CD28 Signals through Acidic Sphingomyelinase

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Summary

T cell receptor recognition of antigen can lead either to T lymphocyte differentiation and proliferation or to a state of unresponsiveness, which is dependent on whether appropriate costimulatory signals are provided to the mature T cell. We have investigated a novel intracellular signaling pathway provided by the costimulatory molecule CD28. CD28 engagement triggers the activation of an acidic sphingomyelinase (A-SMase), which results in the generation of ceramide, an important lipid messenger intermediate. A-SMase activation by CD28 occurred in resting as well as in activated primary T cells or leukemic Jurkat cells. In contrast, ligation of either CD3 or CD2 did not result in A-SMase activation. Overexpression of recombinant A-SMase in Jurkat T cells substituted for CD28 with regard to nuclear factor-κB activation. These data suggest that CD28 provides an important costimulatory signal by activation of an acidic sphingomyelinase pathway.

The generation of an adaptive immune response requires the activation of naive lymphocytes that recognize antigens with their specific receptors. However, triggering of the antigen-specific receptor (TCR) alone appears to be insufficient to activate effector cells and only provides an incomplete stimulatory signal (1, 2). In vitro and in vivo studies of T cell activation have demonstrated that additional cell-associated ligands play a key function during the initial phase. Some of these ligands function to stabilize the physical interaction of the APC and the T cell (3–5), but others provide important costimulatory signals that are crucial for complete activation (1, 6–8).

Costimulatory molecules induce separate signals that complement TCR function. TCR complex-initiated signal cascades comprise cytoplasmic protein tyrosine kinases such as members of the src family (lck, fyn) and the syk/ZAP-70 family as well as protein tyrosine phosphatases such as CD45 (9–18). The CD28 costimulatory signaling is only beginning to be understood (for review see references 19 and 20). The physiological ligands for CD28, B7-1, and B7-2, have been identified as cell-surface molecules expressed by APC (21–25). B7-1 through binding to CD28 can increase cellular tyrosine phosphorylation and activate phosphatidylinositol 3-kinase (PI 3-kinase)[1] (26, 27). Other early signal transduction events observed after CD28 ligation include activation of phospholipase Cγ1 (28), raf-1 kinase (29), $p21^{ras}$ (30), and increase of Ca^{2+} and inositol (1, 4, 5) P_3 (28, 31). Recent studies have shown that CD28 itself becomes phosphorylated at Try191, which is essential for the interaction with the p85 subunit of PI 3-kinase (26, 27). While TCR activation of tyrosine kinases leads to CD28 Tyr191 phosphorylation, the identity of the kinase that phosphorylates CD28 remains unknown. It has been recently proposed that at least two signaling pathways are coupled to CD28 depending on the degree of CD28 phosphorylation and the state of cellular activation (20). This model reconciles previously noted discrepancies of CD28 signal transfer reactions observed with resting T cells versus T cell blasts or transformed T cells. It remains to be shown, however, which of these CD28 signaling pathways functions as a costimulatory signal for the TCR complex.

Costimulatory signals are required for expression of the high affinity IL-2 receptor, IL-2 production, and a proliferative T cell response (1, 2, 8, 32, 33). These events are greatly

L.-M. Boucher and K. Wiegmann contributed equally to this work.

[1] *Abbreviations used in this paper:* A-SMase, acidic sphingomyelinase; CAT, chloramphenicol acetyltransferase; NF, nuclear factor; PI 3-kinase, phosphatidylinositol 3-kinase; SM, sphingomyelin.

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diminished in T cells of mice lacking CD28 cell surface expression subsequent to the targeted disruption of the CD28 gene (34). It has been shown that TCR and CD28 stimulation together lead to the activation of the nuclear factor-κB (NF-κB), which has been directly implicated in the transcription of the IL-2 and the IL-2 receptor α gene (8, 35–37). Recently, we have shown that the TNF-dependent activation of NF-κB is mediated via an acidic sphingomyelinase A (A-SMase) (38, 39). This enzyme catalyzes the breakdown of sphingomyelin to phosphorylcholine and ceramide that eventually triggers the proteolytic degradation of I-κB required for the subsequent nuclear translocation of NF-κb (39, 40). These observations prompted us to investigate whether the A-SMase is also involved in the CD28 signal pathway of NF-κB activation.

Here we show that CD28 triggers A-SMase activation in resting as well as in activated T cells. In contrast, A-SMase is not activated by CD3 ligation. Overexpression of recombinant A-SMase is shown to substitute for CD28 in the NF-κB activation pathway. Evidence is provided that A-SMase triggering by CD28 may represent an important costimulatory signal for T cell activation.

Materials and Methods

Cell Culture and Reagents. The basic culture media consisted of a mixture of Click's/RPMI 1640 (50%/50% vol/vol) supplemented with 5% FCS, 2 mM glutamine, 0.1 mM mercaptoethanol, and 50 μg/ml each of penicillin and streptomycin. The human acute leukemic T cell line Jurkat (obtained from the American Type Culture Collection, Rockville, MD) was maintained in culture medium in a humidified incubator containing 5% CO_2.

Hamster mAb IgG anti-murine CD28 clone 37.51 was obtained from PharMingen (San Diego, CA), and hamster mAb anti-murine CD3 (hybridoma 145-2C11) was kindly provided by Dr. J. Bluestone (Ben May Institute, University of Chicago, Chicago, IL). Murine IgG1 mAb anti-human CD2 (clone 6F10.3), murine IgG2a mAb anti-human CD3 (clone X35), murine mAb IgG1 anti-human CD28 clone 28.2, rabbit IgG anti-hamster $F(ab)_2$ fragment cross-linking Ab, and goat IgG (H+L) anti-murine cross-linking Ab were obtained from Dianova (Hamburg, Germany).

Highly purified recombinant TNF-α was kindly provided by Dr. G. Adolf, Boehringer Research Institute, Vienna, Austria. Monensin, chloroquine, β-glycerophosphate, *p*-nitrophenyl phosphate, leupeptin, pepstatin, and trypsin-chymotrypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, MO). [*N*-methyl-^{14}C]sphingomyelin from bovine origin was obtained from Amersham International (Braunschweig, Germany).

