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Inhibitory activity of a ceramide library on interleukin-4 production from activated T cells

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Abstract—Allergic diseases are hypersensitivity disorders associated with the production of specific immunoglobulin E (IgE) to environmental allergens. Interleukin (IL)-4, produced primarily by $CD4^+$ T cells, is an important stimulus for the switch of the antibody isotype to IgE in both mice and humans. In this study we investigated the inhibitory activity of IL-4 production in activated T cells by screening ceramide derivatives prepared by solid phase combinatorial chemistry. Many ceramide derivatives significantly inhibited IL-4 production in T cells. In particular, ceramide derivatives with a lauroyl group showed strong inhibitory activities on IL-4 production in both phorbol 12-myristate 13-acetate (PMA)-activated EL4 T cells and antigen-primed cells, suggesting that they can be used as compounds for the development of anti-allergic agents.

1. Introduction

Ceramide refers to a family of highly hydrophobic molecules that contain a variable length fatty acid linked to sphingosine or a related long chain base.[1] Ceramide has a number of important physiologic functions that regulate cellular homeostasis including regulation of the stress response, induction of cell differentiation, regulation of cell cycle arrest and apoptosis.[2]

IL-4, a pleiotropic cytokine produced by activated T cells, basophils and mast cells, regulates many cellular and humoural immune responses. Dysregulation of IL-4 expression results in uncontrolled allergic inflammation including asthma and aberrant immune responses to pathogens.[3] IL-4 has a variety of other effects in hepatopoietic tissues. It increases the expression of class II MHC molecules in B cells,[4] enhances expression of CD23,[5] and up-regulates the expression of the IL-4 receptor.[6] IL-4 also has an important role in tissue adhesion.[7]

Previous studies have showed that the glycosphingolipids derived from tumours or parasites exhibited immunosuppressive activity.[8] A role for ceramides in regulating the immune response was suggested based on several observations, including its role in apoptosis, TCR expression, T cell proliferation, T cell activation and cytokine expression.[9] Because ceramides play a diversity role in regulation of immune responses and immunosuppressive activity, especially T cell responses,[10] we synthesize a ceramide library and investigate their inhibitory activities on IL-4 production concerned with allergic inflammation.

2. Results and discussion

A ceramide library was synthesized by a solid phase combinatorial chemistry as previously described (Fig. 1A).[11] A library of 80 compounds was generated using 8 sphingosine-like core structures and 10 acyl groups (Fig. 1B). The synthetic compounds were characterized by high-resolution mass spectrometry and ^1H NMR to confirm their identity and purity.

To examine the effects of the ceramide compounds on IL-4 production, EL4 T cells were stimulated in vitro for 48 h with PMA in the absence or presence of ceramide derivatives. As indicated in Table 1 and Figure 2,

