

Transduced PEP-1-FK506BP Ameliorates Atopic Dermatitis in NC/Nga Mice

So Young Kim^{1,4}, Eun Jeong Sohn^{1,4}, Dae Won Kim^{1,4}, Hoon Jae Jeong¹, Mi Jin Kim¹, Hye Won Kang¹, Min Jea Shin¹, Eun Hee Ahn¹, Soon Won Kwon¹, Young Nam Kim¹, Hyung Joo Kwon², Tae-Yoon Kim³, Kil Soo Lee¹, Jinseu Park¹, Won Sik Eum¹ and Soo Young Choi¹

Immunophilin, FK506-binding protein 12 (FK506BP), is a receptor protein for the immunosuppressive drug FK506 by the FK506BP/FK506 complex. However, the precise function of FK506BP in inflammatory diseases remains unclear. Therefore, we examined the protective effects of FK506BP on atopic dermatitis (AD) in tumor necrosis factor- α (TNF- α)/interferon- γ (IFN- γ)-induced HaCaT cells and 2,4-dinitrofluorobenzene-induced AD-like dermatitis in Nishiki-nezumi Cinnamon/Nagoya (NC/Nga) mice using a cell-permeable PEP-1-FK506BP. Transduced PEP-1-FK506BP significantly inhibited the expression of cytokines, as well as the activation of NF- κ B and mitogen-activated protein kinase (MAPK) in TNF- α /IFN- γ -induced HaCaT cells. Furthermore, topical application of PEP-1-FK506BP to NC/Nga mice markedly inhibited AD-like dermatitis as determined by a histological examination and assessment of serum IgE levels, as well as cytokines and chemokines. These results indicate that PEP-1-FK506BP inhibits NF- κ B and MAPK activation in cells and AD-like skin lesions by reducing the expression levels of cytokines and chemokines, thus suggesting that PEP-1-FK506BP may be a potential therapeutic agent for AD.

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INTRODUCTION

Atopic dermatitis (AD) is a common pruritic and chronically relapsing inflammatory skin disease that is steadily increasing in prevalence. Endogenous and exogenous factors have a role in the manifestation of AD, which seriously affects patients, their families, and socioeconomic aspects of life (Homey *et al.*, 2006). With lifetime prevalence rates of 10–20% in children and 1–3% in adults, AD is a major worldwide public health problem (Schultz-Larsen and Hanifin, 2002). The wide variations in prevalence identified in countries with an ethnically similar population suggest that environmental factors determine the expression of AD (Williams *et al.*, 1999). Skin lesions of AD are well characterized by infiltrating lymphocytes, monocytes/macrophages, eosinophils,

and fully granulated mast cells (Scoter, 1989; Uehara *et al.*, 1990). In 1957, Nishiki-nezumi Cinnamon/Nagoya (NC/Nga) mice were established as an inbred strain from Japanese fancy mice. When NC/Nga mice are kept in an air-uncontrolled conventional circumstance, they spontaneously develop AD-like dermatitis. However, when kept in specific pathogenic-free conditions, these mice remain normal and healthy (Matsuda *et al.*, 1997; Watanabe *et al.*, 2002). The application of 2,4-dinitrofluorobenzene (DNFB), evokes contact hypersensitivity reaction in mice (Tomimori *et al.*, 2004). Furthermore, AD-like dermatitis found in NC/Nga mouse models is accompanied by elevated serum IgE levels, increased expression of Th2 cytokines, eosinophil accumulation in the lesions, and frequent scratching behavior, which are characteristic features of human AD (Matsuda *et al.*, 1997; Vestergaard *et al.*, 1999, 2000).

FK506-binding proteins (FK506BPs) belong to a family of immunophilins named for their ability to bind immunosuppressive drugs. FK506BPs can be associated with chaperones and constitute a major part of receptor complexes. FK506BP 12 is a small peptide with a single FK506BP domain and a molecular size of 12 kDa. FK506BP is involved in multiple biological processes, which involve binding to rapamycin and formation of a complex of rapamycin-mediated phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin downstream signal transduction, which is the major mechanism for applying rapamycin in tumor treatment (Schreiber, 1991; Snyder and Sabatini, 1995; Cameron *et al.*, 1997; Schiene-Fischer and Yu, 2001).

Protein transduction is an established means of delivering proteins both *in vitro* and *in vivo* by protein transduction

¹Department of Biomedical Science and Research Institute of Bioscience and Biotechnology, Hallym University, Chunchon, Korea; ²Department of Microbiology, College of Medicine, Hallym University, Chunchon, Korea and ³Department of Dermatology, College of Medicine, Catholic University, Seoul, Korea

⁴These authors contributed equally to this work.

Correspondence: Soo Young Choi or Won Sik Eum, Department of Biomedical Science and Research Institute of Bioscience and Biotechnology, Hallym University, Chunchon 200-702, Korea. E-mail: sychoi@hallym.ac.kr or wseum@hallym.ac.kr

Abbreviations: AD, atopic dermatitis; DNFB, 2,4-dinitrofluorobenzene; FK506BP, FK506-binding protein 12; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MDC, macrophage-derived chemokine; NC/Nga, Nishiki-nezumi Cinnamon/Nagoya; PBS, phosphate-buffered saline; TARC, thymus and activation-regulated chemokine; TNF- α , tumor necrosis factor- α

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domains or cell-penetrating peptides. Although the exact mechanism of transduction is unclear, many studies have demonstrated that protein transduction allows the delivery of therapeutic proteins *in vitro* and *in vivo* (Morris *et al.*, 2001; Wadia and Dowdy, 2002; Eum *et al.*, 2004; Choi *et al.*, 2006; An *et al.*, 2008).

In this study, we demonstrate that a PEP-1-FK506BP fusion protein can be directly transduced into HaCaT cells and markedly inhibits the expression of cytokines, as well as the activation of NF- κ B and mitogen-activated protein kinase (MAPK) in HaCaT cells. In addition, topical application of PEP-1-FK506BP to NC/Nga mice significantly inhibits AD as determined by a histological examination and serum IgE levels. Therefore, we suggest that topical application of the PEP-1-FK506BP fusion protein may be a potential therapeutic agent for AD.