Inbred BALB/c mice were obtained from Bomholtgard (Rye, Denmark). Mice of both sexes ranging in age from 6 to 10 wk old were used.

Preparation of Peripheral T Cells. Single cell suspensions of spleens and lymph nodes from BALB/c mice were prepared and 1×10^8 cells/ml were diluted in PBS containing 2.5% FCS and 3 mM EDTA. For T cell enrichment negative selecting columns (MTCC-1000; R&D Systems, Minneapolis, MN) were used following the instructions of the manufacturer. Human PBL were obtained from normal donors by leukapheresis and purified on Ficoll-Hypaque gradients. For depletion of adherent cells, human PBL were subjected to plastic adherence for 2 h at 37°C. In some experiments, T cells were activated with 1 μg/ml PHA and cultured for >10 d in the presence of recombinant IL-2 at 10 U/ml. Greater than 98% of these cells expressed $CD3^+/CD4^+$ or $CD3^+/CD8^+$ phenotypes as detected by indirect immunofluorescence analysis (data not shown).

Proliferation Assay. 50,000 Balb/c splenocytes were stimulated in 200 μl of culture media with 0.5 μg/ml anti-CD3 or 1 μg/ml anti-CD28 mAb. At time 0 and 9 h, cells were pulsed with dialyzed acidic SMase from human placenta (Sigma Chemical Co.). A-SMase was dialyzed for 18 h at 4°C against PBS. Thereafter the enzyme activity was quantified against standard, nondialyzed SMase activity. At day 3, cells were pulsed for 8 h with [^{3}H]thymidine (1 μCi/well). Cells were harvested and the amount of [^{3}H]thymidine incorporated was measured using a β-counter.

Sphingomyelinase Assays. Acidic and neutral sphingomyelinase were assayed as recently described (41). Briefly, cells were serum starved for 2 h in culture medium supplemented with 2% BSA. Aliquots of 5×10^6 cells were treated at 37°C for the indicated times with mAb anti-CD28, anti-CD2, or anti-CD3 premixed with cross-linking Ab by incubation for 15 min on ice. The final concentration each of anti-CD28, anti-CD2, or anti-CD3 was 2 μg/ml and that of cross-linking Abs was 8 μg/ml. Stimulation was stopped by immersion of samples in methanol/dry ice (−70°C) for 10 s followed by a 15-s centrifugation in a microfuge. Cells were lysed in two distinct buffer systems as described (41). The protein content of the supernatants was measured using a bicinchoninic acid assay (Pierce Chemical Co., Hamburg, Germany) with BSA as standard. Equal amounts of protein (between 15 and 50 μg) from cellular lysates were added to 52.25 μl of reaction buffer containing 250 mM sodium acetate, 1 mM EDTA (pH 5.0) for A-SMase measurements. For N-SMase assays, the buffer contained 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 250 μM ATP. [*N*-methyl-^{14}C]sphingomyelin (56 mCi/mmol) was added (1.1 μCi/ml final concentration) and the reaction mixtures were incubated at 37°C for 2 h. The reactions were stopped by addition of 800 μl $CHCl_3$/methanol (2:1) and 250 μl H_2O. Finally, they were vortexed and microfuged at 14,000 rpm for 2 min. To quantify the sphingomyelinase activity, 200 μl of aqueous phase, containing the [^{14}C]phosphorylcholine released by the enzyme, was counted using a β-counter (Canberra-Packard, Downers Grove, IL). Alternatively, the amount of [^{14}C]phosphorylcholine produced was analyzed by TLC using a solvent system CH_3OH/0.5% NaCl/NH_4OH (50:50:1). Phosphorylcholine, glycerophosphorylcholine, acetylcholine, and choline were used as standards. The amount of [^{14}C]phosphorylcholine produced was visualized by autoradiography.

Mass Measurements of Ceramide and Sphingomyelin. The neutral lipid cleavage product of SMases, ceramide, was measured by charring densitometry of TLC plates as recently described (38). Briefly, phospholipids were extracted according to the method described by Bligh and Dyer (42). Neutral lipids were separated from phospholipids in a two-phase methanol-hexane system. For examination of ceramide, neutral lipids were separated by TLC using a solvent system of CH_3Cl/CH_3OH/7 N NH_4OH/H_2O (85:15:0.5:0.5). After chromatographic separation of lipids, TLC plates were dried and cooled down to room temperature. Plates were exposed for 15 s to a solution of 10% copper sulphate in 8% aqueous phosphoric acid. The lipids were charred by heating the TLC plate for 10 min at 175°C. Densitometry of charred bands was performed by two-dimensional laser scanning (Personal Densitometer with ImageQuant 3.22; Molecular Dynamics, Krefeld, Germany). Calculations of total masses were performed by use of an exogenous ceramide standard (Sigma Chemical Co.). Sphingomyelin was extracted with the phospholipid fraction and analyzed by TLC using

the solvent system $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (100:60:20:5). Sphingomyelin, phosphatidylcholine, phosphatidic acid, phosphoinositol, phosphoethanol, and lysophosphatidylcholine (Sigma Chemical Co.) were used as standards and visualized by charring densitometry. For quantification of sphingomyelin, charred TLC plates were scanned by two-dimensional laser densitometry.

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared as recently described (39). The protein concentration was measured using a bicinchoninic assay (Pierce Chemical Co.) with BSA as standard. The NF-κB–specific oligonucleotide, containing the two tandemly arranged NF-κB–binding sites of the HIV-1 long terminal repeat enhancer (5'-ATCAGGGACTTTCCGCTGGGGACTTTCCG-3') and its complementary oligonucleotide was synthesized on a DNA synthesizer 381A (Applied Biosystems, Weiterstadt, Germany). The oligo was end labeled with [γ-^{32}P]ATP (Amersham International) using T4 polynucleotide kinase (Boehringer Mannheim, Mannheim, Germany) and annealed to a 10-fold excess of its complementary strand to obtain an end-labeled double-stranded oligonucleotide probe.