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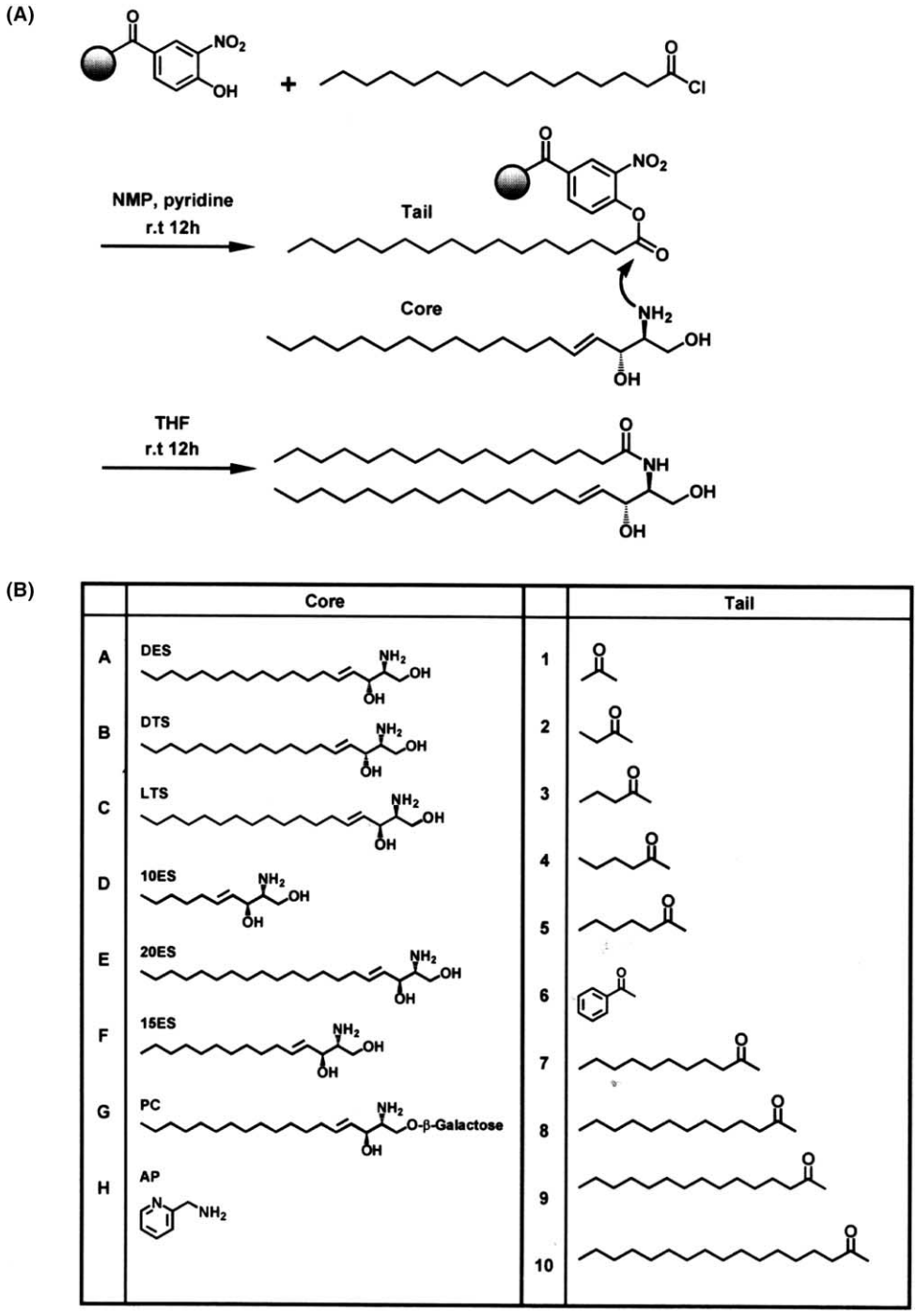

Figure 1. Synthesis and structures of ceramides. Full names for the abbreviations used for compounds; [Cores] DES: D-erythro-sphingosine, DTS: D-threo-sphingosine, LTS: L-threo-sphingosine, 10ES: 10-carbon-sphingosine, 20ES: 20-carbon-sphingosine, 15ES: 15-carbon-sphingosine, PC: psychosine, AP: 2-(aminomethyl)pyridine; [Tails] 1: acetyl, 2: propionyl, 3: butyryl, 4: valeryl, 5: hexanoyl, 6: benzoyl, 7: decanoyl, 8: lauroyl, 9: myristoyl, 10: palmitoyl.

many of ceramide derivatives showed inhibitory activities on IL-4 production by PMA-stimulated EL4 cells. Especially, four ceramide derivatives with a lauroyl group, A8, E8, G8 and H8, were more potent than other effective ceramide derivatives in inhibiting IL-4 production.

To confirm these inhibitory effects, EL4 T cells were stimulated with PMA (1 ng/mL) in the presence of varying amounts of A8, E8, G8 and H8 (1, 5, 10 μM) for 48 h and the levels of IL-4 were determined by IL-4 ELISA. As shown in Figure 3A, each of four ceramide derivatives significantly inhibited IL-4 production from PMA-stimulated EL4 T cells in a dose-dependent manner. To examine whether the IL-4 production inhibited by these ceramide derivatives at the protein level correlates with mRNA transcription, IL-4 mRNA levels were analyzed in PMA-stimulated EL4 cells in the absence or

