

Hyperproduction of IFN- γ by CpG Oligodeoxynucleotide-Induced Exacerbation of Atopic Dermatitis-Like Skin Lesion in Some NC/Nga Mice

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Under conventional conditions, NC/Nga mice spontaneously develop an atopic dermatitis (AD)-like skin lesion accompanied by immunoglobulin E (IgE) hyperproduction and the expression of T helper 2 (Th2) cytokines. CpG DNA activates a strong interferon-gamma (IFN- γ)-dominated T helper 1 (Th1) response, while inhibiting Th2-dependent allergies. In this study, we examined whether CpG oligodeoxynucleotide (ODN) could prevent the development of the skin lesions in NC/Nga mice. Sixteen of 26 NC/Nga mice did not exhibit dermatitis after CpG ODN was administered intraperitoneally every 2 wk for a total of five times. CpG ODN administration induced IFN- γ production, which inhibited the production of Th2 cytokines (interleukin (IL)-4, IL-5, and IL-13) in both spleen and lymph node cells and culminated in a decrease in the serum IgE level. These data suggest that the CpG ODN has a therapeutic effect against AD; however, some mice (10 of 26) treated with CpG ODN exhibited an exacerbation of dermatitis accompanied by the hyperproduction of IFN- γ , although Th2 cytokines were suppressed. These results suggest that the suppression of Th2 cytokines may not completely prevent dermatitis and that IFN- γ may play a role in developing dermatitis in some NC/Nga mice.

Key words: atopic dermatitis/CpG/IFN- γ /NC/Nga
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Atopic dermatitis (AD) is characterized by a chronic inflammatory skin disease with intensely pruritic, subacute, and chronic eczematous plaques (Leung, 1997; Rudikoff and Lebwohl, 1998). Evidence suggests that various factors contribute to the pathogenesis and development of AD; these include immunological abnormalities, barrier dysfunction of the skin (Cooper, 1994), exposure to allergen such as mite (Platts-Mills *et al*, 1983; Sampson and Mccaskill, 1985; Schafer *et al*, 1999), environmental factors (Adinoff *et al*, 1988), and psychotic factors. More than 80% of AD patients have increased level of total immunoglobulin E (IgE) (Johnson *et al*, 1974). The skin lesion shows infiltration of activated CD4⁺ T cells expressing interleukin (IL)-4, IL-5, and IL-13 (Kay *et al*, 1991; Rousset *et al*, 1991). The decreased interferon-gamma (IFN- γ) production by T cells (Reinhold *et al*, 1990; Renz *et al*, 1992; Lester *et al*, 1995) is considered to be linked with IgE hyperproduction and allergen-specific T helper type 2 (Th2) immune responses in AD patients.

The NC/Nga mice have been reported to develop an eczematous AD-like skin lesion when kept under conventional conditions, but not under specific pathogen-free conditions. The skin lesion appears at approximately 7 wk of age and

becomes maximally inflamed at approximately 17 wk of age; the inflammation is characterized by edema, hemorrhage, erosion, and dryness on the ears, face, neck, and back (Matsuda *et al*, 1997; Suto *et al*, 1999). It has been reported that the elevation of serum levels of total IgE is correlated with the severity of the skin lesions and the production of Th2 cytokines and chemokines (Matsuda *et al*, 1997, 1999; Suto *et al*, 1999; Vestergaard *et al*, 1999). The B cells of the NC/Nga mouse have increased sensitivity to the CD40 ligand and IL-4 originating from the constitutive activation of Janus Kinase 3 (JAK3), which results in an acceleration of IgE production (Matsumoto *et al*, 1999). Th2 cytokines are considered to play a major role in the pathogenesis of AD-like skin lesions in NC/Nga mice as well as in that of human AD (Habu *et al*, 2001; Matsumoto *et al*, 2001).

Bacterial DNA and oligodeoxynucleotides (ODNs) expressing unmethylated CpG motifs stimulate B cells, T cells, natural killer (NK) cells, and macrophages to proliferate and/or secrete cytokines, including IL-6, IL-12, IL-18, tumor necrosis factor- α (TNF- α), and IFN- γ (Yamamoto *et al*, 1992; Krieg *et al*, 1995; Klinman *et al*, 1996; Roman *et al*, 1997; Schwartz *et al*, 1999), through the toll-like receptor (TLR)-9-mediated signaling (Hemmi *et al*, 2000; Akira and Hemmi, 2003). CpG ODN are being used as “immunomodulatory agents” because they elicit strong Th1-shifted innate immune responses. In animal studies, CpG ODN have been reported to inhibit allergic responses such as

Abbreviations: AD, atopic dermatitis; IgE, immunoglobulin E; IL, interleukin; ODN, oligodeoxynucleotide; rIFN, recombinant interferon; Th, T helper

pollenosis and asthma (Broide *et al*, 1998; Magone *et al*, 2000), and their effect persists for more than 2 wk after a single administration (Kobayashi *et al*, 1999, 2000; Lipford *et al*, 2000). Furthermore, clinical studies have already begun on pollenosis and other allergic disorders (Creticos and Lichtenstein, 2003).

