

# Rosmarinic acid as a downstream inhibitor of IKK- $\beta$ in TNF- $\alpha$ -induced upregulation of *CCL11* and *CCR3*

<sup>1,2</sup>Jongsung Lee, <sup>1</sup>Eunsun Jung, <sup>1</sup>Youngji Kim, <sup>1</sup>Jiyoung Lee, <sup>1</sup>Junho Park, <sup>1</sup>Seongtaek Hong, <sup>1</sup>Chang-Gu Hyun, <sup>\*</sup><sup>1</sup>Deokhoon Park & <sup>\*</sup><sup>2</sup>Yeong Shik Kim

<sup>1</sup>Department of Skin cell biology, Biospectrum Life Science Institute (BLSI), SK Ventium 101-701, Dangjung Dong, Gunpo City, Kyunggi-do 436-776, Republic of Korea and <sup>2</sup>Natural Products Research Institute, College of Pharmacy, Seoul National University, 28 Yeonkun Dong, Jongro Gu, Seoul 110-460, Republic of Korea

**1** Tumor necrosis factor (TNF)- $\alpha$  is known to induce the expression of *CCL11* and *CCR3* via the activation of NF- $\kappa$ B. *CCL11* (eotaxin), the C–C chemokine, is a potent chemoattractant for eosinophils and Th2 lymphocytes, and *CCR3* is the receptor for *CCL11*.

**2** In order to determine the effects of rosmarinic acid on the TNF- $\alpha$ -induced upregulation of *CCL11* and *CCR3* in human dermal fibroblasts, we performed an enzyme-linked immunosorbent assay for *CCL11* and a Western blot assay for *CCR3*. The TNF- $\alpha$ -induced expression of *CCL11* and *CCR3* genes was attenuated by rosmarinic acid.

**3** In our NF- $\kappa$ B luciferase reporter system, TNF- $\alpha$ -induced NF- $\kappa$ B activation was observed to be reduced by rosmarinic acid. In accordance with this result, rosmarinic acid also inhibited TNF- $\alpha$ -induced phosphorylation and degradation of I $\kappa$ B- $\alpha$ , as well as nuclear translocation of NF- $\kappa$ B heterodimer induced by TNF- $\alpha$ . This suggests that rosmarinic acid downregulates the expression of *CCL11* and *CCR3* via the inhibition of NF- $\kappa$ B activation signaling.

**4** Using the NF- $\kappa$ B luciferase reporter system, Western blot analysis, and IKK- $\beta$  activity assay, we determined that rosmarinic acid inhibits IKK- $\beta$  activity in NF- $\kappa$ B signaling, which upregulates the expression of *CCL11* and *CCR3*. Additionally, TNF- $\alpha$ -induced secretion of soluble intercellular adhesion molecule-1 and soluble vascular cell adhesion molecule-1 molecules was found to be attenuated by rosmarinic acid.

**5** Our results show that rosmarinic acid inhibits the expression of *CCL11* and *CCR3* by suppressing the IKK- $\beta$  activity in NF- $\kappa$ B activation signaling. Further, these results suggest that rosmarinic acid might inhibit the expression of NF- $\kappa$ B promoter-related genes.

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**Keywords:** *CCL11*; IKK- $\beta$ ; nuclear factor- $\kappa$ B; I $\kappa$ B- $\alpha$ ; TNF- $\alpha$

**Abbreviations:** IKK- $\beta$ , I $\kappa$ B kinase  $\beta$ ; MEKK3, MAPK kinase kinase 3; NF- $\kappa$ B, nuclear factor- $\kappa$ B; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; TRAF2, TNF-receptor-associated factor 2

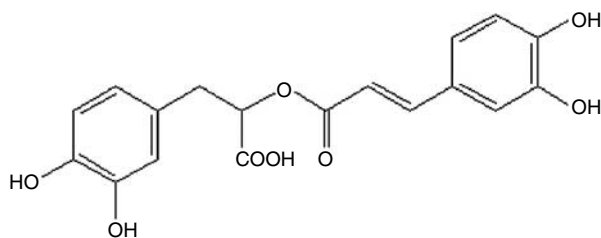
## Introduction

$\alpha$ -*o*-Caffeoyl-3,4-dihydroxyphenyl lactic acid (rosmarinic acid; Figure 1) is a naturally occurring hydroxylated compound. It is widely distributed in the *Labiatae* herbs, which include rosemary, sweet basil, and perilla (Osakabe *et al.*, 2002; del Bano *et al.*, 2003; Kintzios *et al.*, 2003). Rosmarinic acid has broad range of applications, from food preservatives to cosmetics; it also has medicinal use, by virtue of its antimicrobial and antioxidant activities (Van Kessel & Kalter, 1986; Al-Sereiti *et al.*, 1999; Zheng & Wang, 2001). In addition, it has been reported that rosmarinic acid has the ability to block complement fixation and inhibit lipoxygenase and cyclooxygenase activity (Kimura *et al.*, 1987; Sahu *et al.*, 1999; Kelm *et al.*, 2000).

The chemokines are a large family of small proteins involved in the activation and recruitment of specific cell populations during the course of disease (Lukacs *et al.*, 1999). *CCL11*, a CC chemokine, is a potent chemoattractant and an activator

of eosinophils (Ponath *et al.*, 1996), basophils (Ugucioni *et al.*, 1997; Yamada *et al.*, 1997), and Th2 lymphocytes (Gerber *et al.*, 1997; Sallusto *et al.*, 1997). *CCL11* expression was found to be restricted to a few cell types, including eosinophils, bronchial epithelial cells, and dermal fibroblast cells (Gutierrez-Ramos *et al.*, 1999; Huber *et al.*, 2000). In asthmatics, the expression of *CCL11* has been found to be enhanced in these types of cells, and increased expression is associated with disease severity (Mattoli *et al.*, 1997; Ying *et al.*, 1997). Additionally, *CCL11* expression in epithelial cells was found to be increased in atopic dermatitis (Yawalkar *et al.*, 1999), as well as in other inflammatory conditions (Garcia-Zepeda *et al.*, 1996). The expression of *CCL11* is induced by two potent activators, interleukin-4, which is produced by Th2 cells, mast cells, and basophils, and TNF- $\alpha$ , which is produced by monocytes and macrophages (Atasoy *et al.*, 2003). In contrast to most other eosinophil chemoattractants of the CC-chemokine family that generally act on several receptors, *CCL11* only signals through one specific chemokine receptor, namely the G protein-coupled receptor,

\*Author for correspondence; E-mails: pdh@biospectrum.com (D. Park) or kims@plaza.snu.ac.kr (Y.S. Kim)



**Figure 1** Structure of rosmarinic acid ( $\alpha$ -*o*-caffeoyl-3,4-dihydroxyphenyl lactic acid).

CCR3 (Baggiolini *et al.*, 1997). CCR3 is prominently expressed on eosinophils, basophils, Th2-type lymphocytes, and fibroblasts (Ying *et al.*, 1997; Huber *et al.*, 2000). Very little is known regarding how CCR3 is regulated at the transcriptional level, although a recent report demonstrated that CCR3 gene expression is induced by TNF- $\alpha$  (Huber *et al.*, 2002).

