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Title: Immune response against 2, 4-dinitrofluorobenzene-induced atopic dermatitis-like clinical manifestation is suppressed by spermidine in NC/Nga mice

Running head: Spermidine ameliorates AD-like symptoms

Gun-Dong Kim¹, Tae-Ho Kim¹, Yong Seek Park¹, Hyun-Jong Ahn¹, Jeong-Je Cho¹, Cheung-Seog Park¹

¹Department of Microbiology, School of Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea.

Correspondence to : Cheung-Seog Park, Department of Microbiology, School of Medicine, Kyung Hee University, Dongdaemun-Gu, Hoegi-Dong, Seoul 130-701, Republic of Korea.

E-mail: pcs@khu.ac.kr, TEL: 82-2-961-0294 (Office), 0279 (Lab), FAX: 82-2-962-6189

Abstract

Of the biogenic polyamines, spermidine is a natural constituent of living cells and organisms. Spermidine is associated with regulation of cell growth, proliferation and differentiation, and

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with the suppression of oxidation and inflammation. Atopic dermatitis (AD) is a chronic inflammatory skin disease that has a complex and multiple pathogenesis, which include genetic abnormality, modified or abnormal immune response and the production of nitric oxide and reactive oxygen species. We investigated whether spermidine can relieve AD-like clinical manifestation induced by the continual application of 2, 4-dinitrofluorobenzene (DNFB) in NC/Nga mice. Spermidine at concentrations of 1 or 10 mg/kg reduced increasing ear swelling and attenuated edema, hemorrhage and hyperkeratosis in AD-like skin lesions. Repetitive application of DNFB induced inflammatory cell infiltration to skin lesions, whereas intraperitoneal injection of spermidine inhibited DNFB-evoked infiltration of eosinophils, mast cells and T lymphocytes. Furthermore, spermidine suppressed mast cell degranulation and production of interferon-gamma by activated CD4⁺ T cells in AD-like skin lesions. Spermidine may be a potential therapeutic agent for treatment of AD.

Keywords: Atopic dermatitis, polyamines, spermidine, 2, 4-dinitrofluorobenzene, NC/Nga

1. Introduction

The natural polyamines spermidine and spermine are synthesized from putrescine and are present in every living cell of almost all organisms [1, 2]. These polycationic and biogenic amines are essential for cell proliferation and differentiation during cell growth [3].

Polyamines modulate various physiological functions of RNA, DNA, proteins and other acidic substances by direct binding and also by intracellular signal pathways mediated by protein kinases and transcription factor [4-6]. Naturally occurring polyamines are synthesized from both arginine and methionine. Putrescine is produced from L-ornithine via L-arginine

by arginase and ornithine decarboxylase (ODC). Decarboxylated S-adenosylmethionine is formed from methionine by S-adenosylmethionine decarboxylase, and acts with putrescine to produce spermidine and spermine in a retroconversion pathway mediated by spermidine/spermine N1-acetyltransferase [7, 8]. Polyamines have been associated with diverse immune response including pathogen infection, irritation of chemicals or toxicant, tissue injury and inflammation, via secretion of various inflammatory mediators such as cytokines and chemokines [9, 10]. In particular, spermidine displays suppressive effects against lipopolysaccharide (LPS)-stimulated interleukin (IL)-6 and tumor necrosis factor alpha (TNF- α) production of murine BV2 microglial cells due to inhibition of phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPKs) signaling pathways and nuclear factor-kappa B (NF- κ B) activity [11]. In human dermal fibroblasts, spermidine inhibits matrix metalloproteinase (MMP)-2, which is involved in tumor invasion and chronic inflammation through the modulation of histone acetyltransferase (HAT) and histone deacetylase (HDAC) [12]. Furthermore, spermidine suppresses the infiltration of leukocytes and epithelial damage in a murine model of acute colitis and inhibits 12-O-tetradecanoylphorbol-13-acetate (TPA) induced mice skin inflammation [13, 14]. The collective observations prompted us to hypothesize that spermidine may be useful as a therapeutic for atopic dermatitis (AD). AD is a serious inflammatory disorder of the skin with chronic and relapsing characteristics [15]. AD skin lesions have diverse clinical symptoms including edema, erythema, oozing and lichenification that have been implicated in cutaneous hypersensitivity and intense pruritus [16]. AD affects up to 17% of school aged children in the United States [17]. The lifetime global prevalence of AD is increasing [18]. AD has a multitude of factors and a complex pathogenesis that includes a modified immune response, exposure to various exogenous or endogenous factors and genetic abnormality, which has hindered development of a truly efficacious AD treatment [19]. AD skin lesions developed

