

Article type : Regular Article

**Manifestation of atopic dermatitis-like skin in TNCB-induced NC/Nga mice
is ameliorated by topical treatment of substance P, possibly through
blockade of allergic inflammation**

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/exd.13421

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Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disorder characterized by intense pruritus and eczematous lesion. In this study, topically applied substance P (SP) significantly alleviated AD-like clinical symptoms in 2, 4, 6-trinitrochlorobenzene (TNCB)-induced dermatitis in NC/Nga mice. This effect was nullified by pre-treatment of the neurokinin-1 receptor (NK-1R) antagonist CP99994. SP treatment significantly reduced the infiltration of mast cells and CD3-positive T cells as well as inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and thymic stromal lymphopoietin (TSLP), in AD-like skin lesions and decreased the levels of IgE and thymus and activation-regulated chemokine in serum. This SP-induced alleviation of allergic inflammatory responses was also confirmed as reduced activation in the axillary lymph nodes (aLN) and spleen, suggesting the systemic effect of SP on immune responses in TNCB-induced NC/Nga mice. Furthermore, SP-mediated TSLP reduction was confirmed in human keratinocyte culture under pro-inflammatory TNF- α stimulation. Taken together, these results suggest that topically administered SP may have potential as a medication for atopic dermatitis.

Keywords : Atopic Dermatitis, NC/Nga mice, Substance-P, TNCB, TSLP

Introduction

Atopic dermatitis (AD) is characterized by allergic inflammation leading to a chronic relapsing course (1-4). It is caused by allergic skin inflammation, in particular a predominance of the T-helper 2 (Th2)-type immune response, thereby resulting in hyperproduction of IgE (3). The mechanisms underlying the chronicity of allergic inflammation remain unclear, but it is generally accepted that continuous exposure to extrinsic allergens

and itching result in a vicious cycle of the worsening of the skin lesion, causing the allergic inflammatory disease to become chronic.

Thymic stromal lymphopoietin (TSLP) plays a critical role in the initiation of the inflammatory cascade of AD at the interface between keratinocytes and dendritic cells (DCs) and also mediates itching (5). Accordingly, elevated TSLP in atopic skin activates immature DCs to produce chemokines, such as eotaxin-2, thymus and activation regulated chemokine (TARC), and macrophage-derived chemokine (MDC), which attract Th2 cells. These Th2 cells then migrate back to the affected skin and initiate allergic inflammation by activating B cells and triggering IgE production (6). TSLP is mainly produced by epithelial cells and pro-inflammatory cytokines, such as TNF- α and IL-1, upregulate its production. Mast cells are one of the essential sources of TSLP, as well as several proinflammatory cytokines including TNF- α . These cytokines are released by IgE-activated mast cells and also upregulate TSLP expression (7). Consistently, the expression of TSLP is highly increased in the skin of patients with AD, but absent in normal skin (7-13).

SP is an endogenous neuropeptide belonging to the tachykinin family and is known to mediate intracellular signaling, mainly through G-protein coupled receptor (GPCR)-linked NK-1R, in a variety of non-neuronal as well as neuronal tissues, including the peripheral and central nervous systems (14-17). The role of SP in a variety of cell types and diseases is rather controversial. For example, at very high concentrations (10^{-5} M), SP induces histamine release, which is actively implicated in neurogenic inflammation, but at low local concentrations (10^{-11} - 10^{-6} M), SP alleviates AD progression by trophic and immune modulatory functions (18, 19). Recently, several reports have supported the anti-inflammatory and tissue repairing roles of SP in spinal cord injury, demonstrating increases in IL-10 and in the M2 type of macrophages and decreases in TNF- α (20). Furthermore, decreases in TNF- α and IL-17 and increased regulatory T cells (Treg) have been

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demonstrated in a collagen II-induced arthritis animal model (21). SP also improves MSC's inhibitory effect on T cell activation by stimulating tumor growth factor- β (TGF- β) secretion in long term *ex vivo* expanded MSC (22). Furthermore, SP has been newly identified as an injury-inducible messenger mobilizing MSC from the bone marrow for recruitment to the injury site (23, 24). These newly identified physiological roles of SP led us to suggest that SP contributes to wound healing and to the regulation of chronic inflammation, rather than its classically characterized role as a pain-sensing neurotransmitter or mast cell activator.