RESULTS

Purification and transduction of the PEP-1-FK506BP fusion protein into HaCaT cells

A cell-permeable expression vector, PEP-1-FK506BP, containing the human FK506BP cDNA and PEP-1 peptide was constructed. After purification of PEP-1-FK506BP, its intracellular delivery into HaCaT cells was confirmed by

direct fluorescence analysis (Figure 1a). PEP-1-FK506BP was efficiently transduced into cells, whereas no fluorescence signals were evident in negative control cells and in cells treated with control FK506BP. To exclude the possibility that cell fixation with paraformaldehyde may have affected PEP-1-FK506BP transduction by direct fluorescence, FITC-conjugated PEP-1-FK506BP was transduced into unfixed and fixed HaCaT cells. The intracellular distribution of the PEP-1-FK506BP fluorescence signal in unfixed cells was similar to that in fixed cells. These results indicate that cell fixation with paraformaldehyde is not required for PEP-1-FK506BP transduction.

In addition, the transduction efficiency of PEP-1-FK506BP was confirmed by flow cytometry of HaCaT cells (Figure 1b). Various concentrations (0.1–1 μ M) of PEP-1-FK506BP were added to HaCaT cells in culture for 60 minutes, and levels of transduced proteins were measured by flow cytometry. The fusion proteins were transduced into HaCaT cells in a concentration-dependent manner.

To clarify the subcellular localization of transduced proteins into cells, nuclear and cytosolic fractions were prepared from cells transduced with PEP-1-FK506BP and then analyzed by western blotting using anti-histidine, anti-actin, and anti-poly (ADP-ribose) polymerase antibodies. As shown in Figure 1c, PEP-1-FK506BP was detected in the

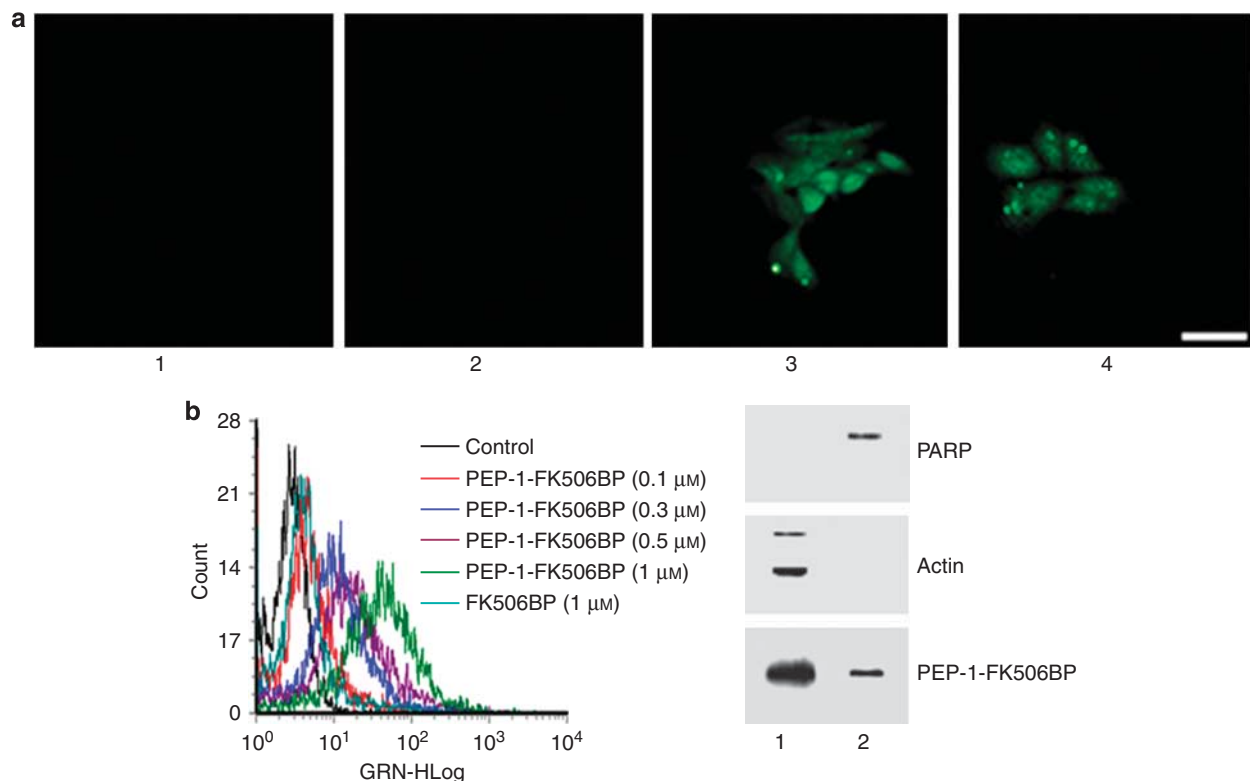


Figure 1. Transduction of PEP-1-FK506BP fusion proteins into HaCaT cells. (a) After FITC-labeled PEP-1-FK506BP and control FK506BP fusion proteins (0.5 μ M) were transduced into HaCaT cells, cells were washed twice with trypsin-EDTA, PBS, and were immediately observed by fluorescence microscopy. Lanes are as follows: lane 1, control cells; lane 2, cells treated with FK506BP; lane 3, unfixed cells treated with PEP-1-FK506BP; lane 4, fixed cells treated with PEP-1-FK506BP. Bar = 50 μ m. (b) Uptake of PEP-1-FK506BP fusion proteins assessed by flow cytometry. (c) Subcellular localization of PEP-1-FK506BP. The nuclear and cytosolic extracts were prepared from transduced cells and analyzed by western blotting. Lanes are as follows: lane 1, cytosolic fraction; lane 2, nuclear fraction. FK506BP, FK506-binding protein 12; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline.

nuclei and in the cytoplasm of transduced cells. However, it was distributed more in the cytosol than in the nuclei.