Electrophoretic mobility shift assays were performed by incubating 6 μg of nuclear extract with 4 μg of poly(dI-dC) (Pharmacia, Freiburg, Germany) in a binding buffer (5 mM Hepes [pH 7.8], 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM dithiothreitol, and 10% glycerol [20 μl final vol]) for 20 min at room temperature. The end-labeled double-stranded oligonucleotide probe (1×10^4 to 5×10^4 cpm) was then added, and the reaction mixture was incubated for 7 min at room temperature. The samples were separated by native polyacrylamide gel electrophoresis in low ionic strength buffer (0.25× Tris-borate-EDTA).

Plasmids, Transfections, and CAT Assays. A 4× κB HIV/Ig-CAT reporter plasmid containing four tandemly arranged NF-κB binding sites of the HIV/Ig enhancer linked to the CAT gene (43) was kindly provided by Dr. R. Schmid (Ulm, Germany). The reporter plasmid was used to measure functionally NF-κB activation in Jurkat cells cotransfected with a human acidic sphingomyelinase expression plasmid pHASMase. To generate pHASMase, the full-length cDNA for human A-SMase (44, kindly provided by Dr. K. Sandhoff) was cloned in both orientations into the high level mammalian expression plasmid pEF-BOS (45, kindly provided Dr. S. Nagata). Jurkat cells were transfected using by DEAE-dextran with 2.5 μg of the 4× κB HIV/Ig-chloramphenicol acetyltransferase (CAT) plasmid and increasing amounts of A-SMase expression plasmid. The native pEF-BOS plasmid was used to keep a total amount of 5 μg DNA transfected. After transfection, cells were grown for 2 h at 37°C in culture medium. Cells were then left either untreated or treated with PMA (20 ng/ml) for 4 h. The actual CAT activities were measured in crude cellular extracts using [^{14}C]chloramphenicol (Amersham International) as substrate followed by TLC to sep-

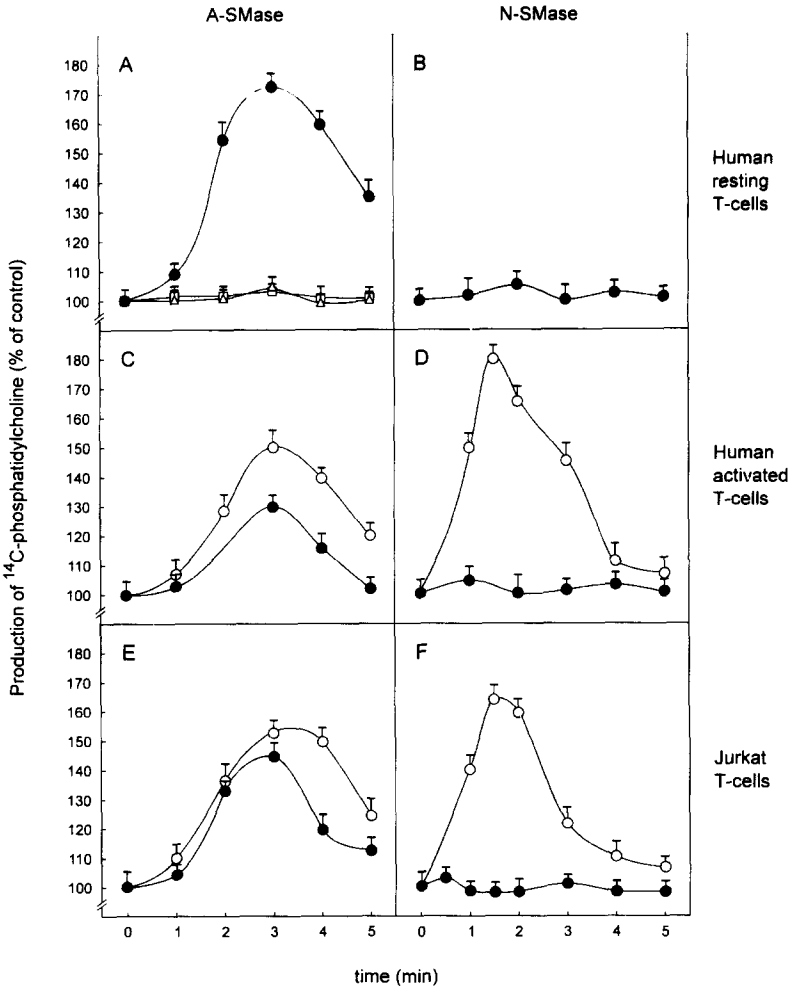


Figure 1. CD28 crosslinking activates acidic SMase in human T cells. Triplicates of 1×10^7/ml primary resting T cells (*A* and *B*), 10-d PHA-stimulated T cells (*C* and *D*), or Jurkat T cells (*E* and *F*) were left either untreated, or were stimulated by CD28 cross-linking (●), or treated with 100 ng/ml recombinant TNF (○). (*A*) Human resting T cells were treated with anti-CD28 alone (2 μg/ml) (△) or incubated with the secondary cross-linking Ab (□). Cellular lysates were prepared as described in Materials and Methods and acidic SMase and neutral SMase were measured using radiolabeled [^{14}C]sphingomyelin. The increase in SMase activity is estimated from the amount of radioactive phosphorylcholine released (100% = amount released at time 0). Basal levels of [^{14}C]phosphorylcholine production were (mg^{-1} protein × h^{-1}): 2.5 nmol (*A*), 207 pmol (*B*), 3.33 nmol (*C*), 195 pmol (*D*), 2.34 nmol (*E*), and 180 pmol (*F*). The bars indicate SEM ($n \geq 3$).

arate the native and acetylated forms, as described (46). For quantification, autoradiographs were analyzed by two-dimensional laser scanning.