NC/Nga mice provide a useful animal model for evaluating potential therapeutic drugs for human AD (25). Moreover, repeated sensitization with TNCB can cause an apparent chronic dermatitis in NC/Nga mice which is similar to human AD (26-28). In this study, using a NC/Nga AD-like dermatitis murine model induced by cutaneous sensitization with TNCB, we evaluated the effect of topical SP treatment in AD-like skin progression. We found that topical SP treatment reduces infiltration of mast cells and T cells in AD-like skin lesions, as well as reducing local inflammation in lymph nodes. These results suggest the efficacy of SP in the treatment of human diseases such as AD. These finding should be further evaluated in future clinical studies.

Materials and Methods

Animals

Male 5-week-old NC/Nga mice were purchased from SLC (Shizuoka, Japan) and maintained under conventional circumstances. Animals were maintained under a 12 h light/12 h dark illumination cycle in an animal holding room and allowed to adapt to the new environment for a period of 1 week before the experiments were started. All animals received

standard chow and water *ad libitum*. All animal studies were performed in accordance with the Animal Experiment Guidelines of Kyung Hee University and were approved by the Animal Use Committee at Kyung Hee University (approval number; KHUASP[SU]-10-010).

Induction of dermatitis

AD-like dermatitis was induced in NC/Nga mice as described by Takahashi *et al* (29) (Fig. 1A). 2,4,6-trinitrochlorobenzene (TNCB, Sigma-Aldrich, St Louis, MO, USA) was dissolved in a mixture of acetone and olive oil at a 3:1 ratio. NC/Nga mice were anesthetized using ketamine (106.25 µg/kg; YuhanYanghang, Seoul, Korea) and Rompun (1.2 mg/kg; Bayer Korea, Seoul, Korea). TNCB solution was topically applied twice a week in a 200 µl volume on the denuded back skin and ears of the mice. TNCB sensitization was performed in two steps: 1% TNCB (higher concentration) solution for 1 week and then 0.4% TNCB (lower concentration) for 4 weeks. After 5 weeks of sensitization, all mice started to show typical AD-like symptoms (AD). AD mice (n= 32) were classified randomly to the vehicle group (AD-VE), which received 250 µl of 20% poloxamer 407 (Sigma-Aldrich) in PBS, the SP group (AD-SP) which received 5 nmol/kg (250 µl of 5×10^{-7} M, 0.16845µg per mice) SP (Calbiochem, Darmstadt, Germany) in vehicle, and the SP with NK-1R antagonist CP99994 group (AD-SP-A) which was pretreated with 3 µmol/kg (300 µl of 2.5×10^{-6} M) NK-1R antagonist CP99994 (Tocris, Bristol, UK) in vehicle 15 min before SP treatment (n=8 for AD, AD-VE, AD-SP, and AD-SP-A, respectively), which correspond to clinically used doses (21, 24, 30). During the drug treatment period, 0.1% TNCB was applied once a week to maintain chronic dermatitis. The mice were kept in the same environment without sensitization and were considered to be the normal (Nor) group (n=5 for Nor).

Evaluation of skin dermatitis severity

The severity of dermatitis in the dorsal skin was evaluated by the scoring atopic dermatitis (SCORAD) index at 0, 1, 2, and 4 weeks after drug application (31). The clinical skin severity of dermatitis was scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe), according to the criteria of the four major clinical symptoms of AD: erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness. SCORAD was evaluated by three individuals in single-blind testing.

Immunohistochemistry

For immunohistochemical staining, specific antibodies (Abs) to TSLP (Abcam, Cambridge, UK, 1:200) was used to stain 5- μ m thick paraffin embedded skin samples, as described previously (32).

Enzyme-linked immunosorbent assay (ELISA)

The levels of TARC in serum were measured using TARC ELISA kits (R&D Systems, Wiesbaden, Germany). All experiments were performed in triplicate with at least three independent experimental replicates.

Analysis of lymph nodes

To evaluate immunological alterations in the entire body, axillary lymph nodes (aLNs) were collected when the mice were sacrificed at 2 weeks. Briefly, aLNs were isolated after incision of the arm skin. Isolated tissues were measured by wet weight and normalized to

body weight of each animal, and then the specimens were fixed in 4 % PFA for histological analysis.

Statistical analysis

Results were expressed as mean $\pm$ standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by a Dunnett's post hoc Multiple Comparison Test using Graph Pad Prism 5.0. (GraphPad Software, Inc. La Jolla, CA, USA). $P < 0.05$ was accepted as significant.