Recently, the inhibition of T-cell receptor (TCR)-mediated signaling has become viewed as one of the many effects of anti-inflammatory drugs. Despite their different points of action, anti-inflammatory drugs such as tacrolimus, pimecrolimus (Paccani *et al.*, 2002), and hydroxychloroquine (Goldman *et al.*, 2000) have been found to inhibit TCR-induced signaling events, including Ca<sup>2+</sup> mobilization, the activation of p38 mitogen-activated protein kinase, and the expression of the CD40 ligand. It was recently determined that rosmarinic acid inhibits the Ca<sup>2+</sup>-dependent pathways of TCR-mediated signaling by inhibiting PLC- $\gamma$ 1 and Itk activities (Kang *et al.*, 2003). Many reports regarding the anti-inflammatory and immunomodulatory effects of rosmarinic acid led us to study the possibility that rosmarinic acid may exert its inhibitory effect on the expression of CCL11 and CCR3. To this end, using human dermal fibroblast cells, we attempted to determine the effects and mechanisms of action of rosmarinic acid on the TNF- $\alpha$ -induced expression of CCL11 and CCR3. In this report, we demonstrated that rosmarinic acid inhibits the IKK- $\beta$  downstream signaling pathway in the TNF- $\alpha$ -induced upregulation of CCL11 and CCR3 in human dermal fibroblasts.

## Methods

### Cell cultures

NIH3T3 mouse fibroblast cells and human dermal fibroblast cells (derived from neonatal foreskin) were obtained from the Amore-Pacific Corporation R&D Center in the Republic of Korea, and were cultured in Dulbecco's-modified Eagle's medium (DMEM) (Hyclone, UT, U.S.A.) containing 10% fetal bovine serum (Invitrogen, CA, U.S.A.), and penicillin-streptomycin at 37°C in a humidified 95% air/5% CO<sub>2</sub> atmosphere.

### Cytotoxicity assay

Human dermal fibroblast cells were cultured in DMEM (Hyclone) containing 10% fetal bovine serum, and penicillin-streptomycin at 37°C in a humidified 95% air/5% CO<sub>2</sub> atmosphere. Cells were seeded on 96-well plates and drug treatment was initiated 24 h after seeding. The general viability

of cultured cells was determined by the reduction of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphonyl)-2H-tetrazolium, monosodium salt) (Dojindo Laboratories, Japan) to a highly water-soluble formazan dye. This assay was performed after the incubation of human dermal fibroblast cells in the presence or absence of TNF- $\alpha$ , along with indicated concentrations of rosmarinic acid, for 14 h at 37°C in a 5% CO<sub>2</sub> atmosphere. To each well, 10  $\mu$ l of WST-8 solution was added. Cells were then incubated at 37°C for 3 h and the absorbance was measured at 450 nm using a spectrophotometer (Power Wave, Bio-tek Inc., VT, U.S.A.). Data are presented as means  $\pm$  s.d. All values were significant ( $*P < 0.05$ ) compared with values for control. The entire experiment was performed in triplicate and results were confirmed by three independent experiments.

### Western blot analysis

Human dermal fibroblast cell lysates were separated by sodium dodecyl sulfate (SDS)-PAGE (16% acrylamide gel) and transferred to Hybond-C membranes. The blots were probed with phospho-I $\kappa$ B- $\alpha$  (1 : 1000), I $\kappa$ B- $\alpha$  (1 : 1000), IKK- $\beta$  (1 : 1000), NF- $\kappa$ B p65 (1 : 1000), Histone H2B (1 : 1000), Flag (1 : 1000), CCR3 (1 : 500), and  $\beta$ -actin (1 : 1000) antibodies, and the proteins were visualized by the Amersham ECL system and quantified using a densitometer. All results were confirmed by three independent experiments.

### Detection of I $\kappa$ B- $\alpha$ phosphorylation and degradation

I $\kappa$ B- $\alpha$  and phosphorylated I $\kappa$ B- $\alpha$  were identified by Western blot analysis using anti-I $\kappa$ B- $\alpha$  or phospho I $\kappa$ B- $\alpha$  antibody, respectively. Human dermal fibroblast cells were incubated in the presence or absence of TNF- $\alpha$  (40 ng ml<sup>-1</sup>), along with indicated concentrations of rosmarinic acid, for 30 min. Alternatively, human dermal fibroblast cells were transfected with TRAF2, MEKK3, or IKK- $\beta$ , and were then incubated for 24 h. At that time, the human fibroblast cells were treated with rosmarinic acid for 14 h. The cultured human dermal fibroblast cells were washed with ice-cold phosphate-buffered saline (pH 7.4), and 0.2 ml of lysis buffer (10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g ml<sup>-1</sup> leupeptin, 10  $\mu$ g ml<sup>-1</sup> aprotinin) was then added. The human dermal fibroblast cells were then harvested. After 30 min of centrifugation at 16,000  $\times$  g at 4°C, the cell lysates were denatured in boiling water for 5 min. Samples of the supernatant were subjected to SDS-PAGE using 16% polyacrylamide gel. After electrophoresis, the proteins were transferred to Hybond-C membrane at 4°C. In order to detect phosphorylated I $\kappa$ B- $\alpha$ , the sheets were immersed in blocking buffer containing 5% skim milk, 20 mM Tris-buffered saline (pH 7.4), and 0.1% Tween-20 for 3 h at 25°C. The phosphorylation and degradation of I $\kappa$ B- $\alpha$  were detected by protein immunoblotting; this process used a 1 : 1000 dilution of mouse monoclonal antibody (phospho-I $\kappa$ B- $\alpha$  (Ser32/36)) or mouse monoclonal antibody (I $\kappa$ B- $\alpha$  (112B2)). Blots were developed *via* enhanced chemiluminescence following incubation with HRP-conjugated secondary anti-mouse IgG monoclonal antibody (1 : 2000 dilution) for 1 h at 25°C, according to the manufacturer's instructions. All results were confirmed by three independent experiments.

### Subcellular fractionation

The preparation of membrane, cytosolic, and nuclear fractions were performed as described previously (Kabouridis *et al.*, 1997). In brief,  $1 \times 10^7$  human dermal fibroblast cells were incubated in the presence or absence of TNF- $\alpha$  ( $40 \text{ ng ml}^{-1}$ ), along with indicated concentrations of rosmarinic acid for 30 min. The cells were then resuspended in 0.5 ml hypotonic solution (25 mM Tris pH 7.5, 5 mM EGTA, 250 mM sucrose,  $25 \mu\text{g ml}^{-1}$  aprotinin, 1 mM PMSF,  $25 \mu\text{g ml}^{-1}$  leupeptin, 5 mM NaF, and 1 mM  $\text{Na}_3\text{VO}_4$ ), and subjected to two successive freeze-thaw cycles. The cell suspension was homogenized on ice using Dounce homogenizer (40 strokes), and the salt concentration was adjusted to 150 mM NaCl. Nuclei were removed by two successive centrifugations at  $480 \times g$  for 5 min at  $4^\circ\text{C}$ . Soluble and particulate fractions were separated by centrifugation at  $100,000 \times g$  for 30 min. Fractionated proteins were resolved by SDS-PAGE and Western blotted with their corresponding antibodies. All results were confirmed by three independent experiments.

