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Mechanistic Study of Saikosaponin-d (Ssd) on Suppression of Murine T Lymphocyte Activation

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ABSTRACT

Saikosaponin-d (Ssd) is a triterpene saponin derived from the medicinal plant, *Bupleurum falcatum* L. (Umbelliferae). Previous findings showed that Ssd exhibits a variety of pharmacological and immunomodulatory activities including anti-inflammatory, anti-bacterial, anti-viral and anti-cancer effects. In the current study we have investigated the effects of Ssd on activated mouse T lymphocytes through the NF-κB, NF-AT and AP-1 signaling pathways, cytokine secretion, and IL-2 receptor expression. The results demonstrated that Ssd not only suppressed OKT3/CD28-costimulated human T cell proliferation, it also inhibited PMA, PMA/Ionomycin and Con A-induced mouse T cell activation in vitro. The inhibitory effect of Ssd on PMA-induced T cell activation was associated with down-regulation of NF-κB signaling through suppression of IKK and Akt activities. In addition, Ssd suppressed both DNA binding activity and the nuclear translocation of NF-AT and activator protein 1 (AP-1) of the PMA/Ionomycin-stimulated T cells. The cell surface markers like IL-2 receptor (CD25) were also down-regulated together with decreased production of pro-inflammatory cytokines of IL-6, TNF-α and IFN-γ. These results indicate that the NF-κB, NF-AT and AP-1 (c-Fos) signaling pathways are involved in the T cell inhibition evoked by Ssd, so it can be a potential candidate for further study in treating T cell-mediated autoimmune conditions. J. Cell. Biochem. 107: 303–315, 2009.

KEY WORDS: SAIKOSAPONIN-D; T LYMPHOCYTE ACTIVATION; NF-κB; NF-AT; AP-1

Saikosaponin-d (Ssd) is a triterpene saponin derived from *Bupleurum falcatum* L. (Umbelliferae), a medicinal plant that has been used for treating various inflammatory and infectious diseases by Chinese and Japanese doctors for over one thousand of years. Other saikosaponins have been isolated and identified from this plant, including saikosaponins-a, -b, -c, -d, -m, -n, -p and –t. Ssd is believed to be the most active among them, with saikosaponin-a (Ssa) the second most active [Wang et al., 2006]. The anti-inflammatory property of Ssd has been demonstrated by its inhibition of mouse ear and paw edema induced by phorbol 12-myristate 13-acetate (PMA) in vivo, and the reduction of cyclooxygenase (COX) and lipoxygenase (LOX) production in vitro [Bermejo Benito et al., 1998].

It has been shown that nuclear factor κB (NF-κB) is an important transcription factor involved in the maturation and survival of T lymphocytes and the induced expression of gene products mediating innate and adaptive immunity, thereby contributing to the control of cell proliferation and apoptosis. The role of NF-κB in adaptive immunity involves a variety of functions, including anti-apoptosis and the induced expression of MHC proteins, co-stimulatory molecules such as B7.1, and cytokines including interleukin-2 (IL-2), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) [Ghosh and Karin, 2002; Karin and Greten, 2005]. The nuclear factor of activated T-cells (NF-AT) is also involved in T cell activation. This NF-AT transcription factor was first described as an inducible regulatory complex critical for transcriptional induction of the IL-2 gene in activated T cells [Shaw et al., 1988], but later, it was shown to regulate the transcription of other genes, including cytokines (IL-4, interferon-γ, TNF-α and granulocytes/macrophage-colony stimulating factor) and regulatory enzymes such as COX-2 [de Gregorio et al., 2001]. NF-AT binds to the DNA either alone or in conjunction with activator protein 1 (AP-1) transcription factors in the nucleus [Macian et al., 2001]. Therefore, the coordinate induction and activation of the transcription factors NF-κB, NF-AT and AP-1 are required for regulation of cytokine gene expression [Crabtree and Clipstone, 1994].

Clinically, T cell activation has been strongly implicated in the mediation of many aspects of autoimmune diseases, such as rheumatoid arthritis [Xiao and Link, 1999]. Once T cells recognize

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the antigen peptide present on the surface of antigen-presenting cells, the naïve T cells differentiate into effector cells and respond vigorously with clone expansion, cytokine production and cytokine receptor expression. This process is closely associated with initiation of the primary autoimmune responses in a variety of autoimmune diseases. Upon T cell activation, IL-2 and IFN-γ appear; they are two essential cytokines produced by T cells and exert powerful immunoregulatory effects. IL-2, a T cell growth factor, is required for T cell proliferation [Smith, 1988]. IFN-γ promotes T cell differentiation and activates macrophages to secrete various cytokines [Belardelli, 1995]. Meanwhile, activation of T cells also leads to induction of several cell surface molecules such as IL-2 receptor (CD25), a very early activation antigen (CD69), and a late activation antigen (CD71) that participates in cell proliferation; levels of this latter antigen correlate with the degree of immune activation. In our previous studies, we demonstrated that Ssd inhibits T cell activation by suppression of both early (CD69) and late (CD71) expressions through the modulation of the protein kinase θ (PKCθ) c-Jun N-terminal kinase (JNK) pathway [Leung et al., 2005]. However, whether and how Ssd suppresses NF-κB signaling through inhibition of inhibitory NF-κB alpha (IκBα) phosphorylation, protein kinase B (Akt) activation and the NF-AT and AP-1 signaling pathways remains to be investigated.

In the current study, we demonstrated that Ssd not only suppressed OKT3/CD28 co-stimulated human T cell proliferation, it also significantly inhibited PMA-induced or PMA/Ionomycin-induced mouse T lymphocyte activation in vitro, and this inhibitory effect was associated with down-regulation of NF-κB signaling of T cells through suppression of IκB kinase (IKK) and Akt activities. Moreover, Ssd was shown to inhibit NF-AT and AP-1 signaling pathways of T cells which are known to play a critical role in the immune response. Cell surface markers like CD25 were also down-regulated together with decreased production of the pro-inflammatory cytokines of IL-2, IL-6, TNF-α and IFN-γ. So, the current study has elucidated the underlying molecular mechanisms by which Ssd contributes to the inhibition of T lymphocyte activation. Our conclusions suggest that Ssd is a strong candidate for further studies of its usefulness in treating T cell-mediated autoimmune conditions.

MATERIALS AND METHODS

REAGENTS

Saikosaponin-d (>98% purity, HPLC) was obtained from the China Chengdu Biotechnology Company Ltd. The antibodies used for surface staining were anti-mouse CD3 phycoerythrin (PE) and anti-mouse CD25 fluorescein isothiocyanate (FITC) obtained from BD Pharmingen (San Diego, USA). Mouse T lymphocytes were stimulated by Concanavalin A (Con A), phorbol 12-myristate 13-acetate (PMA) obtained from Sigma (St. Louis, MO) or ionomycin obtained from Calbiochem (San Diego, CA).

ANIMALS

Male Balb/c mice at the age of 7–10 weeks were obtained from the Chinese University of Hong Kong, Hong Kong, China. The animals were acclimated for ≥1 week under 12 h light and 12 h dark cycle at room temperature of 22°C ± 1°C. Chow diet and water were provided ad libitum. Animal care and treatment procedures conformed to the Institutional Guidelines and Animal Ordinance (Department of Health, Hong Kong Special Administrative Region).

CELL CULTURE

Mouse T lymphocytes were isolated from lymph nodes of Balb/c mice. Single cell suspensions were prepared by mechanical disruption through nylon mesh and each was washed twice by centrifugation (2,000 rpm, 5 min) using RPMI medium. Viable cell counting was conducted by the Trypan blue exclusion method. Mouse T Cells were cultured in RPMI 1640 medium, and human cervix cancer cell line, HeLa obtained from ATCC (Manassas, VA) were maintained in MEM medium, all medium supplemented with 10% fetal bovine serum (FBS) and antibodies with 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen, Paisley, Scotland, UK). All cell cultures were incubated at 37°C in a 5% humidified CO_2 incubator.

T CELL PURIFICATION

T lymphocytes were purified using magnetic cell separation via high affinity negative selection according to standard procedures using the Pan T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The T cell purity, as analyzed by FACS, was always higher than 97%.

CYTOTOXICITY ASSAY

Ssd was dissolved in DMSO at final concentration of 100 mM and stored at −20°C. Cytotoxicity was assessed using the Trypan blue exclusion method. Mouse T lymphocytes (5×10^6) were incubated with DMSO control and various concentrations of Ssd (5–15 μM) at 37°C in a 5% humidified CO_2 incubator for 48 h. After that, viable cell counting was conducted by the Trypan blue exclusion method under light microscope. The percentage of cell viability was calculated using the following formula: Cell viability (%) = Cells $\text{number}_{\text{treated}}$/Cells $\text{number}_{\text{DMSO control}} \times 100$. Data were obtained from three independent experiments.

T CELL PROLIFERATION ASSAY

T cell proliferation was assessed by 5-bromo-2′-deoxy-uridine (BrdU) assay. In brief, the isolated human T lymphocytes (10^5 cells/well) were cultured in triplicates in a 96-well flat-bottomed plate (Costar, Corning Incorporated, Corning, NY) in 100 μl of RPMI 1640 medium supplemented with 10% FBS and then stimulated with anti-OKT-3/anti-CD-28 antibodies in the presence or absence of Ssd (7.5–15 μM) for 72 h. 5-Bromo-2′-deoxy-uridine (BrdU, Roche) was added to the cells 14 h before the end of stimulation at a final concentration of 10 μM. BrdU can be incorporated into the DNA of growing cells during the labeling period; the amount of BrdU incorporated into the DNA can be quantified as an indicator of cell proliferation. In this experiment, BrdU was determined by ELISA according to manufacturer's instruction.

