Ceramide catabolism critically controls survival of human dendritic cells

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Abstract: The regulation of dendritic cell (DC) survival is crucial for the modulation of adaptive immunity. Ceramide is a lipid mediator of the stress response, which accumulates intracellularly during DC differentiation. We found that ceramide levels are tightly regulated in human DCs and that the pharmacological inhibition of enzymes responsible for ceramide catabolism, such as ceramidases and sphingosine kinases, sensitizes DCs to ceramide-induced cell death. It is important that inhibition of sphingosine kinases, during lipopolysaccharide stimulation, causes extensive ceramide accumulation and death of DCs. These data indicate that ceramide catabolism regulates survival of human DCs and reveal novel potential targets for the pharmacological manipulation of the immune response. *J. Leukoc. Biol.* 79: 166–172; 2006.

Key Words: *LPS · ceramidases · sphingosine kinases*

INTRODUCTION

Dendritic cells (DCs) are the major antigen-presenting cells of our organism. In their immature state, DCs reside in the periphery and migrate to the lymph nodes at a slow but constant rate, promoting tolerance [1]. Immature DCs are equipped for sensing the presence of infectious agents and continuously sample the environment through active macropinocytosis, endocytosis, and phagocytosis [2]. The presence of microbes triggers the maturation process, and DC behavior changes drastically; in fact, their capacity to sample the environment is actively blocked, and their egression from tissues is greatly increased, and major histocompatibility complex class II molecules, costimulatory molecules, and cytokines are up-regulated [3]. These events, in which DC survival and lifespan are critical parameters [4], activate the immune system and eventually tailor adaptive immune responses [5].

Ceramide is a lipid mediator of cellular stress responses, evolutionary, conserved from unicellular eukaryotes, which regulates many different processes, ranging from cell-cycle arrest and cell differentiation to cell death [6]. In many circumstances, ceramide is converted into other lipids, such as sphingosine, sphingosine-1-P (S1P), or gangliosides, which are responsible for relevant biological outcomes. In other situations, ceramide directly regulates specific cellular settings, via the activation of different kinases, phosphatases, phospholipases, or proteases [7, 8]. The relevance of ceramide metabolism in molecular medicine is growing, as the potential impact of its pharmacological manipulation becomes appreciated [9].

Lipopolysaccharide (LPS), the main constituent of the membrane of gram-negative bacteria, promotes the maturation of human DCs [2]. We recently showed that DC survival is actively controlled during LPS-induced maturation. In fact, LPS triggers a caspase-like activity, which is required for early phosphorylation of extracellular signal-regulated kinases (ERK) and the up-regulation of the antiapoptotic proteins Fadd-associated death domain-like β-interleukin-1 (IL-1)-converting enzyme inhibitory protein (FLIP) and Bcl-2, sustaining cell survival during the maturation process [10]. We described previously how ceramide cooperates in the maturation process triggered by LPS and how it can contribute to shutting down endocytosis and phagocytosis [11]. Here, we show that catabolism of ceramide is required for survival of human DCs during LPS stimulation.

MATERIALS AND METHODS

Cell culture

DC generation

Peripheral blood mononuclear cells from different healthy donors were isolated on lymphoprep cushions, and monocytes were purified by positive sorting using anti-CD14-conjugated magnetic microbeads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The recovered cells were 99% $CD14^+$, as determined by flow cytometry with anti-CD14 phycoerythrin (PE)-conjugated antibodies (PharMingen, San Diego, CA). To induce DC differentiation, $CD14^+$ cells were then cultured for 4–6 days in six wells (Costar, Corning, NY) at an initial concentration of 500,000/ml in RPMI, 10% fetal bovine serum (FBS), penicillin, streptomycin, glutamine 2 mM, Hepes 10 mM, 1% nonessential amino acids, and 1% sodium pyruvate, supplemented with IL-4 (2.5 ng/ml, R&D Systems, Minneapolis, MN) and granulocyte macrophage-colony stimulating factor (50 ng/ml, a kind gift of Professor Paolo Rossi, University of Rome "Tor Vergata", Italy). Cells were routinely controlled for CD1, CD14, and CD86 expression using the respective PE-conjugated antibodies (PharMingen) and resulted as CD14-$CD1^{high}CD86^{low}$. THP-1 were cultured with RPMI, 10% FBS, penicillin, streptomycin, glutamine 2 mM, Hepes 10 mM, 1% nonessential amino acids, and 1% sodium pyruvate.

