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Inhibition of interleukin-4 production in activated T cells via the downregulation of AP-1/NF-AT activation by N-lauroyl-D-erythro-sphingosine and N-lauroyl-D-erythro-C_{20}-sphingosine

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Abbreviations:
AP-1, activating protein-1
BECS, N-butyryl-D-erythro-C_{20}-sphingosine
BES, N-butyryl-D-erythro-sphingosine
ELISA, enzyme-linked immunosorbent assay
EMSA, electrophoretic mobility shift assay
IL, interleukin
KLH, keyhole limpet hemacyanin
LECS, N-lauroyl-D-erythro-C_{20}-sphingosine
LES, N-lauroyl-D-erythro-sphingosine

ABSTRACT

Allergic diseases are hypersensitivity disorders that are associated with the generation of specific immunoglobulin E (IgE) in response to environmental allergens. Interleukin (IL)-4, which is primarily produced by the $CD4^+$ T cells, is an important stimulus for the switching of the antibody isotype to IgE in both mice and humans. In a previous study, we demonstrated that ceramide derivatives coupled with a lauroyl group exerted strong inhibitory effects on IL-4 production in T cells. In this study, we attempted to characterize the mechanisms underlying the inhibition of IL-4 production in T cells. Two ceramide derivatives, N-lauroyl-D-erythro-sphingosine and N-lauroyl-D-erythro-C_{20}-sphingosine (hereafter abbreviated as LES and LECS, respectively), were shown to significantly inhibit the production of IL-4 in both primary $CD4^+$ T cells and EL4 T thymoma cells in a dose-dependent manner. LES and LECS also inhibited the activity of the IL-4 gene promoter in EL4 cells transiently transfected with IL-4 gene promoter constructs, but this effect was impaired in EL4 cells that had been transfected with an IL-4 promoter construct deleted of a P4 site harboring the AP-1 and NF-AT binding sites. Furthermore, LES and LECS inhibited the DNA binding activities of both AP-1 and NF-AT transcription factors. In addition, LES and LECS were demonstrated to suppress PMA-stimulated PKC activity, although they exerted no significant effects on the protein levels of the conventional PKCs. These results indicate that the ceramides, LES and LECS, may inhibit the production of IL-4 in the activated T cells, via the downregulation of AP-1/NF-AT activation and PKC activity.

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NF-AT, nuclear factor of activated T cell
PKC, protein kinase C
PMA, phorbol 12-myristate 13-acetate

1. Introduction

The ceramide refers to a family of highly hydrophobic molecules that contain fatty acids of variable length, linked to sphingosine or a related long-chain base [1]. Previous studies have shown that ceramide exerts a number of important physiologic effects that regulate cellular homeostasis, including the modulation of the stress response, the induction of cell differentiation, the regulation of cell cycle arrest and apoptosis [2]. The glycosphingolipids derived from tumors or parasites manifested immunosuppressive properties [3,4]. The ceramides were suggested to play a role in the regulation of the immune response, on the base of several observations, including studies of its role in apoptosis, TCR expression, T cell proliferation, T cell activation and cytokine expression [5–7].

IL-4, a pleiotropic cytokine produced by activated T cells, basophils, and mast cells, regulates a variety of the cellular and humoral immune responses [8]. The dysregulation of IL-4 expression has been determined to result in uncontrolled allergic inflammation, including asthma and aberrant immune responses to pathogens [9–11]. Thus far, NF-ATc, c-Maf, and JunB have been shown to directly bind to and/or activate the IL-4 promoter [12–14]. The IL-4 promoters of both mouse and humans harbor five binding elements (designated P0 to P4) for the NF-AT family of transcription factors [15,16]. Both P1 and P4 are immediately flanked by sequences with affinity for proteins of the AP-1 family, thereby allowing for cooperative binding with the NF-AT proteins [17]. Although all P elements contribute to IL-4 gene control to some extent, the P1 and P4 are believed to be the major positive regulatory P elements [18]. AP-1 consists of a dimer consisting of members of the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra1, and Fra2) families. AP-1 can be induced by a variety of cytokines and growth factors [19]. AP-1 binding sites are located in the promoter region of many proinflammatory cytokines, adhesion molecules and cell proliferation growth factors [20,21].

Recently, we reported that ceramide derivatives coupled with a lauroyl group could exert inhibitory effects on IL-4 production in T cells [22]. In this report, we further verified the inhibitory effects of the ceramide derivatives in $CD4^+$ T cells, and attempted to characterize the mechanisms underlying the inhibition of IL-4 production, by using two ceramide derivatives with profound inhibitory activities, LES and LECS.

2. Materials and methods

2.1. Materials and cell culture

Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (St. Louis, MO). KLH was obtained from Calbiochem Co. (St. Louis, MO). The ceramide derivatives used in the experiments were synthesized and characterized by high-resolution mass spectrometry and ^1H NMR to confirm their identity and purity, as previously described [22]. Anti-murine IL-4 mAbs 11B11 and BVD6 were obtained 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 were maintained in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY) 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.

2.2. Isolation of $CD4^+$ T 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, which had been injected into the footpads. $CD4^+$ T cells were positively purified by incubating the cells with anti-mouse CD4 (L3T4) conjugated microbead (Miltenyi Biotech., Germany) for 20 min at 4 °C, and applying into the MACS positive selection column placed in VarioMACS (Miltenyi Biotech.), according to the manufacturer's protocol. After washing with a buffer (1× PBS containing 2 mM EDTA and 0.5% BSA), the $CD4^+$ T cells were eluted and washed with serum-free RPMI-1640 medium, and stimulated for 4 days with KLH in the absence or presence of the ceramide derivatives. The levels of IL-4 and IFN-γ were determined by a sandwich ELISA and RT-PCR.