TRANSCRIPTION FACTOR ASSAY

For NF-κB p65 transcription factor assay, mouse T lymphocytes were pre-incubated at 37°C for 60 min with different concentrations

of Ssd (5–15 μM) followed by 30 min incubation with 20 ng/ml PMA. For AP-1 (c-Fos) and NF-AT transcription factor assay, mouse T lymphocytes were pre-treated with different concentrations of Ssd (7.5–15 μM) for 60 min followed by 120 min incubation with 20 ng/ml PMA plus 1 μM ionomycin. After these treatments, nuclear extracts from the cells were harvested and assayed for NF-κB p65, AP-1 (c-Fos) and NF-AT activity by NoShift II Transcription Factor Assay Kits (Novagen, San Diego, CA). The double-stranded, biotin-labeled oligonucleotides used in these assays contain the consensus sites for NF-κB (provided by the kits), NF-AT (5′-GAT-CGGAGGAAAAACTGTTTCATACAGAAGGCGT-3′; distal NF-AT sites of the human IL-2 promoter), and AP-1 (5′-GATCTGCAT-GAGTCAGACACACA-3′; AP-1 sites of the human IL-2 promoter). In brief, the nuclear extracts (15 μg) were incubated with biotin-end labeled double-stranded oligonucleotide in NoShift Bind Buffer plus poly(dI-dC).poly(dI-dC) and salmon sperm DNA for 30 min on ice. The reaction mixtures were then transferred to the Streptavidin plate and incubated for 60 min at 37°C. After that, the plate was washed with NoShift Wash Buffer and the antibodies anti-NF-κB (p65) (Novagen) for NF-κB assay, anti-NF-ATc1 (BD Pharmingen, San Diego, CA) for NF-AT assay and anti-cFos (Cell signaling, Danvers, MA) for AP-1 assay were added and incubated for 60 min followed by secondary antibody conjugated with HRP for 30 min at 37°C. Finally, the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added and incubated for 10–30 min to develop colorimetric signal, the sample absorbance at 450 nm was recorded by spectrophotometer.

KINASE ASSAY

To determine the effect of Ssd on PMA-induced IKK activation, we performed an immunocomplex kinase assay using K-LISA^{TM} IKKβ-Inhibitor Screening Kit (Calbiochem). In brief, the IKK complex from 100 μg whole-cell extracts (Ssd treated) was precipitated with antibody against IKK-α and IKK-β followed by treatment with protein A/G-Sepharose beads (Amersham Biosciences, Little Chalfont, UK). After overnight incubation, the beads were washed with lysis buffer and assayed using a kinase assay kit containing kinase assay buffer, substrate glutathione *S*-transferase-IκBα (GST-IκBα) in the wells of a Glutathione-Coated 96-Well Plate, which allowed for substrate phosphorylation. For IKK-β inhibitor screening, both GST-IκBα substrate and IKK-β His Tag human recombinant were incubated in the presence of various concentrations of Ssd in the wells of a Glutathione-Coated 96-Well Plate. After incubation at 30°C for 30 min, the reaction was terminated by adding of kinase stop solution. The phosphorylated GST-IκBα substrate was detected using an Anti-Phospho IκBα (Ser^{32}/Ser^{36}) antibody, followed by the HRP-conjugate and color development with TMB substrate. ELISA stop solution was used to stop the color development and the absorbance was read at 450 nm. The absorbance is directly related to the level of IKK activity. To determine the total amount of IKK-α and IKK-β in each sample, 50 μg of the whole-cell protein was resolved on 7.5% SDS–PAGE, electrotransferred to a nitrocellulose membrane, and blotted with anti-IKK-α or anti-IKK-β antibody.

WESTERN BLOT ANALYSIS

Mouse T lymphocytes (1×10^7) were pre-incubated with different concentrations of Ssd (5–15 μM) for 60 min at 37°C. The cells were then treated with 20 ng/ml PMA and harvested 30 min thereafter. For NF-κB p65 nuclear translocation assay, the cells were pre-incubated with different concentrations of Ssd (5–15 μM) at 37°C for 120 min followed by 30 min incubation with 20 ng/ml PMA/2 μM ionomycin or by 45 min incubation with 5 μg/ml Con A. For AP-1 (c-Fos) and NF-AT nuclear expression detection, mouse T lymphocytes were pre-incubated with different concentrations of Ssd (7.5–15 μM) at 37°C for 60 min followed by 120 min incubation with 20 ng/ml PMA/1 μM ionomycin. Subcellular proteins from cytosolic and nuclear fractions of the purified T cells were extracted using NucBuster Protein Extraction Kit (Novagen). The fractionated cytosolic proteins (50 μg) and nuclear extracts (30 μg) were resolved by 10% SDS–PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membrane which was then blocked with 5% dried milk for 60 min. The membrane was then washed three times for 5 min each with wash buffer and incubated with corresponding antibodies overnight at 4°C. Following washing, the membrane was further incubated with HRP-conjugated secondary antibodies for 60 min. The blot was developed using the ECL Western Blotting Detection Reagents (Amersham Biosciences).

For detection of phosphorylation forms of IκBα, mouse T lymphocytes (1×10^7) were incubated first with different concentrations of Ssd (5–15 μM) together with 100 mg/ml *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) (Calbiochem) for 60 min. The cells were then treated with 20 ng/ml PMA and harvested 30 min thereafter. The harvested T cells were lysed by lysis buffer (50 mM Tris–HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1 mM DTT, 1% Triton, 50 mM NaF, 1 mM sodium orthovanadate, 0.5 mM PMSF and 1× proteinase inhibitor mix (Roche Diagnostics, Barcelona, Spain). The whole-cell lysate (50 μg) was subjected to electrophoresis in 10% SDS–PAGE. The primary antibodies used in this study were rabbit antibodies specific for IκBα, p65, Akt, IKK-α/β (Santa Cruz, CA), p-IκBα^{ser32} (Calbiochem) and c-Fos (Cell signaling); mouse antibodies specific for IKK-α, acetyl-lysine, actin (Santa Cruz), p-Akt^{ser473}, p-p65^{ser536} (Cell signaling) and NF-ATc1 (BD Pharmingen); goat antibodies specific for IKK-β (Santa Cruz).

P65 ACETYLATION

To determine the effect of Ssd on PMA-induced acetylation of p65, T cells (1×10^7) were pretreated with different concentrations of Ssd (5–15 μM) for 60 min. The cells were then treated with 20 ng/ml PMA and harvested 30 min thereafter. The harvested T cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in a buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 2 mM NaF, 10% glycerol, 0.2% Triton X-100, 0.2 mM sodium orthovanadate, 0.5 mM PMSF and 1× proteinase inhibitor mix. The resulting whole-cell lysates were incubated with anti-p65 antibody for 60 min and precipitated with protein A/G-Sepharose beads (Amersham Biosciences) overnight at 4°C. Immunocomplexes were then washed with lysis buffer, boiled with SDS sample buffer for 5 min, resolved on SDS–PAGE, and subjected to Western blot analysis with anti-acetyl-lysine antibody.

FLOW CYTOMETRY ANALYSIS OF CD25 CELL SURFACE EXPRESSIONS

The expressions of cell surface molecules in T cell cultures were evaluated by flow cytometry. Cells (1×10^6) were first pretreated with different concentrations of Ssd (5–15 μM) for 60 min, and then followed by the stimulation with 5 μg/ml Con A or 20 ng/ml PMA/2 μM ionomycin. The cells surface expression of CD25 antigens was accessed after 40 h of the culture. At the end of the culture period, the harvested cells ($n = 3$) were washed twice with buffer. Cells were stained with specific antibodies in the combination of CD3-PE and CD25-FITC for 25 min at room temperature in the dark. Cells were then washed with buffer to remove the excess stains and analyzed on a BD FACSCanto flow cytometer using BD FACSDiva software. Compensation standards consisting of separate tubes of lymphocytes stained with positive single-color antibodies for each of the fluorochromes, as well as an unstained sample, were used at each analysis. Lymphocytes were selected by forward and side scatter gating. Surface antigen CD25 was analyzed by gating $CD3^+$ lymphocytes in a CD3 versus side scatter (SSC) dot plot. The percentages of positive CD25 stained T cells ($CD3^+$) were determined in the dot plot. The quadrant of the dot plot was set based on the unstimulated (control) culture.

IMMUNOCYTOCHEMISTRY ASSAY

HeLa cells were grown on glass coverslips in 6-well plates (35-mm diameter). After exposure to 10 μM Ssd and 20 ng/ml PMA plus 1 μM ionomycin, cells were washed once with PBS and then fixed with 4% paraformaldehyde in PBS for 15 min. The fixed cells were washed thrice with PBS and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. The cells were then washed with PBS for two times and then stained with Alexa Fluor® 546 phalloidin (Invitrogen) for 30 min to visualize the F-actin. For AP-1 staining, cells were then incubated with c-Fos (Cell signaling) (1:200) in 3% bovine serum albumin for 2 h. FITC-conjugated goat anti-rabbit IgG (ZYMED, San Francisco, CA) (1:500) antibody was used as the secondary antibody for another 1 h. Excess antibody was removed by multiple washes with TBS. Coverslips were mounted onto microscope slides with FluorSave™ Reagent (Calbiochem), and examined under the Nikon ECLIPSE 80*i* microscope. Images were captured with CCD digital camera Spot RT3™ (Diagnostic Instruments, Inc., Melville, NY).

ENZYME-LINKED IMMUNOSORBENT ASSAY

The levels of IL-2, IL-6, TNF-α and IFN-γ produced in activated mouse T lymphocytes were measured using the IL-2, IL-6, TNF-α and IFN-γ mouse enzyme-linked immunosorbent assay (ELISA) system (Biosource, Invitrogen). Briefly, cells (5×10^6) were first pretreated with different concentrations of Ssd (5–15 μM) for 60 min, and then followed by stimulation with 5 μg/ml Con A or 20 ng/ml PMA/2 μM ionomycin. The cell-free culture supernatants were harvested at 24 and 40 h after incubation. The concentrations of these cytokines in the supernatants were determined according to the procedure provided by the manufacturer. All samples were determined in triplicate.

STATISTICAL ANALYSIS

The results were expressed as means ± SD as indicated. The difference was considered statistically significant when the *P*-value was less than 0.05. Student's *t*-test or one-way ANOVA analysis was used for comparison among different groups.

