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6-Methoxyflavone Inhibits NFAT Translocation into the Nucleus and Suppresses T Cell Activation

Jae-Seon So,*†,1 Gi-Cheon Kim,*†,1 Minkyung Song,*†,1 Choong-Gu Lee,‡ Eunbee Park,*† Ho Jin Kim,§ Young Sup Kim,‖ Chang-Duk Jun,* and Sin-Hyeog Im‡,#

NFAT plays a crucial role in the immune system by regulating the transcription of inducible genes during immune responses. In T cells, NFAT proteins govern various cellular events related to T cell development, activation, tolerance induction, and differentiation. We previously reported the NFAT1-dependent enhancer activity of conserved noncoding sequence (CNS)-9, a distal cis-acting element, in the regulation of IL-10 transcription in T cells. In this study, we developed a T cell–based reporter system to identify compounds that modulate the regulatory activity of CNS-9. Among the identified candidates, 6-methoxyflavone (6-MF) significantly inhibited the enhancer activity of CNS-9, thereby reducing IL-10 expression in T cells without affecting cell viability.

6-MF also downregulated the transcription of NFAT1 target genes such as IL-4, IL-13, and IFN-γ. Treatment of 6-MF inhibited the translocation of NFAT1 into the nucleus, which consequently interrupted NFAT1 binding to the target loci, without affecting the expression or dephosphorylation of NFAT1. Treatment of 6-MF to CD4+ T cells or B cells isolated from mice with atopic dermatitis significantly reduced disease-associated cytokine production, as well as the levels of IgE. In addition, oral administration of 6-MF to atopic dermatitis mice ameliorated disease symptoms by reducing serum IgE levels and infiltrating lymphocytes. Conclusively, our results suggest that 6-MF can be a potential candidate for the development of an effective immunomodulator via the suppression of NFAT-mediated T cell activation. The Journal of Immunology, 2014, 193: 2772–2783.

Nuclear factor of activated T cells is a transcription factor expressed in most immune cells that plays a critical role in inducible gene transcription during immune responses (1). The NFAT family consists of five members, as follows: NFAT1 (NFATc1, NFATc2), NFAT2 (NFATc, NFATc1), NFAT3 (NFATc4), NFAT4 (NFATx, NFATc3), and NFAT5 (TonEBP) (2). All of the members contain a highly conserved DNA-binding domain (2). The activation of NFAT1–4 is regulated by calcium and the calcineurin-signaling pathway, whereas NFAT5 is responsive to hypertonic stress (3). The engagement of TCRs triggers calcium release from intracellular stores and calcium influx across the plasma membrane and activates the calmodulin-dependent serine/threonine phosphatase, calcineurin (4). Dephosphorylation of NFAT proteins by calcineurin induces the rapid translocation of NFAT into the nucleus, which leads to increased transcription of NFAT target genes (5).

In T cells, NFAT proteins govern various cellular events by controlling gene expression related to T cell development, activation, tolerance induction, and differentiation (6, 7). NFAT1, -2, and -4 are present in T cells, and individual or combinatory NFAT-deficient mice showed alteration in T cell responses, Th1/Th2 cell differentiation, and cytokine expression profiles (8–11). In addition, the phenotype of SCID patients, which is characterized by a defect in the T cell immune response, including multiple cytokine production, correlates with the impairment of NFAT activation and relatively short duration of residing NFAT in the nucleus (12, 13). The importance of NFAT in the immune system was strengthened by several studies showing the regulatory activity of NFAT in the transcription of cytokine genes, such as IL-2, IFN-γ, and IL-4, which were induced during T cell lineage commitment following antigenic stimulation (14–16). Cyclosporin A (CsA) or FK506, an inhibitor of calcineurin, has been used as immunosuppressant in autoimmune diseases and transplantation rejection, because of their ability to interrupt the calcium-mediated NFAT signaling cascade (17, 18). However, this treatment has shown a number of side effects, such as nephro- and neurotoxicities, thus limiting their therapeutic use (19). Therefore, many attempts have been made to develop new drugs from natural compounds that can target NFAT function with less toxicity.

Flavonoids are phenolic compounds composed of a three-ring structure with various substitutions and are found ubiquitously in fruits and vegetables (20). Several recent studies have shown that flavonoids have a variety of biological and pharmacological activities such as antioxidantive, antiviral, anti-inflammatory, anticancer, and cardiovascular protective effects (21, 22). Some flavonoid derivatives also have regulatory activity in immune responses, such as modulation of cell-signaling proteins, including
kinases and transcription factors (23, 24). Therefore, flavonoids have been considered as potential target compounds for the development of new therapeutic agents. However, little information is available concerning the exact molecular mechanism(s) underlying the regulatory effects of each flavonoid derivative.

Previously, we elucidated the role of enhancer elements in the regulation of IL-10 transcription in T cells. A distal cis-acting element, conserved noncoding sequence (CNS)-9, showed an enhancer activity and the recruitment of NFAT1 and IFN regulatory factor (IRF)4 to the site that synergistically increased IL-10 transcription in Th2 cells (25). Based on this result, we developed a T cell–based reporter assay system to screen compounds that can modulate the regulatory activity of CNS-9. We identified that 6-methoxyflavone (6-MF) inhibits the enhancer activity of CNS-9, resulting in the downregulation of IL-10 expression in T cells. This inhibitory effect of 6-MF is due to the inhibition of NFAT1 translocation into the nucleus that consequently interrupts the binding of NFAT1 to its target loci. Treatment of 6-MF also downregulated cytokine production by activated T cells in an atopic dermatitis (AD) mouse model. In addition, oral administration of 6-MF ameliorated AD disease symptoms. Conclusively, our results suggest that 6-MF could have effective immunomodulatory activity by suppressing NFAT-mediated T cell activation.

Materials and Methods

Animals

C57Bl/6 and BALB/c mice were purchased from SLC (Shizuoka, Japan). Mice were housed in a specific pathogen-free barrier facility and were used in accordance with protocols approved by the Animal Care and Ethics Committee of Gwangju Institute of Science and Technology.