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Reagents

C-2 (N-acetylsphingosine) and C-16 ceramide; C-8 ceramine D-erythro; sphingosine D-erythro; S1P D-erythro; C-2 dihydroceramide; dihydrosphingosine D-erythro; dihydrosphingosine-1-phosphate (DS1P) D-erythro; D-erythro-N,N-dimethylsphingosine (DMS); D-erythro-2(N-myristoylamino)-1-phenyl-1propanol (MAPP); and oleylethanolamide (NOE) were from Biomol Research Laboratories (Plymouth Meeting, PA). LPS *Escherichia coli* serotype 055:B5 was from Sigma Chemical Co. (St. Louis, MO).

Evaluation of cell death

Cells were recovered at the indicated time, washed with cold phosphate-buffered saline (PBS), and incubated for 20 min with propidium iodide (PI) 5 μg/ml (Sigma Chemical Co.) and Annexin V (Becton Dickinson, San Jose, CA) in the appropriate buffer according to the manufacturer's instructions. Analysis was performed with a FACScan flow cytometer (Becton Dickinson) using the Cellquest software. Dot plot analysis, with Annexin V on the *y*-axis and PI on the *x*-axis, allowed the quantitation of dead cells, including apoptotic and necrotic cells (AnnexinV$^+$ PI$^-$, AnnexinV$^+$ PI$^+$, AnnexinV$^-$ PI$^+$), as a percentage of total cells analyzed [12]. Cells were also stained at the indicated time with Hoechst 33342 (500 ng/ml for 20 min) to score the percentage of DCs undergoing apoptosis. Cell death was therefore evaluated counting the condensed and/or fragmented nuclei with a fluorescence microscope (IX50, Olympus) with adequate filters. Typically, two independent investigators scored apoptotic cells in blind, counting at least 300 cells from four different microscopic fields.

Thin-layer chromatography (TLC) and immunostaining

Ceramide was extracted as described previously for diacylglycerol kinase assay [13] with minor modifications. Briefly, 1–5 × 10^6 cells were disrupted by three cycles of freezing and thawing and homogenized by 30 s sonication at 10 Watts. The aqueous pellet was extracted with chloroform/methanol (2/1, vol/vol) to obtain a final ratio of chloroform/methanol/water 8:4:2.4 (v/v/v). After vortex and 15 min centrifugation at 1000 *g*, the lower phase was recovered and evaporated under nitrogen. The samples were then resuspended in chloroform/methanol (2/1, v/v), loaded on a silica gel 60 HP-TLC plate (Merck, Rahway, NJ), and chromatographed in chloroform/methanol/acetic acid (65:15:5, v/v/v). Plates were treated with 0.5% polyisobutyl-metacrylate in hexane for 90 s and dried. To carry out immunostaining for ceramide, the plate was first treated with PBS, milk 10% for 30 min, and PBS, 0.05% Tween 20, 1% bovine serum albumin for 60 min, to minimize nonspecific staining and incubated overnight with immunoglobulin M (IgM)-enriched mouse anticeramide (1:500, Glycobiotech GmbH, Kukels, Germany) [14, 15] followed by a horseradish peroxidase-conjugated anti-IgM secondary antibody (1:500) for 60 min. Specific bands were detected by enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions.

RESULTS

Ceramide induces DC death in the absence of serum

We initially proposed that endogenous ceramide may act as a mediator of DC maturation [11]. However, others have reported that DCs can be killed by exogenous ceramide [16, 17]. To better analyze whether ceramide can be lethal for DCs, we exposed DCs to exogenous ceramide in the presence or absence of serum. We found that ceramide induces only little cell death in the presence of serum, and massive cell death is induced in the absence of it (**Fig. 1A**).