RESULTS

Ssd SUPPRESSES PMA-INDUCED NF-κB P65 DNA BINDING IN AN INDIRECT AND DOSE-DEPENDENT MANNER

The p50/p65 heterodimer is the most common dimer found in the NF-κB signaling pathway, while p65 is the major component of NF-κB in PMA-stimulated T lymphocytes [Kunsch et al., 1994]. We therefore determined the effect of Ssd on p65 DNA binding activity but not p50. In the pre-experimental study on time kinetics of the drug action, we pretreated T cells with 15 μM Ssd for 1, 2, 4, and 12 h followed by PMA stimulation. Results showed that 60 min pretreatment elicited the maximum inhibitory effect and was therefore the most appropriate duration of time for Ssd treatment (unpublished work). To determine whether the suppression of NF-κB p65 DNA binding by Ssd is dose-dependent, we pre-treated mouse T lymphocytes with different concentrations of Ssd for 60 min and then with stimulation of PMA for 30 min. The NoShift II Transcription Factor Assay showed that PMA-induced NF-κB p65 activation was significantly inhibited by Ssd in a dose-dependent manner (Fig. 1A). Then, we further investigated how Ssd inhibits PMA-induced NF-κB p65 activation in detail.

To determine whether Ssd directly modifies the binding of NF-κB complex to DNA, we incubated nuclear extracts prepared from PMA-stimulated mouse T lymphocytes with Ssd for 60 min and then analyzed p65 DNA binding activity by using the same non-radioactive transcription factor assay. Results demonstrated that although Ssd could inhibit NF-κB p65 activation (Fig. 1A), it did not modify the p65 DNA binding ability of the NF-κB complex even at 100 μM concentration (Fig. 1B). Thus, we concluded that Ssd inhibits NF-κB p65 activation indirectly.

Ssd INHIBITS PMA-INDUCED IκBα DEGRADATION AND PHOSPHORYLATION

Treatment of T cells with stimuli like PMA can initiate PKCθ signal transduction cascades leading to activation of IκB kinase (IKK), thereby activating the NF-κB signaling [Matsumoto et al., 2005]. Activation of NF-κB signaling requires the translocation of NF-κB to the nucleus, and this translocation is preceded by the proteolytic degradation of IκBα [Aggarwal, 2004]. We first examined the proteolytic degradation of IκBα by treatment of PKC activator, PMA, for periods of 10–180 min. Results showed that the proteolytic degradation of IκBα began to increase at 10 min, reached a maximum at 30 min, and decreased thereafter (Fig. 2A). Thus, 30 min stimulation by PMA in T lymphocytes is optimal for the analysis of IκBα degradation. To determine whether the inhibitory effect of Ssd is due to inhibition of IκBα degradation, we pretreated mouse T lymphocytes with various concentrations of Ssd for 60 min and then with PMA stimulation for 30 min, then examined the IκBα degradation. The results indicated that Ssd inhibited PMA-induced IκBα degradation in a dose-dependent manner (Fig. 2B). We further

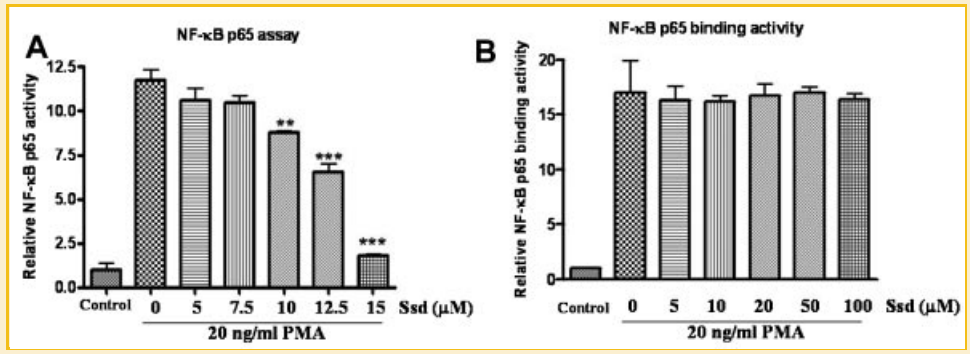

Fig. 1. A: Ssd suppresses PMA-dependent NF-κB p65 activation. Mouse T lymphocytes were pre-incubated at 37°C for 60 min with indicated concentrations of Ssd followed by 30 min incubation with 20 ng/ml PMA. After these treatments, the cells nuclear extracts were harvested and assayed for NF-κB p65 activity by NoShift II Transcription Factor Assay Kits. Results are expressed as relative NF-κB p65 activity over the untreated control. B: Effect of Ssd on DNA binding in vitro. Mouse T lymphocytes were treated with 20 ng/ml PMA at 37°C for 30 min, then the nuclear extracts were prepared and treated with different concentrations of Ssd (up to 100 μM). The NF-κB p65 binding activity was assayed by NoShift II Transcription Factor Assay Kits. Results are expressed as the relative NF-κB p65 binding activity over the untreated control. Mean ± SD are from three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with PMA treatment without Ssd.

determined whether the inhibition by Ssd of PMA-induced IκBα degradation is due to inhibition of IκBα phosphorylation. In this case, we pretreated the T cells with Ssd together with the ubiquitin-proteasome inhibitor, ALLN, at the same time for 60 min, and then stimulated the T cells with PMA. Western blot analysis using an antibody that recognizes the serine-phosphorylated form of IκBα showed that PMA-induced IκBα phosphorylation was suppressed by Ssd in a dose-dependent manner (Fig. 2C).

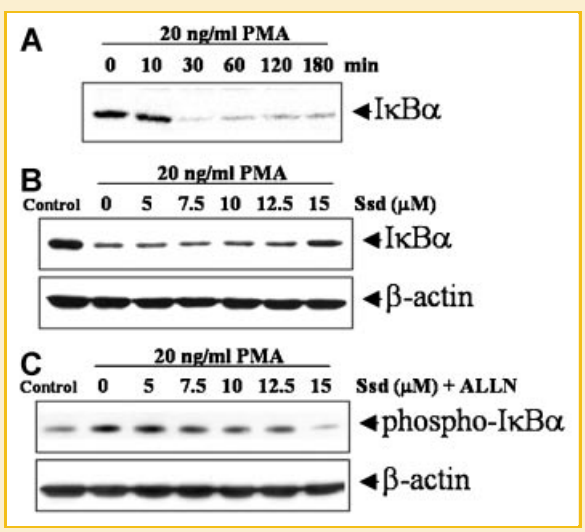

Fig. 2. A: PMA induces the degradation of IκBα. Mouse T lymphocytes were stimulated with 20 ng/ml PMA for different time intervals (10, 30, 60, 120, 180 min). Whole cell extracts were analyzed by Western blotting using antibodies against IκBα. B: Effect of Ssd on PMA-induced degradation of IκBα. Mouse T lymphocytes were pre-incubated at 37°C with indicated concentrations of Ssd for 60 min and then treated with 20 ng/ml PMA at 37°C for 30 min. The IκBα in whole cell extracts were detected by Western blot analysis. C: Ssd inhibits PMA-induced phosphorylation of IκBα. Mouse T lymphocytes were incubated first with indicated concentrations of Ssd together with 100 μM ALLN for 60 min; thereafter, cells were treated with 20 ng/ml PMA for 30 min and whole cell extracts were analyzed by Western blotting using antibodies against phosphorylated IκBα.

Ssd INHIBITS PMA-INDUCED IKK PROTEIN COMPLEX ACTIVATION

IκBα phosphorylation depends on IKK protein complex activity. Because Ssd inhibited the phosphorylation of IκBα in our study, we needed to know whether it directly affects PMA-induced IKKα/β activation. For this purpose, mouse T lymphocytes were treated with Ssd for 60 min before stimulating them with PMA for 30 min. Whole-cell extracts were harvested and 100 μg of the protein was immunoprecipitated with antibodies against IKK-α and IKK-β. Thereafter, an immune complex kinase assay was performed with an IKK-inhibitor screening kit (Calbiochem). Results from the immunocomplexes kinase assay showed that Ssd suppressed PMA-induced activation of IKKα/β in a dose-dependent manner (Fig. 3A). However, neither PMA nor Ssd affected the expression of IKKα and β subunits (Fig. 3B). To evaluate whether Ssd suppresses IKKα/β activity directly by binding to the IKK protein complex or by suppressing the activation of IKKα/β, we performed the IKK-β kinase assay by using an IKK-β inhibitor screening kit (Calbiochem). Since IKK-β appears to be the principal kinase for activation of IKK protein complex and degradation of IκBα by pro-inflammatory stimuli [Hacker and Karin, 2006], we incubated the human recombinant IKK-β and GST-IκBα substrates together with various concentrations of Ssd in vitro. In this kinase assay, the phosphorylated form of GST-IκBα substrate was detected using an anti-phospho IκBα antibody, followed by the HRP-conjugate and color development with TMB substrate. Results revealed that the positive control (IKK inhibitor V) directly suppressed IKK-β activity. However, Ssd did not directly suppress the activity of IKK-β (Fig. 3C). And, the Ssd alone did not affect the expression of IKKα/β subunit nor its downstream signaling molecules like IκBα (Fig. 3D). This suggests that Ssd suppresses IKK protein complex activity either through the regulation of the upstream signaling pathway of IKK or through inhibition of other IKK protein complex subunits.

