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Regulation of ceramide generation during macrophage apoptosis by ASMase and de novo synthesis

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Abstract

The survival of macrophages depends on the presence of specific cytokines that activate survival signalling events, as well as suppressing formation of apoptosis-inducing pathways. We have previously shown that macrophages deprived of macrophage colony stimulating factor (M-CSF) produce ceramide that contributes to apoptosis of these cells, a pathway that is suppressed by exposure to oxidized LDL. In this study we have examined macrophages derived from mice lacking acid sphingomyelinase (ASMase) to ask whether these events are altered due to the impaired ability of these cells to break down sphingomyelin and produce ceramide. We found that these cells do survive better than cells from wild type mice, but they still undergo cell death and some ceramide is formed. We show that the ceramide is being produced by a de novo synthetic pathway. Therefore, ceramide production in M-CSF-deprived macrophages arises from a combination of ASMase activity and de novo synthesis.

Introduction

Ceramide is a key mediator of apoptosis triggered by various stimuli such as ionizing radiation, TNF α , chemotherapeutic agents and Fas ligand [1–4]. Ceramide is thought to induce apoptosis either as a second messenger or by modulating membrane structure and dynamics. Some evidence suggests that ceramide may mediate raft clustering into macrodomains for transmembrane signaling, or alternatively, it may promote mitochondrial membrane permeability and channel formation for cytochrome c release [5].

Ceramide can be generated from the hydrolysis of sphingomyelin (SM) via the activity of sphingomyelinases (SMase). Acid sphingomyelinase (ASMase) is a lysosomal enzyme that

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Conflict of interest

None of the authors has any conflict of interest to report in relation to the work in this manuscript.

is one type of SMase that also includes neutral and alkaline SMases [6]. Deficient ASMase activity is the cause of human type A and B Niemann-Pick disease (NPD) in which SM degradation is impaired [7, 8]. It has been shown that ASMase activity is essential for some forms of ceramide-mediated apoptosis. For example, cells from NPD patients or ASMase^{-/-} mice were resistant to ionizing radiation with regard to ceramide generation and apoptosis [9, 10] [7, 9, 10]. Furthermore, ASMase^{-/-} mice also showed defects in ceramide generation and apoptosis in lung endothelium [7] and throughout the central nervous system [11]. MEF cells derived from these mice are resistant to apoptosis induced by some, but not all, stresses [12]. Interestingly, thymic cells from ASMase^{-/-} mice remain sensitive to apoptosis induced by ionizing radiation [7].

Ceramide can also be produced from the *de novo* synthesis pathway regulated by enzymes such as serine palmitoyltransferase (SPT). SPT catalyzes the condensation of serine and palmitoyl-CoA which is the first step of the pathway. *De novo* synthesis of ceramide has been implicated in responses to TNF α [13], heat shock [14], exogenous ceramide [15], and several chemotherapeutic agents [16, 17]. In addition to these pathways, ceramides can also be generated by the reversed activity of neutral ceramidase [18], or by the sphingosine salvage pathway through the action of ceramide synthases [19].

We showed previously that ASMase activation and ceramide generation are involved in apoptosis of bone marrow-derived macrophages (BMDM) induced by growth factor withdrawal [20, 21]. The regulation of macrophage apoptosis has relevance in understanding of macrophage death that occurs in the absence of survival cytokines, such as during resolution of an inflammatory response. Therefore, to further investigate ceramide production in cells undergoing apoptosis, we used BMDM generated from ASMase^{-/-} mice. In the present study we demonstrate that loss of ASMase confers partial resistance to apoptosis, with less ceramide being generated in response to growth factor withdrawal. In addition, we present evidence indicating that the *de novo* pathway of ceramide synthesis is implicated in the accumulation of ceramide in BMDM undergoing apoptosis.

Materials and methods

Materials

Anti-SPTLC1 antibody was purchased from Santa Cruz Biotechnology while anti-SPTLC2 antibody was from Sigma-Aldrich. *Escherichia coli* diacylglycerol kinase, β -octyl glucoside and all other inhibitors were supplied by Calbiochem. Propidium iodide, protease inhibitor cocktail, non-hydroxyl fatty acid ceramide, ceramide-1-phosphate, Anti-Vinculin antibody and RPMI 1640 medium were purchased from Sigma-Aldrich. Caspases FLICA kit was from Immunochemistry Technologies. Fetal bovine serum (FBS) were obtained from Invitrogen. Reagents required for 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium innersalt (MTS) cell viability assays were purchased from Promega. C2-ceramide, C2-dihydroceramide, cardiolipin were purchased from Avanti Polar. [γ -³²P]ATP, [³H]serine, [¹⁴C]palmitoyl-CoA, [³H]palmitate and [*N-methyl*-¹⁴C] bovine sphingomyelin were purchased from Perkin-Elmer NEN.

