Ceramide Inhibits Antigen Uptake and Presentation by Dendritic Cells

By Federica Sallusto,* Chiara Nicolò,* Ruggero De Maria,‡ Silvia Corinti,* and Roberto Testi‡

Summary

Ceramides are intramembrane diffusible mediators involved in transducing signals originated from a variety of cell surface receptors. Different adaptive and differentiative cellular responses, including apoptotic cell death, use ceramide-mediated pathways as an essential part of the program. Here, we show that human dendritic cells respond to CD40 ligand, as well as to tumor necrosis factor-α and IL-1β, with intracellular ceramide accumulation, as they are induced to differentiate. Dendritic cells down-modulate their capacity to take up soluble antigens in response to exogenously added or endogenously produced ceramides. This is followed by an impairment in presenting soluble antigens to specific T cell clones, while cell viability and the capacity to stimulate allogeneic responses or to present immunogenic peptides is fully preserved. Thus, ceramide-mediated pathways initiated by different cytokines can actively modulate professional antigen-presenting cell function and antigen-specific immune responses.

Materials and Methods

In Vitro Culture of Human DCs. PBMCs obtained by standard Ficoll–Paque method (Organon Teknika, Durham, NC) were separated on multistep Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) and the light density fraction from the 42.5–50% interface was recovered and depleted of CD19+ and CD2+ cells using magnetic beads coated with specific antibodies (Dynal, Oslo, Norway). The remaining cells were cultured in RPMI-1640 supplemented with 2 mM l-glutamine, 1% nonessential aminoacids, 1% pyruvate, 50 μg/ml kanamycin, 5 × 10⁻⁵ M 2-ME (GIBCO BRL, Gaithersburg, MD) + 10% FCS (Hyclone Laboratories, Inc., Logan, UT) in the presence of 50 ng/ml GM-CSF and 1000 U/ml IL-4 (provided by Dr. A. Lanzavecchia, Basel Institute for Immunology, Switzerland). Cultured DCs were routinely >90% CD1+*, HLA-DR+*, CD83+ and were used after 5–7 d of culture.

Ceramide Mass Measurement Assay. After stimulation, lipids were extracted and then incubated with Escherichia coli diacylglycerol kinase. Ceramide phosphate was then isolated by TLC using CHCl₃/CH₃OH/CH₃COOH (65:15:5) as solvent (13, 14). Au...
etaria judaica was isolated from an atopic patient (15). with bicarbonate buffer 0.125 M pH 8. PjE-specific clone P6.2 with 10^2 O-phenilendiamine and Hmin, and the enzyme activity of the lysate was measured using with 0.05% Triton X-100 in 10 mM Tris buffer pH 7.4 for 30 above then four times with PBS alone with one tube change, lysed bottomed microplates. Analyzed on a FACScan

Figure 1. Ceramide production in DCs following CD40L, IL-1β (A), and TNF-α exposure (B). DCs were isolated and cultured as described (4), then left untreated (A, open circles) or stimulated for the indicated times with proper amounts of soluble CD40L (31) (A, closed circles), 10^4 U/ml IL-1β (A, closed triangles), 200 ng/ml TNF-α (B, closed triangles), 200 ng/ml TNF-α with R32W and S86T substitutions (TNF-R1 specific) (B, open circles), and 200 ng/ml TNF-α with D143N and A145R substitutions (TNF-R2 specific) (B, closed circles) (32). Lipids were extracted and incubated with E. coli diacylglycerol kinase for ceramide quantitation (13). Mean data ± one SD were obtained from three experiments from three donors.

authentic ceramide-1-phosphate was identified by autoradiography at Rf 0.25. Quantitative results for ceramide production are expressed as pmol ceramide-1-phosphate/10^6 cells.