Results

Topical application of SP ameliorates AD-like symptoms in TNCB-treated NC/Nga mice

Repeated topical applications of TNCB resulted in the development of contact dermatitis symptoms, which mimic the clinical symptoms of human AD, in NC/Nga mice. The efficacy of each treatment on the amelioration of the AD-like symptoms was evaluated by weekly analysis (Supplemental Fig. 1). Based on the gross appearance of the five test groups, a significant improvement in the AD-like skin lesions was noted, as evidenced by a smooth and clean skin surface in the SP-treated group (AD-SP) at 2 weeks (Fig. 1A). This SP-mediated improvement was nullified by pretreatment with NK-1R blocker CP99994 before SP treatment (AD-SP-A). SP treatment reduced the skin severity score from approximately 7.5 at 0 week to 6.0 ($p < 0.01$) at 1 week and 5.0 ($p < 0.01$) at 4 week, while no treatment and pretreatment of NK-1R blocker resulted in scores greater than 7.0 at 4 weeks (Fig. 1B). The improvement in SCORAD was initiated one week after SP treatment and low score was maintained up to 4 weeks.

A high serum level of IgE is a hallmark of AD symptoms in TNCB-treated NC/Nga mice. We found highly elevated levels of serum IgE with AD manifestation during the 4 weeks of TNCB treatment (Supplemental Fig. 2). Compared to the AD group at 0 week, the serum IgE levels in the AD and AD-VE groups continuously escalated during the 4-week test period under the continuous low dose treatment of TNCB to inhibit spontaneous recovery of AD symptoms. Significant differences in serum IgE levels between the test groups were shown beginning at 2 weeks rather than at 1 week as seen with the SCORAD. These differences in serum IgE levels were partially blocked by pretreatment with the CP99994. In addition, in the AD-SP group, low dose TNCB treatment during the test period resulted in maintenance of serum IgE similar to that of the challenged one.

In addition, ear skin was examined at 1 week of treatment in each group. Histological analysis of each ear showed significant reductions in the epidermal thickness and in infiltrating immune cells after topical treatment with SP comparing to the untreated one (Supplemental Fig. 3A). The thickness of the epidermis in the AD controls was approximately 88 μm , which was about 5-fold thicker than the normal control ($18.3 \pm 4.3 \mu\text{m}$), while SP treatment ($42.8 \pm 8.4 \mu\text{m}$, $p < 0.05$) reduced epidermal thickness by greater than 50%, compared to the AD controls (Supplemental Fig. 3B-C). However, the measured thickness in the AD-VE and AD-SP-A groups was not significantly altered relative to the AD controls.

SP treatment inhibits mast cell recruitment and degranulation in TNCB-induced AD-like lesions

As shown in Fig. 1, topical treatment with SP inhibited the clinical features of AD in NC/Nga mice. In order to investigate the mechanisms of action of SP in the amelioration of AD-like symptoms, infiltration of mast cells and their degranulation in the skin lesions were

examined by toluidine blue staining at 1, 2, and 4 weeks (Fig. 2 and Supplemental Fig. 4). In normal skin, infiltrated mast cells were barely detected and were found to be predominantly in an inactive state (indicated by an arrow head), although a few active state degranulated cells (indicated by an arrow) were observed. Highly infiltrated mast cells, regardless of their degranulation, were observed in AD skin, which was significantly prevented in AD-SP skin from 1 week (Supplemental Fig. 4). This SP-mediated prevention of mast cell infiltration was completely abolished in AD-SP-A skin and sustained up to the 2 and 4 week. Quantitative analysis of the number of infiltrated mast cells revealed approximately three-fold higher levels in the AD and AD-VE groups than in normal skin (AD: 165.6 ± 27.8 , 169.4 ± 12.6 and 155.0 ± 28.4 ; AD-VE: 167.3 ± 21.9 , 153.6 ± 20 and 151.9 ± 13.3 ; Nor: 46.1 ± 1.0 , 41.7 ± 12.6 and 41.5 ± 5.7 cells/field at 1,2 and 4 weeks, respectively). These increases were significantly reduced to approximately 60% by SP treatment (AD-SP: 109.0 ± 16.1 , 93.7 ± 116.8 and 95.3 ± 6.6 cells/field at 1,2 and 4 weeks, respectively). Treatment with the NK-1R antagonist CP99994 reversed this effect, resulting in levels similar to those of the AD groups (AD-SP-A: 153.6 ± 15.5 , 169.5 ± 12.6 and 131.3 ± 13.7 cells/field at 1,2 and 4 weeks, respectively) (Fig. 2A and Supplemental Fig. 4).

