

Capsiate Inhibits DNFB-Induced Atopic Dermatitis in NC/Nga Mice through Mast Cell and CD4+ T-Cell Inactivation

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Capsaicin has many biological effects, such as antioxidant, anticancer, and antiangiogenic effects, but it is rarely used because of its high pungency. Capsiate, a nonpungent capsaicin analog, also has multiple biological effects, similar to those of capsaicin, but does not cause irritation. However, the effect of capsiate on allergic responses and immune cells has not been well studied. In this study, we investigated the effect of capsiate on atopic dermatitis, mouse CD4+ T cells, and mast cell activation. Capsiate inhibited DNFB-induced atopic dermatitis in NC/Nga mice. Topical treatment with capsiate suppressed serum IgE levels and cytokine and chemokine expression in the skin of DNFB-treated NC/Nga mice. In addition, it suppressed the activation of CD4+ T cells and mast cells, which are implicated in allergic diseases. Capsiate inhibited the differentiation of naïve CD4+ T cells into T helper type 1 (Th1), Th2, and Th17 cells. Treatment with capsiate inhibited the expression of pro-inflammatory cytokines and degranulation from activated bone marrow-derived mast cells through the inhibition of extracellular signal-regulated kinase signal pathways. Consistent with these results, treatment with capsiate inhibited passive cutaneous anaphylaxis. Taken together, our results suggest that capsiate might be a good candidate molecule for the treatment of allergic diseases such as atopic dermatitis.

Journal of Investigative Dermatology advance online publication, 7 May 2015; doi:10.1038/jid.2015.117

INTRODUCTION

Capsaicin exhibits antioxidant, anticancer, and antiangiogenic activity (Murakami *et al.*, 2001; Min *et al.*, 2004). Despite these beneficial effects, capsaicin is rarely considered for use as an antioxidant or anticancer drug because of its high pungency that leads to irritation. However, capsiate (4-hydroxy-3-methoxybenzyl (E)-8-methyl-6-nonenoate), isolated from the fruit of a cultivar of *Capsicum annuum* L.(CH-19 Sweet; Sundelin *et al.*, 2005), is a nonpungent capsaicin analog with an ester bond instead of the amide bond between the vanillyl moiety and the fatty acid chain. Importantly, capsiate has multiple biological effects without causing irritation, including an anti-inflammatory effect (Lee *et al.*, 2010). Treatment with capsiate inhibits the expression of UV-induced pro-inflammatory mediators in keratinocytes. Furthermore, topical treatment with capsiate inhibits UVB-induced cyclooxygenase-2 and pro-inflammatory cytokine expression in mouse dorsal skin. Capsiate also has an antiangiogenic effect (Pyun *et al.*, 2008; Lee *et al.*, 2010). Topical treatment with capsiate inhibits the expression of

UVB-induced pro-angiogenic factors (Lee *et al.*, 2010) and blocks tumor-induced and vascular endothelial growth factor-induced angiogenesis (Pyun *et al.*, 2008).

Immune cells are highly involved in inflammation. However, the effects of capsiate on T cells, mast cells, and allergic responses, which are important in the development of allergic inflammation, have not been well studied. Atopic dermatitis (AD) and asthma are allergic diseases that are induced by activated T helper type 2 (Th2) cells and mast cells. Mast cells are activated by the binding of allergen-bound IgE to the FcεRI (Rivera, 2002; Kalesnikoff and Galli, 2008); subsequently, activated mast cells release many biologically active products, such as pre-stored mediators, including histamine, and newly synthesized mediators, including cytokines (IL-4, IL-5, and IL-13, and tumor necrosis factor-α) and arachidonic acid metabolites (leukotrienes and prostaglandins; Theoharides *et al.*, 2007). These released mediators cause allergic inflammatory diseases such as asthma. Thus, the inhibition of mast cell activation or mediators from activated mast cells is the main method for treating or preventing allergic diseases (Rivera and Gilfillan, 2006; Kraft and Kinet, 2007).

CD4+ T cells are also key factors in allergic inflammatory diseases. Th2 cells produce IL-4, which induces B-cell activation and class switching to IgE; increased IL-4 production or induction of the IL-4 signaling pathway causes allergic diseases (Hershey *et al.*, 1997). Recently, it was also reported that IL-17, which is produced by Th17 cells, is implicated in

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Received 19 June 2014; revised 22 December 2014; accepted 6 January 2015; accepted article preview online 25 March 2015

asthma. The messenger RNA and protein levels of IL-17 are increased in the lungs, sputum, bronchoalveolar lavage fluids, and sera of asthmatic patients (Molet *et al.*, 2001; Wong *et al.*, 2001; Chakir *et al.*, 2003; Bullens *et al.*, 2006). Furthermore, Th17 cells are involved in the pathogenesis of AD (Koga *et al.*, 2008). The percentage of Th17 cells was increased in peripheral blood of AD patients, and the severity of AD was also associated with the Th17 cell percentage. Therefore, it seems that IL-17 and Th17 cells are involved not only in autoimmune diseases such as rheumatoid arthritis and psoriasis (Hirota *et al.*, 2007; Lowes *et al.*, 2008) but also in the development of various allergic diseases.

NC/Nga mice are normal when they are kept in pathogen-free conditions. However, repeated treatment with DNFB in the shaved skin of NC/Nga mice induces symptoms of AD-like skin inflammation, such as increased epidermal thickness, enhanced chemokine expression, high levels of serum IgE, and upregulation of Th2 cytokines (Tomimori *et al.*, 2005; Jin *et al.*, 2009; Kim *et al.*, 2011). Thus, DNFB-induced AD is a well-known mouse model of AD.

In the present study, we investigated the anti-allergic effect of capsiate using DNFB-induced AD-like skin inflammation in NC/Nga mice. Our results show that capsiate inhibited DNFB-induced AD-like skin inflammation in NC/Nga mice. In addition, capsiate was also shown to inhibit passive cutaneous anaphylaxis (PCA) in mice. Furthermore, we demonstrated the inhibitory effect of capsiate on mast cell activation as well as on CD4+ T-cell activation and differentiation, suggesting that the anti-allergic effect of capsiate might result from the inhibition of mast cell and CD4+ T-cell activation.

RESULTS

Treatment with capsiate inhibited DNFB-induced AD-like skin inflammation in Nc/Nga mice

To determine whether capsiate could inhibit AD in mice, we induced AD-like skin inflammation with DNFB with or without capsiate treatment (Figure 1a). Figure 1b shows that treatment with capsiate suppressed DNFB-induced AD-like skin inflammation. A histological examination also showed that epidermal thickness was reduced upon treatment with capsiate (Figures 1c and d). In addition, the number of mast cells and CD4+ T cells in the skin also decreased after treatment with capsiate (Figure 1e; Supplementary Figure S1 online). Eosinophil infiltration is also involved in AD pathogenesis, and thus the eosinophil number in mouse skin was determined. Figure 1f shows that the eosinophil number reduced in mouse skin treated with capsiate.

One of the major characteristics of AD is the increased level of serum IgE. Thus, we determined the level of serum IgE using an ELISA. DNFB-treated Nc/Nga mice showed increased IgE levels. However, capsiate-treated Nc/Nga mice showed decreased serum IgE levels, suggesting that capsiate could inhibit AD (Figure 2a). It is also known that the expression levels of cytokines, such as IL-4 and thymic stromal lymphopoietin (TSLP), are increased in AD skin (Homey *et al.*, 2006). Therefore, we determined the levels of IL-4 and TSLP in mouse skin using a real-time PCR experiment. Figure 2b shows that the gene expression levels

of IL-4 and TSLP increased in DNFB-treated mouse skin but decreased in capsiate-treated skin (Figure 2b).