No study on the effect of CpG ODN in AD model mice has been reported. In this study, we examined whether CpG ODN could inhibit Th2 cytokines and prevent the development of the AD-like skin lesions in NC/Nga mice. We used 7-wk-old NC/Nga mice that had not yet developed skin lesions, and CpG ODN was administered intraperitoneally every 2 wk for a total of five times. We observed the skin condition and also measured the levels of different cytokines produced in the spleen and lymph node cells after the repeated administration of CpG ODN.

Results

Prevention and exacerbation of AD-like skin lesion by CpG ODN administration in NC/Nga mice AD-like skin lesions developed in 72.5% (29 of 40) of the NC/Nga mice when they were housed under conventional conditions. When the mice were administered 25 μ g of CpG ODN (25 CpG) or 50 μ g of CpG ODN (50 CpG) intraperitoneally, the frequencies of skin lesions were 70% (seven of 10) and 38.5% (10 of 26), respectively. To evaluate the severity of the skin lesions, the skin was examined, and the lesions were scored in each mouse. The clinical skin score at 15 wk in 50 CpG group was significantly lower than that in the group without treatment (NT group) ($p < 0.05$). There was no significant difference between 25 CpG group and NT group (Fig 1A). Therefore, we administered 50 μ g CpG in further study. To examine whether the CpG ODN treatment can improve the severity of the developing AD-like skin lesions, mice were divided into two groups: those with AD lesions (AD+) and those without AD lesions (AD-). When we selected mice from the AD+ group and evaluated the skin score at 15 wk, the score in the CpG-ODN-AD+ group was higher than that in the NT-AD+ group (Fig 1B). We suspected that the mice in the CpG-ODN-AD+ group did not respond to CpG ODN and examined the production of IgE and cytokines.

Suppression of serum IgE level by CpG ODN administration The serum IgE levels of NC/Nga mice were generally higher than those of BALB/c mice (Fig 2). The serum IgE levels of the CpG-ODN group were significantly lower than those of the NT group in both NC/Nga and BALB/c mice (Fig 2). As shown in Fig 2B, in the CpG-ODN group, no difference in serum IgE levels was observed between the AD- group and AD+ group. In the NT group, the serum IgE level was significantly higher in the AD+ group than in the AD- group. It is noteworthy that when CpG ODN was injected into NC/Nga mice, there was no difference in the serum IgE level between AD- and AD+ groups.

Cytokine production by spleen cells isolated from mice **CpG ODN-administered mice** To examine the effect of CpG ODN administration on cytokine production in NC/Nga mice, an enzyme-linked immuno-spot (ELISpot) assay

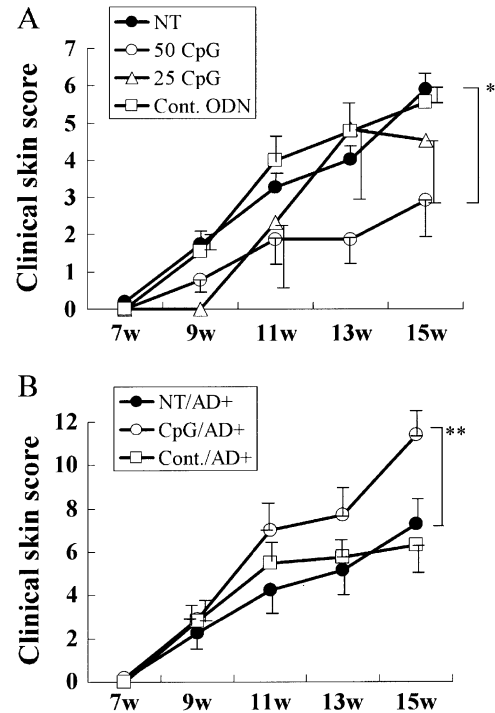


Figure 1

The clinical skin score of atopic dermatitis (AD)-like skin lesion in mice administered CpG oligodeoxynucleotide (ODN) i.p. The clinical skin score is the sum of individual scores judged by the symptoms of itch, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness. (A) NC/Nga mice were left non-treated (NT, $n = 40$), 25 μ g (25 CpG, $n = 10$), 50 μ g (50 CpG, $n = 26$) of CpG ODN and control-ODN (Cont. ODN, $n = 15$) was administered i.p. every 2 wk. (B) The skin score of the CpG ODN-administered AD+ group (CpG/AD+, $n = 10$) was compared with that of the NT group with AD (NT/AD+, $n = 29$) or control-ODN group with AD (Cont./AD+, $n = 11$). The scores represent the mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$ by t test.

targeting IFN- γ and IL-4 and an ELISA targeting IL-5 and IL-13 were performed using spleen cells (Fig 3).