Endocytosis and Antigen Presentation Assays. 2 × 10^5 DCs were resuspended in 200 µl RPMI buffered with 25 mM Hepes + 10% FCS. C2-ceramide and C2-dihydroceramide (Sigma Immunocchemicals, St. Louis, MO) were reconstituted in EtOH at 15 mM and stored at –20°C until use. Diacylglycerol was purchased from Amersham (Buckingham, England). Lucifer yellow (LY), FITC–dextran (DX) (Molecular Probes, Inc., Eugene, OR), or HRP (Sigma Immunocchemicals) were reconstituted in PBS, stored at 4°C, and spun in a microfuge before use to eliminate aggregates. To quantify LY and FITC–DX, cells were washed four times with cold PBS containing 1% FCS and 0.01% NaN3 and analyzed on a FACSscan® (Becton Dickinson, Mountain View, CA), using propidium iodide to exclude dead cells. For horseradish peroxidase (HRP) quantification, cells were washed four times after above then four times with PBS alone with one tube change, lysed with 0.05% Triton X-100 in 10 mM Tris buffer pH 7.4 for 30 min, and the enzyme activity of the lysate was measured using O-phenylendiamine and H2O2 as substrates with reference to a standard curve. Xenopus toxoid (TT)-specific T cell clones KSI40 and KB24 and TT peptide P2 (residues 830-843) were provided by Dr. A. Lanzavecchia. TT antigen was purchased from Connaught (Ontario, Canada).

For antigen presentation assays, 4 × 10^4 T cells were cultured with 10^4 irradiated DCs in 200 µl RPMI with 10% FCS in flat-bottomed microplates. [3H]Thymidine incorporation was measured at day 2. For MLR, 1.5 × 10^4 responding cells from allogeneic adult PBMCs were cultured with different numbers of irradiated DCs. [3H]Thymidine incorporation was measured at day 5. Parietaria judaica pollen (Allergon, Angelholm, Sweden) was extracted with bicarbonate buffer 0.125 M pH 8. PjE-specific clone P6.2 was isolated from an atopic patient (15).

DNA Labeling and Flow Cytometry Analysis. 2 × 10^5 DCs were treated in 200 µl RPMI 10% FCS with 80 µM C2-ceramide for 10 min on ice, then for 1 h at 37°C. After incubation, cells were washed and left in culture for 48 h. Cells were then recovered and processed for propidium iodide staining and FACS® analysis as previously described (13).

Other Reagents. Supernatant from J558L cells stably expressing a chimeric mCD40L–mCD8α construct, provided by Dr. P. Lane, (Basel Institute for Immunology) was used as a source of CD40L. Recombinant human TNF-α with R32W and S86T substitutions (TNF-R1 specific) and TNF-α with D143N and A145R substitutions (TNF-R2 specific) were provided by Drs. W. Lesslauer and H. Loetscher (Hoffman La Roche, Ltd., Basel, Switzerland). IL-1β was provided by Dr. L. Mellén (IRIS, Siena, Italy).

Results and Discussion

Cytokines that Induce Maturation, Signal Ceramide Accumulation in DCs. IL-1β and TNF-α have been shown to induce transient ceramide accumulation in tumor cell lines (16, 17). It was not known whether CD40, like other TNF receptor family members such as TNF-R1 p55, Fas/APO-1, or NGF-R p75 (13, 16, 18), also signaled through ceramide generation. Therefore, we investigated whether cross-linking of CD40 was able to induce ceramide accumulation in cultured immature DCs. Fig. 1 shows that CD40L, as well as IL-1β and TNF-α, engaging TNF-R1 p55 but not TNF-R2 p75, were all potent inducers of ceramide generation in cultured DCs. Because CD40L, IL-1β, and TNF-α trigger in vitro maturation of DCs (5), as does LPS, which is structurally analogous to ceramide itself (11), these results raised the possibility that a common ceramide-mediated pathway, mimicked by LPS, could be responsible for some of the functional changes observed during in vitro maturation of DCs.