Ssd INHIBITS PMA, PMA/IONOMYCIN AND CON A-INDUCED NUCLEAR TRANSLOCATION AND POST-MODIFICATION OF p65

In order to identify the molecular targets of Ssd we further determined if Ssd could affect PMA-induced nuclear translocation

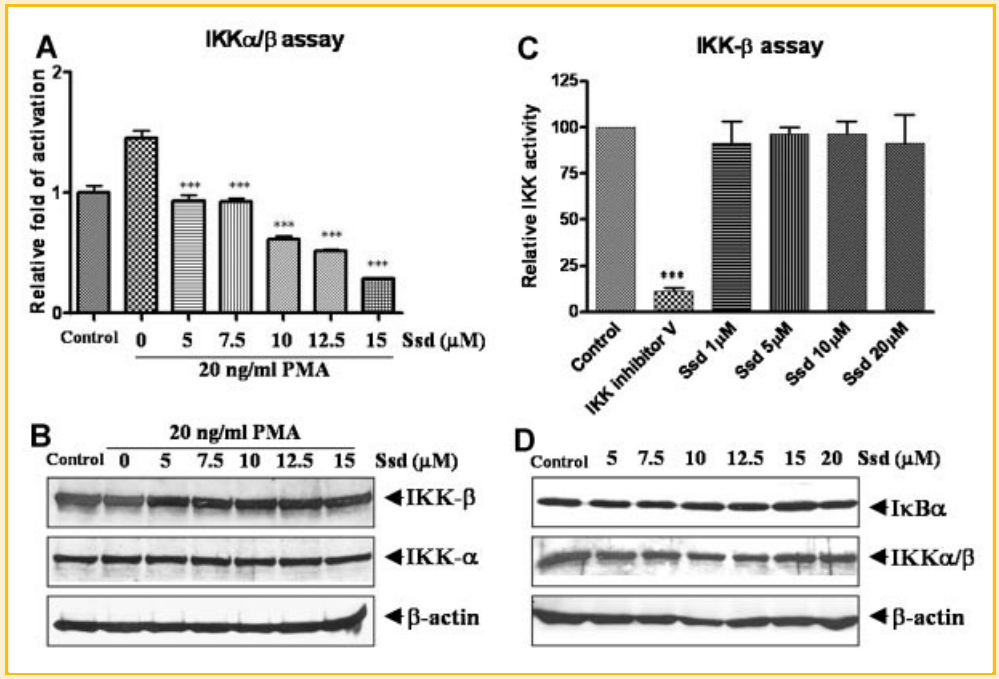

Fig. 3. A: Ssd inhibits PMA-induced IKKα/β activity. Mouse T lymphocytes were treated with indicated concentrations of Ssd for 60 min and then activated with 20 ng/ml PMA for 30 min. Whole cell extracts were harvested and 100 μg of the protein was immunoprecipitated with antibodies against IKK-α and IKK-β. Thereafter, an immune complex kinase assay was performed with IKK-inhibitor screening kit (Calbiochem). $^{***}P < 0.001$, compared with PMA treatment alone. B: Expression level of IKK proteins. To examine the effect of Ssd on the level of expression of IKK proteins, 50 μg of whole cell extract was run on a 7.5% SDS–PAGE, electrotransferred and immunoblotted with the indicated antibodies. C: Inhibition of IKK-β activity by Ssd. IKK-β His-Tag human Recombinant (2 ng) was incubated with GST- IκBα substrate in the presence of increasing concentrations of Ssd and 10 mM ATP. IKK inhibitor V is the positive control provided in the screening kit. The activity was measured by spectrophotometer at OD 450 nm and the results were calculated as relative IKK activity over untreated control. $^{***}P < 0.001$, compared with untreated control treatment. D: The effect of Ssd on IκBα and IKK protein expression. To verify the effect of Ssd alone on the expression levels of IKK proteins and IκBα, mouse T lymphocytes were treated with indicated concentrations of Ssd for 60 min. Cells were then lysed and 50 μg of whole cell extract was run on a 7.5% SDS–PAGE, electrotransferred and immunoblotted with the indicated antibodies. Results are mean ± SD from three independent experiments.

of p65. The mouse T lymphocytes were pretreated with Ssd for 60 min before stimulating them with PMA for 30 min, and then the nuclear extracts were prepared and analyzed by Western blotting using antibodies against p65. For detection of the phosphorylation form of p65, T cells were treated with Ssd for 60 min before PMA stimulation, and then whole cell extracts were analyzed by Western blotting using anti-phospho-p65 antibody. The results demonstrated that Ssd not only suppressed the PMA-induced nuclear translocation of p65, but it also dose-dependently suppressed the PMA-induced phosphorylation of p65 (Fig. 4A). Phosphorylation of p65 is required for its transcriptional activity and subsequent translocation to the nucleus [Zhong et al., 1998]. On the other hand, we also determined whether Ssd can suppress the PMA/Ionomycin or Con A-induced NF-κB activation, mouse T lymphocytes were treated with different concentrations of Ssd for 120 min and then stimulated with PMA/Ionomycin for 30 min or with Con A for 45 min. Both subcellular cytosolic and nuclear fractions were harvested for Western blotting using antibodies against NF-κB (p65). The Western blot data showed that PMA/Ionomycin or Con A-induced NF-κB (p65) nuclear translocation was significantly inhibited by Ssd in a dose-dependent manner (Fig. 4B). The results demonstrated that Ssd could suppress the PMA, PMA/Ionomycin and Con A-induced NF-κB activation through the inhibition of nuclear translocation of NF-κB subunit, p65.

In addition to p65 phosphorylation, the acetylation of p65 plays a key role in activation of NF-κB transcriptional activity [Kiernan et al., 2003]. Thus, we then examined the effect of Ssd on the induction of p65 acetylation by PMA. Mouse T lymphocytes were pretreated with Ssd for 60 min and then with PMA for 30 min; whole-cell extracts were then prepared, and immunoprecipitated with anti-p65 antibody. Western blot analysis using anti-acetyl-lysine antibody indicated that Ssd could dose-dependently suppress PMA-induced acetylation of p65 (unpublished work).

Ssd INHIBITS PMA-INDUCED Akt ACTIVATION

Previous studies have demonstrated that Akt can effectively activate IKK [Ozes et al., 1999] and induce p65 phosphorylation [Sizemore et al., 1999]. Thus, it is predictable that Ssd may suppress PMA-induced Akt activation. To verify this prediction, mouse T lymphocytes were incubated firstly with various concentrations of Ssd for 60 min and then stimulated by PMA for 30 min. Whole cell extracts were then prepared for analyzing the activated form of Akt by using the anti-phospho-specific Akt. Western blot analysis showed that Ssd dose-dependently suppressed PMA-induced Akt activation by down-regulating the phosphorylation state of Akt (Fig. 4C). Subsequently, we examined whether Ssd could affect the association of Akt with IKK. We prepared whole cell extracts from the PMA-treated cells, and then those extracts were immunopre-

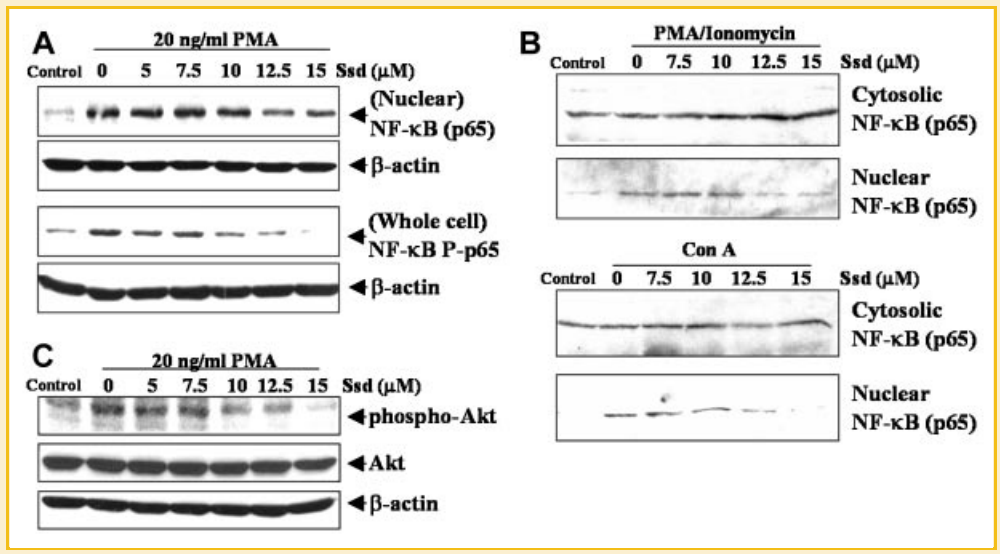

Fig. 4. A: Ssd inhibits PMA-induced phosphorylation and nuclear translocation of NF-κB p65. Mouse T lymphocytes were pre-incubated at 37°C with indicated concentrations of Ssd for 60 min, and then cells were treated with 20 ng/ml PMA at 37°C for 30 min. The whole cell extracts and nuclear extracts were prepared and analyzed by Western blotting using antibodies against P-p65 and p65. B: Ssd suppresses PMA/Ionomycin or Con A induced NF-κB nuclear translocation. Mouse T lymphocytes were pre-incubated at 37°C for 120 min with indicated concentrations of Ssd followed by 30 min incubation with 20 ng/ml PMA/2 μM ionomycin or by 45 min incubation with 5 μg/ml Con A. After these treatments, the subcellular proteins from cytosolic and nuclear fractions were harvested and assayed by Western blotting using anti-p65 antibody. C: Effect of Ssd on PMA-induced Akt activation. Mouse T lymphocytes were incubated first with indicated concentrations of Ssd for 60 min and then treated with 20 ng/ml PMA for 30 min. Whole cell extracts were prepared and analyzed by Western blot analysis using anti-phospho-specific Akt. The same membranes were reblotted with anti-Akt and anti-Actin antibody.

cipitated with anti-IKK-α/β antibody and Western blot analysis was performed using anti-Akt antibody. The result indicated that Ssd suppressed the PMA-induced association between IKK and Akt (unpublished work).