Increased chemokine expression is also a characteristic of AD skin lesions because chemokines recruit immune cells, including CD4+ T cells, into skin lesions. Thus, we investigated the expression levels of CCL11, CCL17, and CCL22 genes in mouse skin and found that treatment with capsiate suppressed these gene expression levels, which were elevated in the mouse skin with DNFB-induced AD-like skin inflammation (Figure 2c). These results confirmed that capsiate could inhibit DNFB-induced skin inflammation.

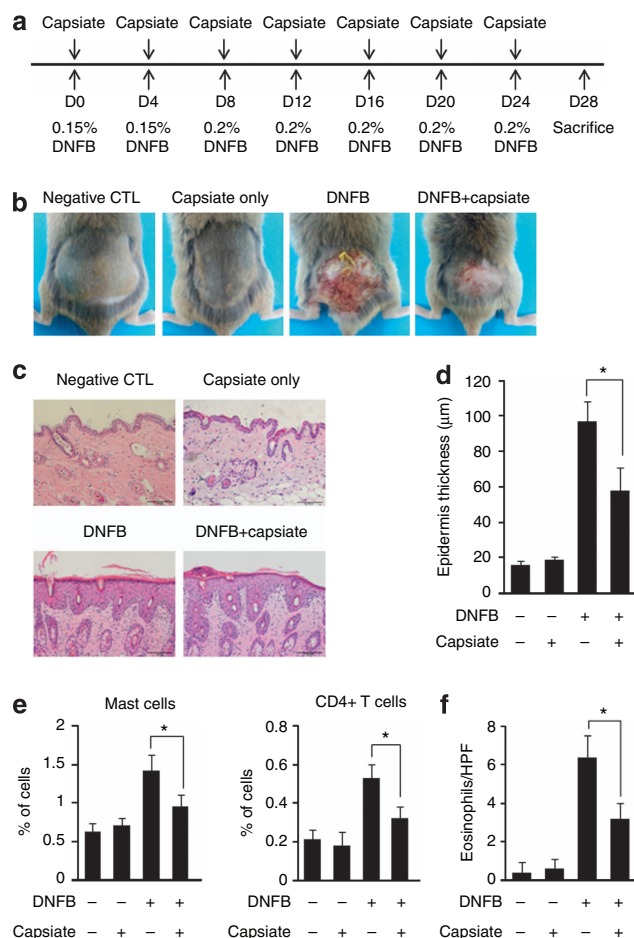


Figure 1. Treatment with capsiate inhibits DNFB-induced AD-like skin inflammation in NC/Nga mice. (a) Experimental scheme for the DNFB-induced AD model. (b) The photographs were taken on day 28. (c) Histology of back skin. Sections recovered from negative control, DNFB-treated (positive control), and DNFB- and capsiate-treated mice were stained with hematoxylin and eosin. Scale bar = 100 μm. (d) The epidermal thickness of the back skin in DNFB/capsiate-treated mice was less than that in DNFB-treated positive control mice. The epidermal thickness of capsiate only-treated mouse skin did not increase. Data represent mean ± SE. (e) Percentages of mast cells (FcεRI+ and CD117+) and CD4+ T cells in skins were determined by flow cytometry after single cell suspension and staining with fluorescence tagged antibodies. (f) Eosinophils were counted in five high-power fields (HPF). Data represent mean ± SE. Data are from three independent experiments, with four mice per group. *P < 0.05. AD, atopic dermatitis.

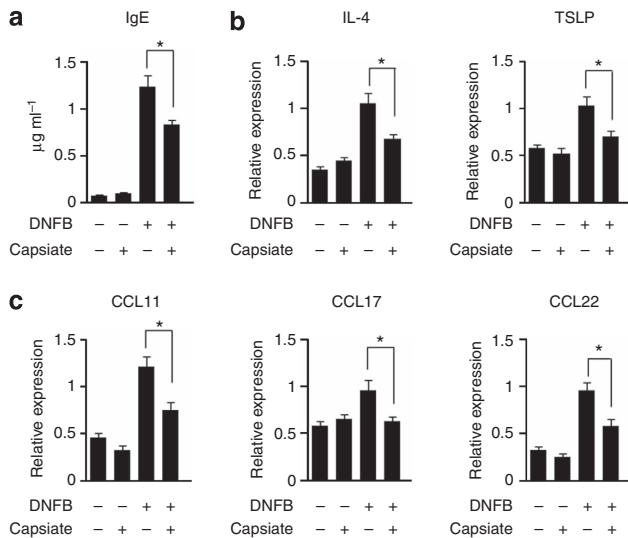


Figure 2. Treatment with capsiate causes decreased levels of serum IgE, cytokine expression, and chemokine expression. (a) Serum IgE level was measured using ELISA as described in the Materials and Methods section. Expression levels of (b) cytokines and (c) chemokines in AD-induced NC/Nga mouse skin were determined by real-time PCR. Data represent mean $\pm$ SE. A representative example of three independent experiments is shown. * $P < 0.05$. AD, atopic dermatitis; TSLP, thymic stromal lymphopoietin.

As it is well known that Th2 cells are implicated in allergic disorders, we determined the percentages of Th2 cells, secreting IL-4 or IL-5, in draining lymph nodes. Figure 3 shows that when AD-like skin inflammation was induced by DNFB, the percentages of IL-4-expressing CD4⁺ T cells and IL-5-expressing CD4⁺ T cells in draining lymph nodes increased to 12.38% and 6.00%, respectively, whereas in the lymph nodes of normal NC/Nga mice 2.57% of the CD4⁺ T cells expressed IL-4 and 0.26% expressed IL-5. On the other hand, capsiate-treated mice showed decreased percentages of IL-4- and IL-5-expressing CD4⁺ T cells (3.94% and 1.15%, respectively) compared with positive control mice.

Recently, it was reported that Th17 cells are also involved in the pathogenesis of AD (Koga *et al.*, 2008). Thus, we determined the percentages of Th17 cells and Th1 cells. Levels of Th17 cells were increased in DNFB-treated mice (6.40%) but Th1 cell levels were only slightly increased (3.07%). Furthermore, capsiate inhibited Th17 cell differentiation in draining lymph nodes of DNFB-treated mice (Figure 3). These results imply that DNFB-induced AD-like skin inflammation may be caused at least by Th2 and Th17 cells, and that capsiate inhibits the AD-like skin inflammation through suppression of Th2 and Th17 cell differentiation. To determine the effect of capsiate itself in unstimulated NC/Nga mice, capsiate was used for treatment without DNFB stimulation. The results showed that capsiate does not have an effect on unstimulated NC/Nga mice.

Capsiate inhibits mast cell activation

Mast cells are one of the major cell types involved in an allergic response. However, there are no reports on the effects

of capsiate on mast cells or the allergic response. To determine the effect of capsiate on mast cell activation, we generated bone marrow-derived mast cells (BMMCs) from mice, as described in the Materials and Methods section (Figure 4a). Activated mast cells express and produce pro-inflammatory cytokines such as IL-1 β , IL-6, IL-17, and tumor necrosis factor- α . Therefore, we determined the levels of pro-inflammatory cytokines in activated BMMCs that were pretreated or not with capsiate. The results showed that pretreatment with capsiate inhibited pro-inflammatory cytokine expression without cytotoxicity (Figure 4b; Supplementary Figure S2A online).

TRPV1 is the receptor of capsiate. Therefore, we determined whether the knockdown expression of TRPV1 in mast cells abrogates the inhibitory effect of capsiate. The inhibitory effect of capsiate was only partially suppressed after transfection of TRPV1 small interfering RNA, probably because transcripts of TRPV1 were not perfectly knocked down or another receptor might be involved in the inhibitory effect of capsiate on mast cell activation (Supplementary Figures S3A and B online).