Vectors, reagents, and Abs

pGL4.17 firefly and pRL-TK Renilla luciferase vectors were purchased from Promega. Expression vectors for NFAT1 and constitutively active NFAT1 (CA-NFAT1) were provided by Dr. Anjana Rao (La Jolla Institute for Allergy & Immunology). The following reagents were used: PMA and ionomycin from Calbiochem; DMSO, 6-MF, and IL-12 from Sigma-Aldrich; human IL-2 from the National Cancer Institute, Preclinical Repository; and murine IL-4 from PeproTech.

The following Abs were used: anti-CD3 (145.2C11), anti-CD28 (37.51), anti-IFN-γ (XMG1.2), anti-IL-12 (C17.8), FITC anti-IL-10 (JES5-16E3), and FITC anti-IL-4 (11B11) from BD Pharmingen; anti–IL-4 (11B11) from the National Cancer Institute, Preclinical Repository; and anti-IRF4 from Santa Cruz Biotechnology; anti-NFAT1 (ab2722), anti-NFAT2 (ab2796), and anti-β-actin (ab3280) from Abcam (Cambridge, U.K.); anti-lamin B from Cell Signaling; and anti–NFAT1-phospho Ser24 from GenTex.

Computational analysis of the IL-10 locus

Genomic sequences spanning 120 kb of the IL-10 gene were analyzed using web-based alignment software, VISTA browser 2.0 (http://pipeline.lbl.gov/cgi-bingateway2), to identify CNS.

Development of stable cell line and screening of natural compounds library

Reporter construct was generated by cloning the regulatory element of the IL-10 locus into the pGL4.17 vector, which contains luciferase and neomycin-resistant gene. EL4 cells were transfected using Lipofectamine 2000 (Invitrogen) or electroporated using culture media containing 900 µg/ml plate-bound anti-CD3ε and 2 µg/ml soluble anti-CD28 for 24 h under Th1-skewing (10 ng/ml IL-12 plus 10 µg/ml anti–IL-4) or Th2-skewing (10 ng/ml IL-4, 10 µg/ml anti–IFN-γ plus 10 µg/ml anti–IL-12) conditions. Then cells were expanded in complete medium containing 100 U/ml human rIL-2 for 7 d. On day 7, differentiated T cells were stimulated with PMA plus ionomycin in the presence or absence of chemicals. For T cell blast preparation, the CD4+ T cells (1 × 10^6) were stimulated with 2 µg/ml plate-bound anti-CD3ε and 2 µg/ml soluble anti-CD28 for 24 h supplemented with 100 U/ml human rIL-2 and expanded with IL-2 for 5 d.

Quantitative real-time PCR analysis

Total RNA was extracted using TRIzol reagent (Molecular Research Center) in accordance with the manufacturer’s protocol. cDNA was synthesized in 20 µl mixture containing 1 µg RNA using the ImProm-II reverse transcription system (Promega). Quantitative real-time PCR (qRTPCR) was carried out using cDNA, SYBR Premix Ex Taq (Takara, Shiga, Japan), primers shown in Table I, and DNA Engine with a Chromo-4 Detector (MJ Research). The data were normalized using the expression level of hypoxanthine-guanine phosphoribosyl transferase.

Flow cytometric analysis

T cells were stimulated for 24 h, and 1 µg/ml brefeldin A (Sigma-Aldrich) was added 14 h before the end of the culture. Cells were washed twice with permeabilization buffer containing 0.5% saponin and 1% BSA. Then cells were incubated with Ab for intracellular cytokine staining at 4°C for 20 min. Cells were washed and resuspended in PBS. At least 20,000 events were analyzed using EPICS XL and EXPO32 software (Beckman Coulter).

Preparation of cell lysates and nuclear extract

For total lysate preparation, Th2 cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, and 1% Nonidet P40) containing protease inhibitors mixture (Roche) and were incubated for 30 min on ice. For nuclear extraction, Th2 cells were stimulated for 1 h, and nuclear extracts were prepared, as previously described (26). Briefly, T cells were washed twice with ice-cold PBS and were resuspended in 500 µl cold buffer A containing protease inhibitor mixture (Roche, Mannheim, Germany) and 0.4% Nonidet P40. The cells were allowed to swell by incubation on ice for 15 min, and then homogenate was centrifuged for 5 min. The nuclear pellet was resuspended in 150 µl cold buffer B. The tube was thoroughly mixed and was placed on a rotatory shaker for 2 h. The nuclear extracts were centrifuged for 5 min. The supernatant containing nuclear proteins was used for experiments. Cytoplasmic and nuclear extract were prepared by NE-PER nuclear and cytoplasmic extraction reagents (Thermo), according to the manufacturer’s protocol.

Immunoblotting

Total lysates or nuclear extracts (20–40 µg) of T cells were subjected to SDS-PAGE and were electrotransferred onto nitrocellulose membranes for immunoblot analysis. Each membrane was incubated with Abs against NFAT1, NFAT2, NFAT1-phospho Ser24, β-actin, or lamin B. Blots were developed using HRP-conjugated secondary Ab (Sigma-Aldrich) and the ECL western blot detection kit (Amersham Pharmacia Biotech).Intensity of individual bands was quantified using ImageJ densitometry software.

Immunocytchemistry

Round glasses were located in a 12-well plate and were coated with anti-CD3 (1 µg/ml) or poly-L-lysine (Sigma-Aldrich) for 12 h at 4°C. Glass was washed with PBS for three times, and then 1 × 10^5 T cells were transfected onto the glass. Cells were transfected with anti-CD28 (2 µg/ml) in the using Hyposmolar Electroporation Buffer (Eppendorf, Hamburg, Germany) with a Multiporator system, in accordance with the manufacturer’s protocol. In the reporter assay, pXPG vector was cotransfected with pRL-TK Renilla vector. After 12 h of plating, cells were stimulated with PMA plus ionomycin in the presence or absence of chemicals for 24 h, and then reporter activity was measured using the dual luciferase assay system (Promega). Firefly luciferase activity was normalized by the activity of Renilla luciferase.