### Luciferase reporter assay

To assay for NF- $\kappa\text{B}$  promoter activity, human dermal fibroblast cells were transfected with NF- $\kappa\text{B}$ -Luc reporter, or with the indicated genes, including *TRAF2*, *MEKK3*, and *IKK- $\beta$* , along with the *Renilla* luciferase expression vector, driven by the thymidine kinase promoter (Promega, WI, U.S.A.) using Superfect™ reagent (Invitrogen, CA, U.S.A.). After 24 h of incubation, the cells were incubated in the presence or absence of TNF- $\alpha$  ( $40 \text{ ng ml}^{-1}$ ), along with indicated concentrations of rosmarinic acid for 14 h. The cells were then harvested and lysed. Supernatants were assayed for luciferase activity. Luciferase activity was determined with a Dual Luciferase Assay system (Promega, WI, U.S.A.) and a LB953 luminometer (Berthold, Germany), and was expressed as a ratio of the NF- $\kappa\text{B}$ -dependent firefly luciferase activity divided by the control thymidine kinase *Renilla* luciferase activity (% control). Results were confirmed by three independent transfections. Data are expressed as the means  $\pm$  s.e.m. \* $P < 0.05$ , compared with untreated controls. ° $P < 0.05$  versus TNF- $\alpha$  ( $40 \text{ ng ml}^{-1}$ ) only or transfected controls.

### CCL11, soluble vascular cell adhesion molecule-1, and soluble intercellular adhesion molecule-1 assay

The concentrations of CCL11 and soluble vascular cell adhesion molecule-1 (sVCAM-1) in the culture medium were measured with a sandwich immunoassay kit, used according to the manufacture's instructions (R&D Systems). The concentration of soluble intercellular adhesion molecule-1 (sICAM-1) was measured by ELISA using a commercially available kit designed for the quantitative measurement of human sICAM-1 (Endogen, MA, U.S.A.). The standard curve was linearized and subjected to regression analysis. The CCL11 concentration of the unknown samples was extracted using the standard curve. The results were expressed as  $\text{pg ml}^{-1}$  of culture medium. Results were confirmed by three independent experiments. Data are expressed as the means  $\pm$  s.e.m. \* $P < 0.05$ , compared with the untreated control. ° $P < 0.05$  versus TNF- $\alpha$  ( $40 \text{ ng ml}^{-1}$ ) only.

### IKK kinase- $\beta$ activity assay

A kinase assay based on phosphorylation of substrate was used to investigate IKK- $\beta$  activity. IKK- $\beta$  was precipitated from whole-cell extracts with antibodies against IKK- $\beta$ , and was then treated with  $20 \mu\text{l}$  protein A/G-Sepharose (Pierce, Rockford, IL, U.S.A.). After 2 h, the beads were washed with lysis buffer and assayed in a kinase assay mixture containing  $50 \mu\text{M}$  HEPES (pH 7.4),  $20 \mu\text{M}$   $\text{MgCl}_2$ ,  $2 \mu\text{M}$  DTT,  $20 \mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]adenosine triphosphate (ATP),  $10 \mu\text{M}$  unlabeled ATP, and  $2 \mu\text{g}$  substrate glutathione-S-transferase (GST)-IKK- $\alpha$  (i.e., residues 1–54 of IKK- $\alpha$  conjugated with GST). After incubation at  $30^\circ\text{C}$  for 30 min, the reaction was terminated by boiling with  $5 \mu\text{l}$   $5 \times$  SDS sample buffer for 5 min. Finally, the protein was resolved on a 10% polyacrylamide gel under reducing conditions, the gel was dried, and the radioactive bands were visualized using a Phosphorimager (Alpha Innotech Corp., San Leandro, CA, U.S.A.). To determine the total amounts of IKK- $\beta$  in each sample,  $30 \mu\text{g}$  of the whole-cell extract protein was resolved on a 7.5% acrylamide gel and electrotransferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk protein for 1 h and was then incubated with anti-*IKK- $\beta$*  for 1 h. The membrane was washed and treated with horseradish peroxidase-conjugated secondary anti-mouse antibody, and the proteins were detected by chemiluminescence analysis (Amersham Biosciences, Bucks, U.K.).

### Materials

Polyclonal goat antisera to human CCR3 and rosmarinic acid were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.) and A.G. Scientific Inc. (CA, U.S.A.) respectively. Antisera to phospho-IKK- $\alpha$ , IKK- $\alpha$ , IKK- $\beta$ , histone H2B, and NF- $\kappa\text{B}$  p65 were purchased from Cell Signaling Technology Inc. (Beverly, MA, U.S.A.). Antibodies against  $\beta$ -actin and Flag M1, TNF- $\alpha$ , pyrrolidone dithiocarbamate (PDTTC), sodium salicylate (salicylic acid, sodium salt) were obtained from the Sigma Chemical Co. (MI, U.S.A.). Protease inhibitor cocktail was purchased from Roche (Indianapolis, IN, U.S.A.). The chemiluminescence kit was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). NF- $\kappa\text{B}$ -Luc reporter plasmids were purchased from Stratagene. Flag-tagged *TRAF2* (Flag-*TRAF2*) and *MEKK3* (Flag-*MEKK3*) expression vectors were constructed by cloning of a fragment which contained the first methionine codon of *TRAF2*, *MEKK3*, into the blunted *XhoI* site of the expression vector, pcDNA3.1. Flag-tagged, constitutively active *IKK- $\beta$*  (S177E, S181E) expression vector was constructed by cloning of a fragment which had been made through site-directed mutagenesis into the blunted *XhoI* site of the expression vector, pcDNA3.1. Flag-tagged *TRAF2*, Flag-tagged *MEKK3*, and constitutive active *IKK- $\beta$*  were a gift from Dr Won (Mogam Biotech Res Inst., Republic of Korea).

### Statistical evaluation

Results are shown as means  $\pm$  s.e.m. and statistical analysis of the results was performed by Student's *t*-test for independent samples. Values of  $P < 0.05$  were considered to show significant differences between means. From concentration–response data, as in Figures 2a, 3a and b, 9a and b, IC<sub>50</sub>