The number of IFN- γ producing cells (Fig 3A) was higher in the CpG-ODN group than in the NT and control-ODN groups in both BALB/c and NC/Nga mice. In contrast, IL-4, IL-5, and IL-13 production (Fig 3B–D) was lower in the CpG-ODN group than in the NT and control-ODN groups in both BALB/c and NC/Nga mice. When the levels of cytokine production between the AD- group and the AD+ group in NC/Nga mice were compared, IFN- γ production was found to be higher in the CpG-ODN-AD+ group than in the CpG-ODN-AD- group (Fig 3A). No difference, however, was observed in the levels of IL-4, IL-5, and IL-13 between the CpG-ODN-AD+ and CpG-ODN-AD- groups (Fig 3B–D). It is noteworthy that the production of Th2 cytokines (IL-4, IL-5, and IL-13) was not activated in the CpG-ODN-AD+ group in spite of developing dermatitis, whereas IFN- γ production was greatly enhanced.

Cytokine production by regional lymph node cells isolated from CpG ODN-administered NC/Nga mice Cytokine production was also examined by ELISpot assay in the regional lymph nodes (LN) from CpG ODN-administered NC/Nga mice (Fig 4). Similar to the systemic immune response observed above, a higher production of IFN- γ

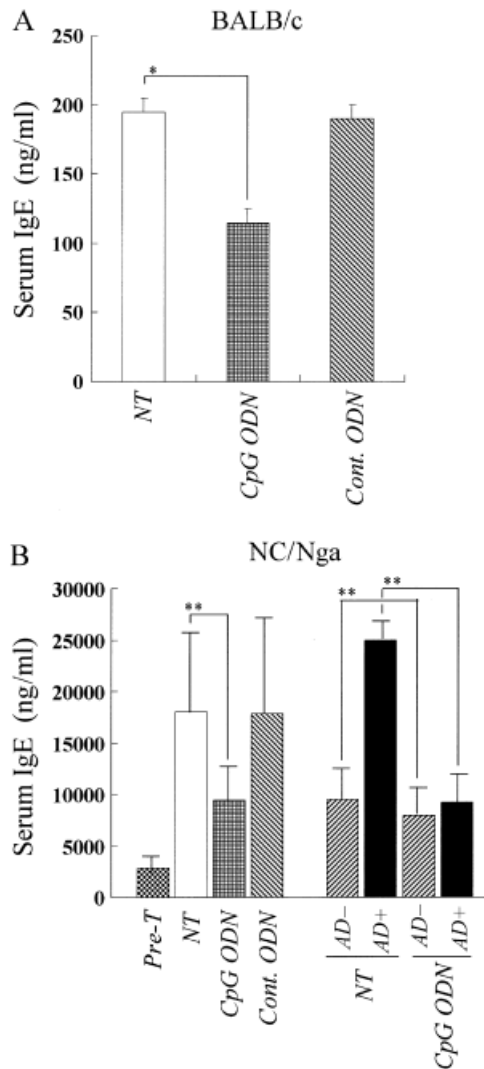


Figure 2
Suppression of serum immunoglobulin E (IgE) level by CpG oligodeoxynucleotide (ODN) administration. The sera were collected from the mice before treatment (Pre-T, $n = 81$) or 2 d after the final treatment. The serum IgE level was measured by ELISA. BALB/c (A, $n = 10$ per group) and NC/Nga (B) mice were left non-treated (NT) ($n = 40$) or were treated with CpG ODN ($n = 26$) or control-ODN (Cont. ODN) ($n = 15$). Each group of NC/Nga mice was subdivided into atopic dermatitis (AD)- group in which the mice showed no clinical symptoms (NT/AD-, CpG/AD-) and AD+ group in which the mice showed AD-like skin lesions (NT/AD+, CpG/AD+) (B). Each data represent the mean $\pm$ SD. * $p < 0.01$, ** $p < 0.0001$ by t test.

(Fig 4A) and a lower production of IL-4, IL-5, and IL-13 (Fig 4B–D) were detected in the CpG-ODN group than in the NT group. When compared with the CpG-ODN-AD- group, a higher level of IFN- γ production was also observed in the CpG-ODN-AD+ group (Fig 4A). No difference was observed in the levels of IL-4, IL-5, and IL-13 between the CpG-ODN-AD+ and CpG-ODN-AD- groups (Fig 4B–D).