To verify that this was not a result of a generic prosurvival effect of serum, we exposed DCs to ceramine, a synthetic ceramide derivative, in which the carbonyl group is replaced by a methylene group. Ceramine maintains the ability of ceramide to act as a second messenger but in contrast to ceramide, cannot be further catabolized [18], thus shifting the balance of sphingolipid mediators inside the cell. As shown in Figure 1B, although control cells and cells treated with ceramide underwent 20% and 22% of cell death, respectively, DCs exposed to ceramine underwent massive cell death (69%), even in the

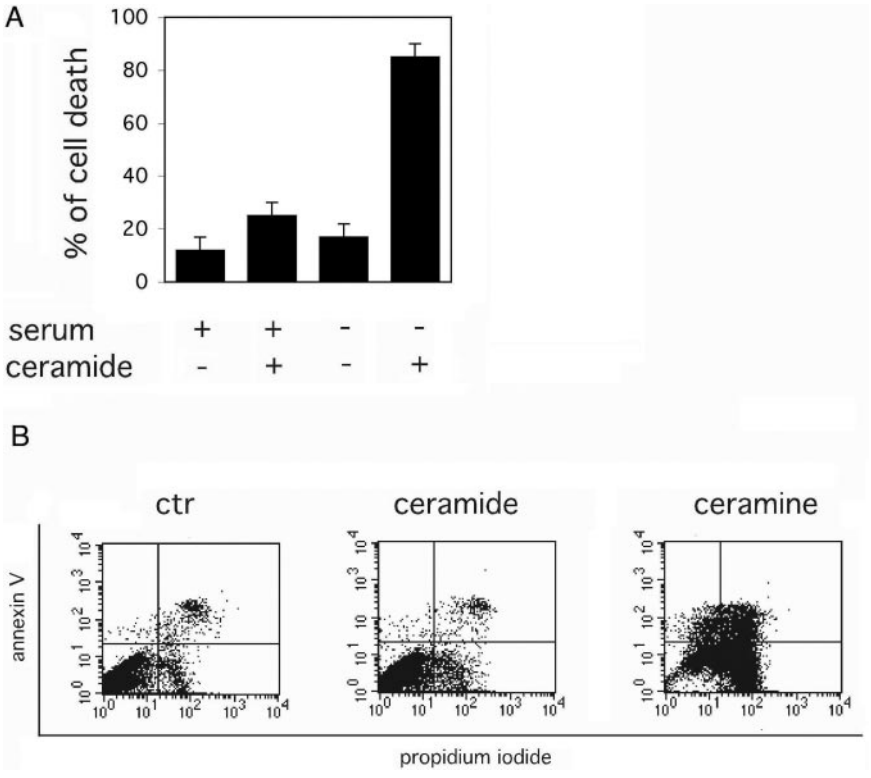

Fig. 1. Ceramide induces DC death in the absence of serum. (A) DCs were exposed to 50 μM ceramide in the presence or absence of 10% serum. Cell death was scored after 24 h by fluorescein-activated cell sorter (FACS) analysis with Annexin V and PI. Mean ± 1 SD from five experiments is shown. (B) DCs in 10% serum were exposed to 50 μM ceramide or 10 μM ceramine. Cell death was scored after 24 h by FACS analysis with Annexin V and PI. In the experiment shown, cell death was 20%, 22%, and 69% for control (ctr), ceramide, and ceramine, respectively. Three independent experiments gave similar results.

presence of serum, suggesting that a prolonged accumulation of ceramide could induce DC death.

Inhibition of acid or neutral/alkaline ceramidase sensitizes DCs to ceramide-induced cell death

As serum can regulate the activities of enzymes responsible for ceramide catabolism [19], it is possible that serum-induced modifications of ceramide are responsible for critical changes in its biological activity. To address this hypothesis, we stimulated DCs with ceramide in the presence and absence of inhibitors of ceramidases, the enzymes responsible for ceramide catabolism. **Figure 2A** shows that NOE, a ceramide analog that acts as an inhibitor of acid ceramidase [20], sensitizes DCs to ceramide-induced cell death (24% vs. 55%).