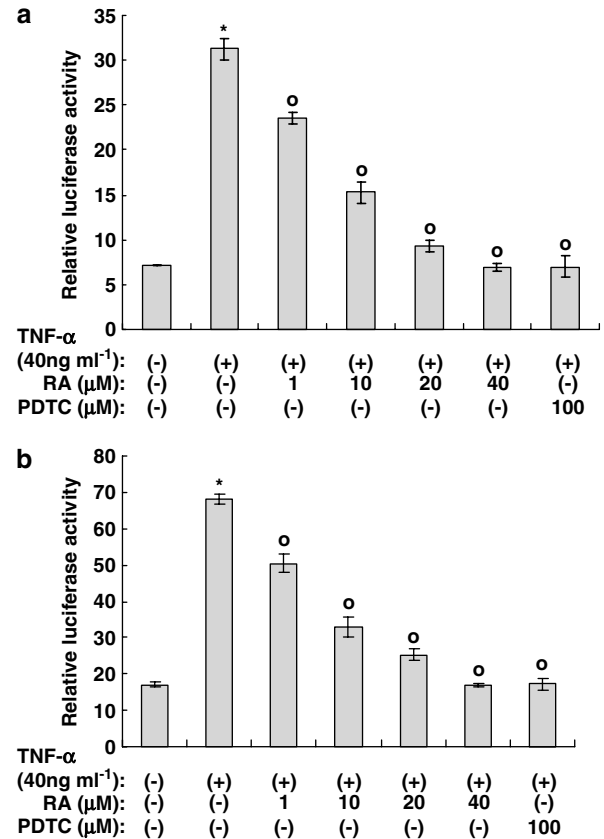
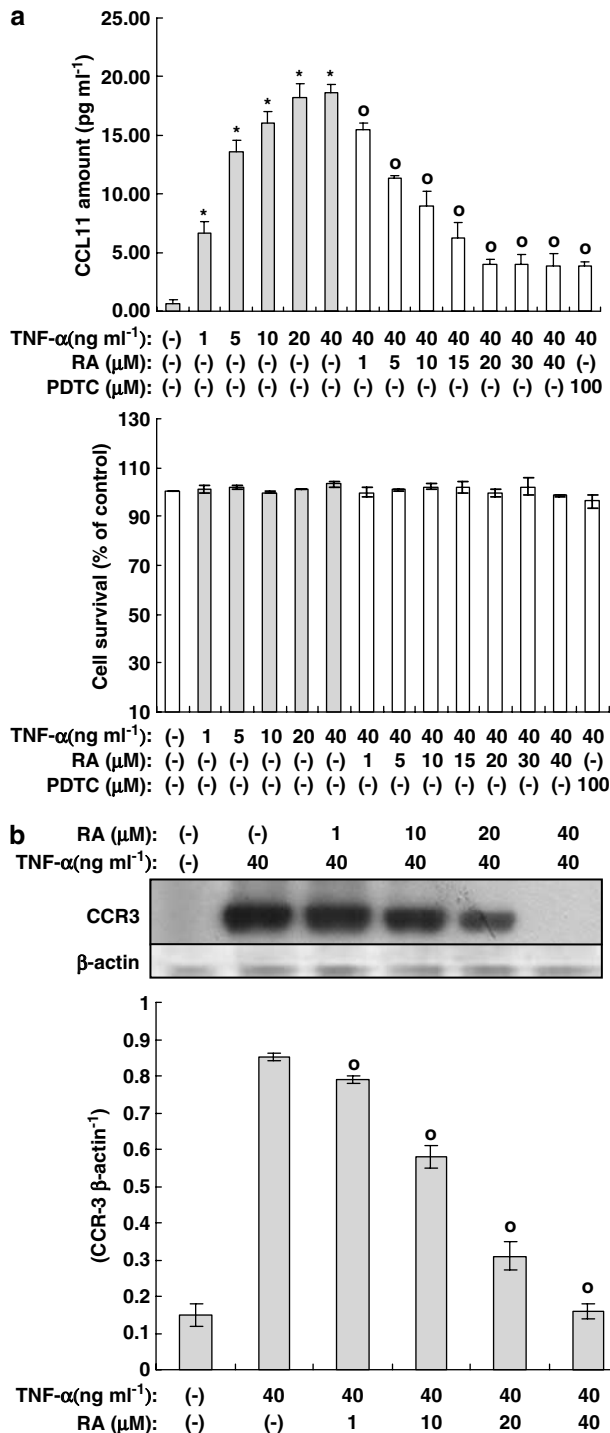
values were calculated after regression analysis (DataFit programme).

**Results**

*Rosmarinic acid inhibits the TNF- $\alpha$ -induced expression of CCL11 and CCR3 in human dermal fibroblast cells*

TNF- $\alpha$  is known to induce the expression of *CCL11* and *CCR3* via NF- $\kappa$ B activation. As previously mentioned, rosmarinic

acid is a hydroxylated compound (Figure 1) and is known to exhibit anti-inflammatory and immunomodulatory actions. Thus rosmarinic acid may exert an inhibitory effect on the



**Figure 3** Rosmarinic acid inhibits NF- $\kappa$ B activation induced by TNF- $\alpha$  in (a) NIH3T3 cells and (b) human dermal fibroblast cells. NIH3T3 cells and human dermal fibroblast cells were transfected with NF- $\kappa$ B-Luc reporters, along with *Renilla* luciferase expression vector driven by the thymidine kinase promoter using Superfect™ reagent. After 24 h of incubation, cells were treated with rosmarinic acid in the presence or absence of TNF- $\alpha$  (40 ng ml<sup>-1</sup>) for 14 h. The cells were then harvested and assayed. *Renilla* luciferase vector was employed as a control for transfection efficiency and the reporter data were processed using the dual luciferase method, as described in Methods. Results were confirmed by three independent transfections. Data are expressed as the means  $\pm$  s.e.m. \* $P$  < 0.05 compared with untreated controls. ° $P$  < 0.05 versus TNF- $\alpha$  (40 ng ml<sup>-1</sup>) only. RA: rosmarinic acid; PDTC: pyrrolidone dithiocarbamate (100  $\mu$ M).

**Figure 2** Rosmarinic acid inhibits expression of the *CCL11* and *CCR3* genes induced by TNF- $\alpha$  in human dermal fibroblast cells. Human dermal fibroblast cells were incubated in the presence or absence of TNF- $\alpha$ , along with indicated concentrations of rosmarinic acid for 14 h. (a) The concentration of CCL11 in the culture medium was measured by a sandwich immunoassay kit (upper panel). The cell survival curve is shown in the lower panel. Results were confirmed by three independent experiments. Data are expressed as the means  $\pm$  s.e.m. \* $P$  < 0.05, compared with untreated controls. ° $P$  < 0.05 versus TNF- $\alpha$  (40 ng ml<sup>-1</sup>) only. RA: rosmarinic acid; PDTC: pyrrolidone dithiocarbamate (100  $\mu$ M). (b) The cultured human dermal fibroblast cells were subjected to Western blot analysis using anti-CCR3 and  $\beta$ -actin antibodies. RA: rosmarinic acid. Bands were visualized by an ECL method and quantified using a densitometer. Similar results were obtained in three independent experiments. Data are expressed as means  $\pm$  s.e.m. of three independent experiments (bar graph). ° $P$  < 0.05 versus TNF- $\alpha$  (40 ng ml<sup>-1</sup>) only.