Cell culture

CD1 and C57BL/6 mice were obtained from the UBC animal facility. ASMase knockout mice were generated in the laboratory of Ed Schuchman of the Mt. Sinai School of Medicine [7].

Bone marrow was harvested from the femurs of 6–8 week old female mice as described [20]. Cells were plated overnight in RPMI 1640 supplemented with 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 100 units/ml penicillin/streptomycin and 10% L-cell conditioned medium as the source of M-CSF. Non-adherent cells were removed and cultured in the above medium until 80% confluence was reached (4–6 days).

Genotyping

ASMase^{-/-} mice were genotyped by PCR [22]. Genomic DNA was mixed with an ASMase sense primer (PS; 5'-AGCCGTGTCCTCTTCCTTAC-3') and two antisense primers, one from within exon 2 of the ASM gene (PA1; 5'-CGAGACTGTTGCCAGACATC-3') and one from within the *neo* cassette (PA2; 5'-CGCTACCCGTGATATTGCTG-3'). Thirty cycles of PCR amplification, each consisting of 1 min at 93 °C, 1 min at 58 °C, and 1 min at 72 °C, were performed. In wild-type mice, a single band of 269 bp corresponding to the undisrupted ASM gene was amplified, while in ASM^{-/-} mice a single band of 523 bp was amplified from the sense and *neo* primers.

Cell viability assay

When 80% confluence was reached, BMDM were harvested using a rubber cell scraper. 5×10^4 cells per well were seeded into 96-well plates and incubated overnight. Cells were then washed with PBS, and drugs in the absence or presence of PTX were added in 100 μ l of the same medium except without M-CSF. At the end of the 24 hour incubation, 20 μ l of MTS/PMS solution (prepared according to the manufacturer's instructions) was added to each microwell. The plate was incubated for 1–4 hours at 37°C and was read using an ELISA plate reader at 490 nm. We previously showed that the bio-reduction rate of MTS is linearly correlated with the number of viable macrophages [23].

Flow cytometric analysis

One million cells were seeded in 6 well plates, incubated under the indicated conditions for 24 hours, harvested with a rubber scraper, and pelleted by centrifugation. To assess DNA fragmentation, cells were fixed with cold 70% ethanol for 10 minutes, and resuspended in propidium iodide staining solution (10 μ g/ml RNaseA, 20 μ g/ml PI, in PBS + 0.1% glucose). To quantify phosphatidylserine externalization, macrophages were incubated with annexin V-FITC according to the manufacturer's instructions. Measurement of caspase activation was carried out with a fluorescent-labeled indicator kit, FLICA (Immunochemistry), and assayed by flow cytometry according to manufacturer's instructions. Cells were analyzed by Beckman Coulter flow cytometer (EPICS XL-MCL) on the FL3 channel for DNA content, on the FL1 channel for FITC fluorescence with ten thousand events counted for each analysis.

Lipid labelling for determination of ceramide levels

Radioactivity in ceramide was assayed after labeling of BMDM with 5 $\mu\text{Ci}/\text{ml}$ of [^3H]palmitate for 24 h in RPMI 1640 without or with 10% FBS and 10% M-CSF. The cells were washed twice with PBS and scraped into 1 ml of methanol, which was then mixed with 1 ml of chloroform and 0.9 ml of 2M KCl, 0.2M H_3PO_4 [24]. The aqueous phase was discarded, and the chloroform phase was dried under nitrogen. Ceramides were isolated by TLC by using Silica Gel 60-coated glass plates developed with chloroform/methanol/acetic acid (9:1:1 by volume) for half their length and then with petroleum ether/diethyl ether/acetic acid (60:40:1 by volume). Lipids were visualized by iodine and identified by co-chromatography with authentic standards. Radioactivity was measured by scraping the corresponding bands from TLC plates and liquid scintillation counting.