Ceramides Down-modulate Macromolecule Uptake by DCs. To test this hypothesis directly, we investigated whether exposure to exogenous cell-permeant C2-ceramide could down-modulate DC antigen uptake ability. DCs capture antigen either via macropinocytosis, a cytoskeleton-dependent type of fluid phase endocytosis initiated by membrane ruffling and formation of large vesicles, or receptor-mediated endocytosis through Fcγ and mannose receptors (5). As shown in Fig. 2, C2-ceramide could inhibit the uptake of three different classical endocytosis markers and their time-dependent accumulation into DCs. Both macropinocytosis, as assessed by LY and FITC-DX, and receptor-mediated endocytosis, as assessed by limiting amounts of HRP, were significantly affected. Comparable results were also obtained using C6-ceramide, a longer acyl chain ceramide analogue (data not shown). By contrast, C2-dihydroceramide, a structural analogue of C2-ceramide that lacks a double bond at the 4–5 position in the sphingoid base, was ineffective. Similarly, exposure to other diffusible signal-transducing lipid mediators such as diacylglycerol, did not affect macromolecule uptake ability of DCs (Fig. 2, A, C, E).

We then tested whether the endogenous production of ceramide would result in a similar inhibition of the endocytic
ability of DCs. Exposure of cultured DCs to exogenous sphingomyelinase, which results in intracellular ceramide accumulation (data not shown), also induced a dose-dependent inhibition of HRP uptake (Fig. 3A) and substantially retarded its time-dependent accumulation (Fig. 3B). Taken together, these results indicated that ceramide could specifically mediate inhibition of macromolecules uptake by DCs.

**Ceramides Down-modulate Soluble Antigen Presentation by DCs.** Cultured DCs are extremely efficient at presenting soluble antigen to specific T cells (2). In vitro maturation of DCs promoted by short term exposure to TNF-α results in a severalfold decrease of the antigen presentation capacity, associated with an increase in T cell stimulatory ability (4). Therefore, we tested whether ceramide could be sufficient for effectively modulating antigen presentation to T cells by using two different soluble antigens, TT and a soluble extract of *P. judaica* pollen (PjE). Cultured DCs were exposed to C2-ceramide and then pulsed with TT or PjE, before being used to challenge antigen-specific T cell clones (15, 19). Fig. 4 shows that C2-ceramide induced a \( \approx 50\)-fold reduction in the ability of DCs to present PjE, and \( \approx 100\)-fold reduction in the ability to present TT to their respective T cell clones (Fig. 4, A and B). By contrast, DCs treated with C2-ceramide were at least as efficient as untreated DCs in presenting nonprocessed antigen, i.e., in presenting an immunogenic TT peptide to the same TT-specific T cell clone (Fig 4C). Ceramide analogue C2-dihydroceramide was ineffective in blocking the response to soluble antigens (Figs. 4, A, B, and C).

**C2-ceramide is known to induce apoptotic cell death when administered to hemopoietic tumor cell lines or to in vivo-activated primary lymphoid cells within 6–12 h (20–22). Therefore, we checked whether the observed changes in antigen-processing capacity were due to loss of cell viability. C2-ceramide–treated DCs cultured for as long as 48 h excluded Trypan blue, displayed normal morphology, and

---

**Figure 2.** C2-ceramide inhibits both fluid phase and mannose receptor mediated endocytosis by DCs. (A, C, E) 2 × 10⁵ DCs were incubated with different concentrations of C2-ceramide (open circles), C2-dihydroceramide (closed circles), or diacetylceramide (closed triangles) for 10 min on ice, then transferred at 37°C and LY (1 mg/ml) (A), FITC–DX (1 mg/ml) (C), or HRP (0.1 μg/ml) (E) were added for 30 min. Results are expressed as percent of maximum uptake. The background (cells pulsed at 0°C) was less than 1% of the uptake at 37°C in all the experiments. (B, D, F) 2 × 10⁵ DCs were pretreated with 80 μM C2-ceramide (open circles) or medium (closed squares) for 10 min on ice, then transferred at 37°C and LY (B), FITC–DX (D) or HRP (F) accumulation was measured at different times. Results are expressed as mean fluorescence intensity (B, D) or as amount of cell-associated HRP (F). Comparable results were obtained using five different DC preparations. The vehicle did not affect endocytosis (data not shown).

