1,2-diacylglycerol. These second messengers induce elevation of intracellular calcium and activation of PKC, respectively. The increase in calcium ultimately results in nuclear translocation and activation of the transcription factor, NFAT (40). PKC and adapter proteins, some of which are also phosphorylated on tyrosines, participate in the activation of Ras and the MAP kinase cascade that ultimately controls the activation of other transcription factors (43, 48). Here, we present evidence that CM production is required for TCR-induced MAP kinase activation, and that CM may act independently or upstream of Ras. However, CM production is not required for TCR-induced tyrosine kinase activation. In fact, like other biochemical messengers of the TCR signal, activation of nSMase requires protein tyrosine kinase activity (data not shown).

Our data indicate that CM is a second messenger molecule essential for the antigen receptor to signal for both activation and cell death. Further investigation into the downstream targets of CM action and on how this pathway is integrated with others to give biological outcomes such as differentiation, activation, anergy, cell death, and effector function will help to elucidate the physiological role of CM in TCR signaling.

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References

1. Wilson, I.A., and K.C. Garcia. 1997. T-cell receptor structure and TCR complexes. *Curr. Opin. Struct. Biol*. 7:839–848.
2. Wange, R.L., and L.E. Samelson. 1996. Complex complexes: signaling at the TCR. *Immunity*. 5:197–205.
3. Berridge, M.J. 1997. Lymphocyte activation in health and disease. *Crit. Rev. Immunol*. 17:155–178.
4. Kappler, J.W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell*. 49:273–280.
5. Kappler, J.W., U. Staerz, J. White, and P. Marrack. 1988. Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. *Nature*. 332:35–40.
6. MacDonald, H.R., R. Schneider, R.K. Lees, R.C. Howe, H. Acha-Orbea, H. Restenstein, R.M. Zinkernagel, and H. Hengartner. 1988. T-cell receptor Vβ use predicts reactivity and tolerance to Mls^a-encoded antigen. *Nature*. 332:40–45.
7. Hengartner, H., B. Odermatt, R. Shneider, M. Schreirer, G. Walle, H.R. MacDonald, and R.M. Zinkernagel. 1988. Deletion of self-reactive T cells before entry into the thymus medulla. *Nature*. 336:388–390.
8. Kisielow, P., H. Bluethmann, U.D. Staerz, M. Steinmetz, and H. Von Boehmer. 1988. Tolerance in T-cell receptor transgenic mice involves deletion of nonmature $CD4^+8^+$ thymocytes. *Nature*. 333:742–746.
9. Sha, W.C., C.A. Nelson, R.D. Newberry, D.M. Kranz, J.H. Russell, and D.Y. Loh. 1988. Positive and negative selection of an antigen receptor in transgenic mice. *Nature*. 336:73–76.
10. Shi, Y., R.P. Bissonnette, N. Parfrey, M. Szalay, R.T. Kubo, and D.G. Green. 1991. In vivo administration of monoclonal antibodies to the CD3 T-cell receptor complex induces cell death (apoptosis) in immature thymocytes. *J. Immunol*. 146: 3340–3346.
11. Smith, C.A., G.T. Williams, R. Kingston, E.J. Jenkinson, and J.J.T. Owen. 1989. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature*. 337:181–184.
12. Murphy, K.M., A.B. Heimberger, and D.Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+$ $CD8^+$ TCR^{lo} thymocytes in vivo. *Science*. 250:1720–1723.
13. D'Adamio, L., L.K. Clayton, K.M. Awad, and E.L. Reinherz. 1992. Negative selection of thymocytes: a novel PCR-based molecular analysis detects requirements for macromolecular synthesis. *J. Immunol*. 149:3550–3553.
14. Berg, L.J., B. Fazekas de St. Groth, A.M. Pullen, and M.M. Davis. 1989. Phenotypic differences between αβ versus β T-cell receptor transgenic mice undergoing negative selection. *Nature*. 340:559–562.
15. Blackman, M.A., J.W. Kappler, and P. Marrack. 1989. Influence of the MHC on positive selection of $V\beta17a^+$ T cells. *Science*. 244:214–217.
16. MacDonald, H.R., R.K. Lees, R. Schneider, R.M. Zinkernagel, and H. Hengartner. 1988. Positive selection of $CD4^+$ thymocytes controlled by MHC class II gene products. *Nature*. 336:471–473.
17. Webb, S., C. Morris, and J. Sprent. 1990. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell*. 63:1249–1256.
18. Kawabe, Y., and A. Ochi. 1990. Selective anergy of $V\beta8^+$, $CD4^+$ T cells in *Staphylococcus* enterotoxin B–primed mice. *J. Exp. Med*. 172:1065–1070.
19. Kawabe, Y., and A. Ochi. 1991. Programmed cell death and extrathymic reduction of $V\beta8^+CD4^+$ T cells in mice tolerant to *Staphylococcus aureus* enterotoxin B. *Nature*. 349:245–248.
20. Lenardo, M.J. 1991. Interleukin-2 programs mouse αβ T lymphocytes for apoptosis. *Nature*. 353:858–861.
21. D'Adamio, L., K.M. Awad, and E.L. Reinherz. 1993. Thymic and peripheral deletion of antigen-specific T cells might cooperate in establishing self-tolerance. *Eur. J. Immunol*. 23: 747–753.
22. Vito, P., E. Lacana, and L. D'Adamio. 1996. Interfering with apoptosis: Ca^{2+}-binding protein ALG-2 and Alzheimer's disease gene ALG-3. *Science*. 271:521–525.
23. Harding, F.A., J.A. McArthur, D.H. Gross, D.H. Raulet, and J.P. Allison. 1992. CD28-mediated signaling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature*. 356:607–609.
24. Bhattacharya, A., M.E. Dorf, and T.A. Springer. 1981. A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication. *J. Immunol*. 127:2488–2495.
25. Nicoletti, I., G. Migliorati, M.C. Pagliacci, F. Grignani, and C. Riccardi. 1991. Simple method for measuring thymocytes apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods*. 139:271–279.
26. Liu, J., S. Mathias, Z. Yang, and R.N. Kolesnick. 1994. Renaturation and TNFα stimulation of 97 kDa ceramide-activated protein kinase. *J. Biol. Chem*. 269:3047–3052.
27. Tomiuk, S., K. Hofmann, M. Nix, M. Zumbansen, and W. Stoffel. 1998. Cloned mammalian neutral sphingomyelinase: functions in sphingolipid signaling? *Proc. Natl. Acad. Sci. USA*. 95:3638–3643.
28. Stoica, B., K.E. DeBell, L. Graham, B.L. Rellahan, M.A. Alava, J. Laborda, and E. Bonvini. 1998. The amino-terminal Src homology 2 domain of phospholipase C gamma 1 is essential for TCR-induced tyrosine phosphorylation of phospholipase C gamma 1. *J. Immunol*. 3:1059–1066.
29. Harel, R., and A.H. Futerman. 1993. Inhibition of sphingolipid synthesis affects axonal outgrowth in cultured hippocampal neurons. *J. Biol. Chem*. 268:14476–14481.
30. Brunner, T., R.J. Mogil, D. LaFace, N.J. Yoo, A. Mahboubi, F. Echeverri, S.J. Martin, W.R. Force, D.H. Lynch, C.F. Ware, and D. Green. 1995. Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature*. 373:385–386.
31. Dhein, J., H. Walczak, C. Baumler, K.M. Debatin, and P.H. Krammer. 1995. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature*. 373:438–441.
32. Ju, S.T., D.J. Panka, H. Cui, R. Ettinger, M. el-Khatib,