Immunoblotting

For immunoblotting whole cell lysates, 1.5 million cells were washed with PBS and lysed in 50 μl of ice-cold 20 mM Tris HCL pH 8.0, 1% NP40, 10% glycerol, 137 mM NaCl, 10 mM NaF supplemented with protease inhibitor cocktail and 200 μM sodium vanadate. The cells were then sonicated for 5 seconds and centrifuged at $23,000 \times g$ for 5 min. The supernatant was collected and assayed for protein concentration. The extracted proteins were adjusted to equal concentration and were boiled in SDS sample buffer for 5 min. 50 μg of cell lysate was loaded in each lane of a 12% SDS-polyacrylamide gel. Transfers were done by semi-dry blotting onto nitrocellulose membranes. The membranes were blocked for one hour in 5% low fat dry milk in Tris-buffered saline with 0.05 % Tween 20 followed by overnight incubation at 4°C with appropriate antibody. Bound antibody was visualized with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies and enhanced chemiluminescence.

Diglyceride Kinase assay for ceramide mass

Ceramide levels were measured using the diglyceride kinase method as described previously [17, 25]. In brief, total cellular lipids were extracted with chloroform/methanol/2 M KCl, 0.2M HCl, resuspended in a micellar solution of 1 mM cardiolipin, 1.5% N-octyl- β -D-glucopyranoside, 0.2 mM DETAPAC, 5 μg of diacylglycerol kinase and 10mM DTT. The reaction was initiated with 1 μCi of [γ - ^{32}P]ATP diluted with unlabelled ATP to give a final concentration of 1 mM. After incubation for 45 min at 30°C , lipids were extracted and separated on Silica Gel 60 TLC plates with chloroform/methanol/2N NH_4OH (65:35:7.5). The plates were dried and redeveloped with chloroform/acetone/ methanol/acetic acid/water (50:20:10:10:5). Ceramide-1-phosphate spots were scraped from the plates and quantitated by scintillation counting. The assay was calibrated with a standard curve of authentic ceramide. Results were normalized to total lipid phosphate. To measure lipid phosphate, the chloroform phase of the cell extract was evaporated under N_2 , and incubated at 180°C for 30 min in 50 μl of 70% HClO_4 . Then 278 μl of H_2O , 55 μl of 2.5% ammonium molybdate, and 55 μl of 10% ascorbic acid were added and incubated for a further 15 min at 95°C . Inorganic phosphate was detected by absorbance at 700 nm and quantified based upon a standard curve of glycerol phosphate.

Microsome preparation for in vitro serine palmitoyltransferase and ceramide synthase assays

Microsomes were prepared by sonication of cell pellets in 50 mM HEPES, pH 7.4, 10 mM EDTA, 5 mM DTT, and 0.25 M sucrose supplemented with protease inhibitor cocktail from Sigma-Aldrich. The preparation was centrifuged at $1000 \times g$, and the resulting supernatant was then ultracentrifuged at $100,000 \times g$. The resultant pellet was suspended by homogenization in 50mM HEPES, pH 7.4, 5 mM DTT buffer containing 20% glycerol to form microsomes for the assays. Protein concentration was determined using the Bio-Rad dye protein assay reagents with a standard curve of bovine serum albumin.

Serine palmitoyltransferase assay

Serine palmitoyltransferase was assayed as described previously [26]. Briefly, enzyme activity in 50–100 μ g of microsomal membranes was determined in 100 mM HEPES (pH 8.3), 5 mM DTT, 2.5 mM EDTA (pH 7.0), and 50 μ M pyridoxal 5'-phosphate. The reaction was initiated by the addition of 200 μ M palmitoyl CoA and 3 μ Ci of L-[3 H]serine, with a final serine concentration of 1 mM. Reactions were incubated for 20 min at 37 °C prior to termination with 1.5 ml of chloroform/methanol (1:2) followed by 1 ml of chloroform and 1.8 ml of 0.5 N NH_4OH with sphinganine as carrier. Lipids were extracted as described previously and quantified by liquid scintillation counting [26].

Statistical analysis

Results were expressed as means $\pm$ SD. Statistical analysis was done by Student's *t*-test as appropriate. A *P* value of less than 0.05 was taken as significant.