**Figure 3.** Endogenously produced ceramide inhibits endocytosis by DCs. 2 × 10⁵ DCs were incubated with *Streptomyces* sp. SMase (open circles) at different concentrations (A) or at 0.1 U/ml (B) for 1 h at 37°C, then HRP was added at the final concentration of 1 mg/ml for 30 min (A) or for different times (B). The results are expressed as percentage of maximum HRP uptake (A) or as the amount of cell associated HRP and compared with untreated cells (closed squares) (B).
did not show any DNA fragmentation by propidium iodide staining and FACS® analysis (Fig. 4, D and E). Moreover, C2-ceramide treatment did not affect the ability of DCs to stimulate allogeneic T cells (Fig. 4 F).

Finally, we investigated whether the endogenous production of ceramide would affect the ability of DCs to present soluble antigen to T cells. Cultured DCs were treated with exogenous sphingomyelinase before being pulsed with TT, or with a TT peptide, and used to challenge a TT-specific T cell clone. Fig. 5 shows that endogenous ceramide production almost completely prevented presentation of soluble TT antigen, but had no inhibitory effect on TT peptide presentation by DCs, to the same TT-specific T cell clone. 

In this paper, we provide evidence that ceramides inhibit the antigen-capturing ability of cultured DCs, thereby suggesting a common molecular basis for CD40L, TNF-α, and IL-1β, or bacterial products such as LPS, to down-modulate antigen presentation by professional APC (5). In fact, we show that CD40L, as well as TNF-α or IL-1β, were all strong inducers of ceramide accumulation in DCs. Ceramides may specifically control antigen capturing and processing by DCs, as other cytokine-mediated differentiation events, i.e., upregulation of LFA1, B7-1, ICAM-1, and MHC molecules, were not consistently affected by ceramide exposure (data not shown). Accordingly, the enhanced immunostimulatory ability of mature DCs could not be promoted by exogenous ceramides, suggesting that additional intracellular mediators participate in the maturation process. Importantly, specific immunoefficiency of DCs can be inhibited without affecting cell viability or the ability to present nonprocessed antigen.

A possible explanation for these findings may reside in the capacity of endogenously released ceramides to interfere with vesicular trafficking. In fact, ceramides have been shown to directly inhibit endocytosis (23) and glycoprotein transport through the Golgi complex in CHO cells (24). Perturbing anterograde transport through the Golgi may prevent newly synthesized MHC class II molecules to reach endosomal compartments to be loaded with peptides de-
rived from hydrolyzed antibiotic. Interestingly, the fungal antibiotic brefeldin A (BFA), a classic inhibitor of both endogenous and exogenous antigen processing and presentation (25, 26), which causes disassembly of the Golgi apparatus (27) and its fusion with the ER and with early endosomes (28, 29), also triggers sphingomyelin hydrolysis resulting in ceramide production (30). Therefore, it is likely that the capacity of BFA to modulate antigen presentation is mediated by endogenously released ceramide.

Ceramides are emerging as intramembrane messengers involved in a variety of cellular adaptive and differentiative responses. Here, we provide evidence for a novel important function of ceramides in highly specialized cells such as DCs, which is modulation of soluble antigen presentation. Moreover, our data suggest that the capacity of ceramides to perturb intracellular membrane trafficking may be exploited by extracellular ligands able to trigger sphingomyelin hydrolysis, or by bacterial products that mimic ceramide, such as LPS, in order to regulate professional APC function and antigen-specific immune responses.

We thank Drs. A. Lanzavecchia, P. Lane, W. Lesslauer, and H. Loetscher for reagents.

This work has been supported by Istituto Superiore di Sanità (Progetto Tuberculosi), Associazione Nazionale Ricerca sul Cancro, CNR (Progetto Citochine), MURST, and European Community (Projects Human Capital and Mobility and Biomed 2). R. De Maria is an AIRC fellowship holder.

Address correspondence to Roberto Testi, Department of Experimental Medicine and Biochemical Sciences, University of Tor Vergata, via Tor Vergata 135, 00133 Rome, Italy.

Received for publication 13 September 1996 and in revised form 9 October 1996.