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chronically by topical stimulation of invading mites, bacteria, irritants, and microorganisms, which leads to the disruption of the skin barrier, immunoglobulin E (IgE)-mediated sensitization and intense pruritus [20]. Furthermore, in AD skin lesions, massive infiltration of leukocytes and hyper production of inflammatory mediators including IL-4, IL-5, IL-13, interferon-gamma (IFN- γ), thymus-and activation-regulated chemokine, thymic stromal lymphopoietin and IgE induces increasing severity of immune dysregulation [21, 22]. A recent study showed that repeated application of 2, 4, 6-trinitrochlorobenzene (TNCB) or 2, 4-dinitrofluorobenzene (DNFB) on NC/Nga mice induces AD-like clinical symptoms similar to those of AD in humans [23]. Presently, we exploited this mouse model to assess the therapeutic efficacy of spermidine for AD. Specifically, we gauged the capability of spermidine to suppress DNFB-induced AD-like clinical symptoms.

2. Materials and methods

2.1. Mice

Six week old, male NC/Nga mice (19–22 g) were purchased from Japan SLC (Shizuoka, Japan) and maintained under specific pathogen-free conditions. Animals were housed in an air-conditioned animal room at 24°C $\pm$ 2°C at a relative humidity of 45% $\pm$ 5%, and were fed a laboratory diet and distilled water. All experimental procedures were conducted in accordance with the United States National Institute of Health guidelines and with the guidelines issued by the ethical committee for animal welfare at Kyung Hee University [KHUASP (SE)-09-005].

2.2. Spermidine treatment

Mice were randomly assigned to four treatment groups: DNFB non-treated group, DNFB treated group, DNFB with 1 mg/kg of spermidine and DNFB with 10 mg/kg of spermidine (n

= 5 per group). Spermidine was dissolved in 1% dimethylsulfoxide (DMSO) in distilled water to a volume of 100 µl and then intraperitoneally injected daily from the day of first DNFB challenge (day 7). Mice in the DNFB non-treated group and DNFB-only treated group received the same volume of aqueous 1% DMSO.

2.3. Allergen sensitization and challenge

DNFB sensitization was induced by the repeated application of 25 µl of 0.15% DNFB in acetone/olive oil (3:1) to the outer and inner surfaces of the right ears, and 100 µl of the same solution was applied to shaved dorsal skin once on days 1 and 4. On days 7, 10 and 13, sensitized mice were challenged with 0.2% DNFB to the dorsal and ear skin surfaces. Control mice were treated with the same volume of vehicle. Increasing ear thicknesses were measured using a Digimatic Indicator thickness gauge (Mitsutoyo, Tokyo, Japan).

2.4. Dermatitis score

The severity of dermatitis was assessed macroscopically, as previously described [24]. The degree of each symptom was scored as 0 (absence), 1 (mild), 2 (moderate), and 3 (severe). This scoring was based on the severity of edema, hemorrhage, oozing, crust, excoriation, and lichenification as described previously [25]. The total dermatitis score (minimum 0 and maximum 18) of the six symptoms of each mouse was taken as the score for that mouse. Assessment was performed by an investigator who was blind to the grouping of the animals.

2.5. Measurement of IFN- γ and IL-4 production

T lymphocytes were isolated from the lymph nodes and CD4⁺ T cells were purified using a Biomag separation column (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated CD4⁺ T cells (1×10^6) were cultured in model 3524 24-well flat-bottom

culture plates (Corning, Corning, NY, USA) in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum and 50 μ M β -mercaptoethanol, then stimulated with 5 μ g/ml of immobilized anti-CD3 (BD Pharmingen, San Diego, CA, USA) and 2 μ g/ml of soluble anti-CD28 antibody for 50 h at 37°C in a 5% CO₂ atmosphere. After incubation, culture supernatants were collected and the production of IFN- γ and IL-4 were quantified by ELISA (Biolegend, San Diego, CA, USA).