Cell lines and T cell differentiation

EL4 mouse lymphoma and HEK 293T cells were purchased from Korean Cell Line Bank (Seoul, Korea) and Invitrogen, respectively. T cells were purified from the lymph nodes and spleen of 8- to 10-wk-old C57BL/6 mice using CD4+ T cell isolation magnetic beads (LS74; Milteny Biotech). T cells were cultured in RPMI 1640 medium (Welgene) supplemented with 10% FBS, g-glutamine, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, HEPES, and 2-ME. For T cell differentiation, the cells (5 × 10^6) were stimulated with 1 µg/ml plate-bound anti-CD3ε and 2 µg/ml soluble anti-CD28 for 24 h under Th1-skewing (10 ng/ml IL-12 plus 10 µg/ml anti–IL-4) or Th2-skewing (10 ng/ml IL-4, 10 µg/ml anti–IFN-γ plus 10 µg/ml anti–IL-12) conditions. Then cells were expanded in complete medium containing 100 U/ml human rIL-2 for 7 d. On day 7, differentiated T cells were stimulated with PMA plus ionomycin in the presence or absence of chemicals. For T cell blast preparation, the CD4+ T cells (1 × 10^6) were stimulated with 2 µg/ml plate-bound anti-CD3ε and 2 µg/ml soluble anti-CD28 for 24 h supplemented with 100 U/ml human rIL-2 and expanded with IL-2 for 5 d.
presence or absence of 6-MF and were fixed by incubation with ice-cold 100% methanol for 10–15 min at room temperature. After washing with PBS (0.25% Triton X-100), cells were blocked with PBST (PBS with 0.1% Tween 20 and 3% BSA) for 30 min at 37°C. Cells were incubated with anti-NFAT1 Ab (Abcam, Cambridge, U.K.) for 12 h at 4°C and then stained with FITC- or tetramethylrhodamine isothiocyanate–conjugated anti-mouse IgG Ab. For counterstaining, cells were incubated with 2% SSC solution (0.3 M NaOH, 0.03 M sodium citrate [pH 7.0]) containing 20 μg/ml RNase, and then nuclei were stained with propidium iodide or DAPI (Invitrogen). Stained cells were examined using a FV1000 confocal microscope (Olympus), and images were processed using Olympus FLUOVIEW software.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation (ChIP) analysis was carried out as previously described with minor modifications (27). Briefly, Th2 cells were stimulated for 1 h and were then cross-linked using 2% formaldehyde. After incubation in lysis buffer, cell lysates were sonicated using Bioruptor (Diagenode, Liege, Belgium) to fragment the DNA. The sheared chromatin was immunoprecipitated using Abs against NFAT1 and rabbit IgG. Complexes of DNA and Abs were precipitated using protein G agarose (Millipore) and then reverse-cross-linked. The presence of selected DNA sequences was assessed using real-time PCR with primers described in Table III. PCR was done on input DNA purified before immunoprecipitation. Data are presented as the amount of DNA recovered relative to the input control. Result of ChIP with isotype IgG was confirmed as a background value and showed <0.001 relative ratio to input.

EMSA
EMSA was performed as previously described with minor modifications (28). Double-stranded probes were labeled with γ-32P-ATP (PerkinElmer) by T4 polynucleotide kinase (NEB) and then purified by G-50 micro column (GE HealthCare). Nuclear extracts of T cells (10 μg) were incubated with 0.1 μg poly(dI-dC) in the binding buffer for 30 min and were subsequently mixed with radiolabeled probes for 30 min. Probe sequences are described in Table III. Unlabeled oligomers used as competitors were added to the mixtures 30 min before probe incubation. For supershift assay, nuclear extracts were preincubated with Abs against target protein. Samples were subjected to 6% nondenaturing PAGE in 0.5× Tris-borate-EDTA buffer.

Induction of experimental AD
Ear surface of BALB/c mice was stripped five times with surgical tape (Nichiban) and was painted with 20 μl 4% 2,4-dinitrochlorobenzene (Sigma-Aldrich) dissolved in acetone/olive oil solution (acetone:olive oil = 1:3) for sensitization. After 3 d, mice ears were challenged with 20 μl 2% dinitrochlorobenzene and 20 μl dust mite extracts (10 mg/ml; Yonsei University) dissolved in PBS containing 0.5% Tween 20 once per week for 6 wk (29). AD mice were treated by oral gavage with DMSO (control), CsA (5 mg/kg/day; Sandimmune, Novartis, NY), or 6-MF (20 or 100 mg/kg/day) five times per week for 6 wk. Ten microliters of each reagent (DMSO, 5 mg/kg/day; CsA, 20 mg/kg/day; 6-MF, 100 mg/kg/day) were individually mixed with 1% hydroxypropyl methylcellulose (Sigma-Aldrich) for drug delivery.

Proliferation assay
CD4+ T cells (2 × 105 cells/well) were stimulated and cultured for 72 h in flat-bottom 96-well plates. After 56-h incubation, 0.5 μCi [3H]thymidine (NEN) was added to each well, and the cells were labeled for additional 16 μCi [3H]thymidine (NEN) was added to each well, and the cells were labeled for additional 16 h. Labeled cells were collected onto glass filters and were counted using a liquid scintillation counter (Beckman). The degree of proliferation was presented as the stimulation index, which was calculated by dividing the cpm of samples with that of unstimulated cells.

Measurement of IgE levels
Serum was obtained from mice 2 and 4 wk after AD induction. Concentration of IgE was measured using 200-fold diluted serum and IgE ELISA kit (BD Biosciences) in accordance with the manufacturer’s protocol. To analyze IgE levels in culture medium, 3 × 105 CD19+ B cells were stimulated with LPS (10 μg/ml) and IL-4 (20 ng/ml) in the presence or absence of 6-MF for 72 h.