Inhibitory effects of PEP-1-FK506BP on TNF- α /IFN- γ -induced activation of NF- κ B and MAPK activation, and cytokine/chemokine expression in HaCaT cells

To determine the effect of transduced PEP-1-FK506BP on tumor necrosis factor- α (TNF- α) (10 ng ml⁻¹)/IFN- γ (10 ng ml⁻¹)-induced activation of NF- κ B, HaCaT cells were incubated for 15 minutes with TNF- α /IFN- γ in the presence or absence of PEP-1-FK506BP. Nuclear extracts from TNF- α /IFN- γ -induced cells were analyzed using an electromobility shift assay. Transduced PEP-1-FK506BP decreased the TNF- α /IFN- γ -induced DNA-binding activity of NF- κ B (Figure 2a). Next, the regulatory effect of PEP-1-FK506BP on the TNF- α /IFN- γ -induced signal cascade of NF- κ B activation was examined. PEP-1-FK506BP inhibited TNF- α /IFN- γ -induced I κ B α phosphorylation and degradation in cells (Figure 2b).

The MAPK signaling cascades are involved in the activation of NF- κ B (Dhar *et al.*, 2002). Appropriately, an experiment investigated the regulatory effect of PEP-1-FK506BP on MAPKs p38 activity, c-Jun N-terminal kinase (JNK), and extracellular signal-related kinase. To examine the effect of PEP-1-FK506BP on TNF- α /IFN- γ -induced MAPK activation, HaCaT cells were incubated for 15 minutes with TNF- α /IFN- γ in the presence or absence of PEP-1-FK506BP and then analyzed by western blot analysis using phospho-specific antibodies against MAPKs (Figure 2c). PEP-1-FK506BP decreased TNF- α /IFN- γ -induced phosphorylation of p38 and JNK in a dose-dependent manner, whereas extracellular signal-related kinase levels were not changed.

Various cytokines and chemokines such as TNF- α , IL-1 β , IL-6, thymus and activation-regulated chemokine (TARC), macrophage-derived chemokine (MDC), and chemokine (C-X-C motif) ligand (CXCL) might be involved in inflammation and AD (Imai *et al.*, 1997; Vestergaard *et al.*, 1999; Sandoval-Lopez and Teran, 2001; De Vry *et al.*, 2005; Murakawa *et al.*, 2006). Accordingly, we examined the effects of the PEP-1-FK506BP fusion protein on cytokine and chemokine production in TNF- α /IFN- γ -stimulated HaCaT cells. Cytokine and chemokine levels increased after treatment with TNF- α /IFN- γ , whereas PEP-1-FK506BP markedly inhibited the TNF- α /IFN- γ -induced mRNA expression of various cytokines and chemokines (Figure 2d). We further investigated the effect of PEP-1-FK506BP on the expression of TNF- α /IFN- γ -stimulated HaCaT cells. Cells were incubated for 24 hours with TNF- α /IFN- γ in the presence or absence of PEP-1-FK506BP, and then culture supernatants were analyzed for secretion levels of cytokine and chemokine protein by ELISA. PEP-1-FK506BP decreased TNF- α /IFN- γ -induced protein expression of cytokine and chemokine in a dose-dependent manner (Figure 2e). These results were consistent with the suggestion that PEP-1-FK506BP inhibits TNF- α /IFN- γ -induced NF- κ B and MAPK activation, as well as various cytokine/chemokine expression levels. Thus, the inhibition of cytokines and cytokine expressions may constitute an effective previously unreported therapeutic strategy for the treatment of AD.

Effect of transduced PEP-1-FK506BP fusion proteins on DNFB-induced NC/Nga mice

Atopic dermatitis-like skin lesions were induced in NC/Nga mice using multiple topical applications of DNFB for 5 weeks (Figure 3a). However, the topical application of PEP-1-FK506BP to mice for 1 hour after DNFB application markedly inhibited DNFB-induced AD (Figure 3b).

To examine the role of PEP-1-FK506BP in inhibiting AD, PEP-1-FK506BP was topically applied to DNFB-treated mice skin. The skin was stained with hematoxylin and eosin and toluidine, and examined using an optical microscope. Figure 4a shows the representative results of histological analyses of the skin lesions of DNFB-treated mice. At 5 weeks, hyperplasia and thickening of the epidermis were evident and cell infiltration was more marked in NC/Nga mice than in control mice. PEP-1-FK506BP transduction significantly inhibited DNFB-induced skin lesions. In addition, the changes were markedly inhibited by protopic, a reference drug. However, control FK506BP showed little to no effect on DNFB-induced skin lesions. Mast cell infiltration was also decreased in PEP-1-FK506BP-treated mice (Figure 4b).

In NC/Nga mice, serum levels are closely correlated with the clinical severity of dermatitis. Therefore, serum levels were measured in DNFB- and PEP-1-FK506BP-treated mice. DNFB-treated mice showed significantly enhanced serum IgE levels than did control mice. Increased serum IgE levels were lower in PEP-1-FK506BP- and protopic-treated mice. In addition, combined PEP-1-FK506BP- and protopic-treated mice showed more markedly decreased serum IgE levels. However, control FK506BP had little to no effect on serum IgE levels (Figure 5).

The effect of PEP-1-FK506BP on cytokine mRNA expression levels in the same conditions was examined by reverse transcription-PCR. Cytokine levels were increased by treatment with DNFB alone. PEP-1-FK506BP significantly reduced the production of TARC, MDC, IL-4, and IL-5, whereas control FK506BP had minimal effects on the levels of these cytokines (Figure 6). These results were consistent with the view that PEP-1-FK506BP can prevent the development of dermatitis in NC/Nga mice.

DISCUSSION

These results demonstrate that transduced PEP-1-FK506BP significantly inhibits the expression of cytokines, as well as the activation of NF- κ B and MAPK in HaCaT cells. Furthermore, topical application of PEP-1-FK506BP to NC/Nga mice markedly inhibited AD-like skin lesions as determined by a histological examination, assessment of serum IgE levels, and expression levels of cytokines. These results implicate PEP-1-FK506BP to be a potential therapeutic agent for AD.