2.6. Measurement of total serum IgE

On day 13, 24 h after the final DNFB application, total serum samples were prepared and quantified using BD OptEIA Set Mouse IgE ELISA kits (BD Pharmingen) according to the manufacturer's instructions. The absorbance was immediately read at 450 nm on an EL 800 ELISA reader (Bio-Tek, Winooski, VT, USA).

2.7. Histological analysis

Right ears and dorsal skins were removed 24 h after final DNFB application and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4). Fixed tissues were washed in sucrose solution and frozen in OCT compound. Cryostat sections (8 μ m) were mounted on slides and dried overnight at room temperature before being fixed in ice-cold acetone for 5 min. Slides were rehydrated in PBS and blocked with 5% normal goat serum in PBS containing 0.3% Tween 20 (washing buffer) for 1 h at room temperature. After washing, sections were incubated with primary anti-CD4 or anti-CD8 antibody overnight at room temperature then washed and incubated with Alexa-Fluor 488-conjugated goat anti-rat antibody for 4 h at room temperature. Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). Sections were mounted and images were captured using an Eclipse 50i fluorescence microscope (Nikon, Tokyo, Japan). Skin sections were also stained with

hematoxylin and eosin for inflammatory cells and mast cells were stained using toluidine blue. Numbers of CD4⁺ T cells, CD8⁺ T cells, eosinophils and mast cells were expressed as average total counts in five fields of 100 μm^2 ($\times 200$) using the CellProfiler Analyst software (free and open-source program) and NIS-Elements imaging software (Nikon, Tokyo, Japan).

2.8. Statistical analyses

Data are presented as the mean $\pm$ S.E.M. of at least three independent experiments performed in triplicate. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test. When not shown, error bars lie within symbols. P-values of < 0.05 were considered statistically significant.

3. Results

3.1. DNFB-induced AD-like clinical symptoms are suppressed by spermidine

ROS and NO are critical in various inflammatory skin diseases including skin cancer, scleroderma and psoriasis [26]. ROS are effectively scavenged by natural polyamines including putrescine, spermine and spermidine [27, 28]. Furthermore, spermidine and spermine inhibit both serotonin- and carrageenan induced paw edema in murine models [29]. Thus, we investigated the therapeutic effects of spermidine on the DNFB-induced AD-like manifest in NC/Nga mice. DNFB-induced ear swelling was gradually increased after sensitization and then significantly increased from days 7 to 13. However, spermidine at a concentration of 1 or 10 mg/kg inhibited DNFB-induced ear swelling (Fig. 1a). Topical repetitive treatment of DNFB produced significant AD-like skin lesions in dorsal skin and right ear including edema, hemorrhage, hyperkeratosis and lichenified lesions, whereas

spermidine at concentrations of 1 or 10 mg/kg improved lesion severity (Fig. 1b and Table. 1).

3.2. Spermidine inhibits infiltration of inflammatory cells to DNFB-induced skin lesions

In AD-like skin lesions, the major characteristic is infiltration of inflammatory cells involving eosinophils and mast cells [30]. Repetitive application of DNFB induces the disruption of the skin barrier and increased production of inflammatory cytokines and chemokines that are important in the chemotaxis of inflammatory cells in AD-like skin lesions. Infiltrated eosinophils secrete inflammatory mediators such as eosinophil peroxidase and major basic protein, that leads to tissue damage and the development hyperplasia and hyperkeratosis to skin lesions [25, 31]. In this study, spermidine at 1 or 10 mg/kg inhibited dermal thickness and infiltration of eosinophils in dorsal skin lesions of NC/Nga mice (Fig. 2). Mast cell activation has been associated with allergic inflammations, such as asthma, rhinitis and AD [32]. Mast cells contain various inflammatory mediators including heparin, histamine, leukotriens, eotaxin and IL-13 that induce AD-like skin lesions [33]. Thus, we investigated the suppressive effect of spermidine on infiltration and degranulation of mast cells to AD-like dermal skin lesions using toluidine blue staining. The numbers of infiltrating and degranulating mast cells were depressed by the administration of 1 and 10 mg/kg spermidine in a dose-dependent manner (Fig. 3). A recent study showed that the allergen captured IgE cross-linked to the high-affinity IgE receptors (FcεRI) on mast cell surface leading to mast cells activation and degranulation of the involving chemical mediators such as heparin and histamine, which are causative factors in immediate allergic symptoms and anaphylaxis [34]. Furthermore, serum hyperproduction of IgE is a well-known hallmark of AD [35]. In the present study, we investigated whether treatment of spermidine inhibited IgE hyperproduction in the AD-like murine model using enzyme-linked immunosorbent assay