Effect of recombinant IFN- γ (rIFN- γ) administration in NC/Nga mice With rIFN- γ administration, 10 of 10 mice developed skin lesions, whereas three of 10 mice developed severe dermatitis, and the others developed moderate dermatitis at 15 wk of age. Although, the group without treatment (NT group) displayed that seven of 10 mice

developed skin lesions (two of seven mice developed severe dermatitis, and the others developed moderate dermatitis). The clinical score of the IFN- γ -administered mice was significantly higher than that of the NT group at 15 wk of age ($p < 0.05$) (Fig 5). Therefore, these results suggest that the hyperproduction of IFN- γ induced the exacerbation of the AD-like skin lesion in NC/Nga mice. Next, we examined the possibility that the mechanisms responsible for dermatitis in the CpG-ODN or rIFN- γ group may differ from those in NT mice. In a comparison of histological sections of skin lesions from the back of NC/Nga mice, it was found that both the dermis and epidermis were thickened, and a cellular infiltration was observed in the dermis (Fig 6). The mean thickness $\pm$ SD of the epidermis was 0.488 ± 0.245 μ m in NT/AD- mice, 4.405 ± 2.420 μ m in NT/AD+ mice, 0.378 ± 0.072 μ m in CpG/AD- mice, 1.421 ± 0.128 μ m in CpG/AD+ mice, and 1.386 ± 1.059 μ m in rIFN- γ mice. We also stained the histological skin sections with toluidine blue for counting mast cells. The numbers of mast cells were increased in the CpG-ODN-AD+ and rIFN- γ groups as well as in the NT/AD+ group (NT/AD-, 268.3 ± 12.3 per mm^2 ; NT/AD+, 433.8 ± 30.5 per mm^2 ; CpG/AD-, 202.5 ± 18.6 per mm^2 ; CpG/AD+, 426.0 ± 26.0 per mm^2 ; rIFN- γ , 391.81 ± 27.1 per mm^2). The skin lesions elicited in the CpG-ODN-AD+ and rIFN- γ groups had histological features very similar to those of the NT/AD+ group. Therefore, we concluded that there were no significant differences in the pathogenesis of skin lesions among the CpG-ODN-AD+, rIFN- γ , and NT/AD+ groups.