2.3. Determination of cell viability and proliferation

Cell viability was determined by the trypan blue exclusion assay. Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) assay. In brief, after each treatment, 10 μl of MTT (5 mg/ml) was added to each well in 96-well plates. After incubation for 4 h at 37 °C, the crystals of viable cells were dissolved with 100 μl of 0.04N HCl in isopropanol. The absorbance of each well was then read at 540 nm using a kinetic microplate reader.

2.4. Cytokine assays

The levels of IL-4 and IFN-γ in the culture supernatants were determined via a sandwich ELISA using mAbs for mouse IL-4 and IFN-γ, as previously described [23]. Murine recombinant IL-4 and IFN-γ (PharMingen) were used as standard for the quantitation of IL-4 and IFN-γ levels in the supernatants. The mAbs for coating the plates and the biotinylated second mAbs

were as follows: for IL-4, 11B11 and BVD6; for IFN-γ, HB170 and XMG1.2. The lower limits of detection were 3 pg/ml for IL-4 and 25 pg/ml for IFN-γ.

2.5. 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 [24]. 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′-ATGGGTCTCAACCCCCAGCTAGT-3′; antisense, 5′-GCTCTTTACGCTTTCCAGGAAGTC-3′), mouse IFN-γ (sense, 5′-AGTTATATCTTGGCTTTTCA-3′; antisense, 5′-ACCGAATAATTAGTCAGCTT-3′) and β-actin (sense, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′; antisense, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′). The PCR reactions were run for 35 cycles for 94 °C (30 s), 58 °C (45 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 ethidium bromide. The sizes of PCR products for IL-4 and β-actin genes were 397 and 349 bp, respectively.

2.6. IL-4 promoter constructs, transient transfection and AP-1/NF-AT minimal promoter assay

The −741/+56 fragment of murine IL-4 promoter was generated by PCR from genomic DNA of DBA/2 mice. The PCR product was cloned into the BamHI/EcoRI sites of the pGEM-7Z and then subcloned into the SacI/XhoI sites of the pGL3-basic luciferase vector (Promega Co., Madison, WI). All the deletion mutants were generated by PCR using an upstream primer containing BamHI site. For transfections, EL4 cells were cultured in RPMI-1640 medium and transfected with indicated plasmid in the presence of Superfectam according to the manufacturer's protocol (Qiagen, Germany). The cells were stimulated with PMA (1 ng/ml) in the absence or presence of the ceramide derivatives. The cells were harvested 24 h later, and luciferase activity was assayed. Results represent as relative fold induction compared to the unstimulated EL4 cells. AP-1 and NF-AT minimal promoters were obtained from J.W. Lee (Baylor College of Medicine, TX). For transfections, EL4 cells were cultured in DMEM medium and transfected with the indicated plasmid in the presence of Superfectam according to the manufacturer's protocol (Qiagen, Germany). The cells were stimulated with PMA (1 ng/ml) in the absence or presence of the ceramide derivatives. The cells were harvested 24 h later, and luciferase activity was assayed.

2.7. Preparation of nuclear extracts and electrophoretic mobility shift assay

EL4 cells were stimulated for 1 h with PMA (1 ng/ml) in the presence of varying amounts of the ceramide derivatives. The nuclear extracts were prepared from the cells, as previously described [25], and aliquots were frozen at −80 °C. Protein concentrations in nuclear extracts were determined by BCA protein assay kit (Pierce Biotech., Rockford, IL). Synthetic oligonucleotides were annealed and end-labeled using [α-^{32}P] dCTP (250 μCi; Amersham) and Klenow enzyme. For binding assays, 10 μg of total nuclear extract was incubated with ^{32}P-labelled oligonucleotide in the presence of a reaction mixture containing 20 mM dithiothreitol, poly(dI-dC), 10× gel retardation assay buffer (GRAB) for 30 min at room temperature. For competition experiments, the extracts were pre-incubated with a 50-fold excess of unlabelled specific or non-specific probes. Protein/DNA binding complexes were separated from free probes using a 4% polyacrylamide gel in 0.5 × Tris-borate-ethylenediaminetetra-acetic acid buffer at 200–250 V for 45 min. Dried gels were exposed to an X-ray film at −80 °C. The base sequences of oligonucleotides used in binding and competition assays were as follows: NF-AT, 5′-CGCCAAAGACGCCAAAGAGGAAAATTTGTTTCATA-3′; AP-1, 5′-GATCTGCATGAGTCAGACACACA-3′; NF-κB, 5′-CCGGTTAACAGAGGGGGCTTTCCGAG-3′; CRE, 5′-GATCCGAGCCCGTGACGTTTACACTCATTCT-3′.

2.8. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was conducted, as previously described [26]. On the day prior to the experiment, 2 × 10^6 cells were plated in 0.5% bovine serum albumin containing growth medium. The cells were stimulated for the indicated times, and sequentially cross-linked with disuccinimidyl glutarate and 1% formaldehyde in serum-free medium for 15 min at 37 °C. The cells were washed, transferred to Eppendorf tubes, and finally solubilized in 400 μl of SDS lysis buffer (1% SDS, 10 mM Tris, pH 8.0, 1 mM EDTA) with a protease inhibitor mixture (Sigma). The samples were then sonicated three times, for 15 s on setting 2 until the DNA fragments measured 300–400 bp or less. Equal amounts of DNA were then immunoprecipitated overnight at 4 °C in ChIP dilution buffer (50 mM NaCl, 1 mM HEPES, pH 7.4, 1% IGEPAL-630, 10% glycerol, 1 mM dithiothreitol) with 20 μg of antibody. The immunoprecipitates were collected using protein-A magnetic beads (Dynal, Inc.) and washed sequentially with ChIP dilution buffer, high salt buffer, LiCl buffer, and TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The DNA was eluted in 1 ml of elution buffer (1% SDS in 0.1 M $NaHCO_3$). The samples were de-cross-linked in 200 mM NaCl at 65 °C, for 1 h. The DNA was phenol extracted, ethanol precipitated, and used for PCR. The primer pairs used in this step were 5′-GTGGCAACCCTACGCTGATAAG-3′ (sense) and 5′-GCTAACAATGCAATGCTGGCAG-3′ (antisense). The PCR products were fractionated via agarose gel chromatography, followed by ethidium bromide staining.