Histological evaluation
Excised ears of each group were fixed in 4% paraformaldehyde for 16 h and were embedded in paraffin wax. Six-micrometer sections were stained with H&E (Sigma-Aldrich) and were observed by microscope.

Statistical analysis
All data are shown as mean values ± SD. Statistical analyses were performed using Student t test in different groups, and p values <0.05 were considered to be significant.

Results
6-MF inhibits enhancer activity of CNS-9
We previously reported a crucial role of distal cis-regulatory element, CNS-9, in IL-10 gene transcription (25). Recruitment of NFAT1 and IRF4 to the CNS-9 effectively increased the activity of IL-10 minimal promoter (25). In this study, we established a T cell–based IL-10 gene reporter assay system to identify compounds that have modulatory effects on IL-10 expression by altering the enhancer function of CNS-9. CNS-9 and minimal promoter region of IL-10 were cloned into the upstream of the luciferase gene in pGL4.17 reporter vector (Fig 1A, 1B). After transfection of EL4 cells with this reporter construct, stable EL4 clones that continuously express luciferase under the control of CNS-9 were identified. Establishment of stable cell line was confirmed by the presence of neomycin-resistant gene, luciferase gene, and CNS-9 element in the genomic DNA extracted from each clone (Fig. 1C). To evaluate whether the stable cell line appropriately responds to stimuli, we examined luciferase activity after stimulation with PMA and ionomycin in the presence or absence of CsA. Luciferase activity was increased by ∼3- to 5-fold upon PMA and ionomycin stimulation; however, this increase was disrupted by CsA (Fig. 1D). Next, we assessed optimal cell number for screening in 96-well plate and selected 6 × 103 cells/well that showed the highest ratio of luciferase activity upon stimulation as compared with that of unstimulated cells (Supplemental Fig. 1A, 1B).

Next, we screened chemical libraries of single compounds derived from natural sources that contain derivatives of several structural analogs of compounds, such as flavone, stilbene, and terpene (Supplemental Fig. 1C). The luciferase activity was measured after 4 h of chemical treatment (Supplemental Fig. 1D). All the screenings were repeated three times, and the compounds that showed reproducibility were primarily selected. Among these compounds, we selected four, namely 6-MF, 7-hydroxyflavone, baicalein, and foronemonetin. These four compounds showed >50% reduction of luciferase activity of stable cell line (Fig 2A). Interestingly, all compounds are flavone derivatives that have flavonoid backbone with ∼250 kDa. The chemical information and structures of the compounds are described in Fig. 2B, 2C, respectively. Then we tested cytotoxicity of these compounds to rule out the possibility that their inhibitory effect is derived from the inhibition of cell viability. None of the selected compounds induced any significant change in the viability of EL4 cells and primary CD4+ T cells at the tested concentration (20 μM) (Fig. 2D, 2E). Among the candidates, we have focused on the activity of 6-MF because it showed the strongest effect in suppressing CNS-9–mediated luciferase activity (Fig. 2A).

6-MF inhibits cytokine expression by activated T cells
We examined whether 6-MF treatment could suppress IL-10 expression in T cells, using the primers shown in Table I. Compared with DMSO-treated EL4 cells, treatment of 6-MF significantly suppressed the stimulation-dependent increase of IL-10 transcription (Fig. 3A, left panel). Furthermore, 6-MF treatment led to almost 50% decrease of IL-10 mRNA level in primary Th2 cells differentiated in vitro (Fig. 3A, right panel). Intracellular IL-10 analysis using flow cytometry confirmed that 6-MF treatment significantly reduced IL-10 production from Th2 cells by up to 60% (Fig. 3B). These results indicate that 6-MF has an inhibitory effect on IL-10 expression by suppressing the enhancer activity of CNS-9.
To examine whether inhibitory effect of 6-MF is restricted to IL-10 regulation, we analyzed the expression of other cytokines in T cells. Similar extent of inhibition was observed on IL-4 and IL-13 expression in 6-MF–treated Th2 cells (Fig. 3C, 3D). Interestingly, 6-MF also reduced IFN-γ transcription in Th1 cells (Fig. 3C, right panel). These results suggest that 6-MF acts as a negative regulator of cytokine expressions in T cells.