Table 1. Inhibition of a ceramide library on IL-4 production

A1	48.83 ± 10.98	C1	84.66 ± 3.37	E1	78.58 ± 7.09	G1	85.64 ± 11.03
A2	88.29 ± 4.28	C2	96.52 ± 6.03	E2	84.77 ± 4.02	G2	88.26 ± 2.48
A3	84.01 ± 9.36	C3	113.36 ± 5.93	E3	99.77 ± 3.36	G3	111.14 ± 4.62
A4	77.22 ± 15.95	C4	114.24 ± 7.96	E4	87.25 ± 5.26	G4	112.84 ± 13.84
A5	79.84 ± 3.64	C5	146.23 ± 2.67	E5	96.92 ± 7.44	G5	92.17 ± 4.51
A6	89.49 ± 5.28	C6	111.28 ± 2.41	E6	90.77 ± 1.75	G6	89.16 ± 3.88
A7	74.69 ± 11.81	C7	92.00 ± 9.08	E7	80.89 ± 10.52	G7	51.46 ± 3.86
A8	34.25 ± 4.93	C8	70.49 ± 5.83	E8	50.33 ± 2.77	G8	47.16 ± 4.51
A9	66.59 ± 8.31	C9	79.10 ± 3.95	E9	85.39 ± 8.96	G9	80.18 ± 4.22
A10	68.00 ± 1.82	C10	80.85 ± 2.72	E10	90.72 ± 5.69	G10	81.76 ± 5.20
B1	78.57 ± 7.03	D1	86.25 ± 7.37	F1	91.31 ± 12.21	H1	93.99 ± 7.41
B2	77.76 ± 3.74	D2	90.39 ± 4.77	F2	89.51 ± 3.72	H2	97.28 ± 2.99
B3	77.96 ± 6.24	D3	83.35 ± 10.41	F3	96.51 ± 6.87	H3	103.77 ± 7.66
B4	83.06 ± 2.97	D4	54.15 ± 7.22	F4	95.54 ± 954	H4	96.08 ± 8.62
B5	111.56 ± 2.41	D5	86.64 ± 5.26	F5	89.98 ± 5.68	H5	90.17 ± 2.91
B6	130.42 ± 3.91	D6	76.71 ± 4.37	F6	93.57 ± 1.75	H6	90.73 ± 5.28
B7	74.44 ± 10.58	D7	96.30 ± 15.15	F7	78.67 ± 12.47	H7	94.20 ± 12.11
B8	63.48 ± 4.43	D8	73.15 ± 0.80	F8	74.96 ± 9.16	H8	48.87 ± 2.62
B9	69.06 ± 1.95	D9	78.09 ± 4.85	F9	86.18 ± 6.69	H9	95.13 ± 8.44
B10	72.47 ± 3.44	D10	77.12 ± 3.45	F10	80.46 ± 7.24	H10	107.33 ± 0.47

EL4 T cells were stimulated with PMA in the absence or presence of 10 μM of each ceramide derivatives for 48 h and the IL-4 levels in the culture supernatants were determined by IL-4-specific ELISA. The data are represented as % inhibition relative to the IL-4 level of PMA-stimulated EL4 cells in the absence of ceramide compounds. The values are the mean ± standard deviations of triplicate determinations.

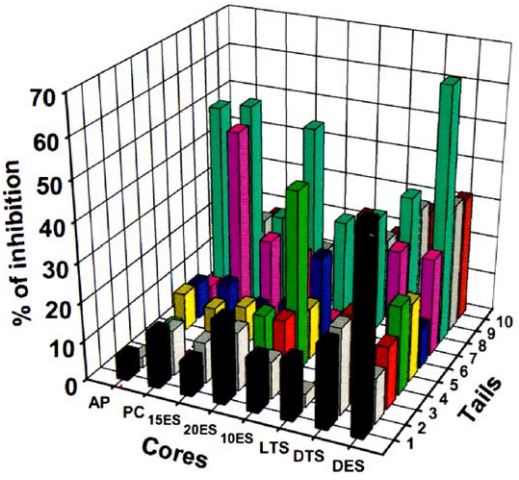

Figure 2. The inhibitory effect of ceramide derivatives on IL-4 production. The *Z* axis is % inhibition relative to the IL-4 level of PMA-stimulated EL4 T cells in the absence of ceramide compounds.

presence of ceramide derivatives with a lauroyl group. As shown in Figure 3B, A8, E8, G8 and H8 inhibited IL-4 mRNA levels in PMA-activated EL4 T cells, indicating that the changes in IL-4 production with A8, E8, G8 and H8 occurred at the transcriptional level. Treatment with A8, D8, E8 and H8 did not affect β-actin mRNA expression by PMA-activated EL4 T cells, suggesting that the inhibitory effect of IL-4 production by A8, E8, G8 and H8 was not the result of a generalized inhibition of cellular activation.