2.9. Preparation of the cell lysates and Western blot analysis

The treated cells were washed twice with the ice-cold phosphate-buffered saline solution and harvested with a plastic scraper. The cells were lysed in lysis buffer (100 mM NaCl, 50 mM Tris–Cl, 1 M EDTA, 10% glycerol, 1% Nonidet P-40, 1 mM NaF, 1 mM Na_3VO_4, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 50 μg/ml PMSF) by incubation on ice. The cell lysates were then centrifuged at 14,000 rpm at 4 °C, and the supernatants were transferred into fresh tubes and stored at −80 °C until required. Protein concentration of the lysates was determined using the

BCA protein assay reagent (Pierce Biotec., Rockford, IL). Proteins of 10 μg were resolved in 10% sodium dodecyl sulfate (SDS) polyacrylamide gels at 30 mA and 200–250 V for 1 h in a Mini-Protein II gel apparatus (Bio-Rad, Richmond, CA). After electrophoresis, the proteins were blotted to the pre-wetting nitrocellulose membrane using a Semi-Phor (Hoefer Scientific Instrument, San Francisco, CA). The membrane was then incubated with washing buffer containing 5% non-fat milk for at least 1 h to block non-specific protein binding. Primary mAb was diluted up to 1:5000 in washing buffer and applied to the membrane for 1 h at room temperature. Following washing, the blots were incubated with the appropriate biotinylated secondary mAb for 1 h at room temperature. Immunoreactive bands were visualized by the enhanced chemiluminescence system (Amersham, Buckinghamshire, UK).

2.10. Protein kinase C activity assay

EL4 cells were lyzed in lysis buffer containing 50 mM Tris (pH 7.5), 2 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1% Triton X-100, 150 mM NaCl, 1 μM dithiothreitol, 1 mM PMSF, 50 mM NaF, 1 mM sodium orthovanadate, 50 μg/ml leupeptin, and 50 μg/ml aprotinin by incubation on ice for 30 min. The lysates were then centrifuged at 14,000 rpm at 4 °C for 20 min. The proteins in 200 μg of the supernatants were incubated with PKC antibody at 4 °C for 2 h. After protein A was added, the mixture was shaken at 4 °C for 1 h and washed with lysis buffer. The antibody-coupled proteins were centrifuged at 5000 rpm for 1 min and reacted with 5 μg myelin basic protein and 0.5 μl [γ-^{32}P]ATP in reaction buffer (0.5 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10 mM $MgCl_2$, 20 mM HEPES (pH7.4), 50 mM ATP, 2 mM dithiothreitol, 2 mM NaF, and 2 mM sodium orthovanadate) at room temperature for 30 min. The reaction mixture was analyzed by electrophoresis on a 15% SDS-PAGE.

2.11. Statistical analyses

The Student's *t*-test and one way ANOVA followed by Bonferroni method were used to determine the statistical differences between values of the various experimental and control groups. *P*-values <0.05 were considered significant.

3. Results

3.1. Inhibition of IL-4 production in activated T cells by LES and LECS

In order to determine whether the two ceramide derivatives, *N*-lauroyl-D-erythro-sphingosine and *N*-lauroyl-D-erythro-C_{20}-sphingosine (hereafter abbreviated as LES and LECS, respectively) (Fig. 1A), had any effect on IL-4 production in CD4$^+$ T cells primed with KLH, the footpads of BALB/c mice were injected with KLH (100 μg/mouse) in alum. Seven days later, CD4$^+$ T cells were purified from the lymph node cells of the immunized mice, and stimulated for 4 days in vitro with KLH in the absence or presence of LES and LECS, after which the levels of IL-4 and IFN-γ in the supernatants were determined. We included two other ceramide derivatives as the controls. These compounds, each with the same core structures, were *N*-butyryl-D-erythro-sphingosine and *N*-butyryl-D-erythro-C_{20}-sphingosine (hereafter abbreviated as BES and BECS, respectively) (Fig. 1A). As shown in Fig. 1B, both LES and LECS significantly inhibited IL-4 production in a concentration-dependent manner, whereas the control ceramide derivatives, BES and BECS, had little effects. In contrast, LES and LECS exerted no effects on the production of IFN-γ, a Th1 cytokine, in the KLH-stimulated CD4$^+$ T cells. Furthermore, LES and LECS inhibited the levels of IL-4 mRNA in the KLH-primed CD4$^+$ T cells, thereby indicating that changes in IL-4 production had occurred at the transcriptional level. The inhibition of IL-4 expression by LES and LECS did not result from reductions in cell proliferation and cell viability, as cell proliferation and viability remained constant throughout the incubation period in the presence of LES and LECS, as demonstrated by MTT assay and trypan blue exclusion assay (data not shown). LES and LECS also resulted in a reduction in the levels of IL-4 production in PMA-activated EL4 T thymoma cells (Fig. 1C).