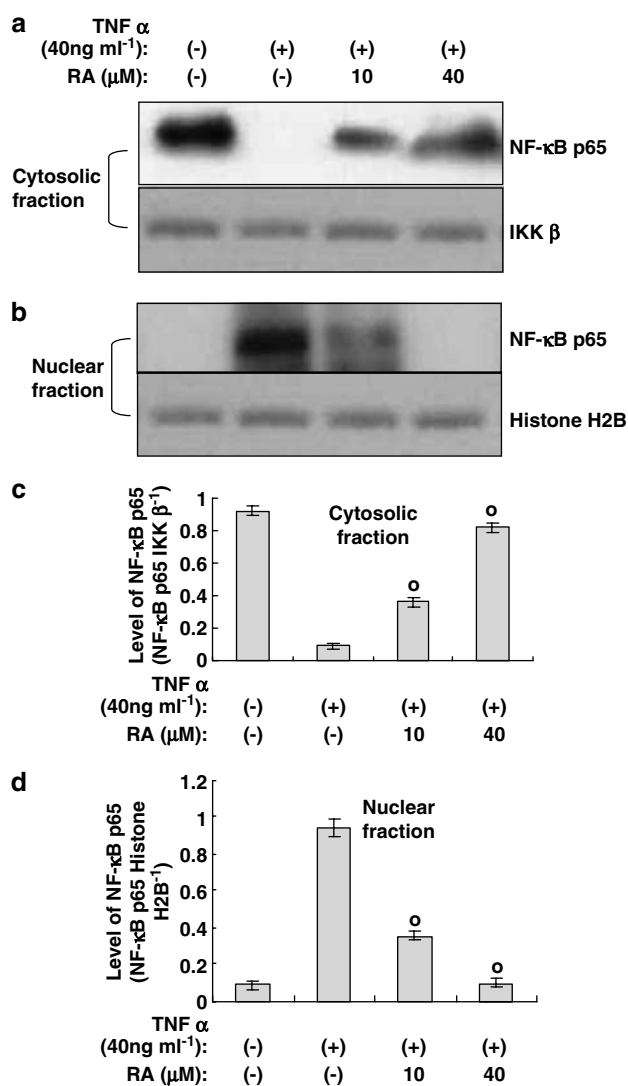
expression of *CCL11* and *CCR3*. As our first step in examining the involvement of rosmarinic acid in the downregulation of *CCL11* and *CCR3* expression, we performed an enzyme-linked immunosorbent assay for *CCL11* and a Western blot assay for *CCR3*. As shown in Figures 2a and b, the TNF- $\alpha$ -induced protein expression of the *CCL11* and *CCR3* genes were attenuated by rosmarinic acid in a concentration-dependent manner. The  $IC_{50}$  of rosmarinic acid on *CCL11* protein expression induced by TNF- $\alpha$  was  $9.1 \pm 1.5 \mu\text{M}$ . Additionally, while rosmarinic acid at  $40 \mu\text{M}$  completely inhibited the protein expression of the *CCR3* gene, expression of *CCL11* protein was not blocked at the same concentration. This result suggests that protein expression of the *CCR3* gene is more dependent on the NF- $\kappa\text{B}$  promoter than the *CCL11* gene. PDTC was employed as a positive control. To assess the possibility that the decreased *CCL11* production was due to a cytotoxic effect of rosmarinic acid, we performed an MTT assay in human dermal fibroblast cells. As shown in Figure 2a, rosmarinic acid showed no significant cytotoxic effect at any of the concentrations tested.

#### Rosmarinic acid inhibits NF- $\kappa\text{B}$ activation induced by TNF- $\alpha$

To investigate whether the inhibitory effect of rosmarinic acid on the TNF- $\alpha$ -induced expression of the *CCL11* and *CCR3* genes is mediated by the suppression of NF- $\kappa\text{B}$  activation, we employed the NF- $\kappa\text{B}$  luciferase reporter in both NIH3T3, mouse fibroblast cells, and human dermal fibroblast cells. As shown in Figures 3a and b, NF- $\kappa\text{B}$  activation induced by TNF- $\alpha$  was inhibited in a concentration-dependent manner in both NIH3T3 cells and human dermal fibroblast cells, which suggests that rosmarinic acid may act on the NF- $\kappa\text{B}$  pathway to inhibit the expression of the *CCR3* and *CCL11* genes. The  $IC_{50}$  of rosmarinic acid on TNF- $\alpha$ -induced NF- $\kappa\text{B}$  activation in NIH3T3 cells and human dermal fibroblast cells was  $10.9 \pm 1.6$  and  $9.5 \pm 1.5 \mu\text{M}$ , respectively. PDTC was employed as a positive control.

#### TNF- $\alpha$ -induced nuclear translocation of NF- $\kappa\text{B}$ heterodimer is inhibited by rosmarinic acid

In order to examine the effect of rosmarinic acid on nuclear translocation of NF- $\kappa\text{B}$  heterodimer induced by TNF- $\alpha$ , we performed a Western blot using nuclear and cytosolic extracts. The cells were stimulated with TNF- $\alpha$  only or with TNF- $\alpha$  plus rosmarinic acid; they were then lysed and separated into nuclear and cytosolic fractions. As controls for cytoplasmic and nuclear fraction, respectively, the levels of IKK- $\beta$  and histone H2B were analyzed and were found to be consistent throughout the test period (Figure 4). In this experiment, while IKK- $\beta$  was detected in the cytosolic fraction, but not in the nuclear fraction, histone H2B existed only in the nuclear fraction, and not in the cytosolic fraction (data not shown). This fact allowed us to confirm the purity of the cytosolic and nuclear fractions. The Western blots of NF- $\kappa\text{B}$  p65 in the lysates of the nuclei and cytosol revealed the nuclear translocation of NF- $\kappa\text{B}$  p65 from the cytosol upon treatment with TNF- $\alpha$ . However, in cases in which cells were incubated in the presence of TNF- $\alpha$  along with rosmarinic acid, nuclear translocation of NF- $\kappa\text{B}$  p65 was decreased (Figure 4), thereby

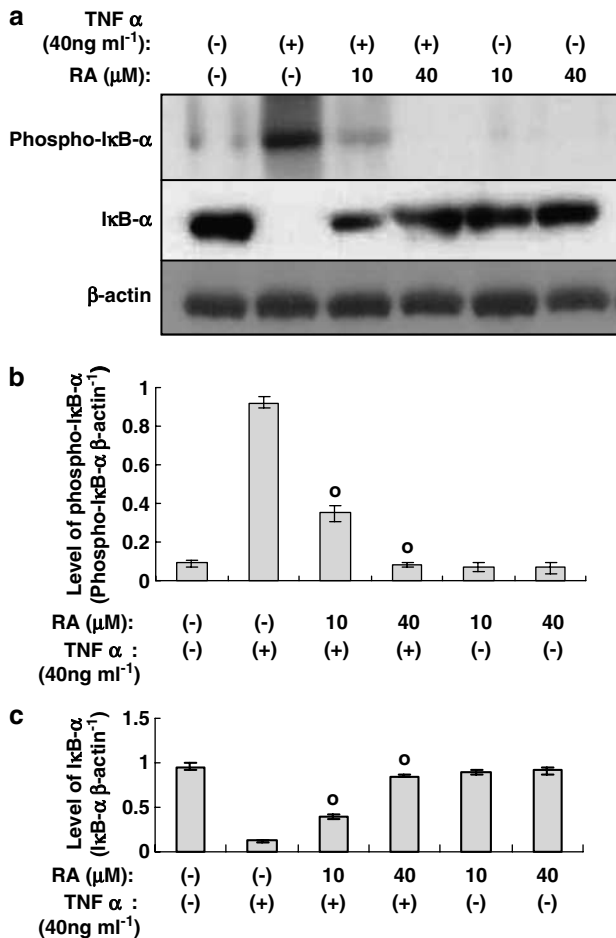


**Figure 4** The nuclear translocation of NF- $\kappa\text{B}$  heterodimer induced by TNF- $\alpha$  is attenuated by rosmarinic acid. Human dermal fibroblast cells were stimulated using control vehicle, TNF- $\alpha$  ( $40 \text{ ng ml}^{-1}$ ), or TNF- $\alpha$  plus rosmarinic acid for 30 min, followed by the isolation of nuclei and cytosol, as described in the Methods. Cytosolic (a) and nuclear (b) fractions were subjected to immunoblotting using anti-NF- $\kappa\text{B}$  p65, IKK- $\beta$ , or histone H2B antibodies. Bands were visualized by an ECL method and quantified using a densitometer (c and d). Similar results were obtained in three independent experiments. Data are expressed as means  $\pm$  s.e.m. of three independent experiments (bar graph).  $^{\circ}P < 0.05$  versus TNF- $\alpha$  ( $40 \text{ ng ml}^{-1}$ ) only.

indicating that its translocation from the cytosol was inhibited by rosmarinic acid.