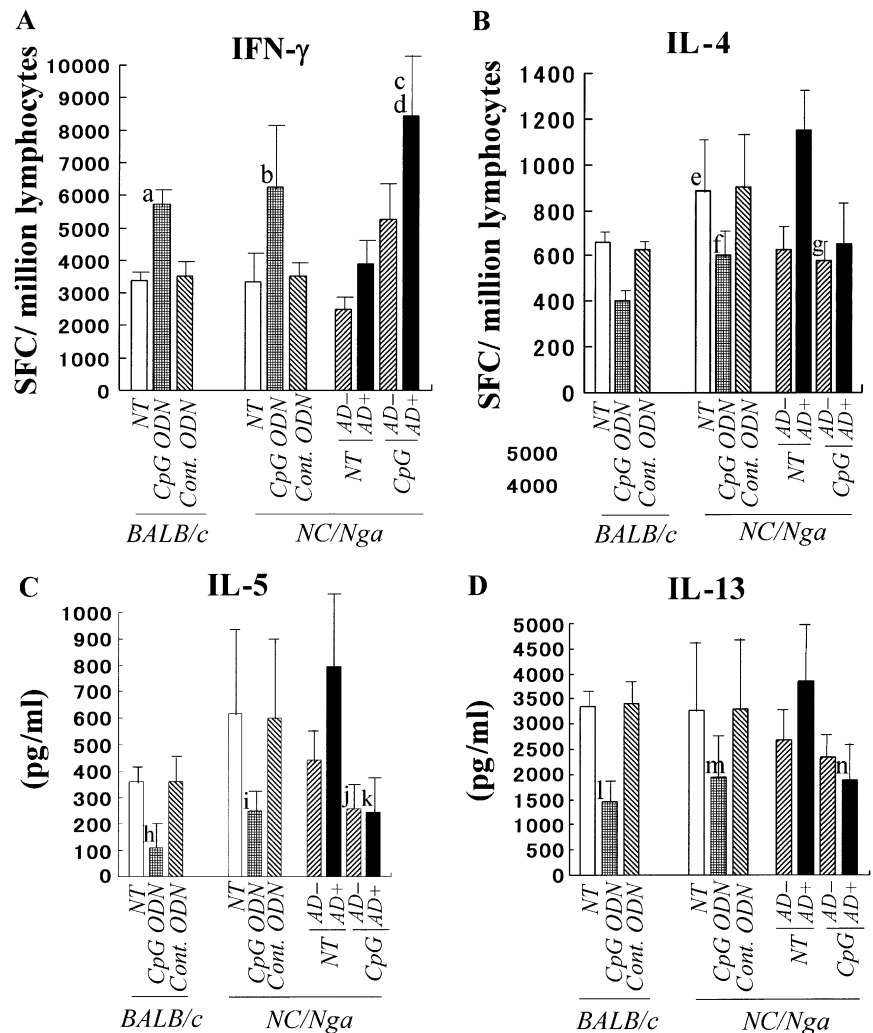
Discussion

This study demonstrates that CpG ODN administration could reduce the frequency and severity of skin lesions in NC/Nga mice (Fig 1A). In fact, CpG ODN treatment induced the Th1 cytokine and reduced serum IgE levels and Th2 cytokines in these mice; this demonstrates the ability of CpG ODN to modulate Th1-dominated immune responses (Figs 2–4). It is known that CpG ODN induce the production of IL-12, which activates NK cells to produce IFN- γ , and promotes the differentiation of naïve T cells into Th1 cells that produce IFN- γ (Krieg *et al*, 1995; Klinman *et al*, 1996). IFN- γ suppresses the proliferation of Th2 cells and inhibits IgE production by B cell. IL-18 is also known to induce IFN- γ production in conjunction with IL-12 (Yoshimoto *et al*, 1997). Therefore, we speculated that the increased IL-12 and IL-18 production by CpG ODN administration induced IFN- γ production. No significant differences in IL-12 and IL-18, production, however, were detected between the CpG-ODN-administered group and the non-treated group in spleen cells, lymph node cells, and serum (data not shown).

It is of significance that CpG ODN administration seemed to increase the severity of AD-like skin lesions in some mice (Fig 1B). In these mice, the production of Th2 cytokines was reduced; however, the production of IFN- γ was higher than that of the CpG-ODN-AD- group (Figs 3 and 4). These results suggest that the suppression of Th2 cytokines may not completely prevent dermatitis in some NC/Nga mice and that IFN- γ may play a role in inducing dermatitis. It was previously reported that IFN- γ plays the role of an inducer

Figure 3

Cytokine production by spleen cells isolated from CpG oligodeoxynucleotide (ODN)-administered mice. Spleen cells were obtained from mice that had been left non-treated (NT, $n=40$), and those administered CpG ODN (CpG ODN, $n=26$) or control-ODN (Cont. ODN, $n=15$). The control mice were BALB/c mice ($n=10$ per group). The cells were incubated with Con A. Enzyme-linked immuno-spot (ELISpot) assay targeting interferon-gamma (IFN- γ) (A) and interleukin (IL)-4 (B) was performed 24 h after stimulation (SFC, spot-forming cells). ELISA targeting IL-5 (C) and IL-13 (D) was also performed using supernatants obtained 48 h after stimulation. Each group was also subdivided into atopic dermatitis (AD)- and AD+ as described in Fig 2. a, NT versus CpG ODN ($p<0.001$); b, NT versus CpG ODN ($p<0.0001$); c, NT/atopic dermatitis (AD)+ versus CpG/AD+ ($p<0.0001$); d, CpG/AD- versus CpG/AD+ ($p<0.01$); e, BALB/c/NT versus NC/NT ($p<0.05$); f, NT versus CpG ODN ($p<0.01$); g, NT/AD+ versus CpG/AD+ ($p<0.001$); h, NT versus CpG ODN ($p<0.0001$); i, NT versus CpG ODN ($p<0.01$); j, NT/AD- versus CpG/AD- ($p<0.05$); k, NT/AD+ versus CpG/AD+ ($p<0.05$); l, NT versus CpG ODN ($p<0.001$); m, NT versus CpG ODN ($p<0.001$); and n, NT/AD+ versus CpG/AD+ ($p<0.01$) by t test. Each data represent the mean $\pm$ SD.



for eotaxin 2 and CC chemokine receptor 3 (CCR3), which were predominantly expressed in skin lesions (Teran *et al*, 2000). The CXC chemokine receptor 3 (CXCR3), which is a receptor for both IFN- γ -inducible protein 10 (γ IP10) and the monokine induced by IFN- γ (Mig), is expressed on eosinophils (Jinquan *et al*, 2000). Moreover, IFN- γ induces TARC (Th2 chemokine) expression in keratinocytes in NC/Nga mice (Vestergaard *et al*, 1999). These reports suggest that it is possible that IFN- γ induce allergic inflammation.

In addition, when recombinant IFN- γ was administered to NC/Nga mice, all the mice developed dermatitis, and the skin lesions at 15 wk of age were more severe than those of the non-treated NC/Nga mice (Fig 5). This result indicates that IFN- γ plays an important role in the development of AD-like skin lesions in NC/Nga mice.