Ssd SUPPRESSES PMA/IONOMYCIN-INDUCED AP-1 (c-FOS) ACTIVATION IN A DOSE-DEPENDENT MANNER

In addition to NF-κB signaling, the transcriptional activity of many lymphokines, including IL-2, depends on the coordinated activation of several other transcriptional factors, such as AP-1 and NF-AT. The activator protein 1 (AP-1) plays a role with NF-κB and NF-AT in immunomodulatory activity of T cells. Thus, we needed to determine if Ssd affects the PMA/Ionomycin-induced activation of AP-1 activity. For this purpose, mouse T lymphocytes were pretreated with different concentrations of Ssd for 60 min and then stimulated with PMA plus ionomycin for 120 min. The NoShift II Transcription Factor Assay showed that PMA/Ionomycin-induced AP-1 (c-Fos) activation was significantly inhibited by Ssd in a dose-dependent manner (Fig. 5A). To examine whether the AP-1 (c-Fos and c-Jun) nuclear expression can be suppressed by Ssd, mouse T lymphocytes were pretreated with different concentrations of Ssd for 60 min before stimulating them with PMA plus ionomycin for 120 min; nuclear extracts were then prepared and analyzed by Western blotting using antibodies against c-Jun and c-Fos. The results demonstrated that Ssd could dose-dependently suppress the PMA/Ionomycin-induced AP-1 activation via down-regulation of c-Fos (Fig. 5B) and c-Jun (unpublished work) in the nuclear region of T cells. Then, we further investigated how Ssd inhibits PMA/Ionomycin-induced AP-1 (c-Fos) expression in nucleus by using immunocytochemistry technique with HeLa cells. HeLa is a human cell line that has been widely used for c-Fos expression study because they provide a discrete compartment for immunofluorescence imaging analysis [Malnou et al., 2007]. In the experiment, we pre-treated HeLa cells with 10 μM of Ssd for 60 min and then with stimulation of PMA plus ionomycin for 120 min. The nuclear expression of AP-1 transcriptional factor was visualized by antibody against its protein subunit (c-Fos). Results demonstrated that there was no AP-1 (c-Fos) expression in the untreated control cells. However, addition of PMA plus ionomycin strongly activated expression of AP-1 (c-Fos) in the nuclear region of the HeLa cells. Interestingly, the pretreatment of Ssd suppressed the nuclear expression of AP-1 (c-Fos) in the stimulated HeLa cells (Fig. 5C). Therefore, it appears that Ssd-mediated suppression on AP-1 activity might be closely correlated with the reduced nuclear expression of c-Fos, the AP-1 subunit in T cells.

Ssd SUPPRESSES PMA/IONOMYCIN-INDUCED NF-AT NUCLEAR TRANSLOCATION AND ACTIVATION IN A DOSE-DEPENDENT MANNER

It has been shown that in the nucleus of T cells activated NF-AT binds to the AP-1 family of transcription factors to increase the rate of transcription of the target genes such as IL-2 or TNF-α [Oum et al., 2002] Moreover, transcriptional activation of NF-AT requires its translocation to the nucleus, where it binds to specific consensus sites in the promoter region of the IL-2 gene [Maggirwar et al., 1997]. TCR signaling that activates NF-AT can be mimicked by a

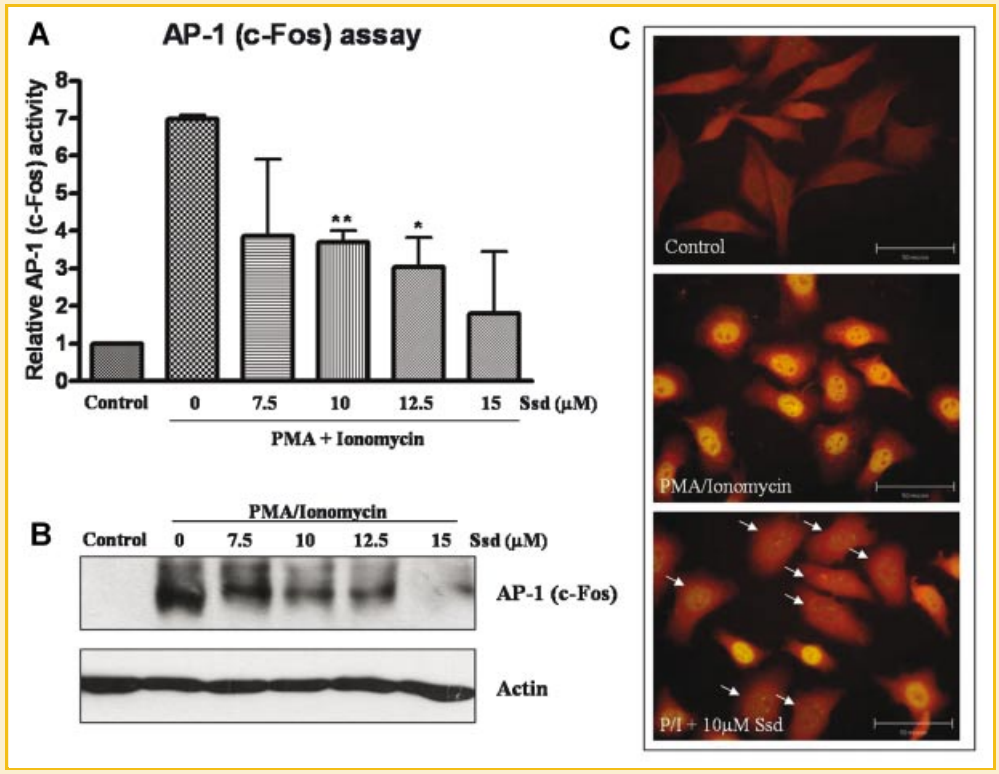

Fig. 5. A: Ssd suppresses PMA/Ionomycin-induced AP-1 (c-Fos) activation. Mouse T lymphocytes were pre-incubated at 37°C for 60 min with indicated concentrations of Ssd followed by 120 min incubation with 20 ng/ml PMA plus 1 μM ionomycin. After these treatments, the cells nuclear extracts were harvested and assayed for AP-1 (c-Fos) activity by using NoShift II Transcription Factor Assay Kits. Results were expressed as relative AP-1 (c-Fos) activity over the untreated control cells. Mean ± SD are from three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$). Significantly reduced compared to PMA plus ionomycin. B: Ssd inhibits PMA plus ionomycin induced nuclear expression of AP-1 (c-Fos). Mouse T lymphocytes were pre-incubated at 37°C with indicated concentrations of Ssd for 60 min, and then cells were treated with 20 ng/ml PMA plus 1 μM ionomycin at 37°C for 120 min. The nuclear extracts were prepared and analyzed by Western blotting technique using antibodies against AP-1 (c-Fos) and actin. C: Analysis of AP-1 (c-Fos) nuclear expression by immunocytochemistry. HeLa cells were pretreated with 10 μM Ssd for 60 min, and then cells were treated with 20 ng/ml PMA plus 1 μM ionomycin (P/I) at 37°C for 120 min. Control cells were treated with solvent (DMSO). Cells were fixed in 4% paraformaldehyde and then co-stained with phalloidin (red) plus anti-c-Fos antibodies (green). Merged images shown are representative of three independent experiments. Arrows indicate the cells with decreased expression of AP-1 (c-Fos) in nuclear region. The scale bars represent 50 μm under 60× magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

combinative stimulation of PMA plus the calcium ionophore, ionomycin. To study whether Ssd inhibits NF-AT activation, we performed the transcription factor assay for the NF-AT activity using nuclear extracts from T cells and Western blotting for nuclear translocation of NF-AT. For this purpose, mouse T lymphocytes were pre-treated with different concentrations of Ssd for 60 min and then with stimulation of PMA plus ionomycin for 120 min. The NoShift II Transcription Factor Assay showed that PMA/Ionomycin-induced NF-AT activation could be significantly inhibited by Ssd in a dose-dependent manner (Fig. 6A). To determine whether the NF-AT nuclear translocation is suppressed by Ssd, mouse T lymphocytes were pretreated with Ssd for 60 min before stimulation with PMA plus ionomycin for 120 min, and then the nuclear extracts were prepared and analyzed by Western blotting technique using antibodies against NF-AT. The results demonstrated that Ssd significantly suppressed the PMA/Ionomycin-induced nuclear translocation of NF-AT in T cells (Fig. 6B). Therefore, it appears that Ssd-mediated suppression on NF-AT activity might be closely correlated with the reduced nuclear expression of NF-AT in T cells.

Ssd SUPPRESSES OKT3/CD28 COSTIMULATION OF HUMAN T LYMPHOCYTE PROLIFERATION AND INHIBITS IL-2 RECEPTOR (CD25) EXPRESSION IN ACTIVATED MOUSE T LYMPHOCYTES

IL-2 and IL-2 receptor are known to be the targeted genes of NF-κB signaling [Coudronniere et al., 2000; Lin et al., 2000]. So, effects of Ssd on the production of IL-2 cytokine [unpublished work and Leung et al., 2005] and IL-2 receptor in PMA/Ionomycin or Con A-stimulated T cells were examined in the cell culture supernatants and on the cell surfaces. To determine if the tested concentrations of Ssd were toxic to the cells, we conducted a cytotoxicity experiment using the Trypan blue exclusion method. The results showed no significant toxicity to those cells at the indicated concentrations of Ssd which covers the range of drug concentrations used in the current studies (Fig. 7A). Besides, these concentrations of Ssd were further found to suppress the OKT3/CD28 costimulation of human T lymphocyte proliferation (Fig. 7B). To determine the effects of Ssd on expression of IL-2 receptor, mouse T lymphocytes were pretreated with Ssd for 60 min and then the cells were stimulated with PMA/Ionomycin or Con A for 40 h. Flow cytometry analysis was performed to measure the cell surface expressions of IL-2

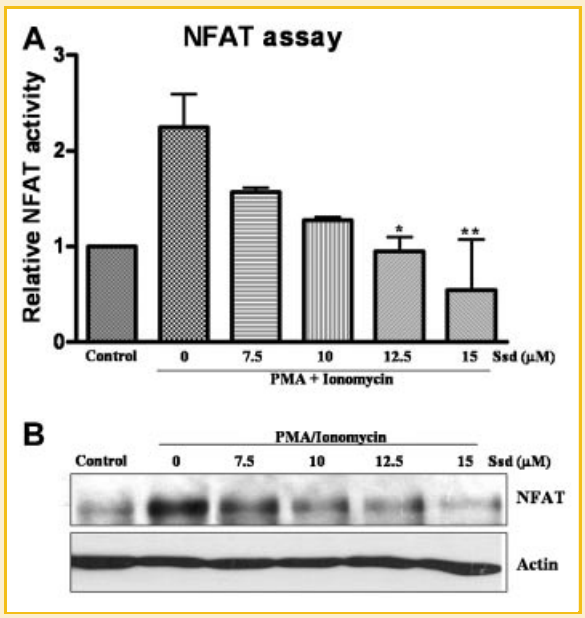

Fig. 6. A: Ssd suppresses PMA/Ionomycin-induced NF-AT activation. Mouse T lymphocytes were pre-incubated at 37°C for 60 min with indicated concentrations of Ssd followed by 120 min incubation with 20 ng/ml PMA plus 1 μM ionomycin. After these treatments, the cells nuclear extracts were harvested and assayed for NF-AT activity by using NoShift II Transcription Factor Assay Kits. Results are expressed as relative NF-AT activity over the untreated control. Mean ± SD are from three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$). Significantly reduced compared to PMA plus ionomycin. B: Ssd inhibits PMA plus ionomycin induced nuclear translocation of NF-AT. Mouse T lymphocytes were pre-incubated at 37°C with indicated concentrations of Ssd for 60 min, and then cells were treated with 20 ng/ml PMA plus 1 μM ionomycin at 37°C for 120 min. The nuclear extracts were prepared and analyzed by Western blotting technique using antibodies against NF-AT.

receptor of T lymphocytes. The results demonstrated that IL-2 receptor was found in a minimal expression in the control cells cultured with medium alone. However, stimulation of mouse T lymphocytes with PMA/Ionomycin or Con A resulted in significant expression of IL-2 receptor (Fig. 7C). More importantly, a significant suppression of IL-2 receptor expression was found in the activated T lymphocytes when they were treated with various concentrations of Ssd (Fig. 7C). These results coincide with our previous IL-2 study with PMA stimulation to the cells [Leung et al., 2005].