Results

ASMase is partly responsible for ceramide generated in response to M-CSF withdrawal in BMDM

We have previously shown that increased ceramide production following M-CSF withdrawal was due to the activity of ASMase [20]. Inhibition of ASMase activity by oxLDL or desipramine [20], or ceramide 1-phosphate [21] increased cell viability. To further elucidate the role ASMase plays in the BMDM apoptosis, we used ASMase knockout mice [7, 27]. As one would predict, in ASMase knockout BMDM cells, the increase in ceramide content resulting from M-CSF withdrawal was less than that in wild type cells (Figure 1A). When the extent of cell death following M-CSF withdrawal was monitored, the lack of ASMase resulted in cells that were more resistant to cell death (Figure 1B). In further characterizing the apoptotic status of BMDMs following M-CSF withdrawal, the ASMase $^{-/-}$ cells were partially protected from apoptosis as reflected by DNA fragmentation (Figure 2A), and they also showed less caspase 9 activation (Figure 2B). It should be noted that in pilot experiments, cells from ASMase $+/+$ heterozygous mice were tested and did not show a significant difference from wild type cells.

Accumulation of ceramide mass from *de novo* synthesis upon M-CSF withdrawal

It is noteworthy that in ASMase $-/-$ cells, ceramide generation was only partially blocked even though these cells are totally deficient in ASMase. To verify that ASMase activity was undetectable in the knockout cells, we performed *in vitro* assays of SMase activity in cell lysates. We confirmed that ASMase activity in wild type cells increased in response to M-CSF withdrawal while this activity was absent in ASMase $-/-$ cells (Fig. 3A). Ceramide generation in response to stimuli such as TNF- α has been attributed to neutral sphingomyelinase (NSMase) activity [28], and therefore we also assayed NSMase in the lysates. NSMase activity in ASMase $-/-$ cells is low and actually decreases in response to M-CSF withdrawal so it cannot account for the ceramide generation by means of SM hydrolysis in ASMase knockout cells (Figure 3B). While suggestive, the lack of increase in NSMase does not completely rule out a role for this enzyme in these processes.

As we failed to find an increase in SMase activity in ASMase $-/-$ macrophages after MCSF deprivation, the obvious alternative explanation for increased ceramide radioactivity was that ceramide synthesis was increased. Ceramide production in response to death stimuli such as daunorubicin [16], etoposide [17], heat shock [14] or photodynamic therapy [29] is reported to be due to accelerated *de novo* synthesis, not increased SM degradation. We have also found that ceramide mass increased rapidly after M-CSF withdrawal and Fumonisin B1 (FB1), an inhibitor of ceramide synthase, blocked the accumulation of ceramide after M-CSF withdrawal (data not shown).

When [3 H] palmitoyl-sphingosine, or C16-ceramide, was monitored following M-CSF withdrawal, the increase observed in the ASM $-/-$ cells was significantly affected by inhibitors of ceramide production. Incubation with FB1 in the absence of M-CSF almost completely abolished the increase in [3 H] C16-ceramide in ASMase $-/-$ BMDM (Figure 4). Incubation with the serine palmitoyl transferase (SPT) inhibitor, myriocin (Myr) also inhibited the M-CSF withdrawal-induced ceramide increase in ASMase $-/-$ BMDM. Both inhibitors also reduced the [3 H] C16-ceramide accumulation in wild type BMDM although not to the level seen in control cells incubated with M-CSF. The lesser effect of the inhibitors on ceramide generation in the wild type cells likely reflects the action of ASMase.

M-CSF withdrawal induces ceramide generation through serine palmitoyltransferase (SPT) activation

A major regulator of the *de novo* ceramide synthesis pathway is SPT. In an independent assay we verified that ceramide mass increased rapidly after M-CSF withdrawal (Figure 5 A and B) and thus the finding that production of ceramide upon M-CSF withdrawal can be attenuated by inhibitors of *de novo* ceramide synthesis led us to investigate the implication of SPT in this pathway. SPT is the rate limiting enzyme for *de novo* ceramide synthesis [30] and its activity has been shown to be required for ceramide increase during etoposide-induced apoptosis [17]. Mammalian SPT is primarily a heterodimer composed of two protein subunits, SPTLC1 (53 kDa) and SPTLC2 (63 kDa). However, recent studies indicate the existence of a third subunit, SPTLC3, with 68% homology to SPTLC2. Nonetheless, the SPTLC3 subunit is almost negligible in hematopoietic cells including macrophages [31]. As shown in Figure 6A, SPT was activated as early as one hour after M-CSF withdrawal and

peaked at three hours. To ensure this observation was not due to increased protein expression of the enzyme, we looked at the two major subunits of SPT in macrophages, SPTLC1 and SPTLC2. Figure 6B demonstrated that the expression of neither changed during the time course.