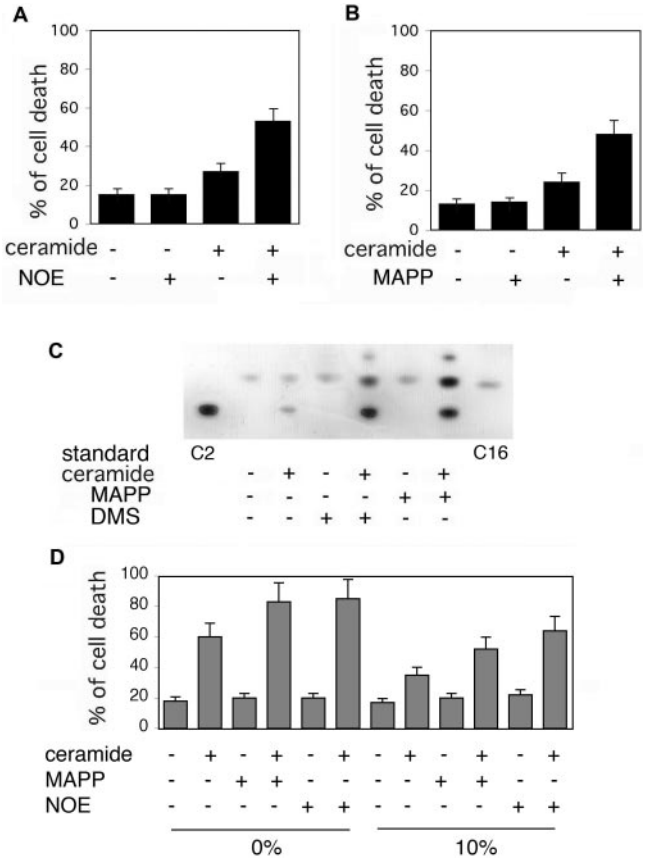

Fig. 2. Pharmacological inhibition of acid or neutral/alkaline ceramidase sensitizes DCs to ceramide-induced cell death. (A) DCs were pretreated or not with 100 μM NOE for 1 h in 10% serum and exposed to 50 μM ceramide. Cell death was scored after 24 h by FACS analysis with Annexin V and PI. Mean ± 1 SD from four experiments is shown. (B) DCs were pretreated or not with 10 μM MAPP for 1 h in 10% serum and exposed to 50 μM ceramide. Cell death was scored after 24 h by FACS analysis with Annexin V and PI. Mean ± 1 SD from three experiments is shown. (C) DCs (4×10^6) in 10% serum were pretreated for 1 h with 10 μM DMS or 10 μM MAPP or not pretreated and exposed to 50 μM C2 ceramide for 6 h. Cellular ceramide was analyzed by TLC and revealed by immunostaining with a specific anticeramide antibody. Data are representative of three independent experiments. Standard C2 and C16 ceramides were used as specific controls. (D) DCs were pretreated with 10 μM MAPP for 1 h or 100 μM NOE for 1 h in the absence of serum, washed, and exposed to 50 μM C2 ceramide in the absence of serum (0%) or in the presence of serum (10%). Cell death was scored after 6 h by Hoechst staining. Data represent the mean ± 1 SD from four independent experiments.

Similarly, as illustrated in Figure 2B, MAPP, another ceramide analog, which acts as an inhibitor of neutral/alkaline ceramidase [21–23], also sensitizes DCs to ceramide-induced cell death (22% vs. 48%).

We then confirmed the activity of the neutral/alkaline ceramidase inhibitor by measuring ceramide content in DCs stimulated with C2 ceramide in the presence or absence of MAPP. As expected, the inhibition of neutral/alkaline ceramidases led to the accumulation of C2 ceramide and interestingly, that of endogenous long chain ceramides (Fig. 2C). In contrast, DCs stimulated with C2 ceramide efficiently catabolized ceramides.

To clarify whether ceramide catabolism is mediated by enzymes present in the serum or by cellular ceramidases, serum-free DCs were pretreated with ceramidase inhibitors MAPP and NOE, washed, and exposed to C2 ceramide in the presence or absence of serum. As illustrated in Figure 2D, when DCs were pretreated with MAPP and NOE in the absence of serum, the inhibition of ceramide catabolism promoted cell death, suggesting that MAPP and NOE act on cellular ceramidases.