3.2. Effects of LES and LECS on PMA-induced IL-4 promoter activity via the targeting of AP-1 and NF-AT transcription factors

In order to identify the region involved in the observed activities of LES and LECS, a series of luciferase reporter constructs were generated, which harbors the IL-4 promoter sequences from positions −741, −251, −220, −88, −58, and −46 to +71 (represented as −741/+71, −251/+71, −220/+71, −88/+71, −58/+71, and −46/+71) relative to the transcription initiation site (Fig. 2A). The EL4 cells were transfected with each of these constructs, and stimulated with PMA in the absence or presence of LES and LECS, after which the luciferase activity was evaluated. As shown in Fig. 2B, the full promoter construct (−741/+71) showed strong stimulation with PMA in the absence of LES and LECS, and the stimulated activity was significantly inhibited by LES and LECS, but not by BES and BECS. In particular, the deletion of sequences to −251 (−251/+71) did not diminish the PMA-dependent promoter activities, although the inhibitory effects of LES and LECS were still observed. However, the deletion of sequence to −220 (−220/+71) resulted in the elimination of the PMA-induced promoter activities that had been inhibited by LES and LECS, indicating that the target site for LES and LECS resided within this region (Fig. 2C). The P4 site contains the NF-AT and AP-1 binding sites. These results indicate that the inhibitory effect of LES and LECS on IL-4 production may be mediated via NF-AT and/or AP-1 binding sites. The NF-AT transcription factor has been consistently determined to be the most critical transcription factor with regard to the regulation of IL-4. Nuclear factor AP-1, which allows for cooperative binding with the NF-AT proteins, is a complex transcription factor consisting of members of the c-Jun and c-Fos protein families.

3.3. AP-1 and NF-AT DNA binding activities inhibited by LES and LECS

In order to further evaluate the involvement of AP-1 and/or NF-AT in the regulation of the IL-4 gene by LES and LECS, EL4

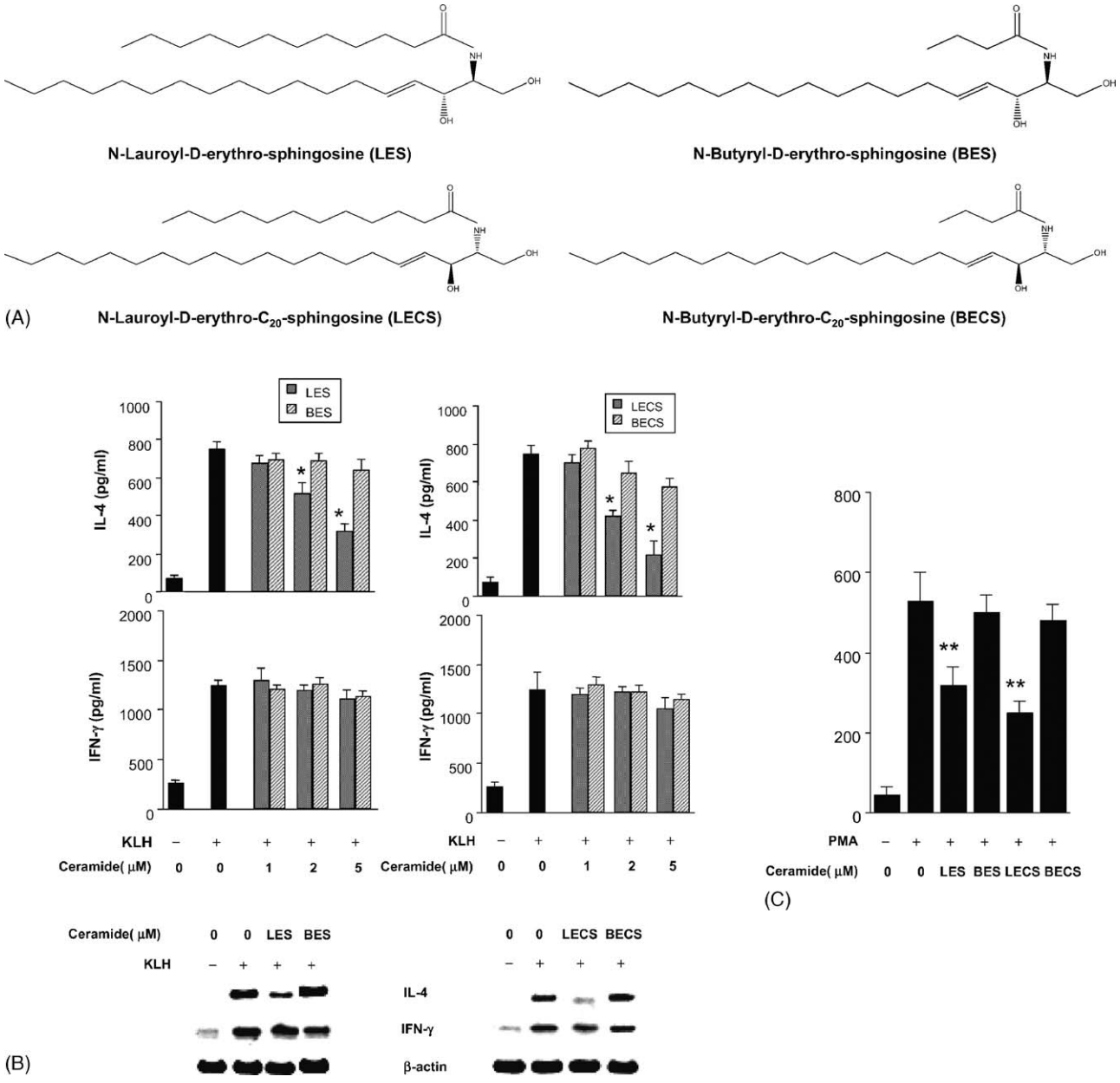

Fig. 1 – LES and LECS inhibit IL-4 production in both activated CD4⁺ T cells and EL4 thymoma cells. (A) Chemical structures of the ceramide derivatives used in the experiments. (B) Isolated CD4⁺ T cells, as described in Section 2, were re-stimulated for 4 days with KLH (100 μg/ml) in the presence of the ceramide derivatives (1, 2, and 5 μM). The cell culture supernatants were harvested and assayed by ELISA for IL-4 and IFN-γ levels. To determine the mRNA levels, CD4⁺ T cells were stimulated for 6 h with KLH (100 μg/ml) in the presence of the ceramide derivatives (5 μM). The cellular RNAs from each of the treatment were extracted and the expression of IL-4, IFN-γ, and β-actin mRNA was analyzed by RT-PCR. $^{*}P < 0.01$, relative to the group treated with KLH alone. (C) EL4 cells were stimulated for 2 days with PMA (1 ng/ml) in the presence of the ceramide derivatives (5 μM). The cell culture supernatants were harvested and assayed by ELISA for IL-4 levels. $^{**}P < 0.01$, relative to the group treated with PMA alone.