FK506BPs are a family of immunophilins that were named for their ability to bind immunosuppressive drugs. To date, 15 mammalian FK506BPs have been found; most are expressed in the brain. Among the FK506BPs, FK506BP12 has been particularly well characterized (Guo *et al.*, 2001; Harrar *et al.*, 2001). In this study, a cell-permeable PEP-1-FK506BP fusion protein was constructed and used to investigate the

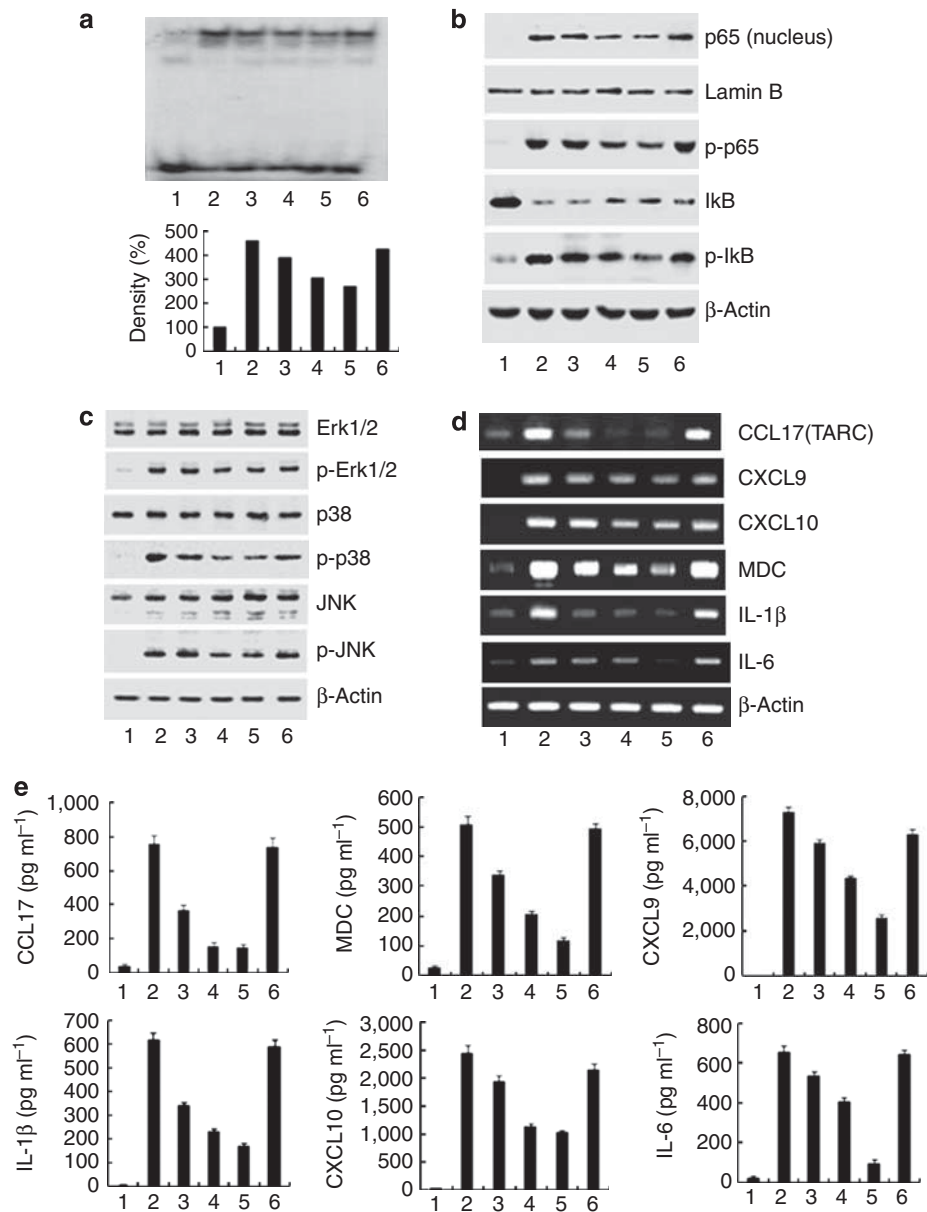


Figure 2. PEP-1-FK506BP-mediated inhibitory effects on TNF- α /IFN- γ -induced NF- κ B, MAPK, and cytokine/chemokine expression in HaCaT Cells. HaCaT cells were stimulated with TNF- α (10 ng ml⁻¹) and IFN- γ (10 ng ml⁻¹) for 15 minutes with or without pretreatment with PEP-1-FK506BP for 1 h. (a) DNA-binding activity of NF- κ B in the nuclear extracts of cells was measured by EMSA and band intensity by densitometer. (b) Phosphorylation and degradation of I κ B α were analyzed by western blotting. (c) Extracts from cells prepared and analyzed for MAPK protein activation by western blotting. (d) Total RNA was extracted, and mRNAs for various cytokines were analyzed by RT-PCR using specific primers. (e) Cells were stimulated with TNF- α (10 ng ml⁻¹) and IFN- γ (10 ng ml⁻¹) for 24 hours. The culture medium of cells was harvested, after which protein production was measured by ELISA. Lanes are as follows: lane 1, control; lane 2, TNF- α (10 ng ml⁻¹) and IFN- γ (10 ng ml⁻¹)-treated cells; lane 3, PEP-1-FK506BP (0.1 μ M)-treated cells; lane 4, PEP-1-FK506BP (0.3 μ M)-treated cells; lane 5, PEP-1-FK506BP (0.5 μ M)-treated cells; lane 6, FK506BP (0.5 μ M)-treated cells. EMSA, electromobility shift assay; ERK, extracellular signal-related kinase; FK506BP, FK506-binding protein 12; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MDC, macrophage-derived chemokine; RT-PCR, reverse transcription-PCR; TNF- α , tumor necrosis factor- α .