Mast cells in their active state were made up only 20% of the total infiltrate in normal skin, while the majority of the infiltrated mast cells were degranulated in AD skin at 2 weeks. Degranulated mast cells are made up a much higher percentage of the total mast cells in the AD and AD-VE groups than in the AD-SP group (AD: 62.2 ± 9.5 vs AD-SP: 41.7 ± 3.9 %, $p < 0.001$ and AD-VE: 62.5 ± 8.4 vs AD-SP: 62.2 ± 9.5 %, $p < 0.001$), while pretreatment with CP99994 before SP treatment (AD-SP-A) nullified the SP-mediated reductions in mast cell infiltration as well as degranulation to the levels similar to the AD and AD-VE groups (Fig. 2B). Similar to the number of infiltrated mast cells, SP treatment inhibited approximately 70% of mast cell activation.

SP reduces chronic dermatitis in TNCB-induced NC/Nga mice

TSLP has a critical role in the initiation of the inflammatory cascade of AD, and TNF- α stimulates the release of TSLP expression (8). Therefore, we examined TNF- α and TSLP expression by immunohistochemistry in AD-like skin samples. The expression of TNF- α and TSLP were not observed in normal mice, and significantly reduced in the AD-SP group compared to the AD, AD-VE and AD-SP-A groups (Fig 3A and Supplemental Fig. 5). We also examined TSLP expression in primary cultured human keratinocytes in order to determine whether SP directly regulates its expression. An SP-mediated reduction of TSLP expression in primary cultured keratinocytes was confirmed under inflammatory conditions induced by TNF- α (Supplemental Fig. 6). Inflammatory stimulation with TNF- α was sufficient to induce TSLP even in cultured keratinocytes. After co-stimulation with SP, significant inhibition of TNF- α -induced TSLP expression was elicited. CP99994 blocked the SP-mediated reduction in TNF- α -induced TSLP expression.

Next, we measured the serum levels of TARC, one of the chemokines secreted by TSLP-activated mature DCs. TARC was markedly reduced in the serum from the AD-SP group, compared to the AD, AD-VE, and AD-SP-A groups (Fig. 3B). Collectively, the topical application of SP in AD mice markedly reduced chronic dermatitis, along with reductions in the expression of TNF- α , TSLP, and TARC.

Since AD is a T cell-mediated chronic inflammatory skin disease, the infiltration of T cells in the skin and the crosstalk between T cells and keratinocytes are important. Therefore, T cell infiltration into the AD-like skin was also evaluated by immunohistochemistry after 2 weeks of treatment when the most significant differences in SCORAD and mast cell infiltration were observed (Supplemental Fig. 7A). The number of CD3-positive cells in the skin (epidermis + dermis) greatly reduced in the AD-SP compared to the AD and AD-VE

(AD-SP: 31.7 ± 7.0 vs AD: 66.5 ± 15.9 cells/field, $p < 0.001$ and AD-SP: 31.70 vs AD-VE: 61.0 ± 6.6 cells/field, $p < 0.01$) (Supplemental Fig. 7B). In particular, the number of CD3 cells infiltrating to the epidermis of the AD-SP group (4.5 ± 2.5 cells/field) was significantly reduced to the levels seen in normal skin (2.3 ± 1.1 cells/field), while AD, AD-VE, and AD-SP-A showed levels approximately five-fold higher than the normal group (Supplemental Fig. 7C).

SP affects local immune responses in TNCB-induced NC/Nga mice

To determine whether topical treatment with SP affects systemic immune responses in TNCB-induced NC/Nga mice, we next examined the secondary lymphoid organs such as aLNs, adjacent to the dorsal skin lesion at the skin-draining site, the mLNs located in the peritoneal area in the mesentery, and the spleen. The sizes of the isolated aLNs and cross-sections evaluated by H&E staining in the AD, AD-VE, and AD-SP-A groups were much larger than those in normal mice, suggesting enhanced immune responses; the aLNs in the AD-SP group were relatively small in comparison (Fig. 4A). These findings were also confirmed by measurements of the cross-section areas (Fig. 4B) and total wet weight (Fig. 4C). However, the weight of the mLNs and spleens was not significantly reduced in the AD-SP mice comparing to the AD-VE and AD-SP-A, implying that the activation of immune cells may be concentrated primarily on the local adjacent lymphoid organs (Supplemental Fig. 8).