Current studies on humans have reported that IFN- γ expression is predominant over Th2 cytokine expression in chronic AD lesions; this indicates a shift from Th2 to Th1 during the chronic period (Grewe *et al*, 1994; Thepen *et al*, 1996; Werfel *et al*, 1996). Another study has shown that both Th2 and Th1 chemokines are elevated in the sera of AD patients (Shimada *et al*, 2004). Akdis *et al* (1999) termed a subgroup of AD patients with normal serum IgE level as the "non-allergic" AD group; this group exhibited a lower level of IL-13 and a higher level of IFN- γ than the "allergic" AD group. Furthermore, it was reported that

AD-like skin lesions can develop in signal transducer and activator of transcription factor 6 (STAT6)-deficient NC/Nga mice in the absence of Th2 cytokines (Yagi *et al*, 2002). Taken together, these data suggest that AD can develop even without the Th2 cytokine and IFN- γ participates in the formation of skin lesions at least in some cases.

Some studies have clinically demonstrated the efficacy of IFN- γ in treating AD. The treatments, however, were evaluated only with the recalcitrant, severe, chronic AD and a decrease in serum total IgE levels was not detected in these patients (Chang and Stevens, 2002).

In this study, CpG ODN administration induced the Th1 cytokine and inhibited Th2 cytokines. Some CpG-ODN-administered mice, however, exhibited an exacerbation of AD-like skin lesions accompanied by the hyperproduction of IFN- γ . We suggest that the level of IFN- γ might play an important role in the development of AD-like skin lesions in NC/Nga mice.

This might be a clue to understanding the mechanism of the pathogenesis of AD. The cautious application of CpG-ODN therapy in AD is necessary.

Materials and Methods

ODN The ODN were kind gifts from D. M. Klinman (US Food and Drug Administration, Rockville, Maryland) and had no detectable