protective effects of PEP-1-FK506BP against AD to clarify the biological function and molecular mechanism of the FK506BP protein in AD. Many studies, including those performed by our group, have demonstrated that protein transduction is an effective protein therapeutic strategy. However, it has been claimed that cell fixation should be

avoided in studies of protein transduction into cells because protein transduction domains are an artifactual redistribution caused by cell fixation (Richard *et al.*, 2003). In this study, no differences were evident in the fluorescence distribution of transduced PEP-1-FK506BP in unfixed and fixed cells. These results demonstrate that cell fixation with paraformaldehyde

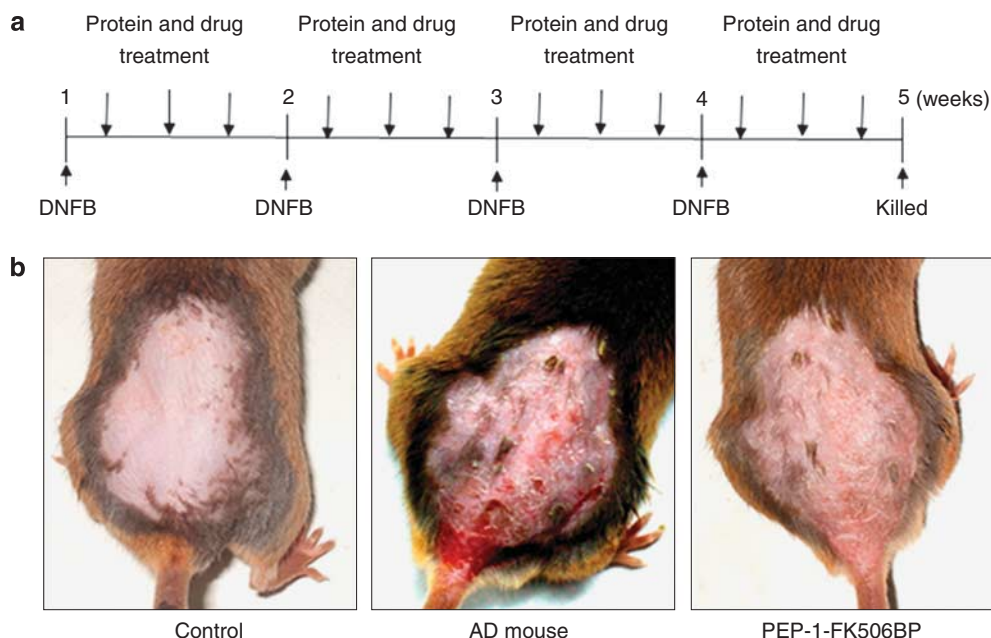


Figure 3. Schedule for DNFB-induced atopic dermatitis in NC/Nga mice and administration of PEP-1-FK506BP. (a) DNFB (0.15%, 150 μ l) in acetone/olive oil (3:1) was applied to denuded sections of the back of mice four times at 7-day intervals. PEP-1-FK506BP fusion proteins (5 μ M) or the prototypic compound were topically applied to the same area of mice three times a week for 4 weeks. (b) Macroscopic observations of skin lesions in PEP-1-FK506BP fusion protein-treated NC/Nga mice. DNFB, 2,4-dinitrofluorobenzene; FK506BP, FK506-binding protein 12; NC/Nga, Nishiki-nezumi Cinnamon/Nagoya.

is not required for PEP-1-FK506BP transduction. In addition, purified PEP-1-FK506BP was efficiently transduced into HaCaT cells.

Both TNF- α and IFN- γ synergistically induce cytokine production in human keratinocytes and in HaCaT cells, which might have a role in the development of AD (Vestergaard *et al.*, 2000). Appropriately, the role of PEP-1-FK506BP in suppressing the production of TARC, CXCL9, CXCL10, MDC, IL-6, and IL-1 β in TNF- α /IFN- γ -stimulated HaCaT cells was examined. Transduced PEP-1-FK506BP significantly suppressed TNF- α /IFN- γ -induced cytokine and chemokine expression levels in a dose-dependent manner. NF- κ B is a transcription factor that controls a number of genes that are important for immunity and inflammation (Barnes and Karin, 1997; Yun *et al.*, 2008). The p50/p65 heterodimer is the most common dimer found in the NF- κ B signaling pathway (Verma *et al.*, 1995). Activation of the NF- κ B signaling cascade results in phosphorylation-mediated degradation of I κ B. The present results offer firm evidence that transduced PEP-1-FK506BP inhibits phosphorylation and subsequent degradation of I κ B. PEP-1-FK506BP suppressed p65 NF- κ B translocation to the nucleus and modulated binding activity. Previous studies have determined that TNF- α /IFN- γ stimulation of keratinocytes activates several intracellular signaling pathways, such as the extracellular signal-related kinase-1/2, JNK, and p38 MAPK pathways. Appropriately, the signal pathway in TNF- α /IFN- γ -stimulated HaCaT cells was examined. Transduced PEP-1-FK506BP inhibited TNF- α /IFN- γ -induced phosphorylation of p38 and JNK, but not that of extracellular signal-related kinase. Lee *et al.* (1994) demonstrated that the p38 kinase acts as a specific target for a class of cytokine-suppressive anti-

inflammatory drugs, which to our knowledge is previously unreported, and has a key role in this regulation. Both the previous and the present results support the suggestion that the NF- κ B and MAPK (p38 and JNK) pathways are capable of modulating the suppression of TNF- α /IFN- γ -induced cytokine and chemokine expressions by PEP-1-FK506BP.

The NC/Nga mouse model is well known as one of the most valuable mouse models representing human AD. In addition, this model has been used for the evaluation of drug candidates developed for treatment of AD (Sasakawa *et al.*, 2001; Matsuoka *et al.*, 2003; Gao *et al.*, 2004). The current results clearly demonstrate that repeated topical application of DNFB results in AD-like skin lesions in NC/Nga mice. However, topical application of PEP-1-FK506BP significantly inhibits AD-like skin lesions induced by DNFB. These results suggest that PEP-1-FK506BP may be a promising topical agent for patients with AD.