Taken together, these data indicate that the activity of ceramidases is required to allow survival of DCs upon ceramide challenge.

Inhibition of sphingosine kinase sensitizes DCs to ceramide-induced cell death

Acid and neutral/alkaline ceramidases catalyze the conversion of ceramide into sphingosine, which acts as a proapoptotic lipid mediator. Sphingosine can be further converted through the action of sphingosine kinases into S1P, a lipid mediator that can prevent cell death [19]. We therefore investigated whether blocking sphingosine kinases could affect cell survival of DCs exposed to ceramide. To this purpose, we used DMS, a sphingosine analog, in which the addition of a methyl group to the shingosine moiety creates a false substrate for sphingosine kinases, thus inhibiting their activity [24]. **Figure 3A** shows that ceramide exposure induces a significant percentage of cell death in DCs only if DCs have been pretreated with DMS (22% vs. 62%). As expected, exposure of DCs to dihydroceramide, an inactive ceramide analog, does not induce cell death alone or in the presence of DMS (Fig. 3A).

We then asked whether ceramide-induced cell death in the presence of DMS occurs because of the accumulation of proapoptotic sphingosine or because of the lack of the synthesis of antiapoptotic S1P. To evaluate DC sensitivity to sphingosine, we exposed DCs, pretreated with DMS or not pretreated, to exogenous sphingosine and to dihydrosphingosine, an inactive sphingosine analog. In neither of these circumstances did sphingosine significantly induce cell death (Fig. 3B), and sphingosine, but not dihydrosphingosine, triggered apoptosis of the lymphoid cell line CEM (data not shown). These results suggest that sphingosine is unlikely to be a proapoptotic mediator in DCs.

We then examined whether S1P could protect DCs against ceramide-induced cell death. To this purpose, DCs pretreated with DMS were exposed to ceramide in the presence or absence of S1P. It is interesting that S1P could not suppress ceramide-induced cell death (Fig. 3C), although it could protect CEM cells from serum starvation-induced cell death (Fig. 3D). Thus,