Capsiate inhibits the extracellular signal-related kinase signal pathway in activated BMMCs

Many signal pathways, such as Syk, PI3K, Akt, and extracellular signal-regulated kinase (ERK), are involved in mast cell activation. To determine which signal pathways are inhibited by capsiate in activated mast cells, BMMCs were activated by dinitrophenol (DNP)-IgE and DNP-human serum albumin (HSA), and treated with or not treated with capsiate. The activated mast cells were collected and stained for pSyk, pAkt, and pERK, followed by flow cytometry and FACS analysis. Figure 4c shows that treatment with capsiate inhibited the phosphorylation of ERK in the BMMCs (from 29.05 to 14.09%). The mean fluorescence intensity also decreased after treatment with capsiate (from 11.07 to 7.68). The results were confirmed by western blot analysis (Figure 4d). The phosphorylations of Syk and Akt were not affected by capsiate (data not shown). Therefore, the inhibition of mast cell activation by capsiate may be the result of suppressed ERK signal pathways.

Capsiate inhibits PCA in mice

We expect that capsiate has an anti-allergic effect *in vivo* because it inhibits mast cell activation. To determine whether capsiate inhibits allergic responses, we induced DNP-specific IgE-mediated PCA in mice. Mice were sensitized by the intradermal injection of DNP-IgE (100 ng per ear). The next day, capsiate was administrated 1 hour before intravenous injection of DNP-HSA (100 $\mu\text{g ml}^{-1}$) containing 4% Evan's blue (Figure 5a). Figure 5b shows that topical treatment with capsiate decreased the activated mast cell-mediated leakage of blood vessels, resulting in a decrease in the amount of diffused dye. Measurement of the extracted dye intensity showed that treatment with capsiate reduced dye intensity by 62.5%, compared with the untreated positive control group (Figure 5c). The increased ear thickness was also abrogated,

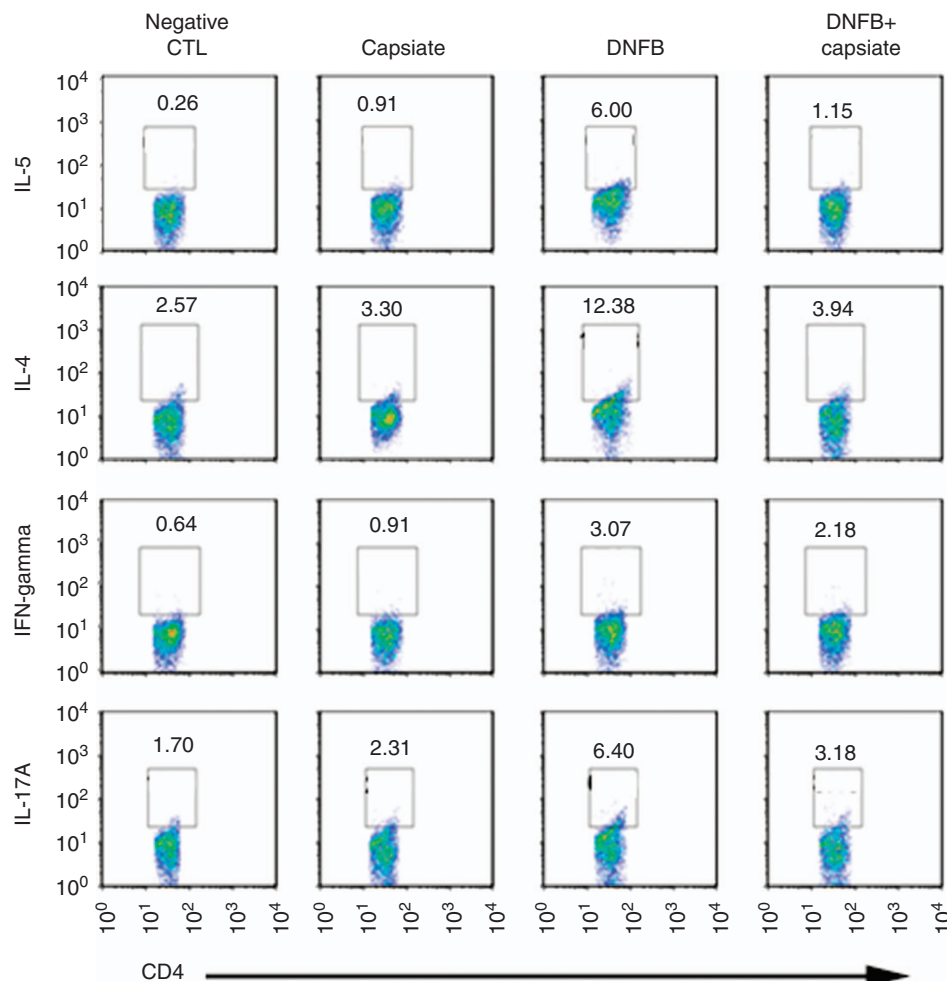


Figure 3. Treatment with capsiate inhibits Th2 and Th17 cell differentiation in draining lymph nodes of AD-induced NC/Nga mice. Development of Th2, Th17, and Th1 cells in draining lymph nodes was determined by intracellular staining and FACS analysis. A representative example of three independent experiments is shown. AD, atopic dermatitis; CTL, control.

from 10.7 to 4.2 μm (Figure 5d). These results suggest that capsiate has an anti-allergic effect through the inhibition of mast cell activation.

Capsiate inhibits CD4+ T-cell proliferation and differentiation

To investigate the role of capsiate on CD4+ T cells, a H^3 incorporation assay was performed. The results showed that treatment with capsiate ($>25 \mu\text{M}$) inhibited anti-CD3/anti-CD28 antibody-stimulated CD4+ T-cell proliferation (Figure 6a). Furthermore, an ELISA experiment also showed that the level of IL-2 in anti-CD3/anti-CD28 antibody-stimulated CD4+ T-cell culture supernatant decreased when $>25 \mu\text{M}$ capsiate was applied (Figure 6c).

To confirm the inhibitory activity of capsiate on CD4+ T-cell proliferation, we carried out a carboxyfluorescein succinimidyl ester (CFSE) dilution assay. Figure 6b shows that treatment with capsiate inhibited CD4+ T-cell proliferation, which is consistent with the results of the H^3 incorporation assay. Previously, we reported that capsiate could inhibit Lck

signaling in activated CD4+ T cells (Lee *et al.*, 2010). Thus, the inhibitory effect of capsiate on CD4+ T-cell proliferation might result from the suppression of the Lck signaling pathway.

CD4+ T cells are implicated in the pathogenesis of atopic dermatitis (Grewe *et al.*, 1998). Therefore, we determined whether capsiate could inhibit CD4+ T-cell differentiation. Naive CD4+ T cells were isolated from mouse spleen and lymph nodes as described in the Materials and Methods section, and cultured under Th1, Th2, and Th17 differentiation conditions. After 4 days in culture, the CD4+ T cells and culture supernatants were collected for real-time PCR and ELISA experiments, respectively. Real-time PCR results showed that treatment with $25 \mu\text{M}$ capsiate inhibited the expression of transcription factors, T-bet, GATA-3, and ROR γ T, which are necessary for Th1, Th2, and Th17 differentiation, respectively. In addition, the expression levels of the signature cytokines of Th1, Th2, and Th17 cells (IFN- γ , IL-4, and IL-17, respectively) were also inhibited when naive