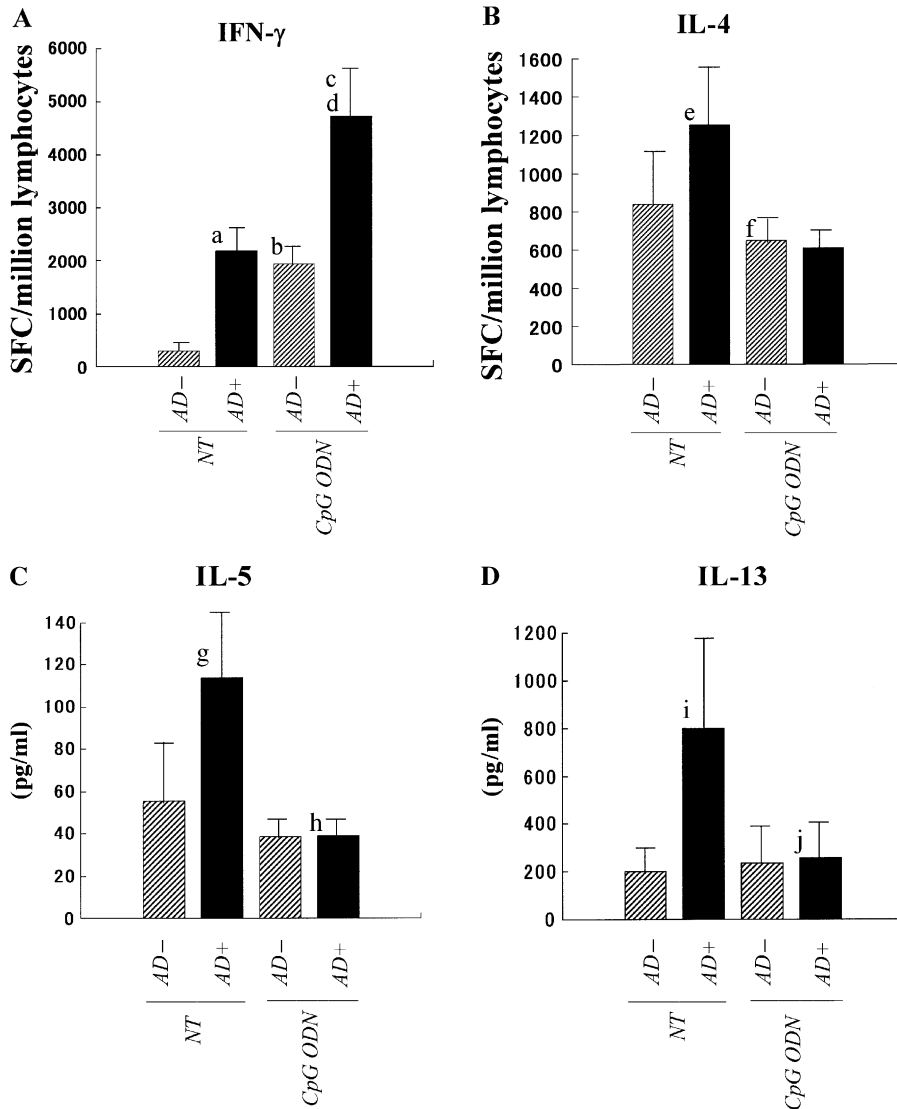


Figure 4
Cytokine production by regional lymph node cells isolated from CpG oligodeoxynucleotide (ODN)-administered NC/Nga mice. Cervical and axillary lymph nodes were isolated from NC/Nga mice that had been left non-treated (NT, $n=40$) or were administered CpG ODN (CpG ODN, $n=26$). Cells were incubated with Con A. Enzyme-linked immuno-spot (ELISpot) assay targeting interferon-gamma (IFN- γ) (A) and interleukin (IL)-4 (B) was performed 24 h after stimulation (SFC, spot-forming cells). ELISA targeting IL-5 (C) and IL-13 (D) was also performed using supernatants obtained 24 h after stimulation. Each group was also subdivided into atopic dermatitis (AD)- and AD+ as described in Fig 2. a, AD- versus AD+ ($p<0.001$); b, NT/AD- versus CpG/AD- ($p<0.001$); c, AD- versus AD+ ($p<0.001$); d, NT/AD+ versus CpG/AD+ ($p<0.001$); e, AD- versus AD+ ($p<0.01$); f, NT/AD+ versus CpG/AD- ($p<0.001$); g, AD- versus AD+ ($p<0.05$); h, NT/AD+ versus CpG/AD+ ($p<0.05$); i, AD- versus AD+ ($p<0.05$); and j, NT/AD+ versus CpG/AD+ ($p<0.05$) by t test. Each data represent the mean $\pm$ SE.

endotoxins (<2.0 EU per mL) as found by the Limulus HS-J Single Test (Wako Pure Chemicals, Osaka, Japan). The sequences of the ODN are as follows; CpG ODN, 5'-GCTAGACGTTAGCGT-3' and 5'-TCAACGTTGA-3' were mixed equally; control ODN, 5'-TCAAGCTTGA-3'.

Mice and treatment Seven-week-old female NC/Nga and BALB/c mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and maintained under conventional conditions.

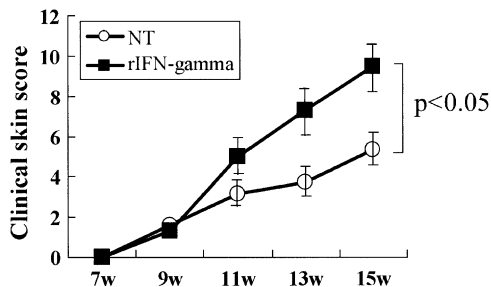


Figure 5
The clinical skin score of atopic dermatitis (AD)-like skin lesions in NC/Nga mice who received recombinant IFN- γ (rIFN- γ) i.p. twice a week from 7 to 15 wk of age. The clinical skin score was calculated as described in Fig 1. The skin score of rIFN- γ -administered group (rIFN- γ , $n=10$) was compared with that of the non-treated group (NT, $n=10$). The scores represent the mean $\pm$ SE.

The mice were administered 50 μ g of ODN intraperitoneally for a total of five times at 2-wk intervals. In further studies, NC/Nga mice were administered 10,000 U of recombinant IFN- γ (Pepro Tech, London, UK) intraperitoneally twice a week from seven to 15 wk of age under conventional conditions. This procedure was approved by the ethical committee of the Yokohama City University.

Clinical skin score The total clinical skin score was defined as the sum of individual scores graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for the symptoms of itch, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness (Leung *et al*, 1997; Suto *et al*, 1999).

ELISA Mouse sera were collected before administration (7 wk of age) and 2 d after the final administration (15 wk of age). The serum IgE level was measured by an ELISA kit (Yamasa, Chiba, Japan). Two days after the final administration, single-cell suspensions were prepared from the spleen and lymph nodes and incubated with 5 μ g per mL of concanavalin A (Con A) (Vector Laboratories, Burlingame, California) in RPMI 1640 (Sigma Chemical, St Louis, Missouri) with 10%, 2-mercaptoethanol, and penicillin/streptomycin at 37°C in 5% CO₂ for 48 or 24 h. The supernatant samples were collected and stored at -80°C until further use. The concentrations of IL-5 and IL-13 were determined by an ELISA kit (R&D System, Minneapolis, Minnesota).