(ELISA). DNFB significantly induced total serum IgE production but spermidine did not suppress IgE hyperproduction when administered at either 1 or 10 mg/kg (Fig. 4).

3.3. Spermidine suppresses infiltration of T lymphocytes and IFN- γ production by activated CD4⁺ T cells

The pathogenesis of AD comprises acute and chronic phases with Th₂ and Th₁ dominant inflammation [36]. In the acute phase of AD, infiltrating T lymphocytes produce Th₂ cytokines, such as IL-4, IL-5 and IL-13, which are important to the synthesis and secretion of IgE, as well as accumulation of inflammatory cells in AD-like skin lesions, whereas Th₁ cells produce IL-2 and IFN- γ that contribute to dermal thickening and hyperkeratosis in chronic phase of AD [37]. Presently, we first examined whether spermidine inhibited T lymphocyte infiltration to AD-like skin lesions using immunofluorescence. Spermidine at both 1 and 10 mg/kg significantly inhibited the infiltration of CD4⁺ and CD8⁺ T cells in dorsal skin lesions (Fig. 5). To investigate whether spermidine suppressed secretion of the Th₁ and Th₂ inflammatory cytokines in the mouse model, ELISA was done to measure IL-4 and IFN- γ production from activated CD4⁺ T cells in draining lymph nodes. Both levels of spermidine decreased the production of IFN- γ in activated CD4⁺ T cells, whereas IL-4 was increased in a dose-dependent manner (Fig. 6).

4. Discussion

AD is a common chronic inflammatory skin disease related with asthma and allergic rhinitis that impairs the quality of life [38]. Topical steroid treatment is an effective remedy for AD. But, continual and long-term application of steroids induces skin thinning and atrophy [39]. Thus, a combination of anti-inflammatory, anti-microbial and emollient therapy is currently

the preferred treatment for most patients [40]. Biogenic polyamines are ubiquitous and cationic small molecules, which are related to diverse cellular functions involving growth, proliferation, anti-oxidation and anti-inflammation [41]. A recent study showed that spermine and spermidine inhibit the secretion of 5-hydroxytryptamine (5-HT, serotonin) on mast cells, which are critical in allergic reactions [42]. Furthermore, spermine suppresses LFA-1 (CD11a/CD18) expression on human leukocytes associated with cellular immunity and inflammation [43]. These observations support the view that polyamines might be a new therapeutic candidate for AD, and prompted the present study of the therapeutic effects of spermidine on DNFB-induced AD-like symptoms in NC/Nga mice. Application of spermidine at a concentration of 1 or 10 mg/kg relived DNFB-induced ear swelling and AD-like skin lesions involving edema, erythema, hemorrhage and hyperkeratosis (Fig. 1 and Table. 1). Repetitive application of DNFB disturbs the physical skin barrier and induces surface expression of adhesion molecules including vascular cell adhesion molecule and intracellular adhesion molecule on inflammatory cells, such as eosinophils, polymorphonucleocytes and mast cells [44]. Following leukocyte adhesion, secretion of inflammatory cytokines and chemokines leads to infiltration of inflammatory cells into AD-like skin lesions and increasing epidermal keratinocytes promotes accumulation of collagen, dermal thickening and tissue damage [45]. In the present study, continual DNFB application induced hyperkeratosis in ear and dorsal skin lesions (Figs. 2a and 2b) and increasing infiltration of eosinophils in NC/Nga mice (Fig. 2c). In contrast, treatment with 1 or 10 mg/kg spermidine suppressed dermal thickening and infiltration of eosinophils. Furthermore, these concentrations of spermidine markedly suppressed infiltration and degranulation of mast cells in AD-like skin lesions (Fig. 3b and 3c) regardless of increasing IgE production of total serum (Fig. 4) as well as IL-4 synthesis by activated CD4⁺ T cells (Fig. 6b). In the acute phase of AD, IL-4 induces isotype class switching that leads to the recombination of variable