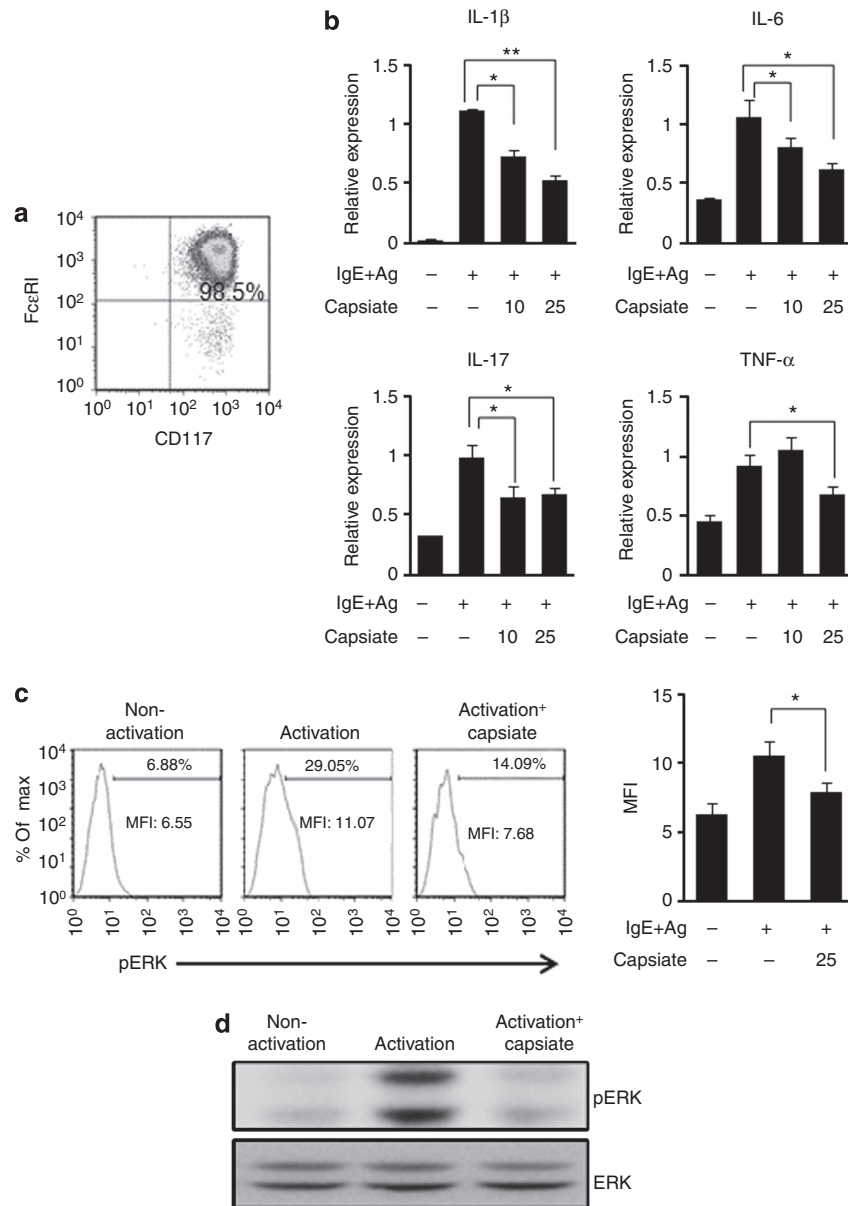


Figure 4. Capsiate inhibits mast cell activation and the ERK, extracellular signal-regulated kinase signal pathway. (a) BMMCs were generated from bone marrow cells from a C57BL/6 mouse as described in the Materials and Methods section. FACS analysis showed that >98% of the cells were FcεRI and CD117 double-positive (mast cells). (b) BMMCs were incubated with anti-DNP IgE for 24 hours. After washing, the cells were pretreated or not with capsiate before activation with DNP-HSA. Total RNA was isolated and the expression levels of IL-1β, IL-6, IL-17, and TNF-α were determined using real-time PCR. mGAPDH levels were used as an internal control. Data represent mean ± SE. (c) BMMCs were treated with anti-DNP IgE for 24 hours. After washing the cells, BMMCs were treated or not with capsiate for 30 minutes and the cells were activated with DNP-HSA. Thirty minutes after activation, the cells were collected and stained with FITC-labeled anti-pERK antibody. After washing the cells, flow cytometry and FACS analysis were performed. (d) pERK was determined by western blot analysis. pERK levels were increased in activated BMMCs, whereas treatment with capsiate inhibited pERK levels. The results are representative of three independent experiments. Ag, antigen; BMMC, bone marrow-derived mast cell; DNP, dinitrophenol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAS, human serum albumin; MFI, mean fluorescence intensity; TNF-α, tumor necrosis factor-α.

CD4⁺ T cells were cultured under Th1, Th2, and Th17 conditions, respectively, with capsiate (Figures 6e, g, and h). Consistently, ELISA showed that the released IL-17 and IFN-γ levels decreased upon capsiate treatment when naive CD4⁺ T cells were cultured under Th17 and Th1 conditions (Figures 6d and f). Interestingly, capsiate did not affect regulatory

T-cell differentiation (Figures 6i and j). We also showed that capsiate is not cytotoxic in CD4⁺ T cells (Supplementary Figure S2B online). Thus, these results suggest that capsiate might inhibit CD4⁺ T-cell-mediated diseases because of its inhibitory activity against CD4⁺ T-cell proliferation and differentiation.

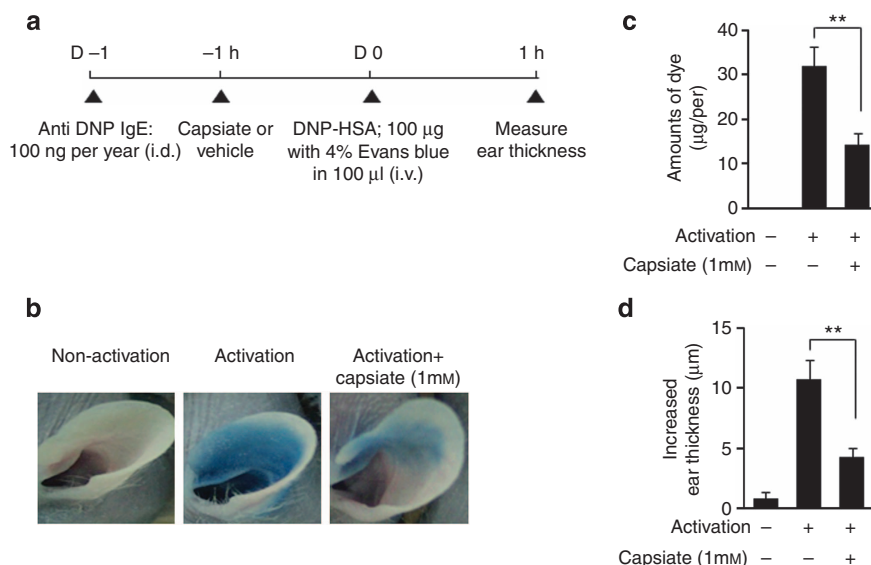


Figure 5. Capsiate inhibits passive cutaneous anaphylaxis (PCA) in mice. (a) Experimental scheme for the PCA experiment. Mice were passively sensitized with 100 ng anti-DNP IgE. Twenty-four hours after the intradermal injection of anti-DNP IgE, capsiate was applied to mouse ears 1 hour before the intravenous injection of 100 µg DNP-HSA containing Evans' blue dye (5%). One hour after DNP-HSA injection (b) images of the ears were taken, and (c) the amount of Evans blue dye and (d) increased ear thickness were measured. Data represent mean $\pm$ SE. The results are representative of two independent experiments. DNP, dinitrophenol; HAS, human serum albumin.