cells were stimulated with PMA in the absence or presence of the ceramide derivatives, and nuclear extracts from each of the treated cells were isolated and analyzed in EMSA using each of the consensus sequences corresponding to the AP-1 and NF-AT sites in the P4 region. As shown in Fig. 3A, the unstimulated EL4 cells exhibited no detectable binding activity for NF-AT or AP-1. The EL4 cells treated with PMA effectively induced NF-AT and AP-1 binding activity. Treatment with LES and LECS inhibited PMA-induced DNA binding activities of both AP-1 and NF-AT in EL4 cells, whereas the control BECS exerted minimal effects. Treatment with BES also decreased the levels of NF-AT DNA binding activity, although the inhibitory effect was relatively low compared with that of LES or LECS.

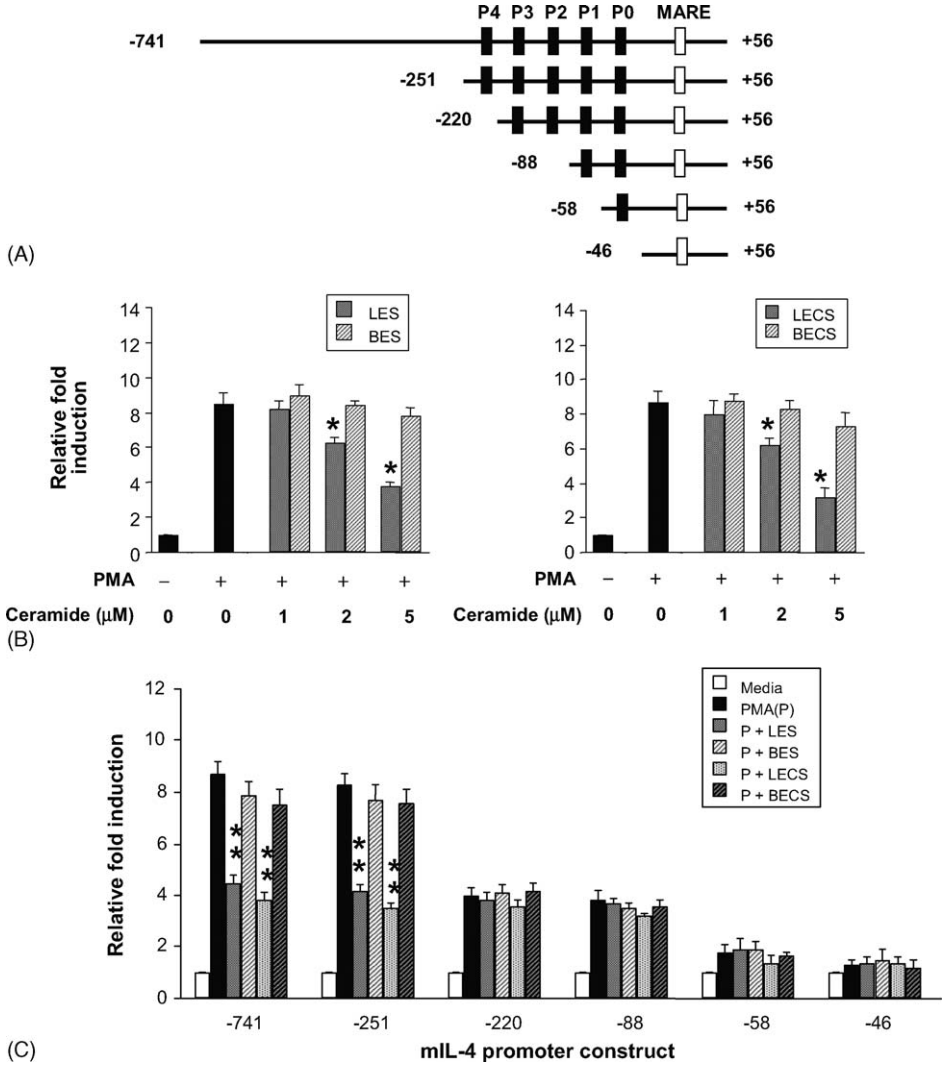

Fig. 2 – LES and LECS inhibit IL-4 gene promoter activity induced by PMA. (A) The schematic diagram of the proximal 750 base pairs of the murine IL-4 gene promoter. P and MARE represent the NF-AT binding sites and c-Maf binding sites, respectively. The P1 and P4 sites include the AP-1 binding site. (B) EL4 cells were transiently transfected with the IL-4 full promoter construct, followed by stimulation with PMA (1 ng/ml) in the absence or presence of the ceramide derivatives (1, 2, and 5 μM). The results are represented as the induction fold over the value obtained with the unstimulated EL4 cells transfected with the promoter construct, which was assigned an arbitrary value of 1. $^{*}P < 0.01$, relative to the group treated with PMA alone. (C) EL4 cells were transiently transfected with the serially deleted IL-4 promoter constructs, followed by stimulation with PMA (1 ng/ml) in the absence or presence of the ceramide derivatives (5 μM). The values represent the mean ± S.D. of triplicate determinations. The data are representative of three independent experiments. $^{**}P < 0.001$, relative to each of the IL-4 promoter constructs treated with PMA alone.

The involvement of AP-1 and NF-AT in the inhibition of IL-4 production was further confirmed in the EL4 cells that were transfected with minimal AP-1 and NF-AT promoter constructs, followed by the treatment with PMA in the absence or presence of LES and LECS. As shown in Fig. 3B, LES and LECS inhibited the PMA-induced minimal promoter activation of AP-1 and NF-AT, whereas BES and BECS exerted no such effects. These results show that the inhibitory effects of LES and LECS on IL-4 production were mediated via the reduced activation of both the AP-1 and NF-AT transcription factors.