References

1. Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol. 9:271–296.
2. Austyn, J.M. 1992. Antigen uptake and presentation by dendritic leukocytes. Semin. Immunol. 4:227–236.
3. Austyn, J.M. 1996. New insights into the mobilization and phagocytic activity of dendritic cells. J. Exp. Med. 183:1287–1292.
4. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. J. Exp. Med. 179:1109–1118.
5. Sallusto, F., M. Cella, C. Danieli, and A. Lanzavecchia. 1995. Dendritic cells use macrophagocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J. Exp. Med. 182:389–400.
6. Kolesnick, R., and D.W. Golde. 1994. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. Cell. 77:325–328.
7. Heller, R.A., and M. Krünke. 1994. Tumor necrosis factor-mediated signaling pathways. J. Cell Biol. 126:5–9.
8. Hannun, Y.A. 1994. The sphingomyelin cycle and second messenger function of ceramide. J. Biol. Chem. 269:3125–3128.
9. Kolesnick, R., and Z. Fuku. 1995. Ceramide: a signal for apoptosis or mitogenesis? J. Exp. Med. 181:1949–1952.
10. Testi, R. 1996. Sphingomyelin breakdown and cell fate. Trends Biochem. Sci. In press.
11. Joseph, C.K., S.D. Wright, W.G. Bormann, J.T. Randolph, E.R. Kumar, R. Bittman, J. Liu, and R.N. Kolesnick. 1994. Bacterial lipopolysaccaride has structural similarity to ceramide and stimulates ceramide-activated protein kinase in myeloid cells. J. Biol. Chem. 269:17606–17610.
12. Wright, S., and R.N. Kolesnick. 1995. Does endotoxin stimu-
21. Jarvis, W.D., R.N. Kolesnick, F.A. Fornari, R.S. Traylor, D.A. Gerwitz, and S. Grant. 1994. Induction of apoptotic damage and cell death by activation of the sphingomyelin pathway. Proc. Natl. Acad. Sci. USA. 91:73–77.

22. De Maria, R., M. Boirivant, M.G. Cifone, P. Roncaiolli, M. Hahne, J. Tschopp, F. Pallone, A. Santoni, and R. Testi. 1996. Functional expression of Fas and Fas ligand on human gut lamina propria lymphocytes. A potential role for the acidic sphingomyelinase pathway in normal immunoregulation. J. Clin. Invest. 97:316–322.

23. Chen, C.-S., A.G. Rosenwald, and R.E. Pagano. 1995. Ceramide as a modulator of endocytosis. J. Biol. Chem. 270:13291–13297.

24. Rosenwald, A.G., and R.E. Pagano. 1993. Intracellular transport of ceramide and its metabolites at the Golgi complex: insights from short-chain analogs. Adv. Lipid Res. 26:101–118.

25. Yewdell, J.W., and J.R. Bennink. 1989. Brefeldin A specifically inhibits presentation of protein antigens to cytotoxic T lymphocytes. Science (Wash. DC). 244:1072–1075.

26. Adorini, L., S.J. Ullrich, E. Appella, and S. Fuchs. 1990. Inhibition by brefeldin A of presentation of exogenous protein antigens to MHC class II-restricted T cells. Nature (Lond.) 346:63–66.

27. Fujiwara, T., K. Oda, S. Yokota, A. Takatsuki, and Y. Ike-hara. 1988. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. J. Biol. Chem. 263:18545–18552.

28. Lippincott-Schwartz, J., L.C. Yuan, J.S. Bonifacino, and R.D. Klausner. 1989. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell. 56:801–813.

29. Wood, S.A., J.E. Park, and W.J. Brown. 1991. Brefeldin A causes a microtubule-mediated fusion of the trans-Golgi network and early endosomes. Cell. 67:591–600.

30. Linardic, C.M., S. Jayadev, and Y.A. Hannun. 1992. Brefeldin A promotes hydrolysis of sphingomyelin. J. Biol. Chem. 267:14909–14911.

31. Lane, P., T. Brocker, S. Hubele, E. Padovan, A. Lanzavecchia, and F. McConnell. 1993. Soluble CD40 ligand can replace the normal T cell–derived CD40 ligand signal to B cells in T cell–dependent activation. J. Exp. Med. 177:1209–1213.

32. Mackay, F., H. Loetscher, D. Stueber, G. Gehr, and W. Lesslauer. 1993. Tumor necrosis factor α (TNF-α)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55. J. Exp. Med. 177:1277–1286.