DISCUSSION

Capsaicin exhibits antioxidant, anticancer, and antiangiogenic effects (Murakami *et al.*, 2001; Min *et al.*, 2004). However, capsaicin has not been used as a drug because of its high pungency that leads to irritation. Capsiate, a nonpungent capsaicin analog, has similar biological effects to those of capsaicin without causing irritation. It has anti-inflammatory and antiangiogenic effects (Pyun *et al.*, 2008; Lee *et al.*, 2010). Both capsaicin and capsiate have been reported to have inhibitory effects on T cells. Capsaicin inhibits Jurkat T-cell activation (Fischer *et al.*, 2001) and capsiate inhibits T-cell and NF- κ B activation (Sancho *et al.*, 2002). However, the effects of capsiate on immune cells have not been well studied. In this study, we demonstrated the effects of capsiate on CD4⁺ T cells and mast cells. Our results show that capsiate could inhibit CD4⁺ T-cell proliferation. This result suggests that capsiate could be used as an immunosuppressive agent similar to cyclosporine to inhibit immune cell-mediated diseases, although more detailed experiments are needed to confirm this hypothesis.

Our results also showed that treatment with capsiate inhibited Th1, Th2, and Th17 cell differentiation, probably via the inhibition of CD4⁺ T-cell activation. CD4⁺ T-cell subsets are involved in the pathogenesis of many diseases. For example, Th17 cells are involved in the pathogenesis of autoimmune diseases, including rheumatoid arthritis (Hirota *et al.*, 2007), psoriasis (Lowes *et al.*, 2008), multiple sclerosis (Tzartos *et al.*, 2008), and uveitis (Amadi-Obi *et al.*, 2007). On the other hand, Th2 cells have been implicated in the pathogenesis of allergic diseases, such as asthma (Ngoc *et al.*, 2005). Therefore, it would be worthwhile to investigate

whether capsiate can inhibit CD4⁺ T-cell-mediated diseases, specifically autoimmune and/or allergic diseases.

We also demonstrated that capsiate inhibits mast cell activation. Mast cells are critical for the induction of allergic diseases, such as asthma, allergic rhinitis, and atopic dermatitis. Mast cells are localized in many tissues, and activation of mast cells induces the release of histamine, tryptase, and pro-inflammatory cytokines, resulting in allergic responses in many tissues. We have demonstrated that treatment of mouse BMMCs with capsiate inhibited IgE-mediated BMMC activation, resulting in the suppression of pro-inflammatory cytokine expression in activated mouse BMMCs. In addition, capsiate inhibited PCA in the mouse ear, suggesting that capsiate has an anti-allergic effect.

Recently, it was reported that capsaicin treatment reduces nasal hyper-reactivity in patients with idiopathic rhinitis (Van Gerven *et al.*, 2014). The result is consistent with ours, which shows that treatment with capsiate ameliorated DNFB-induced AD-like skin inflammation in NC/Nga mice. Capsiate is an analog of capsaicin and shares many biological functions with it. Therefore, it seems that capsiate might be useful in treating not only AD but also other allergic diseases. Interestingly, it was also reported that IL-17 is involved in airway hypersensitivity in asthmatic patients (Molet *et al.*, 2001; Barczyk *et al.*, 2003). Our experimental results show that capsiate has an anti-proliferative effect on CD4⁺ T cells, an inhibitory effect on CD4⁺ T-cell differentiation including Th17 cell differentiation, and anti-allergic effects. Therefore, it will be interesting to determine whether capsiate could inhibit airway hypersensitivity by the double inhibition of Th17 cells and mast cells.

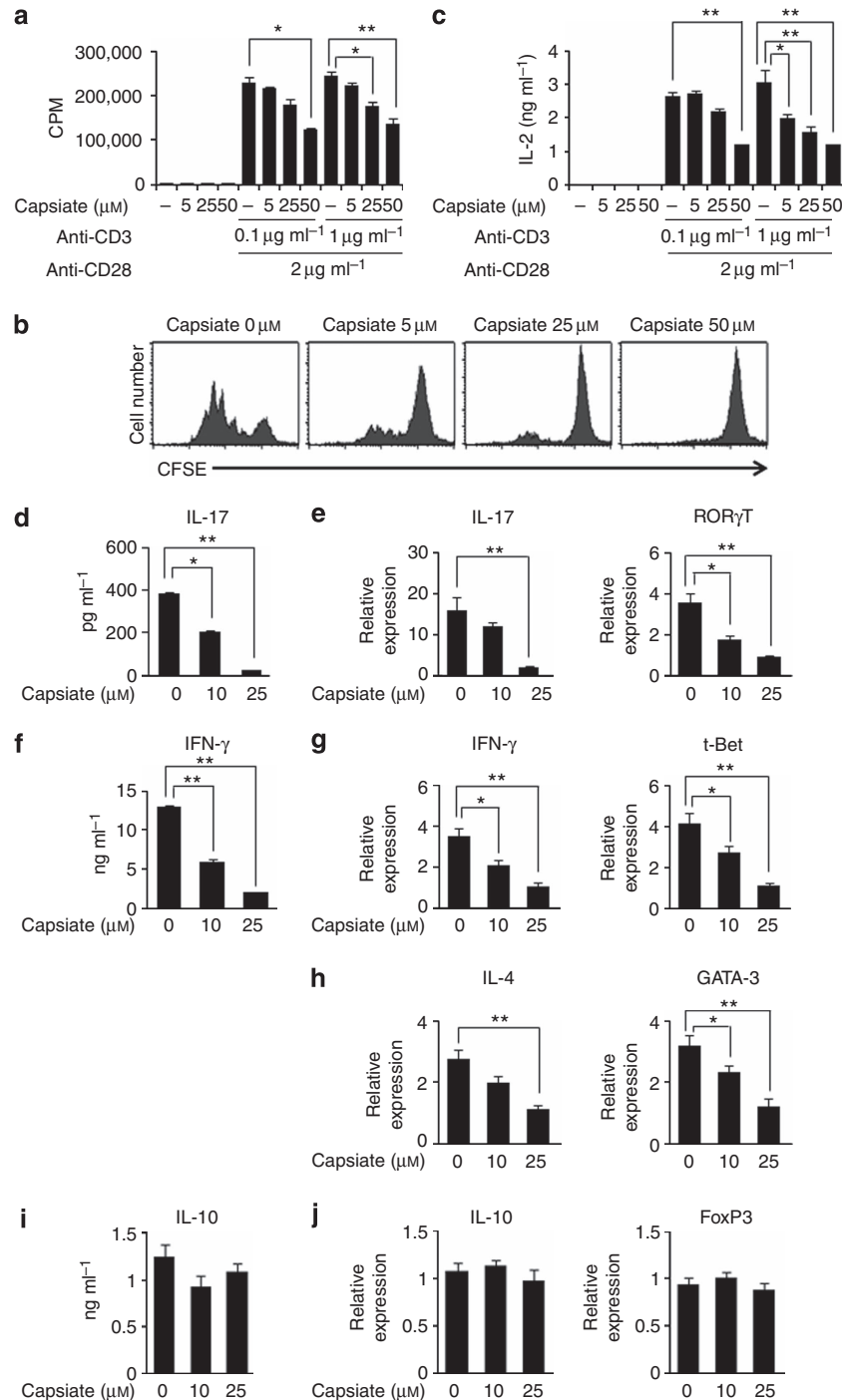


Figure 6. Capsiate inhibits CD4+ T-cell proliferation and differentiation. CD4+ T-cell proliferation was determined by (a) a H³ incorporation assay and (b) a CFSE dilution assay. (c) The IL-2 levels released from activated CD4+ T cells were also determined by ELISA. Purified mouse CD4+ T cells were cultured under Th1, Th2, Th17, or regulatory T cell (Treg) conditions for 4 days. Supernatants were collected for an ELISA experiment to quantify (d) IL-17, (f) IFN- γ , and (i) IL-10 levels. Total RNA was isolated from CD4+ T cells after Th1, Th2, Th17, or Treg differentiation. The mRNA levels of (e) IL-17 and ROR γ T for Th17 cell differentiation, (g) IFN- γ and T-bet for Th1 cell differentiation, and (h) IL-4 and GATA-3 for Th2 cell differentiation, and (j) IL-10 and Foxp3 for Treg differentiation were determined using real-time PCR. mGAPDH levels were used as an internal control. Data represent mean $\pm$ SE. The results are representative of four independent experiments. CFSE, carboxyfluorescein succinimidyl ester; CPM, counts per minute; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Taken together, we demonstrated that capsiate could regulate AD probably through the inactivation of Th2 cells and mast cells. These results imply that capsiate might be a good candidate molecule for the treatment of allergic diseases.