Furthermore, in order to characterize the in vivo nuclear protein binding of AP-1 and NF-AT to the IL-4 promoter region, we conducted a ChIP assay with Abs against the AP-1 Jun and NF-AT, in the presence or absence of the sphingosine derivatives. As shown in Fig. 4, in PMA-stimulated EL4 cells both AP-1 and NF-AT were efficiently coimmunoprecipitated with the IL-4 promoter region. Treatment with LES and LECS decreased PCR band intensity of the IL-4 promoter coimmunoprecipitated with anti-NF-AT antibody, while BES and BECS had no effects. Interestingly, all sphingosine derivatives did not significantly affect PCR band intensity of the IL-4

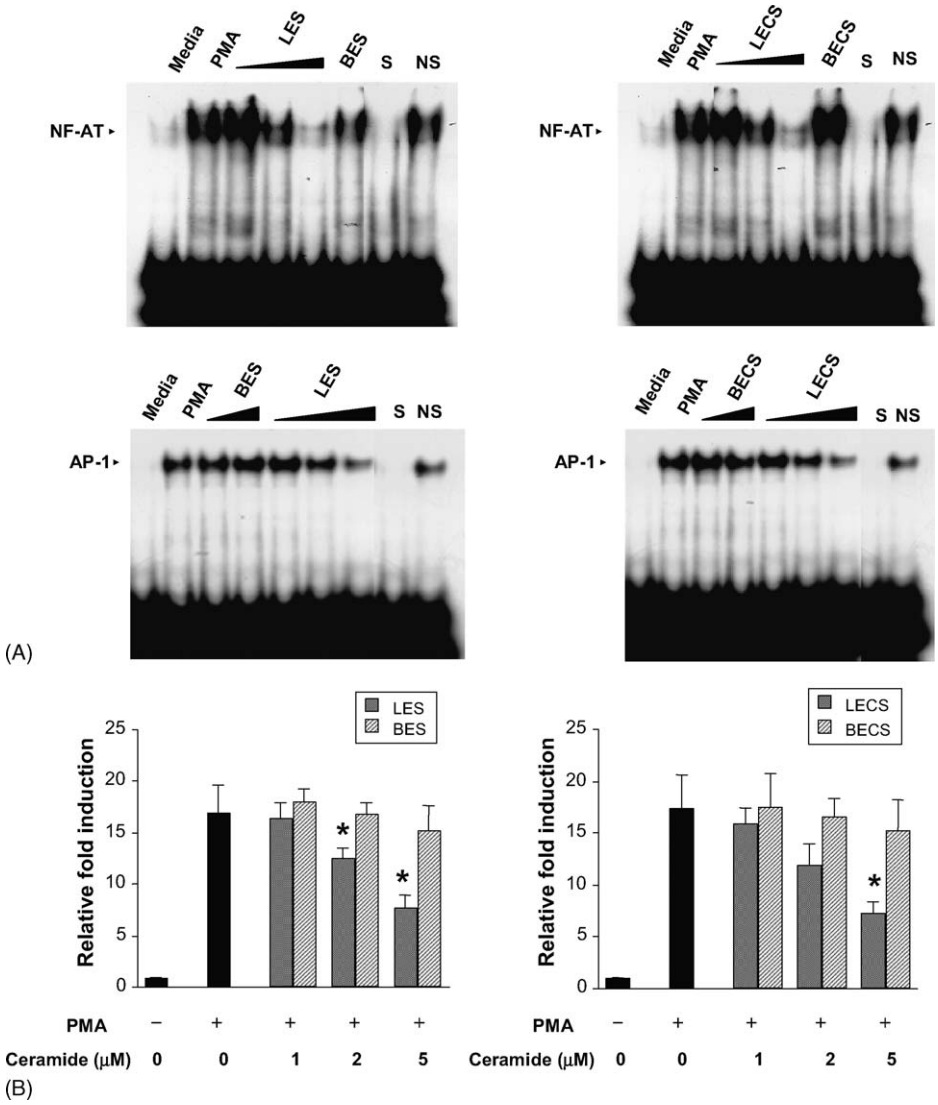

Fig. 3 – LES and LECS inhibit PMA-induced activation of AP-1 and NF-AT. (A) LES and LECS inhibit AP-1 and NF-AT DNA binding activities. Nuclear extracts from the PMA-stimulated EL4 cells in the presence of LES and LECS (1, 2, and 5 μM), or BES and BECS (5 μM) were examined for AP-1 and NF-AT DNA binding activity in the EMSA, using radiolabelled oligonucleotides harboring an AP-1 site or NF-AT site, respectively. S and NS indicate the presence of an unlabelled, specific oligonucleotide (NF-AT and AP-1) and non-specific oligonucleotide (CRE or NF-κB), respectively. (B) LES and LECS inhibit the minimal promoter activities of AP-1 and NF-AT activated by PMA. Transfection of EL4 cells with AP-1 or NF-AT minimal promoter constructs, followed by stimulation with PMA (1 ng/ml) in the absence or presence of the ceramide derivatives (1, 2, and 5 μM). The results are expressed as the induction fold over the value obtained with the unstimulated EL4 cells that had been transfected with the minimal promoter, which was assigned an arbitrary value of 1. The data are representative of three independent experiments. $^{*}P < 0.05$, relative to the group treated with PMA alone.

promoter coimmunoprecipitated with anti-AP-1 antibody (Fig. 4).