To investigate whether the inhibitory effects of four ceramide derivatives on IL-4 production also occurred in primary lymph node cells, BALB/c mice were immunized with KLH (100 μg) in alum. Seven days later, the draining lymph node cells were stimulated in vitro with KLH in the absence or presence of A8, E8, G8 and H8. As shown in Figure 4A, four derivatives significantly inhibited IL-4 production in KLH-primed lymph node cells. A8, E8, G8 and H8 also inhibited IL-4 mRNA levels in KLH-primed lymph node cells, clearly confirming that the changes in IL-4 production with A8, E8, G8 and H8 occurred at the transcriptional level (Fig. 4B). Importantly, ceramide derivatives did not affect the mRNA levels of interferon-γ, a Th1 cytokine (Fig. 4C), suggesting that the inhibitory effect of ceramide derivatives might be specific for IL-4. IL-4 inhibition by ceramide derivatives did not result from a general cytotoxic effect since cells' viability in all cultures remained constant throughout the incubation period in the presence of ceramide derivatives used in the experiment, as demonstrated by trypan blue exclusion test (data not shown).

IL-4, mainly produced in $CD4^+$ Th2 cells, is an important stimulus for the switching of antibody isotype to IgE in both mice and humans.[12] Higher than normal serum IgE is often found in patients with allergic diseases, including allergic asthma. Reduction of IgE is considered as one strategy in the treatment of asthma.[13] In this study, the inhibition of IL-4 production by A8, E8, G8 and H8-treated EL4 cells and primary lymph node cells might result in an inhibition of the IgE level in sera, leading to the reduction of the allergic response. Suplatast tosilate (IPD; Taiho; Tokyo, Japan) is an immunoregulator that suppresses IgE production and eosinophil infiltration through selective inhibition of IL-4 and IL-5 synthesis by Th2-like cells but not IFN-γ production in Th1 cells.[14]

The reason why ceramide derivatives with a lauroyl group were more effective than other ceramide derivatives is not clear. It seems that ceramide compounds with a lauroyl group are beneficial for the higher