6-MF suppresses nuclear translocation of NFAT1 in T cells

To investigate how 6-MF downregulates the transcription of several cytokines in T cells, we analyzed the changes in the expression and activation of transcription factors. As we previously demonstrated, NFAT1 and IRF4 are crucial factors for the enhancer activity of CNS-9 (25). Moreover, NFAT1 is one of the key transcription factors in the regulation of multiple cytokine expression, such as IL-2, IL-4, IL-10, and IFN-γ, upon stimulation (25). First, we analyzed the effects of 6-MF on NFAT1-mediated transactivation using CNS-9 reporter system. As expected, NFAT1 overexpression significantly increased luciferase activity in EL4 cells (Fig. 4A). However, 6-MF treatment significantly suppressed the trans-activity of NFAT1 in a dose-dependent manner, thereby decreasing CNS-9 activity to the basal level (Fig. 4A). Interestingly, treatment of 6-MF also reduced IRF4-mediated luciferase activity (Supplemental Fig. 2A). These results suggest that 6-MF may exert its inhibitory effects by modulating the activity or expression levels of these transcription factors. The transcript and protein level of NFAT1 or IRF4 were measured in 6-MF–treated Th2 cells. The transcript level of NFAT1 or IRF4 was maintained in similar level regardless of 6-MF treatment (Fig. 4B, Supplemental Fig. 2B). Moreover, 6-MF treatment did not change the protein level of NFAT1 or IRF4 in total cell lysates (Fig. 4C, upper panel, and Supplemental Fig. 2C, left panel). Therefore, we investigated whether 6-MF treatment affects nuclear translocation of NFAT1 or IRF4 without altering their expression levels. Interestingly, 6-MF treatment clearly reduced the stimulation-dependent increase of nuclear NFAT1 protein level (Fig. 4C, lower panel). However, no significant change in nuclear level of IRF4 protein was observed in 6-MF–treated Th2 cells (Supplemental Fig. 2C, right panel). Because NFAT1 nuclear translocation is regulated by its phosphorylation status, we further examined whether 6-MF inhibits NFAT1 dephosphorylation, thus limiting nuclear translocation of NFAT1. For this purpose, we detected both phosphorylated and dephosphorylated forms of NFAT1 in the cytoplasmic and nuclear extracts of ex vivo CD4+ T cells and examined nuclear translocation status of NFAT1. Unstimulated ex vivo CD4+ T cells showed both phosphorylated and dephosphorylated forms of NFAT1 in the cytoplasmic and nuclear extracts of ex vivo CD4+ T cells and examined nuclear translocation status of NFAT1. Unstimulated ex vivo CD4+ T cells showed both phosphorylated and dephosphorylated forms of NFAT1 in cytoplasm, whereas any form of NFAT1 was hardly detected in the nuclear fraction (Fig. 4D). In contrast, PMA plus ionomycin stimulation induced NFAT1 dephosphorylation and translocation into the nucleus. CsA is known to disrupt calcineurin activity that consequently inhibits NFAT1 dephosphorylation. Indeed, CsA treatment inhibited nuclear translocation of NFAT1, resulting in a relatively increased amount of accumulated phosphorylated NFAT1 in cytoplasm. Interestingly, 6-MF did not inhibit NFAT1 dephosphorylation; however, its translocation was greatly reduced (Fig. 4D). Similar, but with lesser extent of inhibitory activity of 6-MF on NFAT2 was detected. Because NFAT1-mediated transcriptional activity is dependent on the phosphorylation of S53/S56 site of NFAT1.
transactivation domain, we thus further assessed whether 6-MF inhibits phosphorylation of this site by using Ab that detects 54-phosphoserine (30). 6-MF treatment did not affect phosphorylation state and level of NFATs (Fig. 4D). Collectively, these results suggest that the suppression of cytokine expression upon 6-MF treatment is mediated by the inhibition of nuclear translocation of NFAT1 in T cells without affecting its expression and phosphorylation level.

To further confirm the inhibitory effect of 6-MF on NFAT1 nuclear translocation, we performed immunocytochemistry analysis in primary Th2 cells. In the absence of stimulation, NFAT1 (green) remained in the cytoplasm (Supplemental Fig. 3). However, stimulation with anti-CD3 and anti-CD28 led to NFAT1 activation, resulting in an increase of NFAT1 translocation (yellow) into the nucleus (red) (Supplemental Fig. 3). Then we analyzed nuclear translocation of NFAT1 after treatment of 6-MF. Stimulation of ex vivo CD4+ T cells with either anti-CD3 and anti-CD28 or PMA plus ionomycin induced evident increase of NFAT1 in nucleus, whereas 6-MF treatment significantly inhibited NFAT1 translocation as much as the effects of CsA in both stimulation conditions (Fig. 4E). These results indicate that 6-MF suppresses cytokine expression by inhibiting nuclear translocation of NFAT1 in primary CD4+ T cells.

6-MF inhibits NFAT1 binding to regulatory elements of cytokine genes

We hypothesized that the decreased translocation of NFAT1 by 6-MF treatment may result in the reduction of its binding capacity

Table I. Primer sequences used for qRT-PCR

<table>
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<th>Gene</th>
<th>Sequences (5'→3')</th>
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<td>HPRT</td>
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F, forward; R, reverse; Tm, melting temperature.
FIGURE 3. 6-MF inhibits cytokine expression by activated T cells. (A and C) EL4 T cells and in vitro differentiated Th1 or Th2 cells (2 × 10^6) were pretreated with medium and DMSO vehicle (0.5%) or 6-MF (20 μM) for 1 h, and then the cells were left unstimulated or were stimulated with PMA (50 ng/ml) plus ionomycin (1 μM) for 2 h. Cytokine mRNA level was analyzed using qRT-PCR and was normalized using the expression level of HPRT. Data are presented as a percentage of relative mRNA levels by comparison with vehicle-treated cells. The results are the means of triplicates with SD. All data are representative of three independent experiments with similar results. *p < 0.05, **p < 0.01. (B and D) In vitro differentiated Th2 cells (1 × 10^6) were pretreated with medium and DMSO vehicle or 6-MF for 1 h, and then the cells were left unstimulated or were stimulated with PMA plus ionomycin for 24 h. Intracellular levels of IL-10 and IL-4 were analyzed using flow cytometry. Isotype IgG was used as a control. All data are representative of three independent experiments with similar results.