3.4. *Involvement of PKC in the inhibition of PMA-stimulated IL-4 production by LES and LECS*

Many reports have demonstrated that several PKC isoforms may be involved in the activation of NF-AT [27–29]. In order to investigate any involvement of PKC in the inhibition of IL-4 production by LES and LECS, both PKC protein levels and PKC activity were assessed in the EL4 cells in the absence or presence of the ceramide derivatives. In this report, we focused specifically on the conventional PKC isoforms, such as α, βI, βII, and γ, as these PKC isoforms are phospholipid- and Ca^{2+}-dependent, and are known to be regulated by the ceramides [30,31]. As shown in Fig. 5A, none of the ceramide derivatives used in the experiments affected the protein levels of each of the conventional PKC isoforms in the PMA-activated EL4 cells. However, LES and LECS were determined to inhibit PKC activities, while the

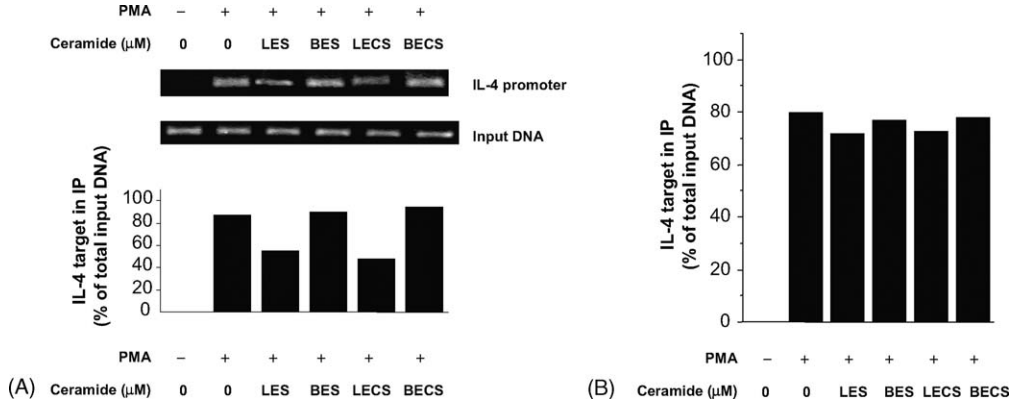

Fig. 4 – LES and LECS decrease PMA-induced in vivo binding of NF-AT to the IL-4 promoter. EL4 cells were incubated for 2 h with 5 μM of each of the derivatives, and cross-linked chromatin was subjected to immunoprecipitation with anti-NF-AT (A) or anti-Jun (B). Immunoprecipitated DNA was amplified by PCR using primers specific for IL-4 promoter. The band intensity of PCR bands was densitometrically analyzed and the data are represented as % of total input DNA. The data are representative of two independent experiments.

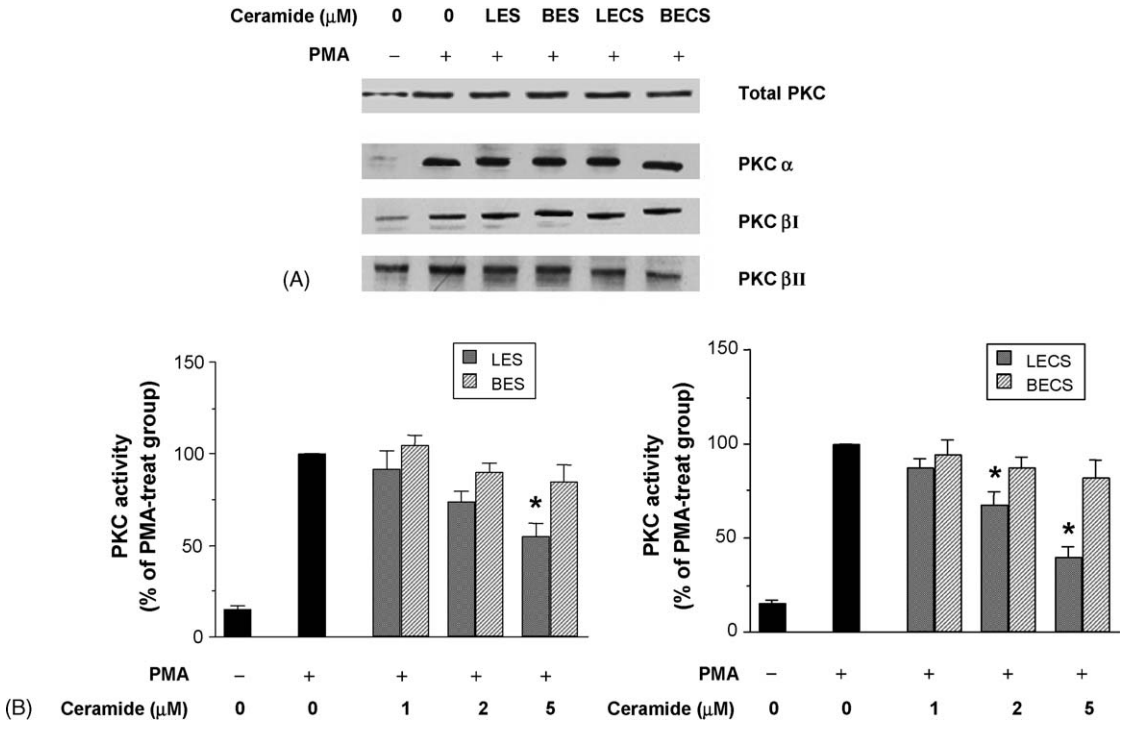

Fig. 5 – Effects of LES and LECS on protein levels of the conventional PKC isoforms and PKC activities. (A) EL4 cells were treated for 2 h with 5 μM of the ceramide derivatives, and the total PKC and the conventional PKC isoforms were evaluated via Western blot analysis using each of anti-PKC isoform mAbs. (B) EL4 cells were treated for 2 h with various concentrations of the ceramide derivatives. The total PKC activity in the treated cells was assessed via PKC kinase assays using γ-^{32}P-ATP, as described in Section 2. Quantitative analysis of PKC kinase activity was conducted using the Tina 2.0 program, normalizing on the basis of total PKC level, respectively. The values represent the % of that in the PMA-stimulated group in the absence of the ceramide derivatives. The values are expressed as the mean ± S.E. mean (n = 3). $^{*}P < 0.01$, relative to the group treated with PMA alone.

control ceramide derivatives, BES and BECS, exerted no such effects (Fig. 5B). This result shows that the reduction in PKC activities may be involved in the LES- and LECS-induced inhibition of IL-4 production in activated T cells.