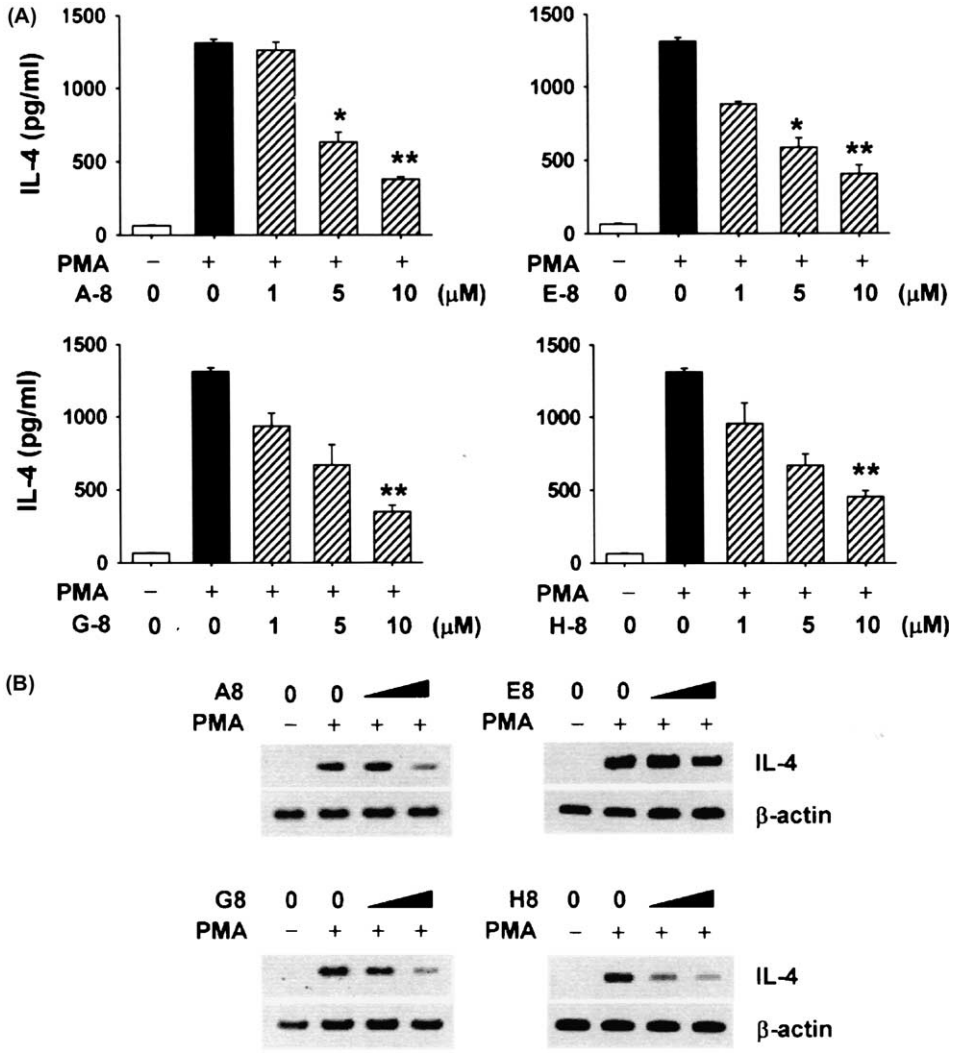

Figure 3. Effects of A8, E8, G8 and H8 on IL-4 production and mRNA expression level in PMA-activated EL4 T cells. (A) EL4 T cells were stimulated with PMA (1 ng/mL) in the absence or presence of varying amounts of A8, E8, G8 and H8 (1, 5, 10 μM) for 48 h. The cell culture supernatants were harvested and assayed for IL-4 levels by ELISA. The values represent the means ± standard deviations of triplicate determinations from one representative experiment. The experiment was repeated more than three times with similar results. $^{*}P < 0.0001$ and $^{**}P < 0.00005$, compared with the respective PMA-treated cells. (B) EL4 cells were stimulated with PMA (1 ng/mL) in the absence or presence of A8, E8, G8 and H8 (1, 10 μM) for 6 h. Cellular RNA from each treatment was extracted and the mRNA expression for IL-4 and β-actin was analyzed by RT-PCR.

potency in inhibiting IL-4 production at the transcriptional level. Several transcription factors have been identified that are critical for IL-4 transcription, including Stat6,[15] NF-ATc,[16] c-Maf,[17] GATA-3[18] and JunB.[19] To date, direct binding to and/or activation of the IL-4 promoter has been demonstrated for NF-ATc,[20] c-Maf[17] and JunB,[19] but not for Stat6[21] or GATA-3.[22] The transcription factor NF-AT is well known to play an essential role in the inducible transcription of IL-4 gene during T cell activation since human and murine IL-4 gene promoters contain at least four NF-AT sites that control their induction in T cells.[23] Activator protein (AP)-1 is necessary for the full high-levels of IL-4 production in atopic Th2 cells and is also an important contributor to the expression of Th2 cytokines, IL-5 and IL-13 as well as IL-4.[24] IL-4 gene promoter contains at least two AP-1 sites, P1 and P4.[25] Additionally, ceramide has been shown to activate a diverse set of protein kinases and protein phosphatases.[26] Therefore, A8, E8, G8 and H8 may regulate IL-4 gene transcription by inhibition activities of transcription factors, such as NF-AT or AP-1, through the modulation of diversity signalling molecules. The structure–reactivity relationships for the inhibitory activity of ceramide are being elucidated.

In conclusion, we have demonstrated that some ceramide derivatives, A8, E8, G8 and H8, strongly inhibited IL-4 production in both EL4 T cells and primary lymph node cells. This effect may explain the anti-allergic effects of A8, E8, G8 and H8. Because the ratio of IFN-γ-secreting Th1 and IL-4-secreting Th2 cells is closely correlated with the outcome of many diseases,[27] A8, E8, G8 and H8 may protect patients from developing diseases caused by unwanted Th2-dominated responses.