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diversity joining in B cells and a switch to IgG₄ or IgE synthesis [46]. Therefore, in this study, increasing IL-4 production may have induced IgE hyperproduction. A recent study showed that spermidine significantly inhibits *N*-formylmethionyl-leucyl-phenylalanine (fMLP)-induced chemotaxis that leads to recruitment of PMNs, while association with other effects of PMNs including adhesion, phagocytosis and actin polymerization are not directly affected by spermidine [47]. Furthermore, IL-4 induces the activity of arginase 1, which is associated with putrescine synthesis, and which is further converted with spermidine and alternatively activated macrophages (M2 macrophages) reactions [43]. Th₁ cytokine and IFN- γ , evoke a M1 macrophage response that promotes tissue damage caused by production of MMPs and inflammatory cytokines, whereas Th₂ cytokine, IL-4 or IL-13 induce M2 macrophages, which leads to tissue repair in lesions [48]. The present results indicate that continual spermidine treatment at a concentration 1 or 10 mg/kg may regulate M2 macrophage response caused by increasing IL-4 induced Th₂ shift, which further leads to the relief of AD-like clinical symptoms (Fig. 6a). In inflammation skin lesions, antigen or allergen captured dermal dendritic cells and epidermal langerhans cells migrate into draining lymph nodes. Migrating antigen presenting cells induce the activation of CD4⁺ T and CD8⁺ T cells from naive T cells and then CD4⁺ T cells leads to the differentiation of Th₁ or Th₂ cells. These cells produce IFN- γ , IL-4, IL-5 and IL-13, which promote the development of an inflammatory state [49]. Our results indicated that spermidine inhibits the infiltration of CD4⁺ T and CD8⁺ T cells in dorsal skin lesions (Fig. 5) and that production of IFN- γ is decreased in a spermidine dose-dependent manner (Fig. 6b). These results further suggested that spermidine ameliorates AD-like clinical manifestations and infiltration of inflammatory cells by inhibiting Th₁ cytokines and degranulation of mast cells. Furthermore, spermidine may regulate M2 macrophage response caused by increasing IL-4. We did not define whether spermidine induces the expression of M2 macrophage response in this study. Thus, further

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research is necessary to investigate the regulation mechanisms of spermidine-induced M2 macrophage response and correlation with IL-4 synthesis in the NC/Nga AD-like model.

Conflict of interest statement

The authors have declared no conflict of interest.

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Figure legends

Fig. 1. Effect of spermidine treatment on AD-like skin lesions induced by DNFB in NC/Nga mice.

AD-like lesions were induced by repeated painting of 0.15% DNFB (acetone : olive oil, 3 : 1) on outer and inner ear surfaces of NC/Nga mice once daily on days 1 and 4. The sensitized mice were further challenged with 0.2% DNFB on both sides of each ear on days 7, 10 and 13. Control animals were treated with an identical volume of acetone : olive oil. Spermidine was injected intraperitoneally daily from the day of first DNFB challenge (day 7). Ear thicknesses were measured with a thickness gauge every day. Values are mean $\pm$ SEM of five animals per group. DNFB (-): acetone : olive oil (3:1) treated; DNFB (+): DNFB + PBS and DMSO 1% treated; DNFB + SPD (1 mg/kg): DNFB + spermidine (1 mg/kg); DNFB + SPD (10 mg/kg): DNFB + spermidine (10 mg/kg) treated. (A) Effect of spermidine treatment on ear swellings of DNFB induced mice. Values are mean $\pm$ SEM of five animals per group. * $p < 0.05$ versus DNFB + SPD groups of mice. (B) Representative photographs captured after treatment completion (fifth treatment) showing comparison of AD like skin lesions.