Results

Activation of A-SMase by CD28 Cross-linking. Human resting peripheral T cells were stimulated with anti-CD28 antibodies in the presence or absence of secondary cross-linking antibodies and assayed for acidic and neutral SMase activities using exogenous radiolabeled sphingomyelin in a micellar system as recently described (41). As shown in Fig. 1 *A*, CD28 cross-linking by anti-mouse IgG antibodies resulted in the rapid induction of acidic SMase activity. Increments of A-SMase activity were detected as early as within 30 s and peaked at 3 min. In contrast, neither anti-CD28 alone nor secondary anti-mouse IgG enhanced A-SMase activity. Notably, CD28 cross-linking failed to activate the neutral SMase (Fig. 1 *B*). Similar kinetics of A-SMase activation were obtained with CD28-cross-linked primary splenic T cells derived from Balb/C mice (data not shown).

Since PI-3 kinase activation seems to depend on the extent of CD28 phosphorylation, that is, on the state of T cell activation, we next investigated the effects of CD28 cross-linking on A-SMase activity in PHA-stimulated cultured human peripheral T cells. As shown in Fig. 1 *C*, CD28 cross-linking of preactivated human T cells stimulated the activation of A-SMase. Notably, the amplitude of A-SMase activation was reproducibly found to be reduced when compared to that obtained with TNF or that observed with CD28-cross-linked resting T cells. Unlike TNF, CD28 failed to induce the activation of neutral SMase (Fig. 1 *D*).

The Jurkat leukemic T cell line has been extensively used for studying CD28 signaling. Jurkat T cells were treated with anti-CD28 and acidic as well as neutral SMase activities were measured. As shown in Fig. 1 *E*, CD28 cross-linking leads to the activation of the A-SMase. The transient activation of A-SMase by CD28 peaked between 2 and 3 min after cross-linking, which parallels the kinetics observed with primary human T cells. In addition, both amplitude and kinetics of A-SMase activation were comparable to the levels induced by TNF. N-SMase activation was not detected in CD28-cross-linked Jurkat T cells (Fig. 1 *F*). These data establish that CD28 can trigger acidic but not neutral SMase in resting T cells as well as in PHA-activated T cells or leukemic T cells.

To confirm A-SMase activation by CD28, water-soluble reaction products of the in vitro micellar assay system were analyzed by TLC. As shown in Fig. 2, CD28 cross-linking elicited an enzymatic activity that exclusively produced [^{14}C]phosphorylcholine identified by a parallel reaction using exogenous bacterial SMase. [^{14}C]choline was not detected, which argues against any phospholipase D activity that might have contributed to SM hydrolysis in the micellar assay system. Furthermore, when mass measurements of ceramide were performed with anti-CD28-stimulated Jurkat T cells, a time-dependent increase of cellular ceramide was detected, which stoichiometrically corresponded to a concomitant breakdown of sphingomyelin (Fig. 3).

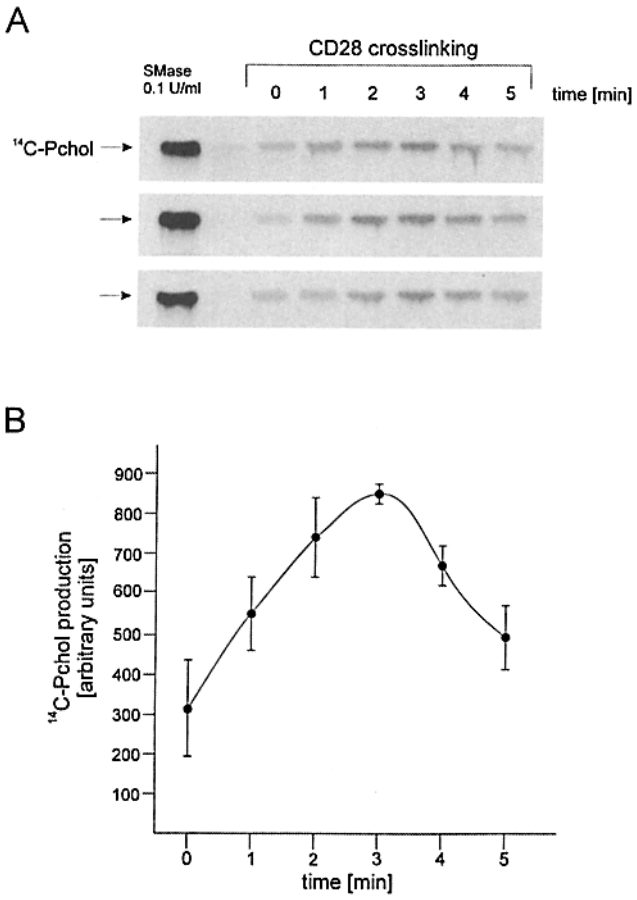
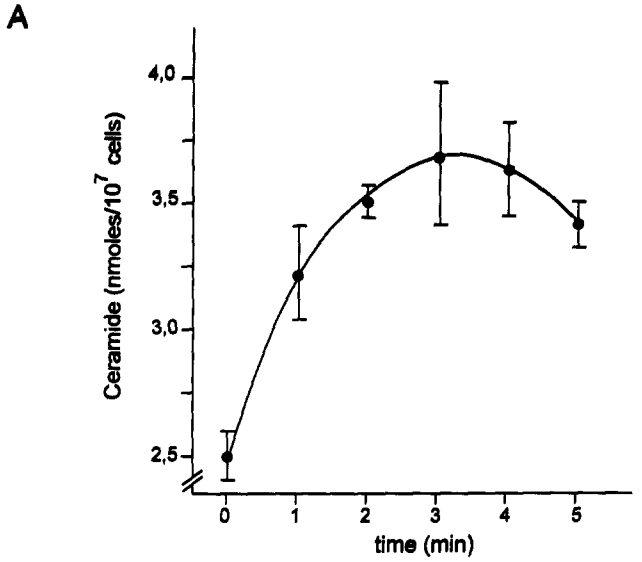


Figure 2. A-SMase activity in CD28-cross-linked Jurkat T cells analyzed by TLC. (*A*) Jurkat T cells were stimulated in triplicates by CD28 cross-linking. At the indicated times, cellular lysates were prepared and incubated with [^{14}C]sphingomyelin for 2 h. The aqueous phase was extracted and analyzed by TLC. In a parallel reaction [^{14}C]sphingomyelin was hydrolyzed by bacterial SMase to produce a radioactive phosphorylcholine standard ([^{14}C]Pchol, *arrow*). The [^{14}C]phosphorylcholine produced was visualized by autoradiography. (*B*) The bands of (*A*) representing the amount of [^{14}C]Pchol produced were quantitated by two-dimensional laser densitometer scanning. The bars indicate SEM (n = 3).