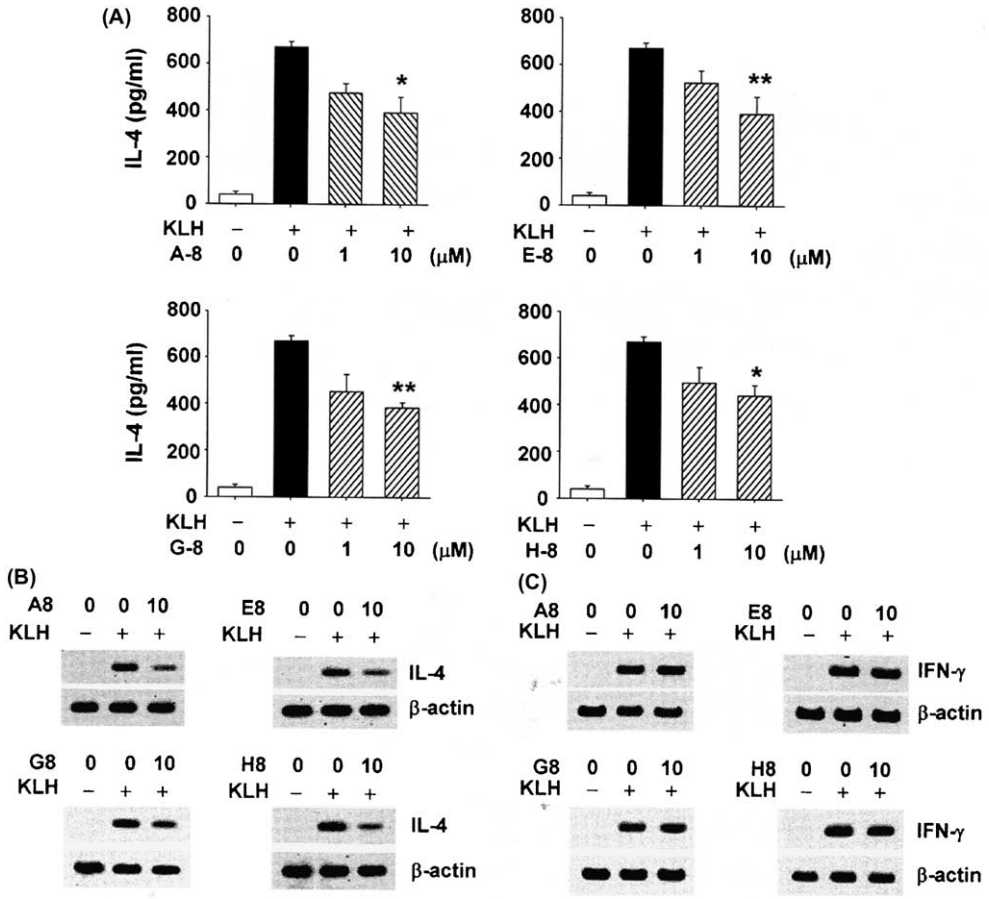

Figure 4. Effects of A8, E8, G8 and H8 on IL-4 production and mRNA expression in KLH-primed lymph node cells. (A) Mice were injected into the footpad with KLH (100 μg) in alum. Seven days later, the lymph node cells were collected and stimulated in vitro for 4 days with KLH (100 μg/mL) in the absence or presence of varying amounts of A8, E8, G8 and H8 (1, 10 μM). $^{*}P < 0.005$ and $^{**}P < 0.0005$, compared with the PMA-treated cells in the absence of ceramide compounds. (B and C) Lymph node cells from KLH-primed mice were re-stimulated for 6 h with KLH (100 μg) in the absence or presence of varying amounts of A8, E8, G8 and H8 (1, 10 μM). Cellular RNA from each treatment was extracted and the mRNA expression for IL-4, IFN-γ and β-actin was analyzed by RT-PCR.

3. Experimental

3.1. General synthetic methods

All chemicals were purchased from Sigma–Aldrich or Acros, and were used without further purification. Products obtained as solids or high boiling-point oils were dried under vacuum. Analytical thin-layer chromatography was performed on precoated silica plates (Merck 60-F 254, 0.25 mm thickness); compounds were visualized by staining with 10% phosphomolybdic acid in ethanol solution or 10% ninhydrin in ethanol solution.