Ceramide-1-phosphate inhibits ceramide generation despite the absence of ASMase by down-regulating SPT activity

We previously showed that ceramide-1-phosphate (C1P) can inhibit ceramide generation observed in BMDM when M-CSF is absent [21], a process involving activation of PKB [32]. Exogenous C1P inhibited ASMase in BMDMs at concentrations that also prevented apoptosis [21]. To determine whether C1P also inhibits *de novo* ceramide synthesis, we treated both ASMase^{+/+} and ASMase^{-/-} BMDM with C1P. Ceramide accumulation was dramatically inhibited and C1P also rescued cells from apoptosis after M-CSF withdrawal in BMDM either with and without ASMase (Figure 7). We then determined whether C1P blocked the ceramide accumulation via down-regulation of SPT activity. We found that while BMDM starved of M-CSF showed an increase in SPT activity of 1.67 ± 0.26 fold (mean $\pm$ SD, n=3), with C1P treatment, SPT activity dropped to 1.08 ± 0.22 fold (mean $\pm$ SD, n=3).

Discussion

In this study, we have shown that in the absence of ASMase, BMDM generated less ceramide and were partially resistant to apoptosis after M-CSF withdrawal. However, there was detectable ceramide generation even though we confirmed there was no ASMase and a decrease in NSMase activity in ASMase^{-/-} cells. We therefore hypothesized that the ceramide accumulation was likely due to *de novo* synthesis in the ASMase^{-/-} cells.

Our observation that the resistance to apoptosis in ASMase^{-/-} BMDM was not complete (Figure 1 & 2) is probably because the role of ASMase in apoptosis is dependent upon the type of stress and may also be cell type specific. This is supported by the observation of Lozano et. al. who showed that ASMase^{-/-} murine embryonic fibroblasts (MEFs) were completely protected from radiation-induced apoptosis but only partially resistant to low serum induced cell death and offered no protection to staurosporine treatment [12]. Moreover, ASMase is essential for chemotherapy-induced apoptosis in oocytes [33] but not required for testicular ceramide production or for the ability of the germ cells to undergo apoptosis [34].

Sumitomo et al [35] reported that etoposide-induced early ceramide increase was due to the transient and rapid activation of the *de novo* pathway, while the ceramide level was sustained in the longer term by the activity of SMase. SPT activity was observed to be activated within 15 minutes by etoposide treatment in Molt-4 cells [17]. Without significant SMase activity in ASMase^{-/-} cells (Figure 3), it is unlikely the ceramide accumulation is from SM hydrolysis. Using inhibitors for the enzymes involved in ceramide *de novo* synthesis pathway, FB1 and Myr, we confirmed that the *de novo* synthesis pathway also contributed to ceramide generation upon M-CSF withdrawal (Figures 4). As demonstrated in Figure 5, ceramide level indeed started to increase after three hours of M-CSF withdrawal and results

of SPT assay (Figure 6) showed its activity started to increase as early as one hour after M-CSF withdrawal. There are reports demonstrating that the changes in ceramide level can be biphasic in certain conditions [36, 37].

Under normal pathways of sphingolipid synthesis, ceramide is considered an intermediate rather than an end product. It serves as a precursor for assembly of more complex sphingolipids such as sphingomyelin and glucosylceramide. Ceramide may accumulate if its conversion to complex sphingolipids is blocked, for example by inhibition of sphingomyelin synthase (SMS) and glucosylceramide synthase (GCS). Interestingly, ceramide itself is reported to inhibit SMS [38]. Whereas SPT and CS reside on the endoplasmic reticulum [39], SMS and GCS are located on the Golgi apparatus and/or plasma membrane [40, 41]. Therefore another regulatory point for accumulation of newly synthesized ceramide during apoptosis in BMDM may be at the level of ceramide transport. Additionally, a limitation of our studies was not knowing the exact nature of the ceramide species being generated under the various conditions. Total analysis of the lipidome may have been one approach to investigate this aspect of ceramide's complex regulation.