D.H. Sherr, B.Z. Stanger, and A. Marshak-Rothstein. 1995. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature*. 373:444–448.
33. Kolesnick, N.R., A. Haimovitz-Friedman, and Z. Fuks. 1994. The sphingomyelin signal transduction pathway mediates apoptosis for tumor necrosis factor, Fas, and ionizing radiation. *Biochem. Cell Biol*. 72:471–474.
34. Hannun, Y.A. 1996. Function of ceramide in coordinating cellular response to stress. *Science*. 274:1855–1859.
35. Woronicz, J.D., B. Calnan, V. Ngo, and A. Winoto. 1994. Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas. *Nature*. 367:277–281.
36. Zheng-Gang, L., S.L. Smith, K.A. McLaughlin, M. Schwartz, and B.A. Osborne. 1994. Apoptotic signal delivered through the T-cell receptor of a T-cell hybrid required the immediate-early gene nur77. *Nature*. 367:281–284.
37. Spiegel, S., D. Foster, and R.N. Kolesnick. 1996. Signal transduction through lipid second messengers. *Curr. Opin. Cell Biol*. 8:159–167.
38. Bose, R., M. Verheij, A. Haimovitz-Friedman, K. Scotto, Z. Fuks, and R.N. Kolesnick. 1995. Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals. *Cell*. 82:405–414.
39. Pushkareva, M., L.M. Obeid, and Y.A. Hannun. 1995. Ceramide: an endogenous regulator of apoptosis and growth suppression. *Immunol. Today*. 16:294–297.
40. Rao, A., C. Luo, and P.G. Hogan. 1997. Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol*. 15:707–747.
41. Weiss, A. 1993. T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. *Cell*. 73: 209–212.
42. Park, D.J., H.V. Rho, and S.G. Rhee. 1991. CD3 stimulation causes phosphorylation of phospholipase C gamma 1 on serine and tyrosine residues in a human T cell line. *Proc. Natl. Acad. Sci. USA*. 88:5453–5457.
43. Cantrell, D.A., J.D. Graves, M. Izquierido, S. Lucas, and J. Downward. 1992. T lymphocyte activation signal. *Ciba Found. Symp*. 164:208–218.
44. Mathias, S., A. Younes, C.C. Kan, I. Orlow, C. Joseph, and R.N. Kolesnick. 1993. Activation of the sphingomyelin signaling pathway in intact EL4 cells and in a cell-free system by IL-1β. *Science*. 259:519–521.
45. Herr, I., D. Wilhem, T. Böhler, P. Angel, and K.M. Debatin. 1997. Activation of CD95 (APO-1/Fas) signaling by ceramide mediates cancer therapy-induced apoptosis. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:6200–6208.
46. Müller, G., M. Ayoub, P. Storz, J. Rennecke, D. Fabbro, and K. Pfizenmaier. 1995. PKC ζ is a molecular switch in signal transduction of TNF-α, bifunctionally regulated by ceramide and arachidonic acid. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:1961–1969.
47. Nishibe, S., M.I. Wahl, S.M. Hernandez-Sotomayor, N.K. Tonks, S.G. Rhee, and G. Carpenter. 1990. Increase of the catalytic activity of phospholipase C-gamma 1 by tyrosine phosphorylation. *Science*. 250:1253–1256.
48. Zhao, H., Y.Y. Li, R.V. Fucini, S.E. Ross, J.E. Pessin, and G.A. Korentzky. 1997. T cell receptor-induced phosphorylation of Sos requires activity of CD45, Lck, and protein kinase C, but not ERK. *J. Biol. Chem*. 272:21625–21634.