#### Phosphorylation and degradation of I $\kappa\text{B}$ - $\alpha$ induced by TNF- $\alpha$ is attenuated by rosmarinic acid

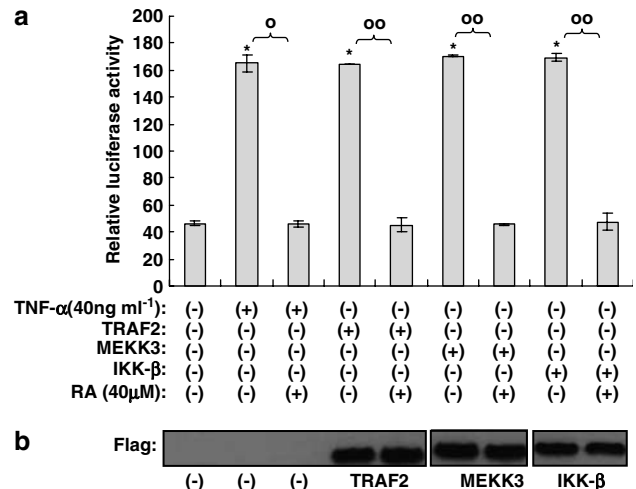
In previous reports, rosmarinic acid has been found to inhibit activation and nuclear translocation of NF- $\kappa\text{B}$ , induced by TNF- $\alpha$ . This result was further confirmed by Western blot analysis, which demonstrated that rosmarinic acid attenuated the phosphorylation and degradation of I $\kappa\text{B}$ - $\alpha$  induced by TNF- $\alpha$ . We employed anti- $\beta$ -actin antibody as a quantitative control (Figure 5a).



**Figure 5** Rosmarinic acid inhibits the phosphorylation and degradation of I $\kappa$ B- $\alpha$  induced by TNF- $\alpha$  in human dermal fibroblast cells. Human dermal fibroblast cells were incubated in the presence or absence of TNF- $\alpha$ , along with the indicated concentrations of rosmarinic acid, for 30 min. The cultured human dermal fibroblast cells were subjected to Western blotting using antiphospho-I $\kappa$ B- $\alpha$  (Ser32/36) Ab or anti-I $\kappa$ B- $\alpha$  Ab (a). RA: rosmarinic acid. Bands were visualized by an ECL method and quantified using a densitometer (b and c). Similar results were obtained in three independent experiments. Data are expressed as means  $\pm$  s.e.m. of three independent experiments (bar graph). <sup>o</sup> $P$  < 0.05 versus TNF- $\alpha$  (40 ng ml<sup>-1</sup>) only.

#### Rosmarinic acid blocks downstream of IKK- $\beta$ in the NF- $\kappa$ B activation pathway induced by TNF- $\alpha$

In our experiments, we identified the inhibitory effects of rosmarinic acid on *CCL11* and *CCR3* gene expression. Two pathways for the induction of NF- $\kappa$ B activity have recently been discovered (Joel & David, 2002). One is a canonical pathway in which TRAF2, MEKK, and IKK- $\beta$  are involved, and the other is a noncanonical pathway in which NIK (NF- $\kappa$ B-inducing kinase) and IKK- $\alpha$  have been implicated. Activation of NF- $\kappa$ B induced by TNF- $\alpha$  is generally known to be mediated by the canonical pathway. Therefore, in order to characterize the action of rosmarinic acid in NF- $\kappa$ B activation induced by TNF- $\alpha$ , a NF- $\kappa$ B luciferase reporter assay was performed using *TRAF2*, *MEKK3*, and *IKK- $\beta$*  in the absence of TNF- $\alpha$ . When each molecule was overexpressed by transient transfection into human dermal fibroblast cells, NF- $\kappa$ B



**Figure 6** A hierarchy of rosmarinic acid that operates in the NF- $\kappa$ B activation pathway. Human dermal fibroblast cells were transfected with NF- $\kappa$ B-Luc reporters or indicated genes, such as TRAF2, MEKK3, and IKK- $\beta$ , along with *Renilla* luciferase expression. After 24 h of incubation, the cells were incubated with 40  $\mu$ M rosmarinic acid for 14 h, after which they were harvested and lysed. Supernatants were assayed for luciferase activity. Luciferase activity was determined using a Dual Luciferase Assay system and a LB953 luminometer; the luciferase activity was expressed as the ratio of the NF- $\kappa$ B-dependent firefly luciferase activity divided by control thymidine kinase *Renilla* luciferase activity (a). Results were confirmed by three independent transfections. Data are expressed as the means  $\pm$  s.e.m. \* $P$  < 0.05, compared with the untreated controls. <sup>o</sup> $P$  < 0.05, <sup>oo</sup> $P$  < 0.05 versus TNF- $\alpha$  (40 ng ml<sup>-1</sup>) only or transfected controls. RA: rosmarinic acid. Western blots using antibody against Flag M1 for Flag-tagged TRAF2, MEKK3, or IKK- $\beta$  show that each of the genes was transcribed and translated following transfection into cells (b).

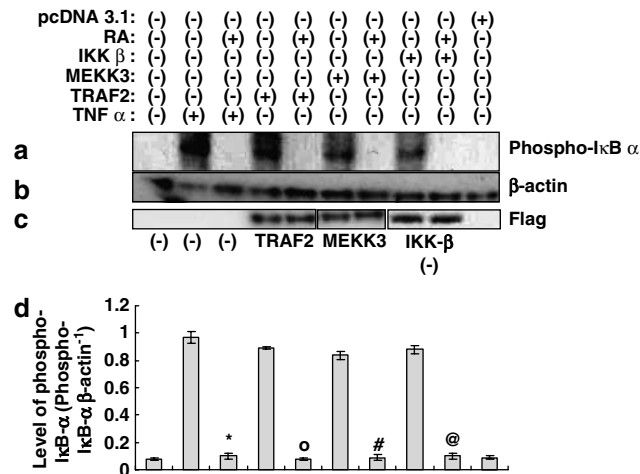
luciferase reporter activity was significantly reduced by rosmarinic acid (Figure 6a). This inhibitory effect of rosmarinic acid was further confirmed by a Western blot assay for phosphorylated I $\kappa$ B- $\alpha$  using TRAF2, MEKK3, and IKK- $\beta$ . As shown in Figure 7a, and also consistent with the results in Figure 6a, rosmarinic acid inhibited I $\kappa$ B- $\alpha$  phosphorylation induced by TRAF2-, MEKK3-, and IKK- $\beta$ . This suggests that rosmarinic acid operates downstream of IKK- $\beta$  in NF- $\kappa$ B-activation induced by TNF- $\alpha$ . Western blot analyses in this experiment were performed using anti- $\beta$ -actin Ab as a quantitative control (Figure 7b).