4. Discussion

In this report, we have demonstrated that the ceramide derivatives containing a lauroyl group, LES and LECS, exerted a

significant inhibitory effect on IL-4 production in both $CD4^+$ T cells and EL4 T cells. In contrast, the ceramide derivatives with a butyryl group rather than a lauroyl group, BES and BECS, exerted no effects on the production of IL-4. A series of transient transfection assays, protein kinase assays, and EMSA experiments showed that LES and LECS inhibited the expression of IL-4 gene via the downregulation of AP-1/NF-AT activation and PKC activity.

IL-4, which is produced primarily in the $CD4^+$ Th2 cells, is an important stimulus for the switching of the antibody isotype to IgE in both mice and humans [32,33]. Elevated serum IgE levels are often encountered in patients suffering from allergic diseases, including allergic asthma. Reduction of IgE levels is one strategy currently utilized in the treatment of asthma [34]. In this study, the LES and LECS-induced inhibition of IL-4 production may result in a decrease of the IgE level in the sera, resulting in an amelioration of the allergic response.

The mechanism by which LES and LECS inhibit the production of IL-4 at the molecular level remains uncertain. We determined that LES and LECS significantly inhibited IL-4 mRNA expression in $CD4^+$ T cells, and this effect occurred in a dose-dependent manner (Fig. 1). LES and LECS also inhibited the activation of the IL-4 gene promoter (Fig. 2), thereby indicating that the inhibition of IL-4 production by LES and LECS occurred at the transcriptional level. In our study, the inhibitory effects of LES and LECS on the PMA-activated IL-4 gene promoter were not observed in the EL4 T cells that had been transfected with a deletion construct of the P4 site harboring the NF-AT and AP-1 binding sites (Fig. 2). This suggests that the inhibitory effects of LES and LECS on IL-4 production might be mediated via increased binding activities of NF-AT and/or AP-1. The NF-AT transcription factor has been previously shown to perform an essential role in the inducible transcription of the IL-4 gene during T cell activation, as both human and murine IL-4 gene promoters harbor at least four NF-AT sites that control their induction in the T cells [35,36]. AP-1 is necessary for optimal high levels of IL-4 production in atopic Th2 cells, and the IL-4 gene promoter harbors at least two AP-1 sites in the P1 and P4 regions. In our study, LES and LECS were shown to inhibit the DNA binding activities of both AP-1 and NF-AT transcription factors (Fig. 3), thereby suggesting that the inhibition of AP-1 activities by LES and LECS coordinately regulate the transcription of IL-4 genes. However, it is unclear how the AP-1 and NF-AT DNA binding activities suppressed by LES and LEBS are coordinately involved in the inhibition of IL-4 production in activated T cells. AP-1, the proinflammatory transcriptional element, is also known to contribute significantly to the expression of Th2 cytokines, IL-5 and IL-13, as well as IL-4. Interestingly, in this study, LES and LECS were not observed to exert any effect on IFN-γ production or mRNA expression levels in $CD4^+$ T cells (Fig. 1). IFN-γ, a cytokine that is secreted preferentially by activated T cells and natural killer cells, is an important regulatory molecule in the immune system [37]. Recent reports have showed that IFN-γ has been implicated in the pathogenesis of several immunological diseases, particularly Th1-mediated diseases, including autoimmune diseases and inflammatory responses [38]. The mechanism underlying the differential effects of LES and LECS on the production of IL-4 and IFN-γ is currently under investigation.

Furthermore, our results showed that LES and LECS reduced PMA-induced PKC activity, although these compounds exerted no observable effects on the protein levels of the conventional PKC isoforms (Fig. 5). PKC consists of 11 isozymes, classified in accordance with their biochemical properties. The conventional PKCs include the α, βI, βII and γ isozymes, and are phospholipid- and Ca^{2+}-dependent. Novel PKCs include δ, ε, η, and ϕ, and are Ca^{2+}-independent, but phospholipid-dependent. The atypical PKCs include the ξ and λ isozymes, and are structurally related, but require neither Ca^{2+} nor phospholipids [39]. Ceramide appears to differentially modulate a variety of PKC isoforms. A recent study indicated that ceramide inactivates both PKCα and $PKC\beta_{II}$ in Molt-4 and Jurkat cells [30]. This inactivation appears to be mediated by the dephosphorylation of PKCα. This is in contrast to the direct inhibitory effects of sphingosine on PKC, which are believed to result from the competitive, but reversible, inhibition of the regulatory domain [40]. Recently, ceramide has been shown to reduce the kinase activity of PKCϕ, as well as that of PKCα [31]. In contrast, ceramide has been also determined to activate PKCξ [41].

It remains unclear as to whether the reduced PKC activity may be involved in the downregulation of AP-1/NF-AT activation by LES and LECS. However, the activation of NF-AT is known to require both PKC and Ca^{2+} signals. Therefore, the PKC isozyme(s) associated with NF-AT activation might belong to a Ca^{2+}-dependent group, or may modulate intracellular levels of calcium. Many reports have implicated several PKC isoforms in the activation of NF-AT [27–29].

In conclusion, the results of the present study demonstrate that LES and LECS significantly inhibit IL-4 production in activated T cells, via the downregulation of AP-1/NF-AT activation and the activities of PKC. As the ratio of IFN-γ-secreting Th1 and IL-4-secreting Th2 cells is correlated closely with the outcome of many diseases [42,43], the ceramide derivatives, LES and LECS, may help to protect patients from diseases caused by undesired Th2-dominated responses, including allergic diseases.

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