MATERIALS AND METHODS

Mice

C57BL/6 mice were obtained from the Korean Research Institute of Biotechnology (Daejeon, Korea). All experiments with the mice were

approved by the Review Board of Experimental Animals Study Committee at the Catholic University and performed following the guidelines provided by the Committee.

Reagents

Roswell's Park Memorial Institute 1,640 was purchased from Gibco BRL (Grand Island, NY). Fetal bovine serum was purchased from Hyclone (Logan, UT). DNP-specific monoclonal IgE, DNP-HSA, and Evans blue were purchased from Sigma Chemical (St Louis, MO). The antibody against the phosphorylated forms of ERK was purchased from BD Biosciences (Franklin Lakes, NJ).

Cell culture

HMC-1 cells were grown in DMEM supplemented with 100 U ml^{-1} penicillin, $100 \mu\text{g ml}^{-1}$ streptomycin, and 10% heat-inactivated fetal bovine serum at 37°C under a humidified atmosphere containing 5% CO_2 .

DNFB-induced AD

AD was induced in Nc/Nga mice by treatment with DNFB as described previously (Tomimori *et al.*, 2005; Jin *et al.*, 2009; Kim *et al.*, 2011). We induced AD-like skin inflammation in mice with minor modification. Briefly, AD was induced by sensitization with topical application of $100 \mu\text{l}$ of 0.15% DNFB in acetone/olive oil (3:1) to the shaved back skin on days 0 and 4, and challenge with 0.2% DNFB on days 8, 12, 16, 20, and 24. On day 28 the mice were killed for analysis.

CD4⁺ T-cell isolation

Mouse CD4⁺ T cells were isolated as previously described (Lee *et al.*, 2011). Briefly, C57BL/6 mouse lymph nodes and spleens were collected and ground into a single cell suspension. Using the T-cell enrichment columns and CD4⁺ MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany), CD4⁺ T cells were isolated. The purity of CD4⁺ T cells was $>95\%$.

Generation of mouse bone marrow-derived mast cells (BMMCs)

BMMCs were generated as previously described, with minor modifications (Frossi *et al.*, 2007; Lee *et al.*, 2014). Briefly, bone marrow cells were obtained from tibias and femurs of 8–12-week-old C57BL/6 mice. Lineage-negative cells were isolated from the bone marrow cells using the lineage-negative cell isolation kit (Miltenyi Biotec, Germany) according to the manufacturer's instructions. The lineage-negative cells were then cultured in Roswell's Park Memorial Institute 1,640 medium supplemented with 10% FCS, 100 U ml^{-1} penicillin, $100 \mu\text{g ml}^{-1}$ streptomycin, nonessential amino acids, and sodium pyruvate in the presence of mIL-3 (10 ng ml^{-1}) and mouse stem cell factor (50 ng ml^{-1}) for 7 weeks at 37°C in a humidified atmosphere with 5% CO_2 . FACS analysis showed that $>97\%$ of the cells were positive for CD117 (c-Kit) and FcεRI.

³H-thymidine incorporation assay

The ³H incorporation assay was performed as previously described (Yu *et al.*, 2012). Briefly, naive CD4⁺ T cells were isolated and activated with an anti-CD3/anti-CD28 antibody (BD Biosciences) for 3 days. The cells were pulsed with ³H-thymidine ($0.5 \mu\text{Ci}/10 \mu\text{l}$ per well) for 12 additional hours and analyzed. The ³H-thymidine

incorporation assay data are mean counts per minute $\pm$ SE of five replicate cultures.

Flow cytometry and intracellular cytokine analysis

Draining lymph nodes were isolated from mice and the lymph nodes were ground for single cell suspension. The cells were activated using anti-CD3 and anti-CD28 antibodies for 3 days and stained with anti-IL-4, anti-IL-5, anti-IL-17 A, and IFN- γ antibodies (BD Pharmingen, San Jose, CA) using a Cytofix/Cytoperm kit (BD Biosciences) as per the manufacturer's protocol. Flow cytometry was performed on a FACSCalibur (BD Bioscience).

CFSE dilution assay

The CFSE dilution assay was performed using the CellTrace CFSE Cell Proliferation Kit (Molecular Probes, Eugene, OR) as described previously (Lee *et al.*, 2013). Briefly, naive CD4⁺ T cells were isolated and stained with CFSE at 37°C for 10 min. After quenching the staining by the addition of ice-cold culture media, cells were incubated for 5 minutes on ice and then collected and washed. The cells were activated with anti-CD3/anti-CD28 antibody (BD Biosciences) for 4 days and analyzed by flow cytometry. The histogram data showing each cell division were obtained using Cell Quest Software (Becton Dickinson, Mountain View, CA).

RNA isolation, complementary DNA synthesis, and real-time PCR

Total RNA was isolated from CD4⁺ T cells and BMMCs using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand complementary DNA was synthesized from $1 \mu\text{g}$ total RNA using a reverse transcription system (Qiagen, Hilden, Germany). The primer sets for IL-1 β , IL-6, IL-17, IFN- γ , and glyceraldehyde-3-phosphate dehydrogenase were purchased (Qiagen). Glyceraldehyde-3-phosphate dehydrogenase messenger RNA was used as an endogenous control. Real-time PCR was performed using the Rotor-Gene 6000 (Corbett, New South Wales, Australia) and a QuantiTect SYBR Green PCR kit (Qiagen). The amplification program consisted of 1 cycle of 95°C for 10 minutes, followed by 45 cycles of 95°C for 20 seconds, 55°C for 20 seconds, and 72°C for 20 seconds.

ELISA

For the detection of cytokines, BMMCs were sensitized with $1 \mu\text{g/ml}$ DNP-IgE overnight. After washing the cells with phosphate-buffered saline, the cells were treated with capsiate for 30 minutes and then activated with 10 ng ml^{-1} DNP-HSA for 24 hours. The supernatants were collected and ELISA was performed as previously described (Hur *et al.*, 2010).

PCA in mice

An anti-DNP-specific IgE (100 ng per ear) was intradermally injected into the mouse ear, followed 24 hours later by topical treatment with capsiate (1 mm per ear). One hour later, the mice were challenged with an intravenous injection of antigen (DNP-HSA; $500 \mu\text{g}$) in $100 \mu\text{l}$ phosphate-buffered saline containing 4% Evans blue. One hour after antigen challenge, the mice were killed and the ears removed to measure the amount of dye extravasated by the antigen. The dye was extracted overnight from the ear in $800 \mu\text{l}$ formamide at 65°C . The

intensity of the absorbance was measured at 620 nm ($n = 5$ mice in each group).

Statistical analyses

Statistical comparisons between groups were made using unpaired two-sided t -tests and differences with $P < 0.05$ (*) and $P < 0.005$ (**) were considered significant. All experiments were performed at least three times.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This research was supported by the Bio and Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science, ICT, and Future Planning (NRF-2013M3A9A9050567).