6-MF inhibits NFAT1 activity regardless of its phosphorylation status

NFAT activation is mediated by sequential events. Stimulation activates calcineurin-dependent dephosphorylation of NFATs that results in their translocation into the nucleus. Therefore, we investigated whether 6-MF inhibits nuclear translocation of NFAT by disruption of the dephosphorylation process. To analyze this possibility, we performed reporter analysis in EL4 cells in the presence of CA-NFAT1 that has multiple serine to alanine mutations in the NFAT1 regulatory domain. Hence, the activity of CA-NFAT1 is independent of its phosphorylation status (30). We reasoned that if 6-MF regulates NFAT1 activation by preventing its dephosphorylation, it would not inhibit the transactivity of CA-NFAT1. Although overexpression of CA-NFAT1 induced much higher increase of luciferase activity than NFAT1 in the absence of stimulation, it showed similar levels of enhancer activity of CNS-9 even after stimulation (Fig. 6A). Interestingly, 6-MF treatment still inhibited luciferase activity of CNS-9 even in the presence of CA-NFAT1 (Fig. 6A). This result indicates that inhibition of nuclear translocation of NFAT1 by 6-MF is not mediated by affecting dephosphorylation of NFAT1. To confirm this result, we analyzed 6-MF activity on the IL-2 promoter reporter construct, which contains three tandem repeats of NFAT-binding sequence. Similar to the result of CNS-9 reporter system, 6-MF reduced the luciferase activity of IL-2 promoter even in the presence of CA-NFAT1 (Fig. 6A). This result indicates that inhibition of nuclear translocation of NFAT1 by 6-MF is not mediated by affecting dephosphorylation of NFAT1. To confirm this result, we analyzed 6-MF activity on the IL-2 promoter reporter construct, which contains three tandem repeats of NFAT-binding sequence. Similar to the result of CNS-9 reporter system, 6-MF reduced the luciferase activity of IL-2 promoter even in the presence of CA-NFAT1 (Fig. 6A). To minimize the effect of endogenously expressed NFAT1, we performed the same experiments in HEK 293T cells. Overexpression of CA-NFAT1 effectively increased the luciferase activity in HEK 293T cells (Fig. 6C, 6D), which was significantly inhibited by 6-MF treatment. As expected, CsA greatly reduced luciferase activity of IL-2 promoter in the presence of NFAT1 in both cell lines; however, it failed to inhibit the activity of CA-NFAT1, which was more evidently observed in HEK 293T cells (Fig. 6C, 6D, black bar). These results imply that the regulatory mechanism of 6-MF on NFAT1 activity might be
different from that of CsA, which inhibits calcineurin activity. We have further confirmed this possibility by immunocytochemistry analysis. HEK 293T cells were transfected with either NFAT1- or CA-NFAT1–expressing constructs in the absence or presence of CsA–NFAT1–expressing constructs in the absence or presence of 6-MF. Similar to our finding with ex vivo CD4+ T cells (Fig. 4E), we also found that overexpressed NFAT1 was localized in the nucleus.

**Table II.** Primer sequences used for chromatin immunoprecipitation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences (5'-3')</th>
<th>Tm (˚C)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS-9</td>
<td>F: CTGAGGGAAAGCCAGCATC</td>
<td>61.63</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>R: TCTGGAAAGTGCCATTCTGTA</td>
<td>60.87</td>
<td></td>
</tr>
<tr>
<td>IL-4 promoter (NFAT binding)</td>
<td>F: AGGCCGATTATGGTGTAATTT</td>
<td>59.83</td>
<td>227</td>
</tr>
<tr>
<td></td>
<td>R: GAGTTAAAGTGCTGAAACCA</td>
<td>59.01</td>
<td></td>
</tr>
<tr>
<td>IL-4 promoter (GATA3 binding)</td>
<td>F: ACTCATTTTCCTGCTTTCAGC</td>
<td>63.86</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td>R: GATTTTGTGCATCCTCGTGG</td>
<td>62.58</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse; Tm, melting temperature.
upon stimulation, which was inhibited by CsA or 6-MF treatment (Fig. 6E, left panel). In contrast, overexpressed CA-NFAT1 was mainly detected in the nucleus regardless of stimulation. CsA treatment did not prevent the translocation of CA-NFAT1 into nucleus. Interestingly, however, 6-MF treatment inhibited the translocation of CA-NFAT1 into nucleus. These results collectively suggest that 6-MF treatment could inhibit NFAT1-mediated transactivity in diverse target genes.

**6-MF inhibits effector function of T and B cells in AD-induced mice**

Next, we evaluated the therapeutic potential of 6-MF as an immunomodulatory agent by inhibiting NFAT1 translocation. AD is known as a chronic inflammatory skin disease mediated by Th2-type immune responses, thereby showing increased levels of IgE and Th2-type cytokines such as IL-4, IL-5, and IL-13 (31). Moreover, the chronic stage of skin inflammation shows production of proinflammatory cytokines such as IFN-γ and TNF-α (32). We tested whether 6-MF treatment could suppress the expression of those cytokines produced by lymphocytes of AD mice (29). CD4+ T cells and CD19+ B cells isolated from AD mice were stimulated with PMA plus ionomycin for 1 h in the presence or absence of 6-MF. Nuclear extracts were prepared, as explained in Materials and Methods. Competitor oligomers or Abs were added, as indicated. Protein binding to the probe was marked with black arrow. CNS-9 probe was designed to contain NFAT binding sites. Probe containing NFAT consensus sequences was used as a positive control. All the data shown are representative of three independent experiments with similar results.

**Figure 5.** 6-MF inhibits NFAT1 binding to the regulatory elements of cytokine genes. (A and B) Th2 cells (1 × 10⁷) were left unstimulated or were stimulated with PMA plus ionomycin for 1 h in the absence or presence of 6-MF. ChIP assay was performed using anti-NFAT1 Ab or isotype IgG Ab. The levels of precipitated DNA normalized by input DNA were measured using qRT-PCR with specific primers of IL-10 CNS-9 region (A) and IL-4 promoter region (B). Results were presented as a relative level by comparing with quantitative level of target locus in IgG sample. IgG was used as a control for the specificity of the Ab. Data are the means of triplicates with SD. All the data shown are representative of three independent experiments with similar results. *p < 0.05. (C) EMSA was performed using specific probes, as indicated in the figure. Th2 cells (1 × 10⁷) were left unstimulated or were stimulated with PMA plus ionomycin for 1 h in the presence or absence of 6-MF: Nuclear extracts were prepared, as explained in Materials and Methods. Competitor oligomer or Abs were added, as indicated. Protein binding to the probe was marked with black arrow. CNS-9 probe was designed to contain NFAT binding sites. Probe containing NFAT consensus sequences was used as a positive control. All the data shown are representative of three independent experiments with similar results.