The pathogenesis of AD causes systemic immunological dysfunction that leads to Th1 and Th2 imbalance, which can differently alter the secretion of cytokines (Leung *et al.*, 2004; Allam *et al.*, 2005; Boguniewica and Leung, 2006). Therefore, it is important to consider the balance of Th1 and Th2 in AD patients. It is well known that AD is a chronically relapsing inflammatory skin disorder, and the clinical phenotype of AD has been classified into extrinsic and intrinsic types, so called allergic and non-allergic types, respectively, because AD has various causes and aspects of pathogenesis. In addition, the incidence of extrinsic and intrinsic AD was ~80 versus 20% in many countries (Novak and Bieber, 2003; Tokura, 2010). In general, intrinsic AD is well characterized by normal IgE levels, lower expression of IL-4 and IL-5, normal skin-barrier functions, and not

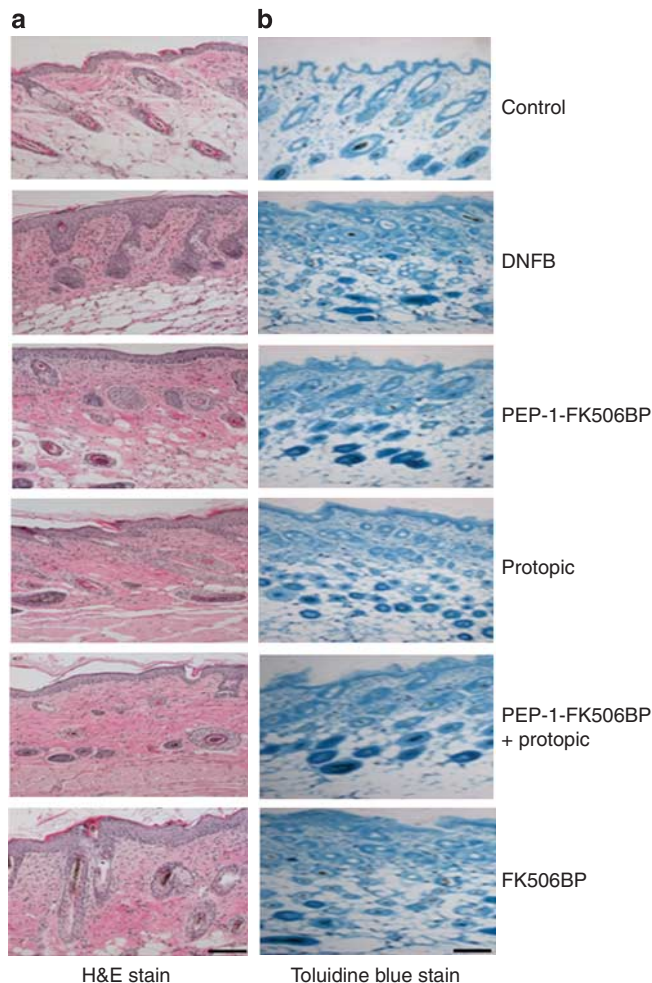


Figure 4. Histological features of NC/Nga mice treated with PEP-1-FK506BP. Atopic dermatitis was induced by repeated topical application of DNFB with or without pretreatment with PEP-1-FK506BP. Tissue samples were excised from the back of each mouse after 4 weeks. Tissue sections were stained with (a) hematoxylin and eosin and (b) toluidine blue. Bars = 100 μ m. DNFB, 2,4-dinitrofluorobenzene; FK506BP, FK506-binding protein 12; H&E, hematoxylin and eosin; NC/Nga, Nishiki-nezumi Cinnamon/Nagoya.

sensitized with allergens. Although the intrinsic types of AD share features of extrinsic AD, it is different from extrinsic AD (Tokura, 2010). However, a majority of AD have been associated with extrinsic types of AD because of allergic factors. Furthermore, AD patients have increased expression of Th2 cytokines (TARC, MDC, IL-4, and IL-5) and increased serum IgE levels (Yamada *et al.*, 1995; Wollenberg *et al.*, 2000; Abramovits, 2005; Beltrani, 2005). Tanaka *et al.* (2007) suggested that topical application is preferred over oral administration for treatment of AD to avoid any unexpected side effects. Other studies have also shown that an ointment applied locally to skin lesions in AD animal models markedly improved the clinical symptoms of severe AD without any significant side effects (Tanaka *et al.*, 2007; Oshio *et al.*, 2009). This study examined the effects of PEP-1-FK506BP on the expression of Th2 cytokine expression levels and serum IgE levels in NC/Nga mice. PEP-1-FK506BP markedly inhibited DNFB-induced IgE levels and Th2 cytokine levels

in NC/Nga mice. Kawasaki *et al.* (2001) demonstrated that TARC antibody therapy reduces the development of allergic airway inflammation and hyperresponsiveness. Thus, TARC may be an important mediator that exaggerates AD. These results indicate that Th2 cytokines (TARC, MDC, IL-4, and IL-5) participate in the pathogenesis of AD-like lesions in NC/Nga mice. In addition, these cytokines could be targets for therapy of AD. Therefore, we suggest that PEP-1-FK506BP may have a potential therapeutic treatment of AD.

In summary, this study demonstrates that transduced PEP-1-FK506BP significantly inhibits the expression of cytokines, as well as the activation of NF- κ B and MAPK in HaCaT cells. Furthermore, topical application of PEP-1-FK506BP to NC/Nga mice markedly inhibits AD-like skin lesions, serum IgE levels, and the expression of cytokines. Therefore, PEP-1-FK506BP may be a potential therapeutic agent for AD.

MATERIALS AND METHODS

Expression and purification of the PEP-1-FK506BP fusion protein

A PEP-1 expression vector was prepared as described previously (Eum *et al.*, 2004). On the basis of the cDNA sequence of human FK506BP, sense and antisense primers were synthesized and PCR was performed. The primer sequences used are listed in Supplementary Table S1 online. The PCR product was ligated into a TA-cloning vector and a PEP-1 vector using T4 DNA ligase, and cloned in *Escherichia coli* DH5 α cells. In addition, a control FK506BP was constructed that expressed the FK506BP fusion protein without PEP-1.