3.2. General procedure for activated ester synthesis using acyl chloride

Activated ester resins were prepared by following literature procedure with a slight modification.[12a] Nitrophenol resin (1 g, 1.73 mmol) was suspended in 1-methyl-2-pyrrolidinone (10 mL). After 10 min, pyridine (1 mL, 12.4 mmol) and the appropriated acyl chloride (4 mmol, except the resin **1**, which was prepared by acetic anhydride) were added to the reaction mixture and stirred overnight at room temperature. The resin was filtered and washed with *N*,*N*′-dimethylformamide (3 × 20 mL), methanol–dichloromethane (1:1, 5 × 20 mL) and dichloromethane (5 × 20 mL) and dried.

3.3. General procedure for ceramide synthesis

An activated ester resin (50 mg, 75 μmol) was added to a core amine (3 μmol) in THF (1 mL) and agitated on a shaker overnight at room temperature. The reaction mixture was filtered and washed with a portion of THF (1 mL) and the filtrate was combined and dried. Completion of the reaction was confirmed by TLC and negative ninhydrin staining. The purity of each compound was checked by TLC and phosphomolybdic acid staining; each gave a single spot.

3.4. Materials, cell culture and mice

Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (St. Louis, MO). KLH was from Calbiochem Co. (San Diego, CA). Anti-murine IL-4 mAb 11B11 and BVD6 were from M. Howard, DNAX Research Institute (Palo Alto, CA) and recombinant murine IL-4 were purchased from PharMingen (San Diego,

CA). Cultures of EL4 thymoma cells and lymph node cells were maintained in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and 1% penicillin/streptomycin at 37 °C in a 5% CO_2 humidified air atmosphere. Six to eight-week-old-female BALB/c mice were obtained from Daehan Animal Inc. (Seoul, Korea), and maintained in pathogen-limited conditions. The mice were treated according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

3.5. In vitro stimulation of lymph node cells

Draining axillary, popliteal and inguinal lymph nodes were removed from mice 7 days after priming with 100 μg KLH absorbed to aluminium hydroxide (alum) adjuvant in the foot-pads as previously described.[28] Single-cell suspensions of lymph nodes were prepared and cultured in vitro with KLH (100 μg/mL) in the absence or presence of either ceramides. At the indicated times as described in the figure legends, the levels of IL-4 in the cell supernatants were determined by sandwich enzyme-linked immunosorbent assay (ELISA), and mRNA levels of IL-4 and interferon-gamma (IFN-γ) in the cells were assayed by reverse transcription-polymerase chain reaction (RT-PCR).

3.6. IL-4 assay

The levels of IL-4 in the culture supernatants were determined by a sandwich ELISA using mAbs for mouse IL-4 as previously described.[29] Briefly, ELISA plates were coated with rat anti-mouse IL-4 (11B11). After coating, serial diluted culture supernatants were added to the plates and incubated overnight at 4 °C. Murine recombinant IL-4 was used as standard for quantitation of IL-4 levels in the supernatants. After washing with washing buffer (1X PBS contained 0.01% Tween 20) and incubated with biotinylated anti-mouse IL-4 (BVD6). The plates were washed and streptavidin-peroxidase at room temperature. After additional washings, *o*-phenylenediamine (OPD) was added and developed. The OD was determined at 490 nm in a Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA).

3.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from the cells and reverse transcribed into cDNA. PCR amplification of the cDNA was preformed as previously described.[30] Total cellular RNA was isolated by the single-step method using the TRIzol reagent (Sigma). The sequences of PCR primers are as follows: mouse IL-4 (sense, 5′-ATG GGT CTC AAC CCC CAG CTA GT-3′; anti-sense, 5′-GCT CTT TAC GCT TTC CAG GAA GTC-3′), IFN-γ (sense, 5′-TGC ATC TTG GCT TTG CAG CTC TTC CTC ATG GC-3′; anti-sense, 5′-TGG ACC TGT GGG TTG TTG ACC TCA AAC TTG GC-3′) and β-actin (sense, 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′; anti-sense, 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′). The PCR reactions were run for 35 cycles for 94 °C (30 s), 57 °C (30 s), 72 °C (30 s) using a PCR Thermal Cycler (MJ Research, Watertown, MA). After the amplification, the RT-PCR products were separated in 1.5% (w/v) agarose gels and stained with EtBr. The sizes of PCR products for IL-4 and β-actin genes were 397 bp and 349 bp, respectively.

3.8. Statistical analysis

Student's *t*-test was used to determine the statistical differences between various experimental and control groups. *P*-values <0.05 were considered significant.

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