C1P is able to promote cell survival and block the ceramide generation despite the absence of ASMase (Figure 7). In addition to its effect on inhibiting ASMase activity in BMDM [21], C1P can also inhibit SPT activity to block ceramide generation in response to serum withdrawal in alveolar NR8383 rat macrophages [42]. We showed that C1P stimulates PI3K to phosphorylate PKB and promote survival [32]. It was demonstrated that besides inhibition of ASMase, PI3K can also activate GCS and SMS to reduce ceramide production [43]. Besides SPT, it is reasonable to expect that in the absence of ASMase, C1P can still activate PI3K/PKB and possibly GCS and SMS to reduce ceramide production.

Ceramide synthesis and metabolism is a complex process. Besides the enzymes discussed here, there are other regulatory enzymes that might be involved in modulating the concentration of this molecule. For example, withdrawal of survival promoting cytokines may block ceramide kinase to reduce production of C1P, which was shown by us and others to be pro-survival [21, 32]. A recent report demonstrated the involvement of dihydroceramide desaturase in cell cycle progression [44]. Another potential regulator is sphingosine kinase which generates the mitogenic metabolite sphingosine-1-phosphate that can inhibit ceramide production and block apoptosis in BMDM upon M-CSF withdrawal [45]. These additional effects could work in concert with ASMase to regulate ceramide levels and cell survival. For example, a recent observation shows that CS activation depends on ceramide generated by ASMase activity [36]. Therefore, although the individual contribution of either pathway of ceramide generation may vary with cell type, they appear to play a complementary role. Finally, we cannot ignore the possibility that some of our findings may be explained by accumulations of sphingomyelin that can cause alterations in endocytosis and lysosomal dysfunction when ASMase activity is lost [46].

In summary, our results have demonstrated a partial role for ASMase in the generation of ceramide in BMDM cells following M-CSF starvation, and a resulting decrease in apoptosis of these cells. Our investigations have shown that ceramide generation still occurs in M-CSF-starved cells lacking ASMase, and this is occurring by *de novo* synthesis of ceramide.

In BMDM, we can conclude that several pathways are operating to regulate ceramide generation, which are suppressed by the presence of cytokines such as M-CSF.

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Abbreviations

ASMase	acid sphingomyelinase
BMDM	bone marrow-derived macrophages
C1P	ceramide 1-phosphate
M-CSF	macrophage colony stimulating factor
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt]
SPT	serine-palmitoyltransferase
PI3K	phosphatidylinositol 3 kinase
PKB	protein kinase B

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Highlights

M-CSF withdrawal from macrophage increases ceramide

Loss of acid sphingomyelinase reduces apoptosis following M-CSF withdrawal

Only a partial reduction in ceramide production is observed

Ceramide is also produced following M-CSF withdrawal by synthetic pathways

Figure 1. ASM deficiency confers partial resistance to M-CSF withdrawal-induced apoptosis and ceramide increase

(A) ASMase ^{+/+} and ASMase ^{-/-} BMDM were incubated with [³H]palmitate and without M-CSF for 24 hours. Cells labeled in the presence of M-CSF served as the control. Ceramide was then isolated by TLC and counted. Radioactivity in ceramide relative to that in control cells was then calculated for each experiment. Data are means $\pm$ SD of six independent experiments done in duplicate. The absolute amount of radioactivity in ceramide in control conditions were a factor of incorporation and specific activity of batches of [³H]palmitate and control levels ranged from 6850 to 18168 cpm in ASMase ^{+/+} and from 6990 to 15048 cpm in ASMase ^{-/-} cells. (B) BMDM from ASMase ^{+/+} and ASMase ^{-/-} were seeded at 5×10^4 cells/well and in 96-well plates overnight to allow cells to adhere. Cells were cultured in the absence of growth factors for 24 hours before adding MTS. Cell survival in the presence of M-CSF was the reference for 100% survival. Data represent means $\pm$ SD of three experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$ vs. ASM^{+/+} cells.

Figure 2. ASM deficiency confers partial resistance to M-CSF withdrawal-induced DNA fragmentation and caspase 9 activation

(A) BMDM from ASMase $+/+$ and ASMase $-/-$ were incubated in the presence or absence of M-CSF for 24 hours. Cells were then stained for sub-diploid DNA with propidium iodide (PI) and analyzed by flow cytometry as described in Materials and Methods. (B) BMDM from ASMase $+/+$ and ASMase $-/-$ were incubated with or without M-CSF for 24 hours, stained for activation of caspase 9 and analyzed by flow cytometry. Results shown are representative of two independent experiments showing similar results.