ELISpot assays The frequencies of IFN- γ - or IL-4-producing cells were determined 2 d after the final administration by using an

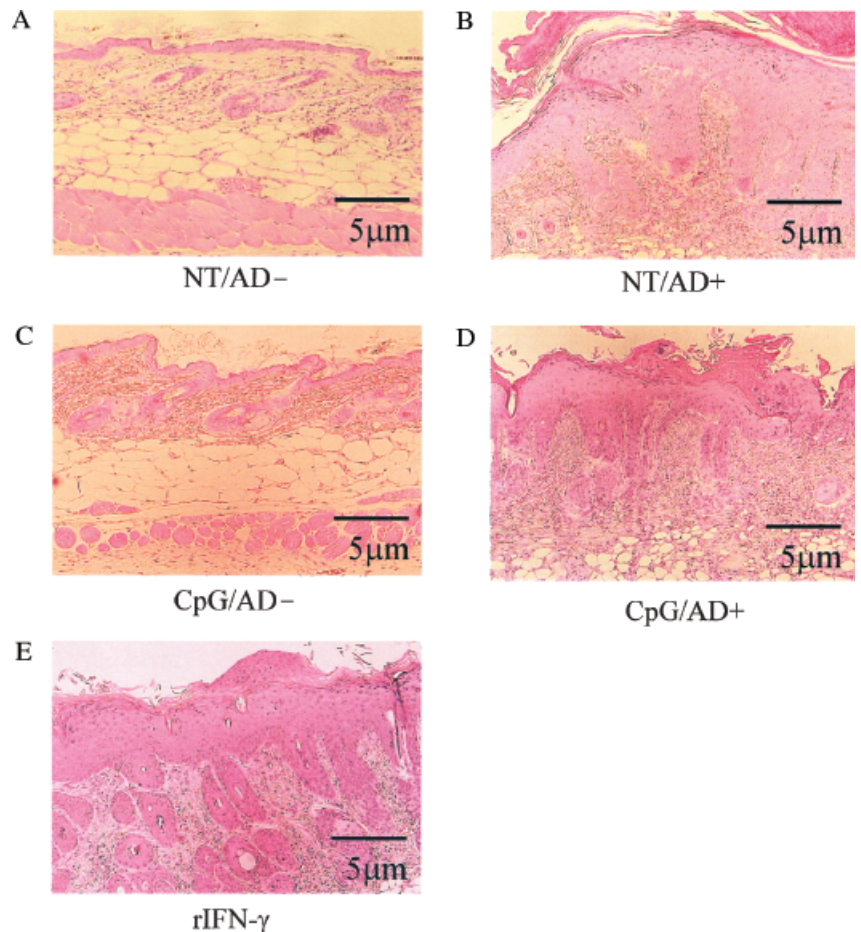


Figure 6

Histologic analysis of dermal skin from the non-treated (NT), CpG oligodeoxynucleotide (ODN), and recombinant IFN- γ (rIFN- γ) groups. The dermal skin was resected from the back in the NT/atopic dermatitis (AD)- group (A) and CpG/AD- group (C). The skin lesions were resected from the back in the NT/AD+ group (B), CpG/AD+ group (D), and rIFN- γ group (E). The sections were stained by hematoxylin and eosin. Magnification $\times 100$.

ELISpot assay kit (MabTech, Nacka, Sweden). In brief, Multi-Screen-IP sterile plates (Millipore, Bedford, Massachusetts) were coated with 10 μ g per mL of purified rat anti-mouse IFN- γ monoclonal antibody (mAb) (AN18) or IL-4 mAb (11B11) in PBS and incubated overnight at 4°C. After blocking with PBS containing 0.025% Tween 20 and 5% bovine serum albumin, single-cell suspensions (10^5 cells) isolated from the spleen or lymph node were added and incubated with 3 μ g per mL of Con A at 3°C for 24 h. After washing, the plates were incubated with 0.5 μ g per mL of biotinylated anti-mouse IFN- γ (R4-6A2) or IL-4 antibody (BVD6) at 37°C for 2 h, followed by alkaline phosphatase streptavidin treatment at room temperature for 1.5 h. Finally, the plates were treated with BCIP/NBT membrane phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) at room temperature for 20 min, and the reaction was stopped by washing with distilled water. The numbers of spots were counted by a computer-assisted video image analysis (Carl Zeiss, Thornwood, New York). The results were expressed as spot-forming cells (SFC) per million cells.

Dermal histology The dermal skin was resected, fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, stained with either hematoxylin & eosin (HE) or toluidine blue (TB) solution, and examined by light microscopy for histologic changes. The thickness of the epidermis in HE sections and the cell numbers in TB sections were expressed as the total counted in five fields. Each value was obtained from five mice per group.

Data analysis Statistical analysis of the experimental data was performed by the two-tailed Student's *t* test. The values of the clinical skin score were expressed as mean $\pm$ standard error (SE). The other values were expressed as mean $\pm$ standard deviation (SD).

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