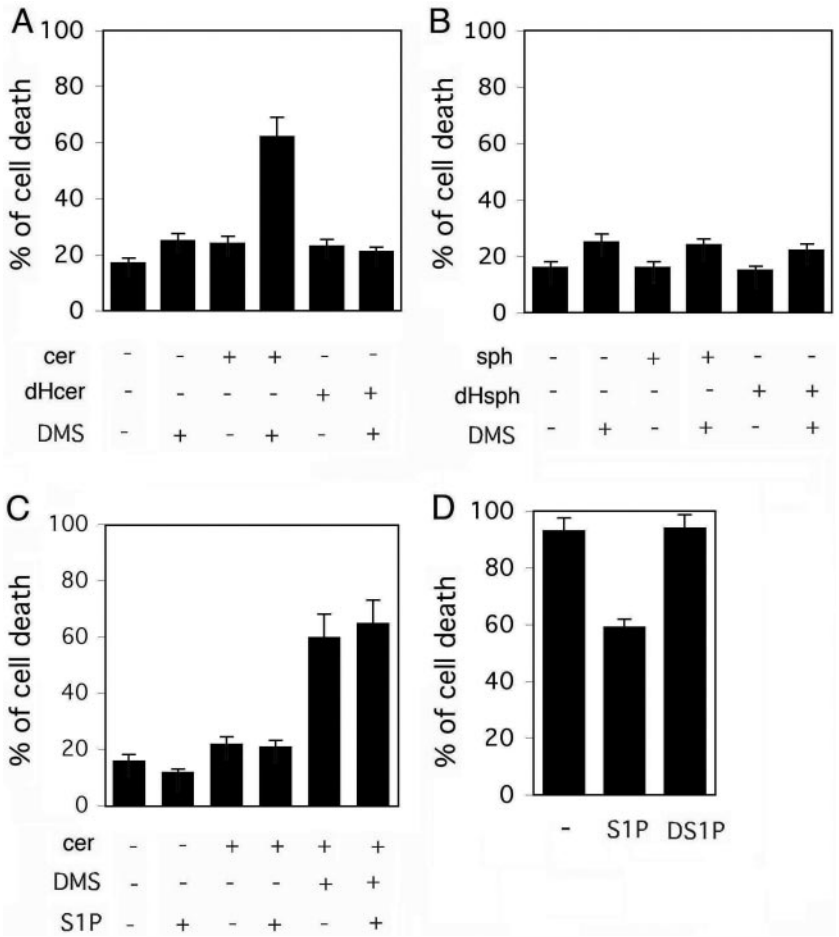

Fig. 3. Pharmacological inhibition of sphingosine kinase sensitizes DCs to ceramide-induced cell death. (A) DCs were pretreated or not with 10 μM DMS for 1 h in 10% serum and exposed to 50 μM ceramide (cer) or 50 μM dihydroceramide (dHcer). Cell death was scored after 24 h by FACS analysis with Annexin V and PI. Mean ± 1 SD from four experiments is shown. (B) DCs in 10% serum were exposed to 10 μM sphingosine (sph) or to 10 μM dihydrosphingosine (dHsph) in the presence or absence of 10 μM DMS. Cell death was scored after 24 h by FACS analysis with Annexin V and PI. Mean ± 1 SD from three experiments is shown. (C) DCs were pretreated for 1 h with 5 μM S1P and then exposed to 50 μM ceramide in the presence or absence of 10 μM DMS in 10% serum. Cell death was scored after 24 h by FACS analysis with Annexin V and PI. Mean ± 1 SD from three experiments is shown. (D) CEM cells were cultured in media without serum in the presence or absence of 5 μM S1P or 5 μM DS1P. Cell death was scored after 48 h by FACS analysis with Annexin V and PI. Mean ± 1 SD from two experiments is shown.

the product of sphingosine kinases cannot grant survival to DCs. Moreover, DMS-treated DCs could accumulate C2 ceramide and endogenous long-chain ceramides after stimulation with C2 ceramide (Fig. 2C).

Taken together, our data suggest that in this system, the products of ceramide catabolism are not directly responsible for mediating its biological effects and point to the ceramide itself as the critical death mediator in DCs.

Inhibition of sphingosine kinase during LPS stimulation induces DC death

We therefore investigated whether preventing ceramide catabolism during DC differentiation would cause changes in ceramide levels and affect cell viability. We previously reported that LPS triggers a transient increase of ceramide content in DCs [11]. To investigate whether ceramide accumulation could be enhanced, by preventing its catabolism, DCs were exposed to LPS, with or without DMS pretreatment, which increased the levels of ceramide accumulating during LPS stimulation (**Fig. 4A**) and sensitized DCs to cell death (Fig. 4B). As we noticed some degree of variability among different donors, we performed similar experiments using Thp-1, a myeloid cell line. LPS stimulation of Thp-1 cells provided analogous results. In fact, DMS pretreatment increases the ceramide cell content (Fig. 4C) and induces 60% of cell death upon LPS stimulation (Fig. 4D).

DISCUSSION

Ceramide is a ubiquitously and abundantly expressed sphingolipid. As an evolutionary, conserved mediator of the cellular stress response, ceramide can act as a second messenger, transducing signals that control processes as different as cell cycle, cellular differentiation, and cell death. The different outcomes are a result of specific cellular settings that dictate the way in which a cell senses ceramide accumulation. These differences depend on where the ceramide is produced, on the amount that is accumulated, on the time in which ceramide is produced, and on the expression of specific gene products [25]. Herein, we show that ceramide catabolism regulates the survival of DCs. In fact, pharmacological inhibition of ceramidases or sphingosine kinases sensitizes human DCs to ceramide-induced cell death.