Ssd SUPPRESSES PRO-INFLAMMATORY CYTOKINE EXPRESSION IN ACTIVATED MOUSE T LYMPHOCYTES

Since IL-6, IFN-γ and TNF-α are the most potent pro-inflammatory cytokines expressed under regulation of NF-κB signaling during T cells activation [Karin and Greten, 2005], to determine the inhibitory effects of Ssd on the production of those pro-inflammatory cytokines, we pretreated mouse T lymphocytes with Ssd for 60 min and then stimulated the cells with PMA/Ionomycin or Con A for 24 h. ELISA was performed to measure the levels of IL-6, IFN-γ and TNF-α cytokines in the culture supernatants of the activated T lymphocytes (Fig. 8). Results showed that both IFN-γ and TNF-α were not detected in the culture medium of the control cells; while a tiny amount of IL-6 was detected in the medium of the control cells. Stimulation of mouse T lymphocytes with PMA/Ionomycin or Con A led to significant production and secretion of IL-6, IFN-γ and TNF-α cytokines. However, pretreatment of Ssd with various concentrations on the cells resulted in a significant decreased production of those cytokines in a dose-dependent manner.

DISCUSSION

Ssd is one of the saponin components of the medicinal herb, *B. falcatum* L., which has potent anti-inflammatory and immunomodulatory properties. Previous studies indicated that Ssd can modulate T cell functions as well as inhibit lymphocyte proliferation in response to T cell mitogens [Ushio and Abe, 1991]. However, the results of some reports demonstrated that Ssd at low concentrations had immune stimulatory properties in both peritoneal macrophages and splenic T lymphocytes [Ushio et al., 1991; Kato et al., 1994]. Interestingly, the same author, Ushio, further reported that Ssd, which itself has no mitogenic activity, could decrease spleen cell proliferation in response to T-cell mitogen stimulation [Ushio and Abe, 1991]. These controversial results could be explained by the cell type-oriented differential modulatory actions of Ssd [Kato et al., 1995]. Nevertheless, the underlying mechanisms by which Ssd affects T cell signaling pathways are not fully understood. Therefore, the current study aimed to demonstrate the underlying mechanisms by which Ssd manifests its suppressive effect on NF-κB, NF-AT and AP-1 activation. We know that the TCR signaling that activates NF-κB, NF-AT and AP-1 can be mimicked by a combinative stimulation of PMA plus the calcium ionophore, ionomycin [Sancho et al., 2004]. In the experiments, we used PMA, ionomycin and Con A as the T cell stimulators. The results clearly demonstrated that Ssd markedly suppressed the functions of PKC activator (PMA) mediated activation of IκBα phosphorylation, IκBα degradation, Akt phosphorylation, IKK activity, Akt-IKK association, p65 phosphorylation, p65 nuclear translocation and p65 acetylation, which in turn lead to the suppression of NF-κB p65 activation.

Previous works in our laboratory showed that Ssd could interfere with the membrane translocation of PKCθ and inhibit the phosphorylation of IκBα in PMA-stimulated T lymphocytes [Leung et al., 2005]. PKCθ is selectively expressed in T cells and skeletal muscles, a phenomenon that has been revealed in several studies to be an essential member of kinase in the NF-κB activation cascades of T cells. PKCθ exerts its activating effect on NF-κB by triggering IKK activity; it also stimulates the trans-activating function of NF-κB by physical and functional cooperation with Akt1 [Bauer et al., 2001]. In the current study, we further demonstrated that Ssd inhibited NF-κB p65 activation by suppressing IKKα/β activity. Moreover, we found that Ssd suppressed IKK complex phosphorylation-dependent activation (unpublished work). Furthermore, Akt has been shown to associate with IKK and activate IKK [Ozes et al., 1999]. Interestingly, we also demonstrated that Ssd suppressed PMA-induced Akt activation phosphorylation as well as Akt-IKK association. These results thus indicated that Ssd may inhibit IKK activation through suppression of Akt activation. Surprisingly, our new data suggest that Ssd does not directly inhibit the activity of IKK. Moreover, we

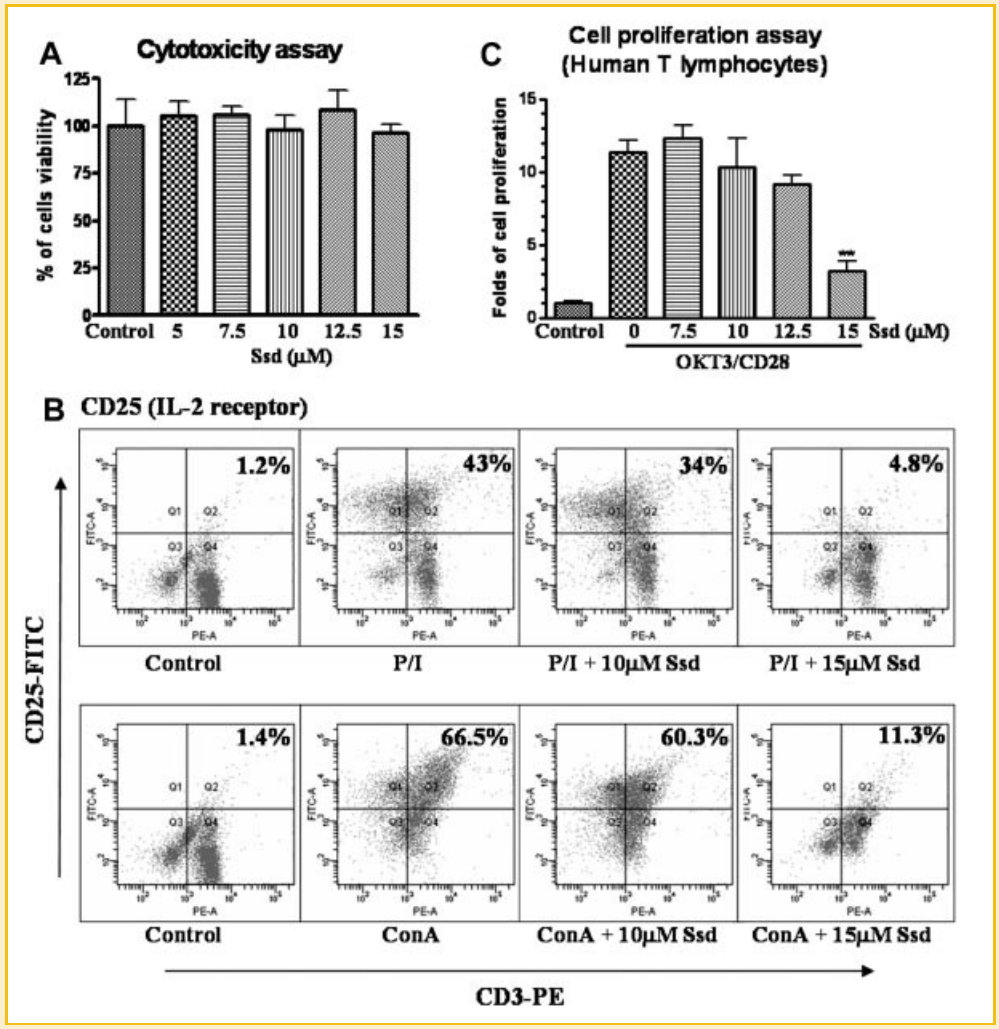

Fig. 7. A: Cytotoxicity assay of Ssd. Mouse T lymphocytes were incubated at 37°C with indicated concentrations of Ssd. After 48 h, the number of live cells was counted using Trypan blue exclusion method. B: Ssd inhibits anti-OKT-3/anti-CD28 costimulation of human T lymphocyte proliferation. Human T lymphocytes (10^5) were co-stimulated with immobilized anti-OKT-3 (5 μg/ml) and anti-CD28 (1 μg/ml) antibodies in the presence or absence of Ssd for 72 h, BrdU was added to the cells 14 h before the end of stimulation and the BrdU incorporation was measured using plate reader at 450 nm. Data in each column represents the mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$. C: Flow cytometry data in dot plot format showing IL-2 receptors (CD25) on CD3-PE T cells. Mouse T lymphocytes were pretreated with Ssd (10 and 15 μM) for 60 min first and then followed by stimulation with PMA (20 ng/ml) + ionomycin (2 μM) or Con A (5 μg/ml) for 40 h. Cells were double stained with (CD3-PE + CD25-FITC) antibodies and then analyzed by flow cytometry. The unstimulated cells served as control. Values represent percentage of double positive cells ($CD25^+$ gated into $CD3^+$ T lymphocyte population). Similar results were obtained in two independent experiments.

found that Ssd also suppressed the phosphorylation of p65. Because both IKK and Akt have been implicated in the phosphorylation of p65 [Sizemore et al., 1999, 2002], it seems feasible that Ssd could suppress p65 phosphorylation by inhibiting both Akt and IKK activation. Furthermore, the phosphorylation of p65 has been shown to be needed for its transcriptional activity [Zhong et al., 1998]. However, we found that Ssd had no direct effect on the binding of NF-κB to the DNA even at 100 μM concentration. Therefore, these results imply that Ssd suppresses the PMA-induced NF-κB signaling through the regulation of upstream signaling, such as the PKCθ-Akt signaling cascade, rather than by direct inhibition of NF-κB p65 DNA binding.