**Table III.** Probe sequences used for EMSA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFAT</td>
<td>F: GATCGCGGAAAAGAGGAATTTTCTTTCATACAG</td>
</tr>
<tr>
<td></td>
<td>R: CTTGATGAACAAATTTCGCCCAAATTGCGGATC</td>
</tr>
<tr>
<td>CNS-9</td>
<td>F: AGGAGGCGGAAAGAGGAATTTTCTTTCATACAG</td>
</tr>
<tr>
<td></td>
<td>R: GCGGCTTTTCCTTGCCTGCTTTTCCTTGCCTGCTTCT</td>
</tr>
</tbody>
</table>

F: forward; R: reverse.

Stimulated in the presence or absence of 6-MF, and then the effects of 6-MF treatment on lymphocyte proliferation and cytokine and IgE production were analyzed. qRT-PCR analysis was performed using the primers shown in Table I. As shown in Fig. 7A, 6-MF treatment significantly downregulated the expressions of Th1- and Th2-type cytokines from CD4+ T cells stimulated with PMA plus ionomycin. Moreover, 6-MF treatment inhibited proliferation of T cells and B cells by up to ~55 and 40%, respectively (Fig. 7B).

Elevated IgE level in serum is a diagnostic marker of AD. Consistent with this, experimental mice model showed increased IgE level after 2 wk of disease induction (data not shown). Therefore, we assessed whether 6-MF has a regulatory activity on IgE production by B cells. B cells isolated from mice with AD produced considerable amounts of IgE. This was further increased by stimulation with LPS and IL-4 (Fig. 7C). Interestingly, 6-MF treatment on B cells effectively decreased IgE production (Fig. 7C). Because T cells are important for activation, differentiation, and immune responses of B cells, we examined the effect of 6-MF on IgE production from B cells after coculturing with T cells. T cells isolated from AD mice were activated by stimulation with anti-CD3 and anti-CD28 in the presence or absence of 6-MF for 24 h and were washed with T cell media three times. Then B cells were cocultured with these T cells in the presence of LPS and IL-4 for 72 h. Coculturing of B cells with activated T cells increased IgE production by ~2-fold as compared with unstimulated T cells (Fig. 7D). However, 6-MF–treated T cells could not induce an increase in IgE production by B cells (Fig. 7D). These data indicate that 6-MF acts as an immunomodulator by inhibiting NFAT1-mediated lymphocyte activation.
Oral administration of 6-MF ameliorates AD

Immunosuppressive effects of 6-MF on lymphocytes led us to test whether 6-MF can suppress the disease onset and progression of AD. The induction of experimental AD and its treatment strategy are depicted in Fig. 8A. CsA was employed as a positive control to compare the therapeutic effects of 6-MF. CsA or 6-MF treatment significantly reduced AD symptoms, including erythema, horny substance, dryness, and scaling (data not shown). We also performed H&E staining on atopic ears excised from different treatment groups (Fig. 8B). In comparison with control mice, 6-MF treatment significantly reduced the infiltration of lymphocytes and granulocytes, which was well reflected by decreased thickness of the epidermis (Fig. 8B). 6-MF treatment also significantly decreased the IgE levels with comparable efficacy to CsA treatment (Fig. 8C). These results indicate that 6-MF has a therapeutic potential to ameliorate AD progression.

Discussion

In this study, we developed a novel screening system that targeted NFAT-mediated transactivation of CNS-9 and identified a novel function of 6-MF as an inhibitor of the nuclear translocation of NFAT1. Treatment of 6-MF suppressed the expression of known NFAT1 target genes, such as IL-10, IL-4, and IFN-γ, by inhibiting NFAT1 nuclear translocation without altering its expression level. Furthermore, 6-MF treatment inhibited the effector function of T and B cells isolated from AD-induced mice. Oral administration of 6-MF significantly ameliorated AD progression and disease severity.

Previous studies showed that natural phenolic compounds possess a variety of biological properties, such as antioxidant, anti-inflammatory, and anticancer activities (21, 22). Recent studies also indicated that natural polyphenols can act as modulators of signal transduction by inhibiting the activity of various enzymes and transcription factors (23, 24). However, the action mechanisms, including identification of the molecular target(s) underlying these biological activities, are not yet fully understood. In this study, we have identified a small, novel natural product inhibitor of NFAT. Previously, we elucidated the molecular mechanism of IL-10 transcription in T cells (25, 33, 34). A distal cis-acting element, CNS-9, acts as an enhancer by recruiting NFAT1 and IRF4, thus increasing IL-10 transcription in Th2 cells (25). Based on this information, we have developed a genetically modified cell-based screening system
that targets CNS-9 regulatory factors and have performed screening of a chemical library derived from plant extracts. We identified four compounds that efficiently inhibited IL-10 reporter activity (Fig. 2A) as well as IL-10 expression, namely 6-MF, 7-hydroxyflavone, baicalein, and formononetin. Interestingly, all the hit compounds possess a common flavone moiety (Fig. 2C). Among these hit compounds, 6-MF was the most effective in suppressing CNS-9–mediated luciferase activity and IL-10 expression in EL4 T cells and primary Th2 cells (Fig. 3A, 3B) without inducing cytotoxicity (Fig. 2D, 2E). Interestingly, the inhibitory effect of 6-MF on cytokine expression was not restricted to IL-10. Cells were stimulated as indicated, and thymidine incorporation was measured at the end of the 72-h culture. (C) B cells were stimulated for 48 h, and IgE level in the culture supernatants was measured using ELISA. (D) T cell–dependent IgE production by B cells was measured. Preactivated T cells by anti-CD3/anti-CD28 stimulation in the presence or absence of 6-MF were cocultured with B cells in the presence of LPS and IL-4. IgE level in the culture supernatants was measured using ELISA. All the data are the means of triplicates with SD. All the data are representative of three independent experiments that produced similar results. *p < 0.05, **p < 0.01.