#### Rosmarinic acid inhibits IKK- $\beta$ activity in NF- $\kappa$ B activation signaling induced by TNF- $\alpha$

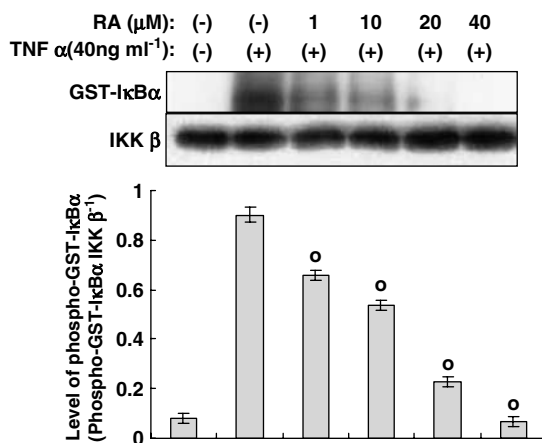
In order to further elucidate the action of rosmarinic acid, we performed an *in vitro* IKK- $\beta$  kinase assay using immunoprecipitated IKK- $\beta$  from human dermal fibroblast cells incubated in the presence or absence of TNF- $\alpha$  (40 ng ml<sup>-1</sup>), along with the indicated concentrations of rosmarinic acid for 30 min and GST-I $\kappa$ B- $\alpha$  as a substrate. As shown in Figure 8, IKK- $\beta$  activity was reduced by rosmarinic acid. This result indicates that rosmarinic acid directly inhibits IKK- $\beta$  kinase activity. Immunoblot analysis of cell extracts from untreated cells and cells treated with rosmarinic acid or TNF- $\alpha$  revealed no significant changes in IKK- $\beta$  protein levels (Figure 8, bottom row).

*Rosmarinic acid inhibits the TNF- $\alpha$ -induced expression of sVCAM-1 and sICAM-1 in human dermal fibroblast cells*

We previously determined that rosmarinic acid inhibits the expression of *CCR3* and *CCL11* through the inhibition of

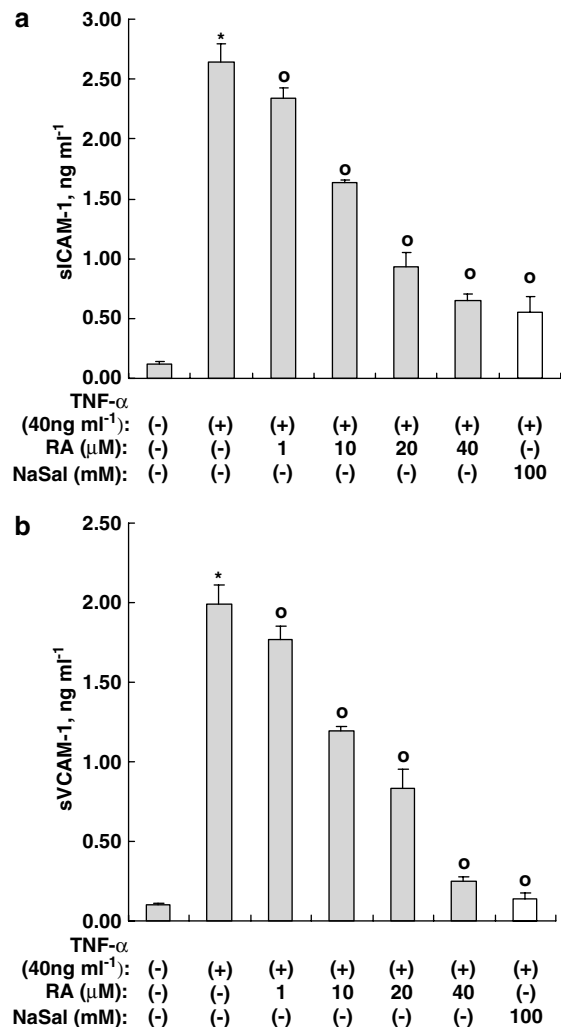


**Figure 7** Rosmarinic acid operates downstream of IKK- $\beta$  in NF- $\kappa$ B activation signaling. Human dermal fibroblast cells were transfected with TRAF2, MEKK3, or IKK- $\beta$ , and were then incubated for 24 h. At that time, the human fibroblast cells were treated with 40  $\mu$ M rosmarinic acid for 14 h. The cultured human dermal fibroblast cells were subjected to Western blot analysis using mouse monoclonal antibody (phospho-IkB- $\alpha$  (Ser32/36)) (a) and  $\beta$ -actin antibody (b). Bands were visualized by an ECL method and quantified using a densitometer (d). Similar results were obtained in three independent experiments. Data are expressed as means  $\pm$  s.e.m. of three independent experiments (bar graph). \* $P$  < 0.05 versus TNF- $\alpha$ -treated control,  $^{\circ}$  $P$  < 0.05 versus TRAF2-transfected control, # $P$  < 0.05 versus MEKK3-transfected control, @ $P$  < 0.05 versus IKK- $\beta$ -transfected control. Western blots using antibody against Flag M1 for Flag-tagged TRAF2, MEKK3, or IKK- $\beta$  show that each of the genes was transcribed and translated following transfection into cells (c).



**Figure 8** Effect of rosmarinic acid on I $\kappa$ B kinase (IKK)- $\beta$  activity induced by TNF- $\alpha$  in human dermal fibroblast cells. IKK- $\beta$  was immunoprecipitated from cells incubated in either the presence or absence of TNF- $\alpha$  (40 ng ml $^{-1}$ ), along with the indicated concentrations of rosmarinic acid, for 30 min; the kinase assay was then performed (top row). Western blotting was performed to evaluate the total IKK- $\beta$  level (bottom row). Bands were visualized by an ECL method and quantified using a densitometer. Similar results were obtained in three independent experiments. Data are expressed as means  $\pm$  s.e.m. of three independent experiments (bar graph).  $^{\circ}$  $P$  < 0.05 versus TNF- $\alpha$  (40 ng ml $^{-1}$ ) only.

NF- $\kappa$ B signaling induced by TNF- $\alpha$ . To further confirm this, we studied the effects of rosmarinic acid on the expression of intercellular adhesion molecule-1 (*ICAM-1*) and *VCAM-1*, which are encoded by NF- $\kappa$ B target genes. These molecules are known to be involved in the transendothelial migration of leukocytes (Pierce *et al.*, 1996). As shown in Figure 9, the expression of both *ICAM-1* and *VCAM-1* was reduced by rosmarinic acid. The IC $_{50}$  of rosmarinic acid on TNF- $\alpha$ -induced expression of *sVCAM-1* and *sICAM-1* was 14.6  $\pm$  2.4 and 15.8  $\pm$  1.6  $\mu$ M, respectively. These results indicate that the anti-inflammatory properties of rosmarinic acid can be accounted for by the inhibition of the NF- $\kappa$ B pathway.



**Figure 9** Rosmarinic acid inhibits the expression of *VCAM-1* and *ICAM-1* genes induced by TNF- $\alpha$  in human dermal fibroblast cells. Human dermal fibroblast cells were incubated in the presence or absence of TNF- $\alpha$ , along with indicated concentrations of rosmarinic acid, for 14 h. The concentration of sICAM-1 (a) and sVCAM-1 (b) in the culture medium was measured by a sandwich immunoassay kit (upper panel). Results were confirmed by three independent experiments. Data are expressed as the means  $\pm$  s.e.m. \* $P$  < 0.05, compared with untreated controls.  $^{\circ}$  $P$  < 0.05 versus TNF- $\alpha$  (40 ng ml $^{-1}$ ) only. RA: rosmarinic acid; NaSal: sodium salicylate (20 mM).