A.

B.

Figure 3. Ceramide generated in ASMase $-/-$ BMDM is not due to SM hydrolysis

BMDM were cultured in the presence or absence of M-CSF for 24 hours. Lysates were assayed for ASMase (A) and NSMase (B) activity. Results were means $\pm$ SD of at least three independent experiments. * $p < 0.05$ vs. control in ASM+/+ cells; ** $p < 0.01$ vs. ASM+/+ cells.

Figure 4. Inhibitors of the *de novo* ceramide synthesis pathway are able to block ceramide production in ASMase^{-/-} BMDM

ASMase^{+/+} and ASMase^{-/-} BMDM were incubated with [³H]palmitate and without M-CSF for 24 hours in the absence or presence of 100 nM Myriocin (Myr) or 50 μM Fumonisin B1 (FB1). Ceramide was then isolated by TLC and radioactivity determined by scintillation counting. Cells incubated in the presence of M-CSF served as control. Radioactivity in ceramide relative to that in control cells was then calculated. Data are means ± SD of three independent experiments done in duplicate. *p<0.05, **p<0.01 vs. N. N: no M-CSF. The absolute level of ceramide in a typical experiment in control cells from which M-CSF was withdrawn was on the order of 2000 dpm per 2 × 10⁶ dpm total lipid in ASMase^{-/-} cells, and approximately 3000 dpm in ASMase^{+/+} cells.

Figure 5. Time course for the change in ceramide mass after M-CSF withdrawal
 BMDM were cultured without M-CSF for 0 to 9 hours. At the indicated times, the cells were harvested, and ceramide mass was quantified using the diglyceride kinase assay and normalized to lipid phosphate. ^{32}P -labeled lipids were separated by TLC and quantified with a phosphorimager. Panel A shows a representative scan from one of three independent experiments. Panel B shows means $\pm$ SD of three experiments done in duplicate. * $p < 0.05$ vs. time zero. The measurement was relative to the levels at 0 time, prior to withdrawal of M-CSF.

Figure 6. SPT is activated during M-CSF withdrawal

(A) BMDM were cultured in the absence of M-CSF for indicated time. BMDM cultured in the presence of M-CSF were used as control. The microsomes were isolated and used to determine the *in vitro* SPT activity as described in Materials and Methods. Data are expressed as fold change in the absence of M-CSF relative to control and as means $\pm$ SD of at least three independent experiments. * $p < 0.05$ vs. time zero. The range of absolute values obtained in typical experiments was between 3 – 10 dpm per μ g protein, with 50 – 100 μ g protein used per assay. The measurement was relative to the levels at 0 time, prior to

withdrawal of M-CSF. (B) BMDM were lysed after being starved without M-CSF for the indicated time. Then whole cell lysates were probed for the expression of SPTLC1 and SPTLC2. Vinculin was used as loading control. Figure is representative of three independent experiments showing similar results. (C) BMDM in normal culture were deprived of M-CSF for 3 hours. The 'M-CSF added' group were deprived of M-CSF for 2.5 hours and then M-CSF was added back for 30 minutes. SPT activity assay was performed using microsomes, and the control shows level of background activity in a parallel assay with not microsomal extract. (* $P < 0.05$)

Figure 7. C1P can inhibit ceramide generation and promote cell survival independent of ASMase
 (A) For ceramide level, ASMase +/+ and ASMase-/- BMDM were labeled with [³H]palmitate when M-CSF was withdrawn in the absence or presence of 30 μ M C1P for 24 hours. Cells labeled in the presence of M-CSF served as control cells. Ceramide was isolated by TLC and radioactivity determined by liquid scintillation counting. Data are means $\pm$ SD of three independent experiments done in duplicate. * p <0.05 vs. MCSF-. (B) BMDM from ASMase +/+ and ASMase-/- mice were seeded at 5×10^4 cells/well and in 96-well plates overnight to allow cells to adhere. Cells were cultured in the absence of growth factors with or without 30 μ M C1P for 24 hours before adding MTS. Cell survival in the presence of M-CSF was the reference for 100% survival. Data represent means $\pm$ SD of four experiments performed in triplicate (closed bars). * p <0.01, vs. MCSF-.