SUPPLEMENTARY MATERIAL

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

REFERENCES

- Amadi-Obi A, Yu C-R, Liu X et al. (2007) TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. *Nat Med* 13:711–8
- Barczyk A, Pierzchala W, Sozanska E (2003) Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir Med* 97:534–7
- Bullens DM, Truyen E, Coteur L et al. (2006) IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir Res* 7:135
- Chakir J, Shannon J, Molet S et al. (2003) Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF- β , IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* 111:1293–8
- Fischer BS, Qin D, Kim K et al. (2001) Capsaicin inhibits Jurkat T-cell activation by blocking calcium entry current I_{CRAC} . *J Pharmacol Exp Ther* 299:238–46
- Frossi B, Rivera J, Hirsch E et al. (2007) Selective activation of Fyn/PI3K and p38 MAPK regulates IL-4 production in BMDC under nontoxic stress condition. *J Immunol* 178:2549–55
- Grewe M, Bruijnzeel-Koomen CA, Schopf E et al. (1998) A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol Today* 19:359–61
- Hershey GK, Friedrich MF, Esswein LA et al. (1997) The association of atopy with a gain-of-function mutation in the alpha subunit of the interleukin-4 receptor. *N Engl J Med* 337:1720–5
- Hirota K, Yoshitomi H, Hashimoto M et al. (2007) Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J Exp Med* 204:2803–12
- Homey B, Steinhilber M, Ruzicka T et al. (2006) Cytokines and chemokines orchestrate atopic skin inflammation. *J Allergy Clin Immunol* 118:178–89
- Hur S, Lee YS, Yoo H et al. (2010) Homoisoflavanone inhibits UVB-induced skin inflammation through reduced cyclooxygenase-2 expression and NF-kappaB nuclear localization. *J Dermatol Sci* 59:163–9
- Jin H, He R, Oyoshi M et al. (2009) Animal models of atopic dermatitis. *J Invest Dermatol* 129:31–40
- Kalesnikoff J, Galli SJ (2008) New developments in mast cell biology. *Nat Immunol* 9:1215–23
- Kim SY, Sohn EJ, Kim DW et al. (2011) Transduced PEP-1-FK506BP ameliorates atopic dermatitis in NC/Nga mice. *J Invest Dermatol* 131:1477–85
- Koga C, Kabashima K, Shiraishi N et al. (2008) Possible pathogenic role of Th17 cells for atopic dermatitis. *J Invest Dermatol* 128:2625–30
- Kraft S, Kinet JP (2007) New development in Fc ϵ R1 regulation, function and inhibition. *Nat Rev Immunol* 7:365–78
- Lowes MA, Kikuchi T, Fuentes-Duculan J et al. (2008) Psoriasis vulgaris lesions contain discrete populations of Th1 and Th17 T cells. *J Invest Dermatol* 128:1207–11
- Lee EJ, Jeon MS, Kim BD et al. (2010) Capsiate inhibits ultraviolet B-induced skin inflammation by inhibiting Src family kinases and epidermal growth factor receptor signaling. *Free Radic Biol Med* 48:1133–43
- Lee YS, Amadi-Obi A, Yu C-R et al. (2011) Retinal cells suppress intraocular inflammation (uveitis) through production of interleukin-27 and interleukin-10. *Immunology* 132:492–502
- Lee YS, Cheon IS, Kim BH et al. (2013) Loss of extracellular superoxide dismutase induces severe IL-23-mediated skin inflammation in mice. *J Invest Dermatol* 133:1134–44
- Lee YS, Hur S, Kim TY (2014) Homoisoflavanone prevents mast cell activation and allergic responses by inhibition of Syk signaling pathway. *Allergy* 69:453–63
- Murakami K, Ito MHH, Tsubouchi R et al. (2001) Antioxidant effect of capsaicinoids on the metal-catalyzed lipid peroxidation. *Biomed Res* 22:15–7
- Min JK, Han KY, Kim EC et al. (2004) Capsaicin inhibits in vitro and in vivo angiogenesis. *Cancer Res* 64:644–51
- Molet S, Hamid Q, Davoine F et al. (2001) IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* 108:430–8
- Ngoc LP, Gold DR, Tzianabos AO et al. (2005) Cytokines, allergy and asthma. *Curr Opin Allergy Clin Immunol* 5:161–6
- Pyun BJ, Choi S, Lee Y et al. (2008) Capsiate, a nonpungent capsaicin-like compound, inhibits angiogenesis and vascular permeability via a direct inhibition of Src kinase activity. *Cancer Res* 68:227–35
- Rivera J (2002) Molecular adapters in Fc ϵ R1 signaling and the allergic response. *Curr Opin Immunol* 14:688–93
- Rivera J, Gilfillan AM (2006) Molecular regulation of mast cell activation. *J Allergy Clin Immunol* 117:1214–25
- Sancho R, Lucena C, Macho A et al. (2002) Immunosuppressive activity of capsaicinoids: capsaicin derived from sweet peppers inhibits NF- κ B activation and is a potent anti-inflammatory compound in vivo. *Eur J Immunol* 32:1753–63
- Sundelin K, Roberg K, Grenman R et al. (2005) Effects of cytokines on matrix metalloproteinases expression in oral squamous cell carcinoma in vitro. *Acta Otolaryngol* 125:765–73
- Theoharides TC, Kempuraj D, Tagen M et al. (2007) Differential release of mast cell mediators and the pathogenesis of inflammation. *Immunol Rev* 217:65–78
- Tomimori Y, Tanaka Y, Goto M et al. (2005) Repeated topical challenge with chemical antigen elicits sustained dermatitis in NC/Nga mice in specific-pathogen-free condition. *J Invest Dermatol* 124:119–24
- Tzartos JS, Friesen MA, Craner MJ et al. (2008) Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol* 172:146–55
- Van Gerven L, Alpizar YA, Wouters MM et al. (2014) Capsaicin treatment reduces nasal hyperactivity and transient receptor potential cation channel subfamily V, receptor (TRPV1) overexpression in patients with idiopathic rhinitis. *J Allergy Clin Immunol* 133:1332–9
- Wong CK, Ho CY, Ko FWS et al. (2001) Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN- γ , IL-4, IL-10 and IL-13) in patients with allergic asthma. *Clin Exp Immunol* 125:177–83
- Yu C-R, Lee YS, Mahdi RM et al. (2012) Therapeutic targeting of STAT3 (Signal Transducers and Activators of Transcription 3) Pathway inhibits experimental autoimmune uveitis. *PLoS One* 7:e29742

Fig S1

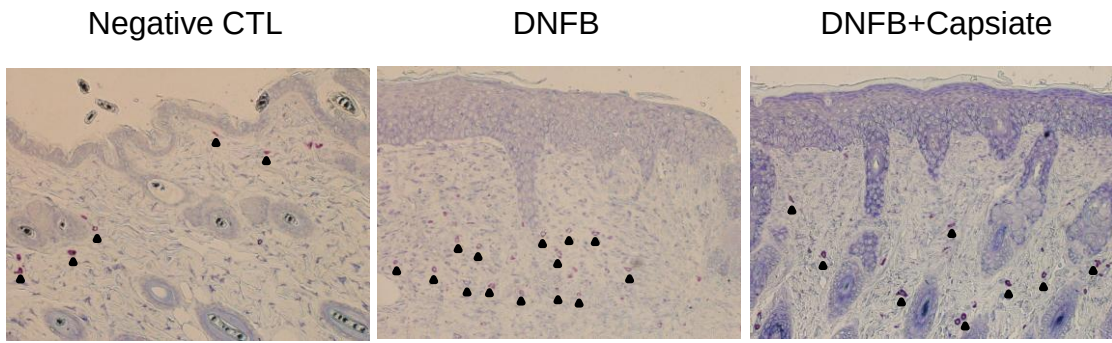


Figure S1. Treatment with capsiate inhibits DNFB-induced mast cell infiltration and degranulation in NC/Nga mice. Toluidine O staining was performed to determine the number of mast cells and the degranulation. The number of mast cells increased when the skin of NC/Nga mice was stimulated; additionally, treatment with capsiate inhibited the mast cell recruitment and degranulation