The synthesis of ceramide is initiated through the action of serinpalmitoyltransferase, which catalyzes the formation of ketosphinganine from serine and palmitoyl-coenzyme A. Ketosphinganine is subsequently reduced by ketosphinganine reductase and acylated by dihydroceramide synthase leading to dihydroceramide, which is finally converted into ceramide by dihydroceramide desaturase [26]. Once formed, ceramide can be further converted into sphingomyelin through the action of sphingomyelin synthase or in glucosylceramide through the action of glucosylceramide synthase. Ceramide can be released from sphingomyelin through the action of specific sphingomy-

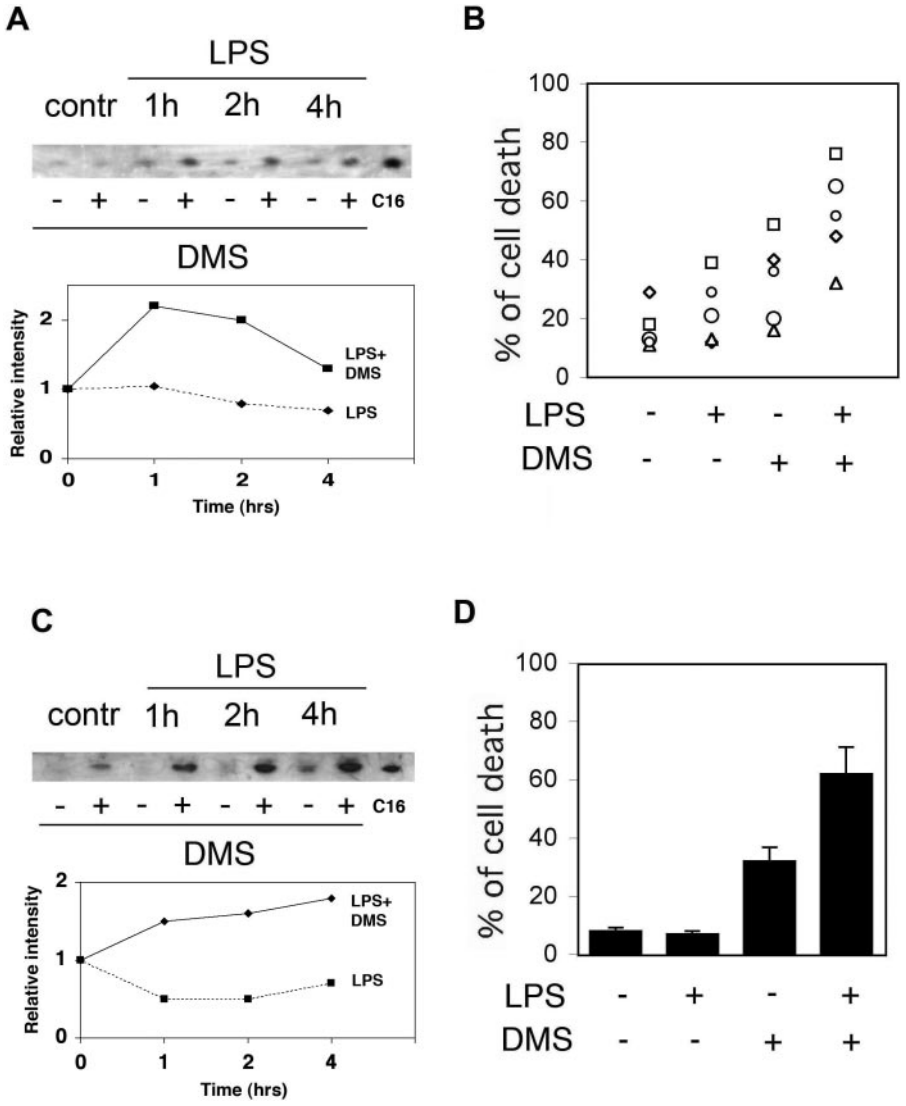

Fig. 4. LPS-induced ceramide accumulation induces cell death. (A) DCs (1.5×10^6) in 10% serum were pretreated for 1 h with 10 μM DMS or not pretreated and exposed to 1 μg/ml LPS for 1, 2, and 4 h. Cellular ceramide was analyzed by TLC and revealed by immunostaining with a specific anti-ceramide antibody. Standard C16 ceramide was used as a specific control. One representative experiment out of three yielding similar results is shown. (Lower panel) Densitometric quantification of DC ceramide content after LPS exposure in the presence or absence of DMS. (B) DCs at 10^5/ml in 10% serum were pretreated with 10 μM DMS for 1 h and then exposed to 1 μg/ml LPS. Cell death was scored after 48 h by FACS analysis with Annexin V and PI. Data from five different donors are shown. (C) Thp-1 (5×10^6) cells in 5% serum were pretreated for 1 h with 10 μM DMS or not pretreated and exposed to 10 μg/ml LPS for 1, 2, and 4 h. Cellular ceramide was analyzed by TLC and revealed by immunostaining with a specific anti-ceramide antibody. Standard C16 ceramide was used as a specific control. One representative experiment out of four yielding similar results is shown. (Lower panel) Densitometric quantification of Thp-1 cell ceramide content after LPS exposure in the presence or absence of DMS. (D) Thp-1 cells in 5% serum were pretreated for 1 h with 10 μM DMS and exposed to 10 μg/ml LPS. Cell death was scored after 8 h by FACS analysis with Annexin V and PI. Mean ± 1 SD from four experiments is shown.

elinases. The catabolism of ceramide is mediated by the action of ceramidases, which convert ceramide into sphingosine [27]. Three different ceramidases have been reported to date: an acid ceramidase, an acid-like ceramidase, and a neutral/alkaline ceramidase. Sphingosine can be further converted into S1P through the action of sphingosine kinases, and two different sphingosine kinases have been reported to date. Sphingosine and S1P can act as a second messenger in cells, but they play opposing roles; that is, sphingosine promotes cell death, and S1P inhibits it [28].