Finally, Jurkat T cells were preincubated for 1 h with the endolysomotropic agents monensin, chloroquine, and NH_4Cl. These agents have been shown to be inhibitory to the activation of the A-SMase following TNF stimulation (41), leaving the N-SMase unaffected. As shown in Fig. 4, each of the endolysomotropic agents completely abolished the activation of A-SMase by CD28 crosslinking. These findings therefore strongly support that CD28 cross-linking results in exclusive activation of A-SMase without engagement of the neutral sphingomyelinase pathway.

One of the most prominent effects of CD28 is to synergize with the TCR to optimally activate T cells. Strikingly, cross-linking of either CD3 or CD2 did not result in A-SMase activation (Fig. 5). Thus, the A-SMase may represent a costimulatory pathway, by which CD28 complements signaling through the TCR complex. To examine whether CD28 costimulation can be substituted for by exogenous SMase, mouse splenocytes were pulsed at 0 and at 9 h with increasing



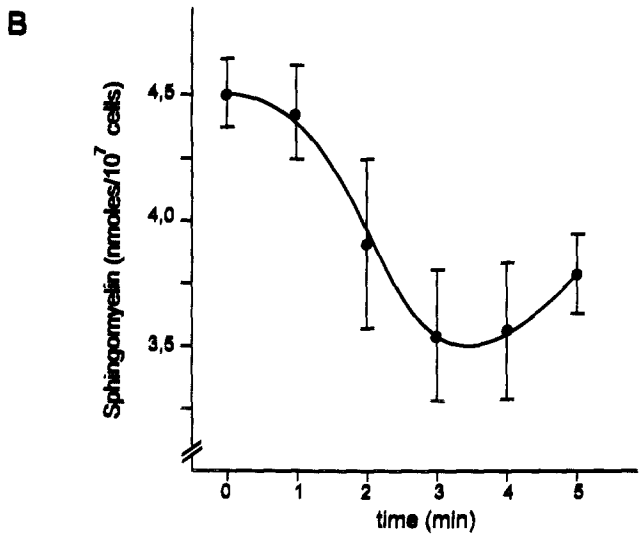


Figure 3. SMase activity in CD28-cross-linked Jurkat T cells analyzed by mass measurements of ceramide and sphingomyelin. Jurkat T cells were stimulated in triplicates by CD28 cross-linking. At the indicated times, neutral lipids and phospholipids were extracted and separated by TLC. To visualize neutral lipids and phospholipids, TLC plates were charred. For quantification, the charred bands were read by two-dimensional laser densitometry. Calculations of total masses were performed using exogenous ceramide and sphingomyelin as standards. The bars indicate SEM (n = 3).

concentrations of A-SMase from human placenta (Fig. 6). Exogenous A-SMase synergized with CD3 cross-linking to induce a dose-dependent proliferative response, which was comparable to that observed with cells treated with a combination of anti-CD3 and anti-CD28 mAb. Cells not exposed to any mAb and cells stimulated with anti-CD28 alone remained in a resting state, ruling out non-specific mitogenic

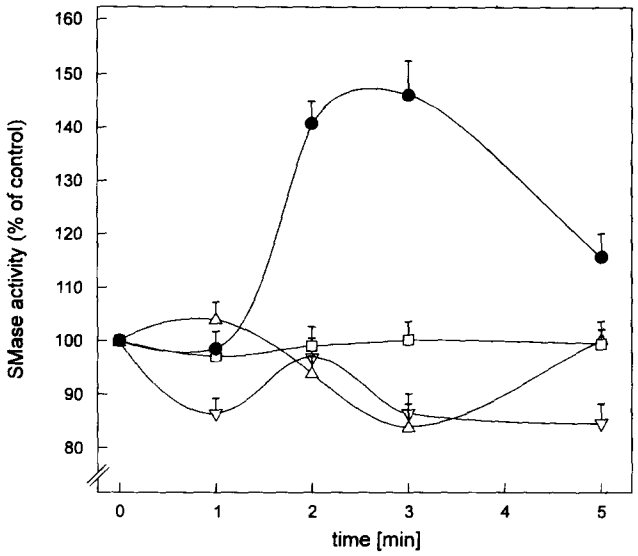


Figure 4. CD28-dependent activation of acidic SMase is inhibited by endolysomotropic agents. Jurkat cells were incubated for 1 h in the absence (●) or presence of either monensin (10 μg/ml) (△), chloroquine (2 mM) (□), or NH_4Cl (100 mM) (▽). Notably, chloroquine diminished the basal A-SMase activity by 45%. Cells were then left either untreated or stimulated by CD28 cross-linking. Acidic SMase activities were measured. Bars indicate SEM (n = 3).

effects of the SMase treatment. In addition, heat-inactivated A-SMase did not induce cell proliferation (data not shown), indicating the requirement for enzymatic activity. At concentrations >7 μg/ml exogenous A-SMase appeared to be cytotoxic.