## Discussion

To our knowledge, this study is the first to investigate the regulatory effects of rosmarinic acid on the TNF- $\alpha$ -induced expression of the *CCL11* and *CCR3* genes. We discovered that rosmarinic acid inhibits the expression of the *CCL11* and *CCR3* genes by suppressing NF- $\kappa$ B promoter activity; we also found that rosmarinic acid operates downstream of IKK- $\beta$  in the TNF- $\alpha$ -induced upregulation of *CCL11* and *CCR3*.

We demonstrated that rosmarinic acid inhibits the TNF- $\alpha$ -induced expression of the *CCL11* and *CCR3* genes by human dermal fibroblast cells, a cell type that acts as a fundamental component of the inflammatory response (Figure 2). Although rosmarinic acid completely inhibited expression of the *CCR3* gene at a concentration of 40  $\mu$ M, expression of the *CCL11* gene was not blocked at this concentration. This result suggests that expression of the *CCR3* gene is more dependent on the NF- $\kappa$ B promoter than the *CCL11* gene. To be precise, the different inhibitory effects of rosmarinic acid on *CCR3* and *CCL11* may be attributed to the fact that, while expression of the *CCL11* gene is dependent on STAT 6 and NF- $\kappa$ B, expression of the *CCR3* gene is only dependent on NF- $\kappa$ B (Matsukura *et al.*, 1999). We used TNF- $\alpha$  in this study because the NF- $\kappa$ B pathway was previously reported to play a key role in the regulation of *CCR3* and *CCL11* (Huber *et al.*, 2002). To this end, the NF- $\kappa$ B luciferase reporter assay and Western blot analysis for phosphorylated I $\kappa$ B- $\alpha$  or I $\kappa$ B- $\alpha$  were performed in the initial steps of the investigation. Using this approach, we were able to demonstrate that the TNF- $\alpha$ -induced phosphorylation and degradation of I $\kappa$ B- $\alpha$ , and NF- $\kappa$ B activation were inhibited by rosmarinic acid. Nuclear translocation of the NF- $\kappa$ B heterodimer induced by TNF- $\alpha$  was also attenuated by rosmarinic acid. These findings indicated that rosmarinic acid inhibited the activation of NF- $\kappa$ B induced by TNF- $\alpha$  and suggested that rosmarinic acid may inhibit the expression of the *CCL11* and *CCR3* genes. This is demonstrated in Figure 2a and b.

Here, we show that rosmarinic acid regulates TNF- $\alpha$ -induced expression of the *CCL11* and *CCR3* genes by inhibiting NF- $\kappa$ B activation. As NF- $\kappa$ B activation induced by TNF- $\alpha$  is known to be mediated by the canonical pathway, we performed a luciferase reporter assay using molecules involved in the NF- $\kappa$ B activation signal, such as *TRAF2*, *MEKK3*, and *IKK- $\beta$* . We also performed Western blot analysis for phosphorylated I $\kappa$ B- $\alpha$  Ab in order to clarify the mechanism(s) of action of rosmarinic acid with regard to the NF- $\kappa$ B activation pathway, induced by TNF- $\alpha$ . In this study, we introduced wild-type cDNA for *TRAF2* or *MEKK3*, and the constitutive active form of *IKK- $\beta$* . Several reports have shown that overexpression of the wild-type *MEKK3* gene or wild-type *TRAF2* gene activates NF- $\kappa$ B signaling (Yang *et al.*, 2001; He *et al.*, 2004; Lee *et al.*, 2005). A report by Mercurio *et al.* (1997) also showed that overexpression of the constitutively active form of *IKK- $\beta$*  activates NF- $\kappa$ B signaling. It is thought that, while the role of TRAF2 and MEKK3 in NF- $\kappa$ B activation is the recruitment of IKK to TNFR 1 by TRAF2 and the activation of IKK by MEKK3, the role of *IKK- $\beta$*  is to directly activate I $\kappa$ B- $\alpha$  (Yang *et al.*, 2001).

When each molecule was overexpressed by transient transfection in human dermal fibroblast cells, NF- $\kappa$ B luciferase reporter activity was significantly reduced by

treatment with rosmarinic acid. The phosphorylation level of I $\kappa$ B- $\alpha$  induced by all of the tested genes was also noticeably attenuated by rosmarinic acid (Figures 6 and 7). This indicates that rosmarinic acid operates downstream of IKK- $\beta$  in the TNF- $\alpha$ -induced activation of NF- $\kappa$ B. This finding was strengthened by an examination of the *in vitro* IKK- $\beta$  kinase activity (Figure 8), which indicated that IKK- $\beta$  activity induced by TNF- $\alpha$  is inhibited by rosmarinic acid.

Recent experiments based on the use of conditional IKK- $\beta$  loss-of-function mutations indicate that IKK- $\beta$  activity is required for the inactivation of a severe inflammatory reaction that leads to multi-organ failure in response to ischemia-reperfusion (Chen *et al.*, 2003). IKK- $\beta$  activity is also required to prevent a considerable number of different cell types from undergoing apoptosis (Karin & Lin, 2002). These results indicate that rosmarinic acid, an IKK- $\beta$  inhibitor, could be useful for the treatment of inflammatory diseases (Barnes & Karin, 1997; Ghosh *et al.*, 1998; Neurath *et al.*, 1998). However, as an IKK- $\beta$  inhibitor, an expected side effect inherent to rosmarinic acid would be increased susceptibility to the induction of programmed cell death. Until now, several compounds have been identified as IKK- $\beta$  inhibitors. Among these are aspirin, salicylates, sulindac, sulfasalazine, thalidomide, cyclopentenone prostaglandins, quinazoline analogue,  $\beta$ -carbolin analogue, and imidazoquinoxaline derivatives (Karin *et al.*, 2004). These compounds have been reported as nanomolar- or micromolar-range inhibitors of IKK- $\beta$  kinase activity, and have demonstrated inhibitory activity in functional cell-based assays. The compounds have also shown efficacy in experimental models. Like these compounds, rosmarinic acid has an IC<sub>50</sub> of  $\sim$ 12  $\mu$ M against the I $\kappa$ B- $\alpha$  kinase activity of IKK- $\beta$ , which suggests the potential use of rosmarinic acid in the treatment of inflammatory diseases. However, until the safety and efficacy profiles of rosmarinic acid are determined, it is not clear whether this compound could be used in the treatment of inflammatory disorders.

One such inflammatory disorder is atopic dermatitis, a common pruritic inflammatory skin disease, which often begins in infancy and frequently affects individuals with a personal or family history of atopic disease (Beltrani, 1996). Increased serum immunoglobulin E levels and blood eosinophilia are also often associated with this condition. Although the pathogenesis of atopic dermatitis is still poorly understood, there are reports suggesting that chemokines are involved in the modulation of tissue inflammation (Schroder *et al.*, 1996; Baggiolini *et al.*, 1997). In a recent report, the transcriptional and translational expression levels of *CCL11* and *CCR3* were significantly increased in lesional skin from atopic dermatitis, but not in nonatopic controls. (Yawalkar *et al.*, 1999). Therefore, the inhibitory effect of rosmarinic acid on the TNF- $\alpha$ -induced expression of the *CCL11* and *CCR3* genes and the antibacterial effect of rosmarinic acid against *Staphylococcus aureus*, a pathogen commonly associated with atopic dermatitis (data not shown), suggest that rosmarinic acid may hold potential as a treatment for this inflammatory disorder.

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