The contribution of ceramidases in protecting cells from ceramide-induced cell death has been reported. In fact, the overexpression of acid ceramidase protects fibroblasts from tumor necrosis factor (TNF)-induced ceramide accumulation and cell death [20], and the fibroblasts of Farber's disease patients, which have an inherited deficiency in acidic ceramidase, are more sensitive to ceramide-induced cell death [29]. The role of sphingosine kinases in cell survival and transformation has been investigated extensively [19, 30]. Accordingly, the inhibition of sphingosine kinase to increase the levels of ceramide and sensitize cancer cells to chemotherapeutic drugs has been exploited [9]. Castillo and Teegarden [31] previously described the protective role of ceramide catabolism. It is interesting that in murine fibroblasts, the prosurvival effect of S1P seems to be significant, underscoring the specificity of signal pathways engaged by human DCs. For the first time, we show that these enzymes sustain the survival of human DCs, underscoring the role of ceramide metabolism in human DCs and suggesting that the control of these enzymes could be exploited to regulate the DC lifespan.

It is noteworthy that DCs survive for only a short time (1–2 days) in lymph nodes but that it takes a longer time (4–5 days) to achieve an adaptive immune response, implying that successive rounds of migration are required to mount a proper response [32]. Several evidences support the importance of DC survival in the control of immune responses. For example, DCs with an increased lifespan induce stronger immune responses and even autoimmunity [5, 33, 34]. Although the mechanisms by which the DC lifespan controls the strength of an adaptive immune response are not clear, they could be manipulated to augment the efficacy of DC-based anticancer vaccines [35]. Conversely, it is intuitive that a decrease in the DC lifespan could be exploited in all those circumstances in which DCs are

aberrantly activated. It is intriguing that several stimuli such as CD40L, TNF, and LPS, which induce the intracellular accumulation of ceramide, promote maturation and activate anti-apoptotic programs in DCs [36–38]. Here, we show that ceramide catabolism is critical to allow DC survival, as pharmacological inhibition of sphingosine kinases strongly elevates ceramide levels during LPS stimulation, therefore inducing DCs as well as Thp-1 cells to undergo cell death. Moreover, as in our experimental system, sphingosine could not induce cell death, and S1P could not protect from it, it is likely that ceramide, accumulated following LPS stimulation, triggers DC death directly.

These data highlight the importance of a tight regulation of ceramide levels during LPS-induced maturation of human DCs, suggesting that early LPS-induced accumulation of ceramide is actively controlled by the action of the enzymes involved in ceramide catabolism. Whether regulatory mechanisms control quantitative thresholds, specific compartmentalization, or other unknown downstream targets remains to be determined.

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