Overexpression of Recombinant A-SMase Substitutes for CD28 Costimulatory Signals. On a molecular basis, TCR and CD28 costimulatory signaling has been shown to converge at the NF-κB (35, 47), which eventually precipitates in an increase of IL-2 production, IL-2 receptor expression, and enhancement of T lymphocyte proliferation. Confirming previous reports (35, 47), incubation of mouse primary T cells with either anti-CD3 or anti-CD28 alone did not result in significant NF-κB activation (Fig. 7). In contrast, combined anti-CD3 and anti-CD28 treatment revealed a strong synergism. The specificity of the retarded complexes was demonstrated by competition analysis with wild-type and mutated HIV-κB oligonucleotides (data not shown). No synergism of CD3 and CD28 cross-linking was observed with Jurkat T cells (Fig. 7 *B*). Instead, CD28 cross-linking strongly enhanced phorbol-ester-induced NF-κB activation as previously described (35, 47).

We next tested whether expression of recombinant human A-SMase can substitute for CD28 signaling. Transfection of Jurkat T cells with a human A-SMase expression plasmid, pHASMase (Fig. 8 *A*), resulted in elevated nuclear NF-κB activity (Fig. 7 *C*). The kinetics of pHASMase-directed A-SMase activities in Jurkat T cells presented in Fig. 8 *B* indicated that recombinant A-SMase was active within the first hour after transfection. Two-dimensional laser scanning revealed a 1.8-, 2.0-, and 2.1-fold increase of NF-κB activity

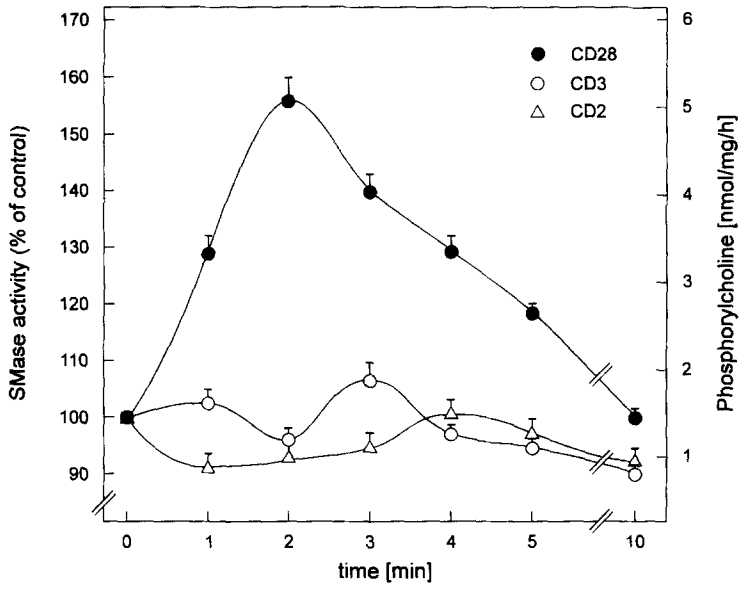


Figure 5. CD3 and CD2 fail to activate A-SMase in resting T cells. Human resting T cells were stimulated in triplicates by cross-linking of either CD28 (●), CD3 (○), or CD2 (△). At indicated times cellular lysates were prepared and analyzed for A-SMase activity. The bars indicate SEM (n = 3).

at 1 h, 2 h, or 4 h after transfection, respectively (data not shown). This suggests that A-SMase on its own can function as a weak activator of NF-κB. A control plasmid, pHASMase$_{rev}$, containing the A-SMase cDNA in opposite orientation, did not change NF-κB activity.

To address functionally the costimulatory potential of A-SMase, pHASMase and an NF-κB-CAT reporter plasmid were cotransfected into Jurkat T cells. As shown in Fig. 9, A-SMase dose-dependently enhanced transcription from the HIV/Ig-κB element. In addition, A-SMase cooperated with PMA-induced NF-κB activation, which corresponds well with the synergistic effects of CD28 and PMA on nuclear translocation of NF-κB (Fig. 7). In contrast, CD28 cross-linking alone did not result in NF-κB activation, nor did it synergize with the overexpressed A-SMase. CD28 ligation slightly increased PMA-induced NF-κB activation, which is likely due to a presumably higher frequency of CD28-cross-linked cells compared to the fraction of cells successfully transfected with the pHASMase plasmid.

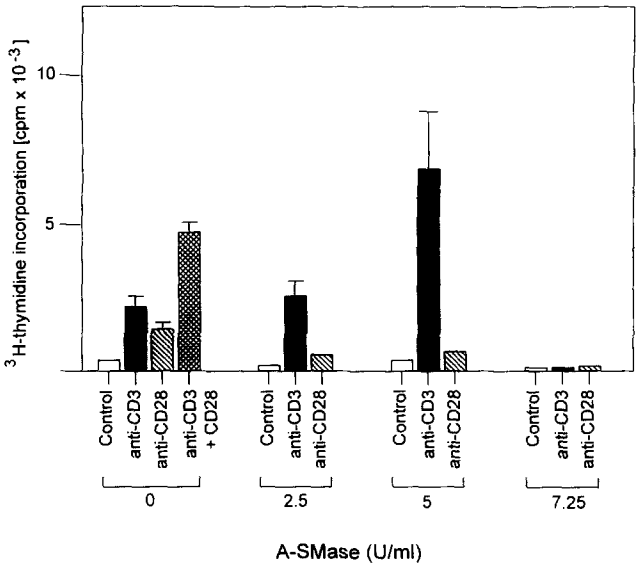


Figure 6. Cellular response to exogenous A-SMase. Triplicates of mouse spleen cell cultures were left untreated or stimulated for 3 d with anti-CD3 (0.5 μg/ml) or anti-CD28 (1 μg/ml). Cells were pulsed at time 0 and 9 h with A-SMase at the indicated concentrations. The rate of proliferation was measured by [^{3}H]thymidine incorporation. The bars represent SEM (n = 3). The results are representative for three independent experiments.

Discussion

CD28 engagement has been shown to have potentially great impact on the generation of an appropriate adaptive immune response (1, 2). This crucial interdependence between the TCR and CD28 may represent one possible mechanism for peripheral immune tolerance. Here we show that CD28 can induce rapid and transient activation of the acidic sphingomyelinase pathway in primary T cells. Because CD3 triggering did not elicit any ceramide production by A-SMase, this lipid messenger system can be viewed as a distinct costimulatory pathway.