To produce PEP-1-FK506BP fusion proteins, a plasmid was transformed into *E. coli* BL21 cells and grown in 100 ml of Luria-Bertani media at 37°C to an optimal density at a 600 nm value of 0.5–1.0 and induced with 0.5 mM isopropyl- β -D-thio-galactoside at 37°C for 3–4 hours. Harvested cells were lysed by sonication at 4°C, and clarified cell extracts were purified using a nickel ion-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography according to the manufacturer's instructions. Protein concentration was estimated by the procedure by Bradford (1976) using bovine serum albumin as a standard.

Cell culture and transduction of the PEP-1-FK506BP fusion protein

HaCaT cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 μ g ml⁻¹ streptomycin and 100 Units ml⁻¹ penicillin) at 37°C under humidified conditions of 95% air and 5% CO₂. For transduction of PEP-1-FK506BP, HaCaT cells were grown to confluence in wells of a 6-well plate. After cells were treated with various concentrations of PEP-1-FK506BP for 1 hour, they were treated with trypsin-EDTA and washed with phosphate-buffered saline (PBS). Cells were harvested for preparation of cell extracts to perform western blot analysis.

Fluorescence analysis

After purification of PEP-1-FK506BP, FITC labeling was carried out according to the manufacturer's instructions (Pierce, Rockford, IL), and cells were grown on glass coverslips and treated with 0.5 μ M of PEP-1-FK506BP fusion proteins. After incubation for 1 hour at 37°C, cells were washed twice with PBS and trypsin-EDTA, and fixed with

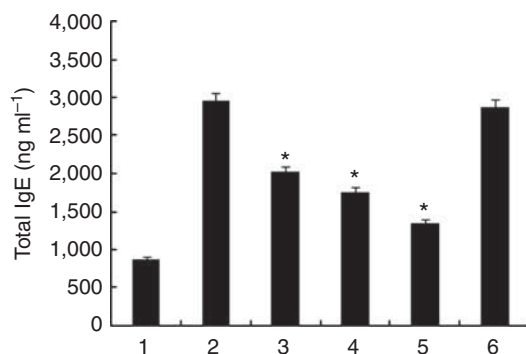


Figure 5. Inhibitory effect of PEP-1-FK506BP on serum IgE levels in NC/Nga mice. AD was induced by repeated topical application of DNFB with or without pretreatment with PEP-1-FK506BP. The serum levels of IgE in NC/Nga mice were measured by ELISA. * $P < 0.05$ compared with mice treated with DNFB alone. Lanes are as follows: lane 1, control; lane 2, DNFB; lane 3, PEP-1-FK506BP; lane 4, protopic; lane 5, PEP-1-FK506BP + protopic; lane 6, FK506BP. AD, atopic dermatitis; DNFB, 2,4-dinitrofluorobenzene; FK506BP, FK506-binding protein 12; NC/Nga, Nishiki-nezumi Cinnamon/Nagoya.

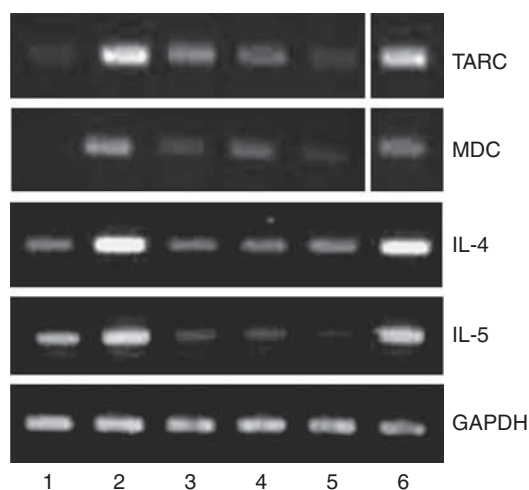


Figure 6. Inhibitory effect of PEP-1-FK506BP on DNFB-induced cytokines in NC/Nga mice. AD was induced by repeated topical application of DNFB with or without pretreatment with PEP-1-FK506BP. Total RNA was extracted from lymph nodes in NC/Nga mice. Cytokine mRNA was analyzed by RT-PCR using specific primers. Lanes are as follows: lane 1, control; lane 2, DNFB; lane 3, PEP-1-FK506BP; lane 4, protopic; lane 5, PEP-1-FK506BP + protopic; lane 6, FK506BP (lane 6 of TARC and MDC were not run consecutively). AD, atopic dermatitis; DNFB, 2,4-dinitrofluorobenzene; FK506BP, FK506-binding protein 12; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MDC, macrophage-derived chemokine; NC/Nga, Nishiki-nezumi Cinnamon/Nagoya; RT-PCR, reverse transcription-PCR; TARC, thymus and activation-regulated chemokine.

4% paraformaldehyde for 10 minutes at room temperature. The distribution of fluorescence was analyzed using a fluorescence microscope (Carl Zeiss, Jena, Germany).

Flow cytometry

HaCaT cells (2×10^5 cells per well) were plated in wells of 6-well plates and grown to 90% confluence. PEP-1-FK506BP was added to the culture medium for 1 hour. After removing the culture medium,

cells were treated with trypsin and washed with ice-cold PBS to remove nonspecific extracellular proteins bound to the cell surface. Cells were suspended in 0.5 ml PBS and analyzed for fluorescence using Guava Easy Cyte (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. A total of 10,000 cells were analyzed for each sample.

Subcellular fractionation of transduced cells

Nuclear and cytosolic fractions were prepared as described previously (Nare *et al.*, 1999; Park *et al.*, 2002). Transduced HaCaT cells were washed with PBS, acid washed with 0.2 M glycine-HCl, pH 2.2, and trypsinized for 10 minutes at 37°C. Cells were harvested after washing with cold PBS and pelleted. Cells were then resuspended in 1 ml of NP-40 buffer by gentle pipetting and incubated on ice for 10 minutes. Cells were spun through a sucrose cushion at 1,000 g for 10 minutes and cytosolic fractions collected from the supernatants. Pellets were washed with 1 ml NP-40 buffer to completely remove cytosolic fractions. The nuclei were lysed in a lysis buffer (50 ml Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, $100 \mu\text{g ml}^{-1}$ phenylmethylsulfonyl fluoride, 1% Triton X-100). The resulting nuclear and cytosolic lysates were analyzed by western blotting.