Several chemokines, interleukins and hematopoietic growth factors are regulated by NF-κB, NF-AT and AP-1 activation [Maggirwar et al., 1997; Oum et al., 2002; Gao et al., 2004]. Most anti-inflammatory compounds could suppress T cell activation via inhibition of those signaling pathways [Marquez et al., 2004; Sancho et al., 2004; Jhun et al., 2006; Yea et al., 2006; Park et al., 2007] and therefore they are used commonly for mechanistic studies of anti-inflammatory drugs as well as the new drug screening and discovery. In the NF-κB signaling pathway, the PKCθ-Akt activated IKK protein complex plays a critical role; it acts downstream of the early signaling events such as IκBα phosphorylation and degradation, ultimately, resulting in the release of NF-κB transcription factor. The NF-κB targeted genes encode cytokines and cytokine receptors, which promote further activation and proliferation of mature T lymphocytes [Su and Karin, 1996; Whitmarsh and Davis, 1996; Vockerodt et al., 2001]. In fact, our results demonstrated that

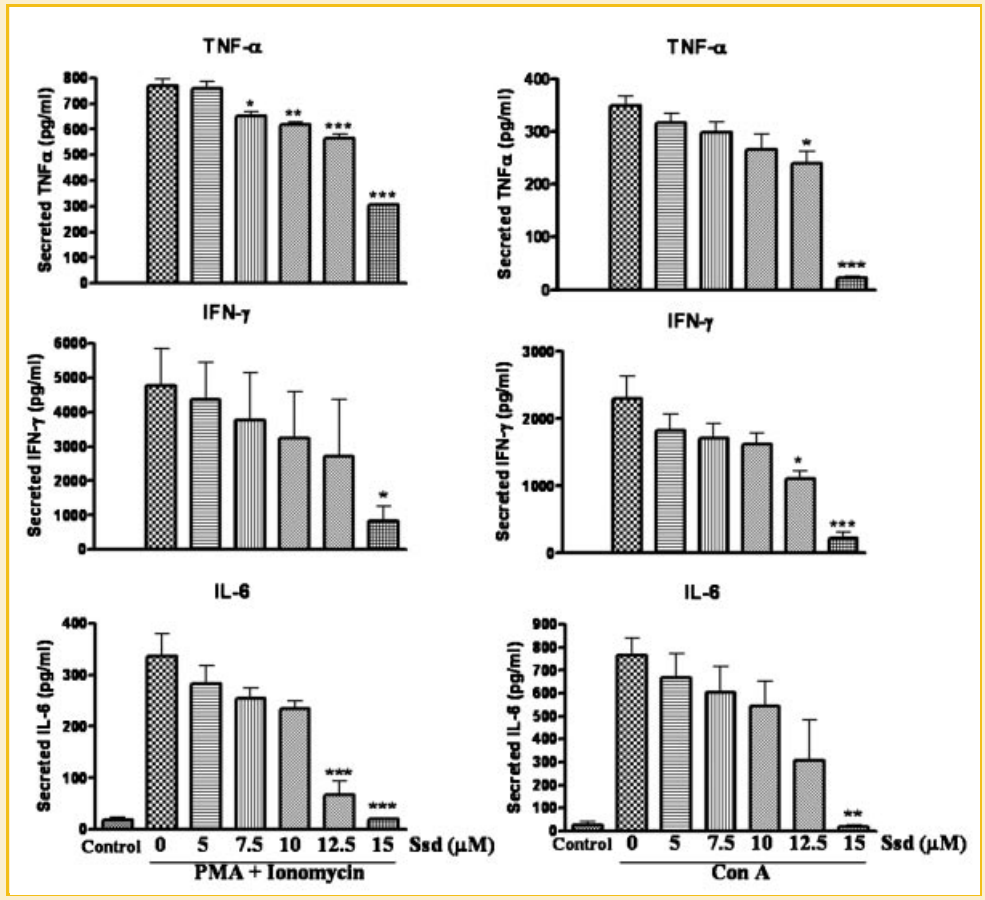

Fig. 8. Effect of Ssd on the production of pro-inflammatory cytokines in the activated mouse T cells. Mouse T lymphocytes were pretreated with Ssd (5–15 μM) for 60 min first and then followed by stimulation with PMA (20 ng/ml) + ionomycin (2 μM) or Con A (5 μg/ml) for 24 h. IL-6, TNF-α and IFN-γ concentrations in cell culture supernatants were determined by ELISA. Results are mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the levels of Con A or PMA/Ionomycin-treated cells without Ssd.

Ssd at non-cytotoxic concentrations not only inhibited OKT3/CD28 costimulation of human T lymphocytes proliferation, it also effectively suppressed the PMA/Ionomycin or Con A-induced NF-κB activation, which in turn suppressed IL-2 receptor expression and cytokine (IL-2, IL-6, TNF-α, and IFN-γ) production during T lymphocyte activation. This thereby explains the immunomodulatory and anti-inflammatory effects of Ssd.

In addition to NF-κB signaling suppression, we report here that Ssd inhibited the activation of NF-AT and AP-1 (c-Jun/c-Fos) transcription factors in mouse T lymphocytes, suggesting that Ssd may target a common element upstream of these transcription factors such as Cot/Tpl2 kinase by which the drug inhibits T cell activation. Cot/Tpl2 has been already demonstrated to participate in the signaling pathway that activates NF-κB, NF-AT and AP-1 transcription factors [Lin et al., 1999; Chiariello et al., 2000; de Gregorio et al., 2001]. In our previous studies it was showed that Ssd suppressed PMA-induced JNK activation but not ERK activation of T cells [Leung et al., 2005]. The current study has demonstrated that Ssd suppressed c-Jun and c-Fos nuclear expression. Therefore, we postulate that Ssd can down-regulate c-Jun/c-Fos expression via the inhibition of JNK and other members of the MAPK family like p38 [Tanos et al., 2005; Jhun et al., 2006]. It is needed in the future work to examine whether Ssd can suppress PMA/ionomycin-induced p38 activation.

Besides, it has been described that both NF-κB and NF-AT participate in COX-2 gene transcriptional regulation, the calcium/calcineurin pathway being essential for COX-2 transcription in T cells [Iniguez et al., 2000]. Over-expression of COX-2 is commonly induced under angiogenesis and it can be detected in the joints of rheumatoid arthritis patients [Woods et al., 2003]. Interestingly, there are two NF-AT binding sites identified in the COX-2 promoter region in T cells, and they are essential for COX-2 expression [Iniguez et al., 2000]. We have currently showed that NF-AT was also inhibited by Ssd, indicating it is likely that COX-2 gene inhibition mediated by Ssd [Recio et al., 1995; Bermejo Benito et al., 1998] is the consequence of the combined inhibitory effect of both NF-AT and NF-κB signaling pathways. In this sense, this COX-2 inhibitory activity of Ssd could explain its usage in treating cancers, rheumatoid arthritis and atherosclerosis. For further future study, it is worthwhile for us to analyze the ionomycin/calcium-dependent dephosphorylation of NF-AT in the presence of Ssd. If the dephosphorylation of NF-AT is inhibited by Ssd, the calmodulin/calcineurin complex must be directly affected by Ssd. Otherwise the nuclear import of NF-AT is inhibited by Ssd, which indicates that

other transcriptional factors such as c-Jun/c-Fos may be involved in the underlying molecular mechanisms by which Ssd influences T lymphocyte activation.

IL-2 plays a crucial role in the progression of antigen activated T cells from G_1 to G_2-M phase of the cell cycle [Gately et al., 1991; Nguyen et al., 2000]; while TNF-α and IFN-γ are required for the cytotoxic cells production and cytolysis [Gao et al., 2004]. Thus, the inhibition of T cell activation by Ssd may involve suppression of the production of these cytokines. Moreover, IL-2 and IFN-γ are Th1-type cytokines. They have been implicated in the pathogenesis of several immunological disorders, particularly the Th1-mediated diseases [Schulze-Koops and Kalden, 2001]. Therefore, the immunosuppressant effect of Ssd may have a potential use in the treatment of several autoimmune diseases associated with an increase in IFN-γ such as multiple sclerosis, rheumatoid arthritis, psoriasis and autoimmune diabetes [Skurkovich and Skurkovich, 2003]. In addition, the inflammatory cytokines IL-6 and TNF-α can promote tumor growth. Evidence shows that IL-6 and TNF-α can be produced by tumor-associated inflammatory cells, and that this production promotes the occurrence and growth of tumors; while down-regulation of these cytokines may prevent both inflammation and tumor initiation [Karin and Greten, 2005]. Therefore, the pro-inflammatory stimuli like TNF-α will be used to activate cells in order to further elucidate the anti-inflammatory mechanism of Ssd in our future study.

In conclusion, our studies demonstrated that Ssd not only inhibited OKT3/CD28 costimulation of human T lymphocyte proliferation, it also suppressed PMA, PMA/Ionomycin and Con A-induced NF-κB, NF-AT and AP-1 signaling and inhibited PMA/Ionomycin and mitogen-induced lymphocyte activation and production of pro-inflammatory cytokines and their related receptors. Therefore, we conclude that NF-κB, NF-AT and AP-1 signaling pathways are strongly involved in suppression of T cell activation evoked by Ssd treatment. These findings herald a novel approach for further studies of Ssd as a potential agent in treating inflammatory and autoimmune conditions.

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REFERENCES

Aggarwal BB. 2004. Nuclear factor-kappaB: The enemy within. Cancer Cell 6:203–208.

Bauer B, Krumbock N, Fresser F, Hochholdinger F, Spitaler M, Simm A, Uberall F, Schraven B, Baier G. 2001. Complex formation and cooperation of protein kinase C theta and Akt1/protein kinase B alpha in the NF-kappa B transactivation cascade in Jurkat T cells. J Biol Chem 276:31627–31634.

Belardelli F. 1995. Role of interferons and other cytokines in the regulation of the immune response. APMIS 103:161–179.