Lipid second messenger systems are emerging as important signaling components in a variety of cellular systems. One possible entry for A-SMase action in T cell activation likely resides in the activation of the nuclear transcription factor NF-κB. This notion is confirmed by our observation that overexpression of recombinant A-SMase mimicked CD28 effects on NF-κB activation in Jurkat T cells. NF-κB controls the expression of a number of genes involved in T cell activation. In particular, NF-κB has been shown to regulate the expression of the genes encoding IL-2 and IL-2 receptor-α chain that are instrumental for T cell proliferation. This suggests that A-SMase represents a major mediator of CD28-induced T cell proliferation. The mitogenic effects of exoge-

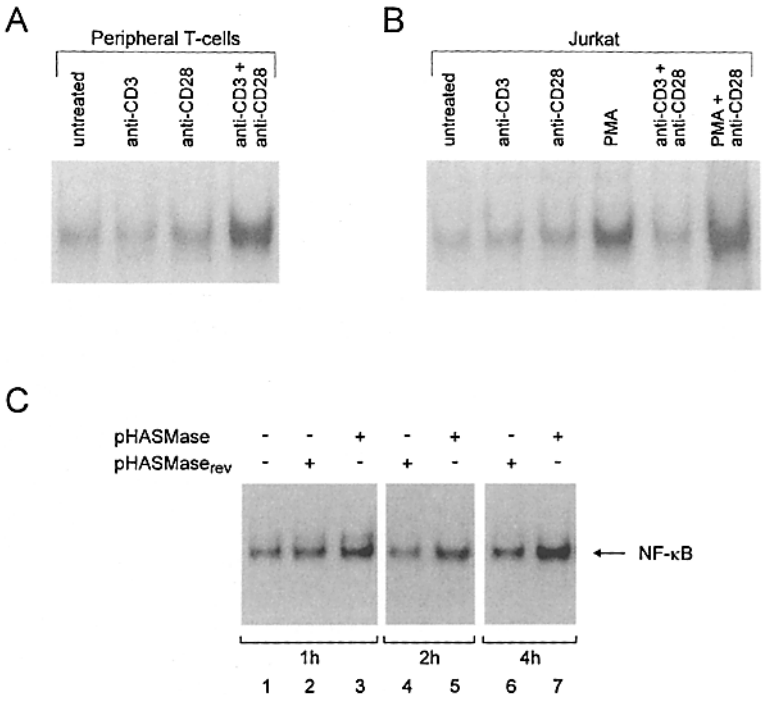


Figure 7. Costimulator effects of CD28 and A-SMase on NF-κB activation in mouse peripheral T cells and Jurkat T cells. Mouse peripheral T cells (*A*) or Jurkat T cells (*B*) were left untreated or treated for 8 h in the presence of anti-CD28 (1 μg/ml) and/or anti-CD3 (0.5 μg/ml) or PMA (5 ng/ml) at 37°C. (*C*) Jurkat T cells were transfected for indicated periods of time with A-SMase cDNA cloned into pEF-BOS in both orientations (see Fig. 8 *A*). Nuclear proteins were extracted and assayed for NF-κB activity.