Fig S2

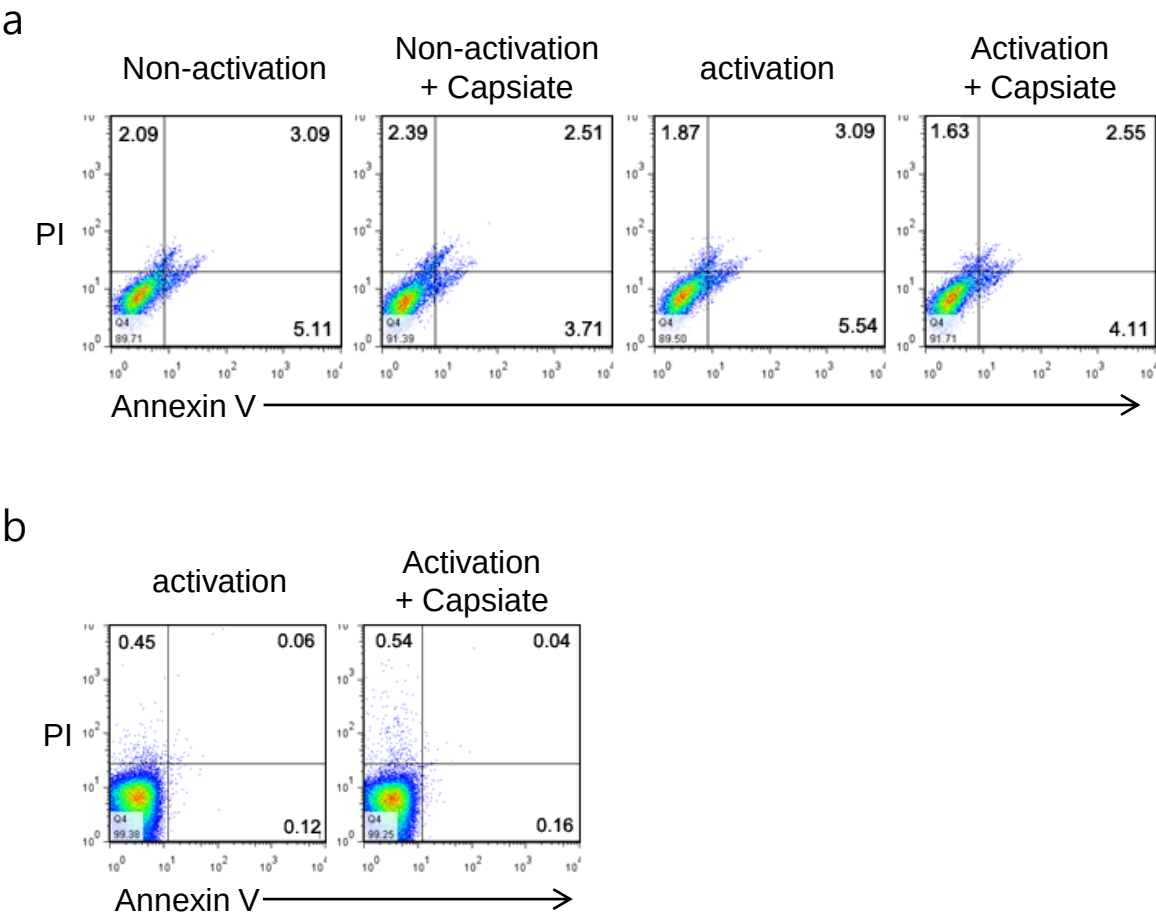


Figure S2. Treatment with capsiate into BMMCs and CD4+ T cells does not induce apoptosis. In order to determine whether capsiate is toxic in (a) BMMCs and (b) CD4+ T cells, the cells were treated with or without capsiate, and BMMCs and CD4+ T cells were stained with PI and Annexin V. A representative example of three independent experiments is shown.

Fig S3

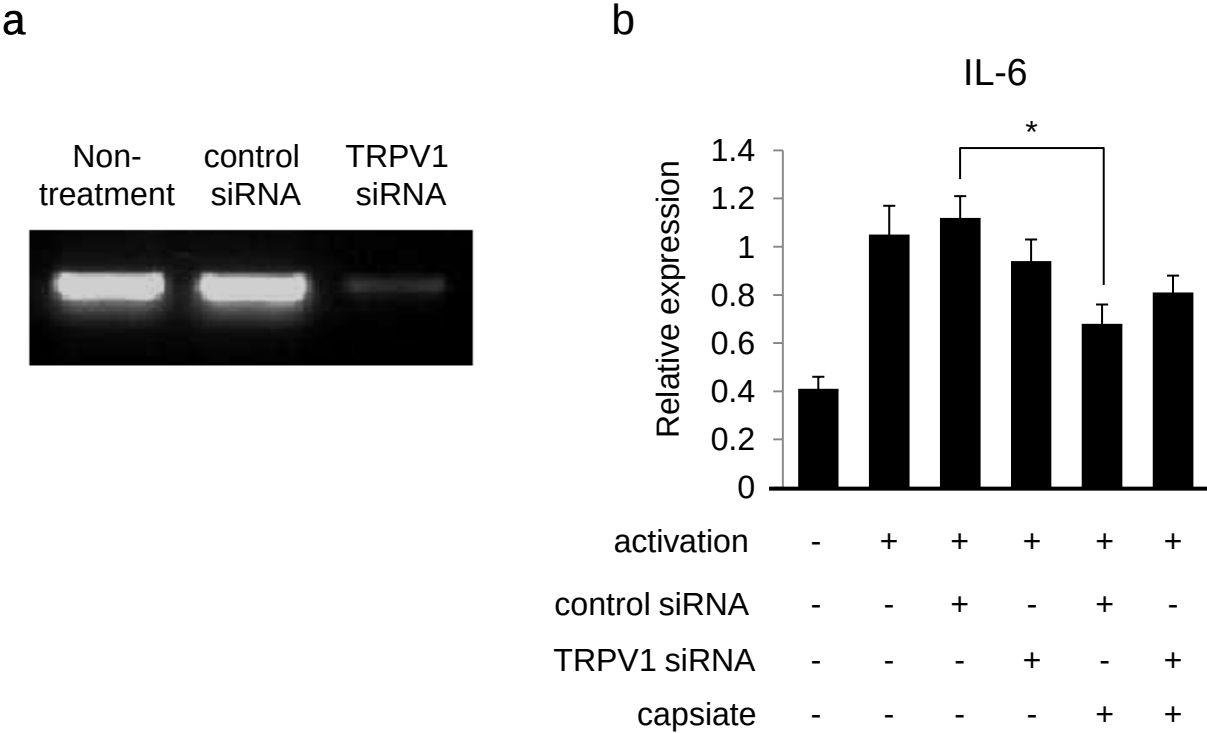


Figure S3. Capsiate partially inhibited mast cell activation when the expression of TRPV1 was knocked down in mast cells. (a) The expression of TRPV1 in HMC-1 was knocked down using siRNA against a TRPV1 transcript. (b) HMC-1 was transfected with control or TRPV1 siRNA. HMC-1 was treated with or without capsiate 24 h after siRNA transfection. One hour later, the cells were activated with PMA/A23187 for 6 h. The cells were collected, and real-time PCR was performed to determine mast cell activation. The data represent mean $\pm$ SE. The results are representative of two independent experiments.