In order to further evaluate the activation of the immune response in the aLNs, proliferating lymphoid cells were examined by CD3 and PCNA immunohistochemistry of the medulla and germinal center, which are the T cell and B cell zones, respectively (Supplemental Fig. 9A-C). In the medullas of the AD, AD-VE, and AD-SP-A groups, elevated numbers, up to 33-fold, of CD3 cells were detected; this level was significantly

reduced in the SP-treated group. Moreover, PCNA-positive proliferating cells were also greatly reduced up to 5-fold in the germinal center zones of AD-SP mice. These results demonstrate that topical treatment with SP not only reduced cutaneous inflammation, but also suppressed immune responses in the adjacent lymph nodes.

Topical treatment with SP reduced IgE levels, infiltration of mast cells and secretion of TNF- α *in vivo*. Therefore, we carried out *in vitro* assays to examine the direct effects of SP in immune cells, such as RBL-2H3 cells (a rat-derived mast cell line) and U266 cells (a human derived B cell line). We performed mast cell migration assay using a transwell system and a β -hexosaminidase assay to examine degranulation of mast cell by SP treatment (Supplemental Fig. 10). Moreover, we examined the cytotoxicity assay and determined the amount of IgE in response to various concentration of SP (Supplemental Fig. 11). Collectively, SP did not stimulate RBL-2H3 migration and degranulation as well as U266 cell proliferation and production of IgE according to concentration of SP *in vitro*.

Discussion

In the present study, topical treatment with SP on the skin of TNCB-induced chronic dermatitis in NC/Nga mice, which is similar to human AD skin, markedly reduced the typical symptoms (Fig. 1 and Supplemental Fig. 2). This alleviation of AD symptoms was accompanied by reduced allergic and cutaneous inflammation (Figs. 2-3 and Supplemental Fig. 3-5). As one possible mechanism for the beneficial role of SP in the alleviation of AD symptoms in TNCB-sensitized NC/Nga mice, marked reductions in TNF- α , TSLP, and TARC were detected (Fig. 3 and Supplemental Fig. 5) and SP-mediated blockade of TNF- α -induced TSLP production was shown in cultured human keratinocytes (Supplemental Fig. 6). These immune modulatory effects of SP were also found in a secondary lymphoid organ, the aLNs

adjacent to the atopic dorsal skin (Fig. 4). In addition, the concentration of SP used for topical treatment in this study reduced the symptoms of AD, specifically the thickness of ear skin and the infiltration of immune cells (Supplemental Fig. 3). SP may be considered as a potential medication for AD-like dermatitis.

The manifestations of AD in TNCB-sensitized NC/Nga mice are caused by recurrent allergen exposure and resulting chronic inflammation of the skin (33). The allergic response itself may be difficult to completely eradicate unless allergen exposure is completely eliminated, since memory plasma B cells are present (34, 35). If low doses of continuous environmental allergen exposure are inevitable, clinical treatment should aim to minimize the cascade of events associated with the cutaneous allergic response. Topical treatment with SP significantly prevented this cascade by directly reducing TSLP and TNF- α expression in AD-like skin. In addition, it has been reported that topically applied SP decreases TNF- α and is accompanied by enhanced healing of full thickness open excision wounds in rats (36). In the case of intravenous treatment with SP, an SP-induced TNF- α reduction has been consistently observed in various experimental models, including spinal cord injury and collagen II-induced rheumatoid arthritis (20, 21).

SP treatment also decreased TNF- α levels in the dermal compartment of AD skin (Supplemental Fig. 5), possibly due to reduced infiltration and degranulation of mast cells, as mast cells are the major source of TNF- α (Fig. 2 and Supplemental Fig. 4). Despite the ability of SP to induce acute mast cell activation and mediate neurogenic inflammation, several studies also have suggested that the modulation of neuropeptide activities is associated with the pathogenesis of AD (37-39). At the concentration of SP used for AD-like dermatitis treatment in this study, SP did not affect mast cell degranulation and migration (Supplemental Fig. 10), contrary to the findings of a previous report that was evaluated at the 100-fold