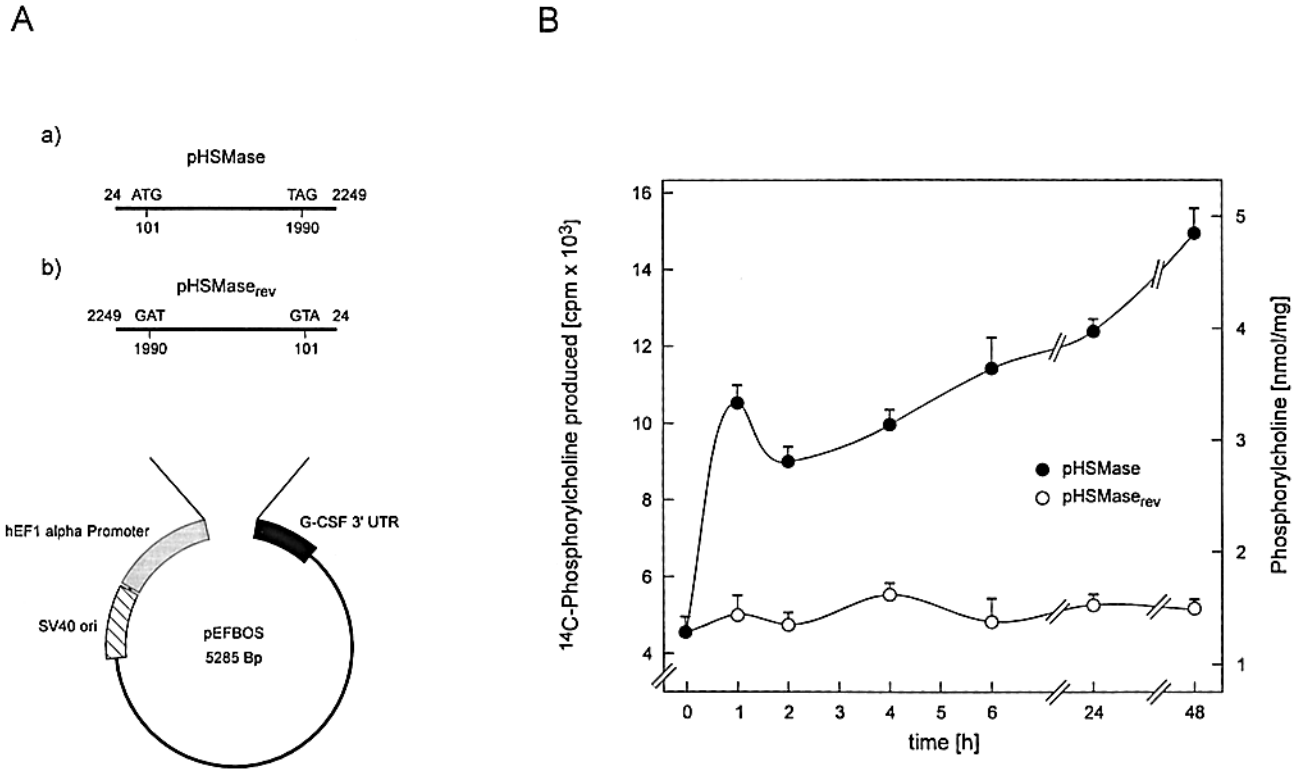


Figure 8. Expression of human A-SMase cDNA in Jurkat T cells. (*A*) The cDNA for human A-SMase (44) was cloned in both orientations into the expression plasmid pEF-BOS (45) to generate pHASMase and pHASMase$_{rev}$, respectively. (*B*) Jurkat T cells were transfected in triplicates with 5 μg of either pHASMase or pHASMase$_{rev}$. At the indicated times, cellular lysates were prepared and analyzed for A-SMase activity. The bars indicate SEM (n = 3).

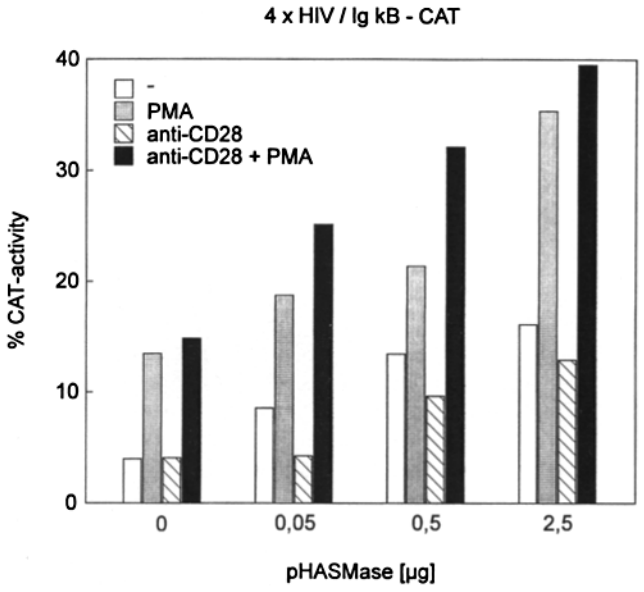


Figure 9. Overexpression of acidic SMase in Jurkat cells leads to activation of NF-κB. Jurkat T cells were cotransfected with 2.5 μg of HIV/Ig-κB-CAT reporter construct along with increasing concentrations of pHAS-Mase. After 2 h, cells were left either untreated (*Open bars*) or stimulated for 4 h at 37°C with PMA (20 ng/ml, *gray bars*), CD28 cross-linking (*hatched bars*), or a combination of PMA and CD28 cross-linking (*filled bars*). CAT activity of crude cellular extracts was measured using radiolabeled chloramphenicol. The results shown are derived from one representative experiment (n = 4).

nous A-SMase in the presence of anti-CD3 mAb (Fig. 6) appear to confirm the costimulatory nature of endosomal A-SMase activation by CD28 cross-linking. We wish to emphasize, however, that this interpretation deserves a precaution. Sphingomyelin (SM) is asymmetrically distributed in the plasma membrane: >95% of SM is found at the outer leaflet of the plasma membrane (48). Exogenous SMase treatment may thus cause extensive SM hydrolysis leading to membrane damage. This notion is illustrated by the cytotoxicity produced by A-SMase used at high concentrations (Fig. 6). Furthermore, SMases are also known as bacterial toxins like hemolysins, which destroy eukaryotic membranes (49). Thus, it cannot be excluded that the mitogenic effects of exogenous A-SMase may be secondary, at least in part, to a cellular stress response. Notwithstanding, expression of recombinant A-SMase clearly activated transcription from a NF-κB promoter. The internal treatment with A-SMase, which more closely resembles endogenous A-SMase action, suggested that A-SMase can mediate CD28-induced T cell activation and proliferation through activation of NF-κB.

A number of different types of CD28-dependent signal transfer reactions have been described. However, the specific signaling pathways complementing TCR function have not yet been identified. Costimulation by CD28 may be brought about by several pathways: (*a*) CD28 ligation may provide additional, distinct signaling systems like A-SMase; (*b*) CD28 may enhance the amplitude or duration of a TCR-triggered signal, thereby reaching a threshold to activate further downstream signaling cascades; and (*c*) CD28 may provide signals similar to the TCR complex yet at different time points. This may result in repetitive stimulation required to maintain a proliferative or functional activation status. Unlike A-SMase, other signaling pathways ascribed to CD28 appear to be also engaged by the TCR. For example, PI 3-kinase is also coupled to TCR signaling through tyrosine kinases, such as lck (50). Further, PLCγ1 and raf-1 kinase are inducible by cross-linking of either CD28 or TCR (28, 29). Given that the TCR and CD28 obviously share quite a number of signaling cascades, synergistic effects may involve any of the above-mentioned cooperative pathways. The activation of A-SMase may be essential to maintain some degree of specificity of the CD28 signal. It is important to emphasize that CD28 ligation led to A-SMase activation in resting T lymphocytes as well as in T cell blasts or transformed Jurkat T cells. Thus, A-SMase activation occurs independent of the T cell activation status. This is in contrast to the activation of PI 3-kinase, which requires TCR-induced phosphorylation of the cytoplasmic tail of CD28 at residue Tyr 191 (26, 27).

Interestingly, CD28 cross-linking did not result in activation of neutral sphingomyelinase. Neutral sphingomyelinase has been implicated in activation of phospholipase A_2 and arachidonic acid degradation (41). Arachidonic acid metabolites, such as leukotrienes or prostaglandins, have extensively been implicated in cytotoxicity and inflammatory processes. Proinflammatory cytokines like TNF and IL-1 activate both acidic and neutral sphingomyelinase (41). The stimulation of A-SMase in the absence of neutral sphingomyelinase activation is thus a property of CD28, which distinguishes this costimulatory molecule from cell surface receptors for proinflammatory cytokines.

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