Bermejo Benito P, Abad Martinez MJ, Silvan Sen AM, Sanz Gomez A, Fernandez Matellano L, Sanchez Contreras S, Diaz Lanza AM. 1998. In vivo and in vitro antiinflammatory activity of saikosaponins. Life Sci 63:1147–1156.

Chiariello M, Marinissen MJ, Gutkind JS. 2000. Multiple mitogen-activated protein kinase signaling pathways connect the cot oncoprotein to the c-jun promoter and to cellular transformation. Mol Cell Biol 20:1747–1758.

Coudronniere N, Villalba M, Englund N, Altman A. 2000. NF-kappa B activation induced by T cell receptor/CD28 costimulation is mediated by protein kinase C-theta. Proc Natl Acad Sci USA 97:3394–3399.

Crabtree GR, Clipstone NA. 1994. Signal transmission between the plasma membrane and nucleus of T lymphocytes. Annu Rev Biochem 63:1045–1083.

de Gregorio R, Iniguez MA, Fresno M, Alemany S. 2001. Cot kinase induces cyclooxygenase-2 expression in T cells through activation of the nuclear factor of activated T cells. J Biol Chem 276:27003–27009.

Gao X, Kuo J, Jiang H, Deeb D, Liu Y, Divine G, Chapman RA, Dulchavsky SA, Gautam SC. 2004. Immunomodulatory activity of curcumin: Suppression of lymphocyte proliferation, development of cell-mediated cytotoxicity, and cytokine production in vitro. Biochem Pharmacol 68:51–61.

Gately MK, Desai BB, Wolitzky AG, Quinn PM, Dwyer CM, Podlaski FJ, Familletti PC, Sinigaglia F, Chizonnite R, Gubler U. 1991. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). J Immunol 147:874–882.

Ghosh S, Karin M. 2002. Missing pieces in the NF-kappaB puzzle. Cell 109(Suppl):S81–S96.

Hacker H, Karin M. 2006. Regulation and function of IKK and IKK-related kinases. Sci STKE 357:re13.

Iniguez MA, Martinez-Martinez S, Punzon C, Redondo JM, Fresno M. 2000. An essential role of the nuclear factor of activated T cells in the regulation of the expression of the cyclooxygenase-2 gene in human T lymphocytes. J Biol Chem 275:23627–23635.

Jhun BS, Lee JY, Oh YT, Lee JH, Choe W, Baik HH, Kim SS, Yoon KS, Ha J, Kang I. 2006. Inhibition of AMP-activated protein kinase suppresses IL-2 expression through down-regulation of NF-AT and AP-1 activation in Jurkat T cells. Biochem Biophys Res Commun 351:986–992.

Karin M, Greten FR. 2005. NF-kappaB: Linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 5:749–759.

Kato M, Pu MY, Isobe K, Iwamoto T, Nagase F, Lwin T, Zhang YH, Hattori T, Yanagita N, Nakashima I. 1994. Characterization of the immunoregulatory action of saikosaponin-d. Cell Immunol 159:15–25.

Kato M, Pu MY, Isobe K, Hattori T, Yanagita N, Nakashima I. 1995. Cell type-oriented differential modulatory actions of saikosaponin-d on growth responses and DNA fragmentation of lymphocytes triggered by receptor-mediated and receptor-bypassed pathways. Immunopharmacology 29:207–213.

Kiernan R, Bres V, Ng RW, Coudart MP, El Messaoudi S, Sardet C, Jin DY, Emiliani S, Benkirane M. 2003. Post-activation turn-off of NF-kappa B-dependent transcription is regulated by acetylation of p65. J Biol Chem 278:2758–2766.

Kunsch C, Lang RK, Rosen CA, Shannon MF. 1994. Synergistic transcriptional activation of the IL-8 gene by NF-kappa B p65 (RelA) and NF-IL-6. J Immunol 153:153–164.

Leung CY, Liu L, Wong RN, Zeng YY, Li M, Zhou H. 2005. Saikosaponin-d inhibits T cell activation through the modulation of PKCtheta, JNK, and NF-kappaB transcription factor. Biochem Biophys Res Commun 338:1920–1927.

Lin X, Cunningham ET, Jr., Mu Y, Geleziunas R, Greene WC. 1999. The proto-oncogene Cot kinase participates in CD3/CD28 induction of NF-kappaB acting through the NF-kappaB-inducing kinase and IkappaB kinases. Immunity 10:271–280.

Lin X, O'Mahony A, Mu Y, Geleziunas R, Greene WC. 2000. Protein kinase C-theta participates in NF-kappaB activation induced by CD3-CD28 costimulation through selective activation of IkappaB kinase beta. Mol Cell Biol 20:2933–2940.

Macian F, Lopez-Rodriguez C, Rao A. 2001. Partners in transcription: NFAT and AP-1. Oncogene 20:2476–2489.

Maggirwar SB, Harhaj EW, Sun SC. 1997. Regulation of the interleukin-2 CD28-responsive element by NF-ATp and various NF-kappaB/Rel transcription factors. Mol Cell Biol 17:2605–2614.

Malnou CE, Salem T, Brockly F, Wodrich H, Piechaczyk M, Jariel-Encontre I. 2007. Heterodimerization with Jun family members regulates c-Fos nucleocytoplasmic traffic. J Biol Chem 282:31046–31059.

Marquez N, Sancho R, Macho A, Calzado MA, Fiebich BL, Munoz E. 2004. Caffeic acid phenethyl ester inhibits T-cell activation by targeting both nuclear factor of activated T-cells and NF-kappaB transcription factors. J Pharmacol Exp Ther 308:993–1001.

Matsumoto R, Wang D, Blonska M, Li H, Kobayashi M, Pappu B, Chen Y, Lin X. 2005. Phosphorylation of CARMA1 plays a critical role in T Cell receptor-mediated NF-kappaB activation. Immunity 23:575–585.

Nguyen T, Wang R, Russell JH. 2000. IL-12 enhances IL-2 function by inducing CD25 expression through a p38 mitogen-activated protein kinase pathway. Eur J Immunol 30:1445–1452.

Oum JH, Han J, Myung H, Hleb M, Sharma S, Park J. 2002. Molecular mechanism of NFAT family proteins for differential regulation of the IL-2 and TNF-alpha promoters. Mol Cells 13:77–84.

Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 401:82–85.

Park KR, Lee JH, Choi C, Liu KH, Seog DH, Kim YH, Kim DE, Yun CH, Yea SS. 2007. Suppression of interleukin-2 gene expression by isoeugenol is mediated through down-regulation of NF-AT and NF-kappaB. Int Immunopharmacol 7:1251–1258.

Recio MC, Just MJ, Giner RM, Manez S, Rios JL, Hostettmann K. 1995. Anti-inflammatory activity of saikosaponins from Heteromorpha trifoliata. J Nat Prod 58:140–144.

Sancho R, Macho A, de La Vega L, Calzado MA, Fiebich BL, Appendino G, Munoz E. 2004. Immunosuppressive activity of endovanilloids: N-arachidonoyl-dopamine inhibits activation of the NF-kappa B, NFAT, and activator protein 1 signaling pathways. J Immunol 172:2341–2351.

Schulze-Koops H, Kalden JR. 2001. The balance of Th1/Th2 cytokines in rheumatoid arthritis. Best Pract Res Clin Rheumatol 15:677–691.

Shaw JP, Utz PJ, Durand DB, Toole JJ, Emmel EA, Crabtree GR. 1988. Identification of a putative regulator of early T cell activation genes. Science 241:202–205.

Sizemore N, Leung S, Stark GR. 1999. Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. Mol Cell Biol 19:4798–4805.

Sizemore N, Lerner N, Dombrowski N, Sakurai H, Stark GR. 2002. Distinct roles of the Ikappa B kinase alpha and beta subunits in liberating nuclear factor kappa B (NF-kappa B) from Ikappa B and in phosphorylating the p65 subunit of NF-kappa B. J Biol Chem 277:3863–3869.

Skurkovich B, Skurkovich S. 2003. Anti-interferon-gamma antibodies in the treatment of autoimmune diseases. Curr Opin Mol Ther 5:52–57.

Smith KA. 1988. Interleukin-2: Inception, impact, and implications. Science 240:1169–1176.

Su B, Karin M. 1996. Mitogen-activated protein kinase cascades and regulation of gene expression. Curr Opin Immunol 8:402–411.

Tanos T, Marinissen MJ, Leskow FC, Hochbaum D, Martinetto H, Gutkind JS, Coso OA. 2005. Phosphorylation of c-Fos by members of the p38 MAPK family. Role in the AP-1 response to UV light. J Biol Chem 280:18842–18852.

Ushio Y, Abe H. 1991. The effects of saikosaponin on macrophage functions and lymphocyte proliferation. Planta Med 57:511–514.

Ushio Y, Oda Y, Abe H. 1991. Effect of saikosaponin on the immune responses in mice. Int J Immunopharmacol 13:501–508.

Vockerodt M, Tesch H, Kube D. 2001. Epstein-Barr virus latent membrane protein-1 activates CD25 expression in lymphoma cells involving the NFkappaB pathway. Genes Immun 2:433–441.

Wang YL, He SX, Luo JY. 2006. [Progress in research on antitumor activity of saikosaponin and its mechanism]. Zhong Xi Yi Jie He Xue Bao 4:98–100.

Whitmarsh AJ, Davis RJ. 1996. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J Mol Med 74:589–607.

Woods JM, Mogollon A, Amin MA, Martinez RJ, Koch AE. 2003. The role of COX-2 in angiogenesis and rheumatoid arthritis. Exp Mol Pathol 74:282–290.

Xiao BG, Link H. 1999. Antigen-specific T cells in autoimmune diseases with a focus on multiple sclerosis and experimental allergic encephalomyelitis. Cell Mol Life Sci 56:5–21.

Yea SS, Jeong HS, Choi CY, Park KR, Oh S, Shin JG, Yun CH. 2006. Inhibitory effect of anethole on T-lymphocyte proliferation and interleukin-2 production through down-regulation of the NF-AT and AP-1. Toxicol In Vitro 20:1098–1105.

Zhong H, Voll RE, Ghosh S. 1998. Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol Cell 1:661–671.