Fig. 2. Histological features of NC/Nga mice treated with spermidine.

NC/Nga mice were treated with 0.15% DNFB (acetone : olive oil, 3 : 1) on shaved back skins on days 1 and 4, and further challenged with 0.2% DNFB on days 7, 10 and 13. After fifth treatment, skin lesions were photographed and cryosections were prepared with H&E staining for histological analysis. H&E stained cryosections of back skin samples to compare effect of spermidine treatment on skin inflammation. Bar = 100 μ m. (A) Epidermal thickness (μ m) (n

= 24) and (B) Eosinophils (nucleus with 2 lobes and intense pink staining) in H&E stained sections were counted under a microscope and confirmed using the CellProfiler Analyst software (free and open-source program) and NIS-Elements imaging software (Nikon, Tokyo, Japan) (C) Arrows indicate eosinophils. The number of cells was expressed as the mean $\pm$ SEM of five sections. * $p < 0.05$ vs. DNFB (+) mice.

Fig. 3. Reduced mast cell infiltration and degranulation in dorsal skin of AD-like skin lesions.

Mast cells in toluidine blue-stained sections. (A) Quantitative data for total mast cells (B) and degranulated mast cells (n = 5 sections each). (C) DNFB (-): acetone : olive oil (3:1) treated; DNFB (+): DNFB + PBS and DMSO 1% treated; DNFB + SPD (1 mg/kg): DNFB + Spermidine (1 mg/kg); DNFB + SPD (10 mg/kg): DNFB + Spermidine (10 mg/kg) treated. Data are means $\pm$ SEM (n = 5). * $p < 0.05$ vs. DNFB (+) mice. Bar = 100 μ m.

Fig. 4. Effect of spermidine on IgE production of DNFB sensitized NC/Nga mice.

IgE was tested by ELISA in serum obtained from DNFB sensitized NC/Nga mice. Spermidine was injected intraperitoneally daily from the day of first DNFB challenge (day 7). Total IgE in the indicated groups were measured 24 h after final administration. DNFB (-): acetone : olive oil (3:1) treated; DNFB (+): DNFB + PBS and DMSO 1% treated; DNFB + SPD (1 mg/kg): DNFB + spermidine (1 mg/kg); DNFB + SPD (10 mg/kg): DNFB + spermidine (10 mg/kg) treated. Data are mean $\pm$ SEM (n = 5). * $p < 0.05$ vs. DNFB (+) mice.

Fig. 5. CD4⁺ and CD8⁺ T cell infiltration is suppressed by spermidine in dorsal skin lesions of DNFB sensitized mice.

NC/Nga mice were treated with 0.15% DNFB (acetone : olive oil, 3 : 1) on shaved back skins on days 1 and 4, and further challenged with 0.2% DNFB on days 7, 10 and 13. Control animals were treated with an identical volume of acetone/olive oil. Spermidine was injected

intraperitoneally daily from the day of first DNFB challenge (day 7). After final treatment with DNFB, immunofluorescent staining was done with the cryosections of back skin samples with Alexa-Fluor 488-conjugated goat anti-rat secondary antibody. Representative pictures showing activation of CD4⁺ T cells and CD8⁺ T cells (A) after DNFB treatment which was inhibited by spermidine dose-dependently. CD4⁺ T cells (B) and CD8⁺ T cells (C) were counted. DNFB (-): acetone : olive oil (3 : 1) treated; DNFB (+): DNFB + PBS and DMSO 1% treated; DNFB + SPD (1 mg/kg): DNFB + spermidine (1 mg/kg); DNFB + SPD (10 mg/kg): DNFB + spermidine (10 mg/kg) treated. The number of cells was expressed as the mean $\pm$ SEM of five sections. * $p < 0.05$ vs. DNFB (+) mice. Bar = 100 μ m.

Fig. 6. Effect of spermidine on IL-4 and IFN- γ production by CD4⁺ T cells from DLN of DNFB-treated NC/Nga mice.