FIGURE 7. 6-MF inhibits the effector function of T and B cells isolated from atopic mice. Experimental AD was induced in BALB/c, as described in Materials and Methods. CD4+ T cells and CD19+ B cells were isolated from AD mice. CD4+ T cells were stimulated with PMA (50 ng/ml) plus ionomycin (1 μM) or anti-CD3 Ab (1 μg/ml) and anti-CD28 Ab (2 μg/ml), as indicated. CD19+ B cells were stimulated with PMA (50 ng/ml) plus ionomycin (2 μM) or LPS (50 μg/ml) and IL-4 (25 ng/ml), as indicated. DMSO (0.5%) or 6-MF (20 μM) was pretreated 1 h before stimulation. (A) After 2-h stimulation, cytokine mRNA level was analyzed using qRT-PCR and was normalized by the expression level of HPRT. Results are expressed as a percentage of relative mRNA level by comparison with the mRNA level of DMSO-treated T cells. (B) Proliferation of T cells (left panel) and B cells (right panel) isolated from AD mice was measured. (C) B cells were stimulated for 48 h, and IgE level in the culture supernatants was measured using ELISA. (D) T cell–dependent IgE production by B cells was measured. *p < 0.05, **p < 0.01. The Journal of Immunology 2781

6-MF also inhibited IRF4-mediated transcriptional activity without interfering with its nuclear translocation and expression level (Supplemental Fig. 2). Although the underlying mechanism of the inhibition of IRF4 transcriptional activity by 6-MF treatment is still unclear, there are several possibilities. 6-MF treatment may inhibit the functional synergism between NFAT1 and IRF4 in upregulating the enhancer activity of CNS-9. IRF4 often functions as a context-dependent transcription factor depending on its binding partner. Indeed, the IRF4 alone showed a weak transactivity, whereas its transactivity was synergized when it was coupled with NFAT1 (25). In addition, the finding that 6-MF can inhibit the activity of CA-NFAT1 further suggests that 6-MF may not interrupt the dephosphorylation of NFAT1, but somehow regulates the translocation of NFAT1 via another mechanism (Fig. 6). The inhibitory effect of 6-MF may not be limited to NFAT1 because 6-MF treatment also inhibited nuclear translocation of NFAT2 (Fig. 4D). Although the dose-dependent inhibitory effect of 6-MF on the nuclear translocation of NFAT was observed (Figs. 4, 6), modification of its chemical structure is needed to improve its activity. In addition, further studies are needed to delineate the precise molecular mechanism of 6-MF that interferes with the nuclear translocation of NFAT, but we believe that this type of detailed investigation lies beyond the scope of current study.

Flavonoids have been reported to exert beneficial effects in many diseases, including cancer, cardiovascular disease, and neurode-
Generative disorders (21, 22, 38). Their biological potential is mainly dependent on their antioxidant properties associated with free hydroxyl groups (39). A flavone subclass, in which all hydroxyl groups are capped by methylation, showed higher metabolic stability and membrane transport in the intestine and liver, thus improving oral bioavailability (40, 41). Recent studies have suggested the potential of methoxyflavone as a therapeutic agent. 7-Methoxyflavone showed a greater inhibitory effect on PGE2 production from LPS-stimulated RAW cells, with the highest membrane permeability when compared with similar flavones possessing hydroxyl groups or multiple methoxyl groups (42). Another group reported that 5-methoxyflavone enhanced TRAIL-induced apoptosis in human leukemic cells by upregulating the expression of cell death receptors and mitochondrial pathways (43).

The biological activity of 6-MF and its regulatory mechanism have not been reported. The inhibitory effects of 6-MF on the nuclear translocation of NFAT1 without altering its phosphorylation status have a great potential as an immunomodulator in controlling NFAT-mediated T cell responses. In fact, the potential of NFAT as a therapeutic target has been supported by the findings that SCID is related to the impairment in NFAT activation and to a relatively short duration of nuclear residence (12, 13). Patients with the SCID phenotype are characterized by a defect in the T cell immune response, such as altered cytokine production. Thus, in a retrospective point of view, NFAT could be an effective target for ameliorating graft rejection in transplanted hosts and autoimmune diseases caused by inappropriate T cell immunity (19). Calcineurin is the phosphatase known to activate NFAT in the immune system; therefore, calcineurin inhibitors, such as CsA and FK506, have been widely used for this purpose (17, 18). Despite their clinical significances, the application of these drugs is restricted only to serious clinical situations due to their severe side effects (19). Several groups have reported selective inhibitors as alternatives to CsA (44–47). Because NFAT1 is not the only transcription factor regulated by calcineurin, these inhibitors were demonstrated as selective inhibitors of the calcineurin-NFAT signaling pathway without disrupting general calcineurin phosphatase activity. In addition, some natural compounds, such as quercetin and kaempferol, have been reported to have calcineurin-inhibitory activity with less toxicity and relatively reduced doses (48, 49). Considering the common characteristics of newly reported inhibitors of the calcineurin–NFAT signaling pathway, 6-MF may have several advantages over classical calcineurin inhibitors, as follows: 1) it could inhibit translocation of NFAT1 without affecting dephosphorylation, which implies that it may not inhibit the enzymatic activity of calcineurin; 2) it is a natural compound with low cytotoxicity; and 3) it has a relatively small size. Our study applying 6-MF in T cells and B cells isolated from an AD model also suggests that 6-MF suppresses the proliferation of activated T cells, reduces cytokine production from T cells, and possibly regulates B cell activity indirectly, via inhibition of T cell activity (Fig. 7). Furthermore, oral administration of 6-MF to AD mice shows beneficial effects, such as reduction in the general symptoms of AD and serum IgE levels (Fig. 8).

In conclusion, in this study, we describe that 6-MF inhibits NFAT-mediated T cell activation via the inhibition of NFAT1 translocation into the nucleus that consequently interrupts NFAT1 binding to its target genes. The therapeutic potency of 6-MF was also assessed using the AD mice model by ameliorating a range of AD symptoms. Thus, our results show that 6-MF could be a potentially effective immunomodulatory agent against diseases involving NFAT-mediated T cell dysregulation.

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Disclosures
The authors have no financial conflicts of interest.
References