Western blot analysis

Sample proteins were electrophoretically transferred to a nitrocellulose membrane, after which the membrane was blocked in 5% nonfat milk in Tris-buffered saline (20 mM Tris, 0.2 M NaCl, pH 7.5) containing 0.05% Tween-20 for 2 hours. The membrane was incubated for 1 hour at room temperature with anti-histidine antibody (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:400) in Tris-buffered saline containing Tween-20. After washing, the membrane was incubated for 1 hour with a proper secondary antibody conjugated to horseradish peroxidase diluted 1:10,000 in Tris-buffered saline containing Tween-20. The membrane was incubated with a chemiluminescent substrate and exposed to Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ). The same membrane was stripped and re-probed with an anti-actin antibody (cytosolic marker; Oncogene, Cambridge, MA) or an anti-poly (ADP-ribose) polymerase antibody (nuclear marker; Biomol, Plymouth Meeting, PA).

Measurement of protein production

HaCaT cells were exposed to $\text{TNF-}\alpha$ (10 ng ml^{-1}) and $\text{IFN-}\gamma$ (10 ng ml^{-1}) in the presence or absence of PEP-1-FK506BP for 24 hours, and protein levels of cytokine and chemokine in the supernatants were measured using ELISA kits (R&D Systems, Minneapolis, MN).

Electromobility shift assay

HaCaT cells were pretreated with PEP-1-FK506BP for 60 minutes before $\text{TNF-}\alpha$ (10 ng ml^{-1}) and $\text{IFN-}\gamma$ (10 ng ml^{-1}) treatment for 15 minutes, after which nuclear extracts of HaCaT cells were prepared and analyzed for NF- κB -binding activity by electromobility shift assay as described previously (Song *et al.*, 2008). An NF- κB consensus oligonucleotide (Promega, Madison, WI) was used in the assay. The complementary oligonucleotide was annealed and end labeled with $[\gamma\text{-}^{32}\text{P}]$ ATP using T4 polynucleotide kinase. The electromobility shift assay was performed in a total volume of

40 µl at 37°C. Overall, 10 µg of nuclear extracts was equilibrated for 15 minutes in a binding buffer (10 mM Tris-HCl, pH 8.0, 75 mM KCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 0.25 mM dithiothreitol) and 1 µg of poly dI/dC. A ³²P-labeled oligonucleotide probe (20,000 c.p.m.) was then added and the reaction incubated at 37°C for an additional 30 minutes. Both bound and free DNA were then resolved by electrophoresis on a 6% native polyacrylamide gel in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA).

Reverse transcription-PCR analysis

After total RNA was isolated from cells using a Trizol reagent kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, RNA (2 µg) was reversibly transcribed with reverse transcriptase and oligo-(dT) primer. PCR amplification of TARC, CXCL9, CXCL10, MDC, IL-1β, IL-6, and β-actin cDNA aliquots was performed with the following sense and antisense primers. All the primer sequences used are listed in Supplementary Table S1 online. PCR was performed using a PCR premix kit (Intron Biotechnology, Seoul, Korea) for each primer and was terminated by heating to 72°C for 5 minutes. PCR products were resolved on a 1% agarose gel and visualized with ultraviolet light after ethidium bromide staining.

Induction and histology of AD-like skin lesions

Female 6-week-old NC/Nga mice were purchased from the Joong-Ang Experimental Animal Center (Seoul, Korea). All experimental procedures involving animals and their care conformed to the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research and Quarantine Service of Korea and were approved by the Hallym Medical Center Institutional Animal Care and Use Committee (Permit number: Hallym 2009-30). AD was induced by the topical application of 150 µl of 0.15% DNFB in acetone/olive oil (3:1) to the hair-removed back of mice four times at 7-day intervals. PEP-1-FK506BP (5 µM) and protopic were topically applied to the same area on mice three times per week for 4 weeks. Mice (*n* = 20 per group) were killed and blood was collected from the vena cava. Skin tissues from the backs of mice were excised and subjected to histological examination. The serum levels of IgE in NC/Nga mice were quantified using a mouse IgE ELISA (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's instructions.

For histological analysis, skin biopsies were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin and toluidine blue. Stained tissue sections were examined to analyze the infiltration of inflammatory cells using standard bright-field optics (Carl Zeiss).

Statistical analyses

Data are presented as mean ± SD. Comparison between the groups was made with analysis of variance, followed by Dunnett's test. A value of *P* < 0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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Supplemental Table S1. Primers for PCR and RT-PCR analysis

FK506BP	Sense	CTCGAGATGGGAGTCCAGGAAACCATC
	Antisense	GGATCCTCATTCCAGTTTTAGAAAGCTCCAC
TARC	Sense	CTTCTCTGCAGCACATCC
	Antisense	AAGACCTCTCAAGGCTTTG
CXCL9	Sense	TGCAAGGAACCCAGTAGTGA
	Antisense	GGTGGATAGTCCCTTGGTTGG
CXCL10	Sense	GAACCTCCAGTCTCAGCACC
	Antisense	GCTCCCCTCTGGTTTTAAGGAGAT
MDC	Sense	AGGACAGAGCATGGCTCGCCTACAGA
	Antisense	TAATG GCAGGGAGGTAGGGCTCCTGA
IL-1 β	Sense	AAAAGCTTGGTGATGTCT GG
	Antisense	TTTCAACACGCAGGACAGG
IL-4	Sense	GCTTCCAAGGTGCTTCGCAT
	Antisense	CATTTGCATGATGCTCTTTAGGCT
IL-5	Sense	CAAAAAGAG AAGTGTGGCGAGG
	Antisense	TAGATAGGAGCAGGAAGCCCCG
IL-6	Sense	AGAGT AGTGAGGAACAAGCC
	Antisense	TACATTTGCCGAAGAGCCCT
β -actin	Sense	GCGGGAAATCGTGCGTGACATT
	Antisense	GATGGAGTTGAAGGTAGTTTCGTG