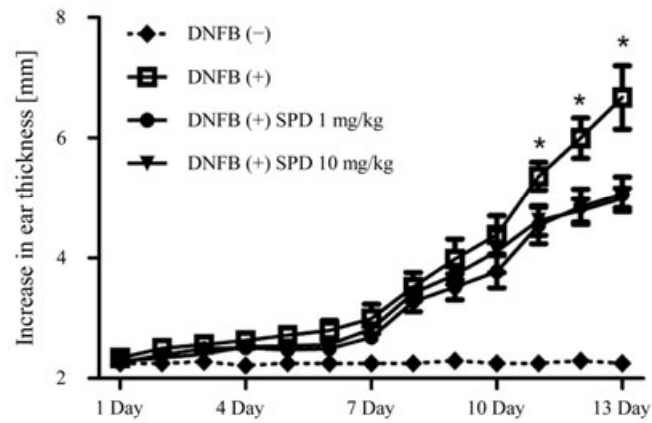
Lymph nodes were excised 24 h after final DNFB administrations and CD4⁺ T cells were purified. The purified CD4⁺ T cells (1×10^6 cells/ml) were stimulated with plate bound anti-CD3 antibody (2C11; 5 μ g/ml) plus a soluble form of purified anti-mouse CD28 antibody (2 μ g/ml) for 50 h. The production of (A) IL-4 and (B) IFN- γ after T cell activation were quantified by ELISA. DNFB (-): acetone : olive oil (3:1) treated; DNFB (+): DNFB + PBS and DMSO 1% treated; DNFB + SPD (1 mg/kg): DNFB + spermidine (1 mg/kg); DNFB + SPD (10 mg/kg): DNFB + spermidine (10 mg/kg) treated. Results are based on triplicate experiments. Data are mean $\pm$ SEM (n = 5). * $p < 0.05$ vs. DNFB (+) mice.

Table. 1. The severity of AD was macroscopically evaluated

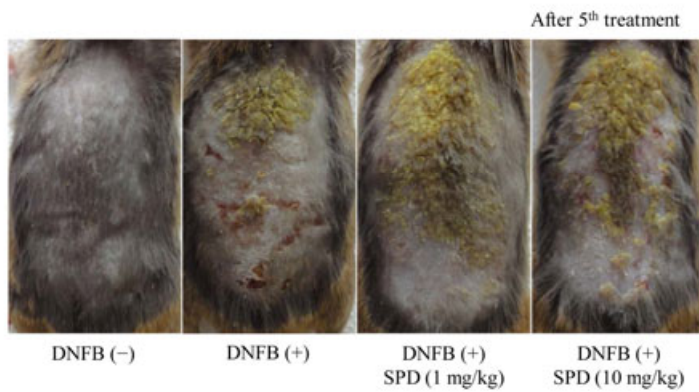
After 5 th treatment		
Group	Dermatitis Score	NO. of Mice
DNFB (-)	0 ± 0.00	5
DNFB (+)	9.8 ± 1.57	5
DNFB (+) SPD 1 mg/kg	7.4 ± 0.95 *	5
DNFB (+) SPD 10 mg/kg	7.6 ± 1.26 *	5

The degree of each symptom was graded from 0–3 (0, absence; 1, mild; 2, moderate; 3, severe). This scoring was based on the severity of edema, hemorrhage, oozing, crust, excoriation, and lichenification. Overall dermatitis score was determined from the sum of all individual scores. Data are presented as mean ± SEM of five determinations. One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test was used for comparing differences among multiple groups. *p < 0.05 vs. DNFB (+) groups.

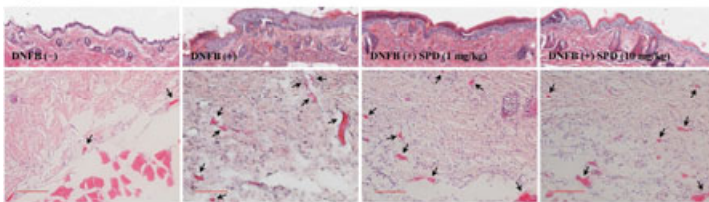
A



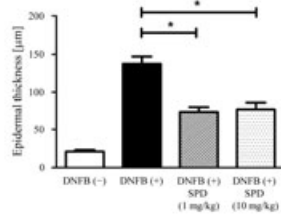
B



A



B



C