higher SP concentrations (18). SP also did not activate B cell activity (Supplemental Fig. 11). Therefore, the reduced infiltration of mast cells and their degranulation may not be due to the direct effect of SP on mast cells, but may rather reflect a less vigorous immune response in the early stages. All the responses to SP in AD-like chronic dermatitis of TNCB-sensitized NC/Nga mice were mediated by NK-1R activation, which was blocked by pretreatment of NK-1R antagonist. However, the IgE in the serum was not reduced to the level of normal unchallenged animal by SP treatment, possibly due to continuous exposure of low dose allergen during the SP treatment period. In other word, SP may reduce allergen-mediated cutaneous and local cascades of immune response at the early stage but not be effective in terminating systemically pre-established allergic response itself.

Recently, TSLP has been regarded as a master switch for allergic inflammation (6). TSLP can perform its biological functions in various types of cells. TSLP activates T cells in mice as well as induces B cell proliferation and activation in humans (8, 40-42). Moreover, TSLP can stimulate the production of Th2 cytokines in human mast cells in combination with IL-1 and TNF- α (8). In patients with AD, elevated TSLP production has been reported (43). This may be caused by TNF- α -mediated chronic dermatitis and due to elevated Th2 cytokines. In this study, we clearly showed the regulation of TSLP expression by SP treatment in TNF- α -treated keratinocytes, as well as in AD-like skin in NC/Nga mice (Supplemental Fig. 5-6). These effects in the down-regulation of TNF- α , mediated by reduced mast cell infiltration and TSLP expression in the epidermis could synergistically ameliorate cutaneous inflammation in AD-like skin.

In conclusion, SP treatment alleviated typical AD symptoms in a TNCB-sensitized chronic dermatitis model in NC/Nga mice, as demonstrated by reduced allergic and cutaneous inflammation. Therefore, we propose that SP may be a potential medication for AD.

Author contribution

Hyeongwon Choi, Dong-jin Kim, Seungwoo Nam and Sunki Lim performed this research. Ki Sook Park, Hyun Sook Hong and Min Kyung Shin designed the research study. Jae-Sung Hwang contributed essential reagents or tools. Eunkyung Chung and Youngsook Son wrote the manuscript.

Funding resources

This work was supported by a Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI13C1479) grant given to Prof. Youngsook Son & Professor Min Kyung Shin.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Abbreviations

AD, atopic dermatitis; aLN, axillary lymph nodes; GPCR, G-protein coupled receptor; MDC, macrophage-derived chemokine; NK-1R, neurokinin-1 receptor; SP, substance P; TARC, thymus and activation regulated chemokine; TGF- β , tumor growth factor- β ; TNCB, 2, 4, 6-trinitrochlorobenzene; TNF- α , tumor necrosis factor- α ; TSLP, thymic stromal lymphopoietin.

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Figure legends

Figure 1. Topical treatment with SP ameliorates TNCB-induced AD manifestations in NC/Nga mice. (A) Representative photographs of the back skin surface of the NC/Nga mice at 2 weeks. Typical signs of AD with erosion and scaling were observed in the AD, AD-VE, and AD-SP-A groups. Smooth and clean skin surfaces without erosion and erythema were detected in the AD-SP group. (B) Evaluation of clinical skin severity scores (SCORAD). SCORAD was expressed as the summation of each score based on four symptoms: erythema, hemorrhage, scaling, and dryness (no sign = 0, mild = 1, moderate = 2, severe = 3). The significance of these differences was determined using a Dunnett's post hoc Multiple Comparison Test following ANOVA. *P<0.05, **P<0.01.

Figure 2. Topically administered SP inhibits mast cell recruitment and degranulation in atopic skin. Skin tissue samples were excised from the backs of each mouse after treatment. Infiltrated mast cells were stained with toluidine blue in skin tissue sections at 1, 2 and 4 weeks. (A) The number of infiltrated mast cells between the epithelium and subcutaneous

layer at 1, 2 and 4 weeks was counted under a microscope at x200 magnification with an average number obtained for each group (5-8 mice) in ten fields (2.74 mm²). (B) The ratio of degranulated mast cells among the total infiltrated mast cells at 2 weeks is shown. Each value represents the mean $\pm$ S.D. The significance of differences was determined using Dunnett's post hoc Multiple Comparison Test following ANOVA. *P<0.05, **P<0.01, ***p<0.001.

Figure 3. SP treatment reduces allergic inflammation. (A) Immunohistochemical analysis of TSLP expression in the epidermis of atopic-like skin 2 weeks after drug treatment. (B) Measurement of TARC levels in the serum collected at 2 weeks. The concentration of TARC was measured by ELISA (n = 5 for Nor, n = 8 for AD, AD-VE, AD-SP, and AD-SP-A). The significance of differences was determined using Dunnett's post hoc Multiple Comparison Test following ANOVA. **P<0.01, ***p<0.001. Scale bar = 100 μ m.

Figure 4. SP treatment reduces local immune response.

(A) Representative pictures of the isolated aLNs (upper) and cross-sections are shown after H&E staining (lower) at 2 weeks. Scale bar = 1 cm (upper) and 1 mm (lower). (B) The area of the cross sectioned aLNs was measured under microscopy (Nikon Eclipse Ti). (C) The weight of aLNs was measured after 2 weeks and the relative increases from the value of normal mice are shown. Each value represents the mean $\pm$ S.D. The significance of differences was determined using Dunnett's post hoc Multiple Comparison Test following ANOVA. *P<0.05, **P<0.01, ***p<0.001.

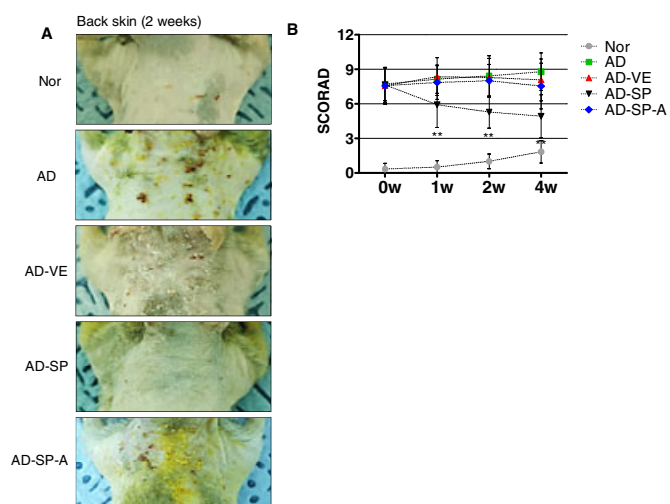
Figure 1.

Figure 2.

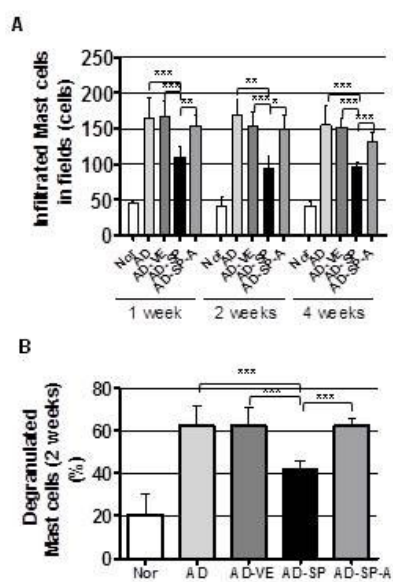


Figure 3.

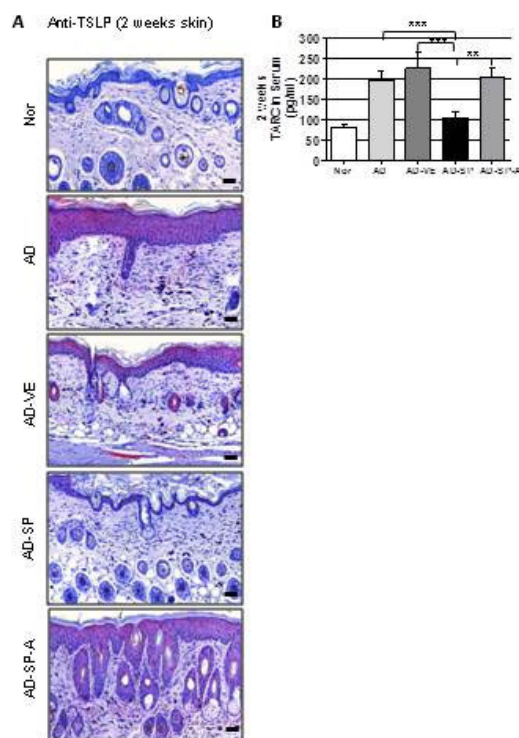


Figure 4.