

# Repeated Topical Challenge with Chemical Antigen Elicits Sustained Dermatitis in NC/Nga Mice in Specific-Pathogen-Free Condition

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NC/Nga mice are known to develop skin lesions resembling to atopic dermatitis (AD) in conventional but not in specific-pathogen-free (SPF) condition. An epicutaneous application of 2,4-dinitrofluorobenzene (DNFB) increased skin thickness in C3H as well as NC/Nga mice in SPF environment, and the response was enlarged by repeating the challenge at weekly intervals. Although the skin reaction in C3H mice was ameliorated when the challenge was discontinued after the fifth application, the reaction in NC/Nga mice was sustained at least for 3 wk. Analyses of cytokine production by CD4<sup>+</sup> cells from the draining lymph node proximal to the lesions revealed that, unlike C3H mice, NC/Nga mice fail to induce T helper 2 (Th2) cytokine interleukin-4 (IL-4), whereas the level of Th1 cytokine interferon- $\gamma$  in NC/Nga mice is equivalent to that of C3H mice. In addition, NC/Nga mice highly expressed IL-12, a cytokine-preventing formation of Th2 response, whereas C3H mice did not. Administration of anti-IL-12 antibody reduced duration of dermatitis in DNFB-treated NC/Nga mice. Taken together, our data suggest that IL-12 plays a role in the persistent skin reaction in NC/Nga mice. The action of IL-12 might be mediated by the decrease in IL-4 production.

Key words: atopic dermatitis/cytokine/hapten  
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Atopic dermatitis (AD) is a chronic eczematous skin disorder with dry and itchy skin. Patients with AD often have a family history of other allergic diseases including asthma and hay fever. In acute lesions of AD, there is a significant increase in the number of cells expressing T helper 2 (Th2) cytokines such as interleukin-4 (IL-4) and IL-5 (Yamada *et al*, 1995). However, delayed-type allergic reaction and Th1 immune response are also likely involved in pathogenesis in AD. For example, the skin lesions of AD are histologically indistinguishable from those of allergic contact dermatitis (Akdis *et al*, 2000). In addition, expression of the Th1 cytokine interferon- $\gamma$  (IFN- $\gamma$ ) predominates in chronic skin lesions of some patients with AD (Grewe *et al*, 1995; Werfel *et al*, 1996) and correlates to the severity of the disease (Grewe *et al*, 1994).

An epicutaneous application with chemical antigens such as 2,4-dinitrofluorobenzene (DNFB) evokes contact hypersensitivity reaction in mice that had previously been sensitized with the same agents. Interestingly, repetition of the topical application with such agents induces not only a shift in kinetics of the skin reaction from delayed-type to immediate-type response (Kitagaki *et al*, 1995; Nagai *et al*, 1997), but also change the cytokine milieu from Th1 to Th2 profile (Kitagaki *et al*, 1997; Nagai *et al*, 1997). This exper-

imental system is therefore useful for studying role of Th1 and Th2 in skin inflammation.

NC/Nga mice were established as an inbred strain from Japanese fancy mice in 1957, and have recently been shown to spontaneously develop AD-like dermatitis with immunoglobulin (Ig)E hyperproduction in air-uncontrolled, conventional circumstance (Matsuda *et al*, 1997; Watanabe *et al*, 2002). NC/Nga mice, however, show no skin lesion when raised in specific-pathogen-free (SPF) condition. The other strains such as BALB/c mice never develop dermatitis even if they are housed with NC/Nga mice, suggesting an involvement of inheritable factor in the pathogenesis of the mice.

In this study, the effect of topical challenge with chemical antigen on skin reaction was investigated using NC/Nga mice in order to elucidate the role of Th1 and Th2 responses in AD. The results showed that repeated painting with DNFB elicits skin lesion in NC/Nga mice under SPF condition, like in other strains, and that the skin inflammation in NC/Nga mice exclusively prolongs even after the challenge was discontinued. Our data also suggested that IL-12-mediated deficiency in IL-4 expression might be associated with the prolongation of the dermatitis in DNFB-treated NC/Nga mice.

## Results

**Change in ear thickness of NC/Nga and C3H mice following DNFB treatment** An epicutaneous application of DNFB to ear elicits a transient skin reaction in C3H mice

Abbreviations: AD, atopic dermatitis; anti-TCR, anti-T-cell receptor; DNFB, 2,4-dinitrofluorobenzene; IFN, interferon; Ig, immunoglobulin; IL, interleukin; SPF, specific-pathogen free; Th2, T helper 2

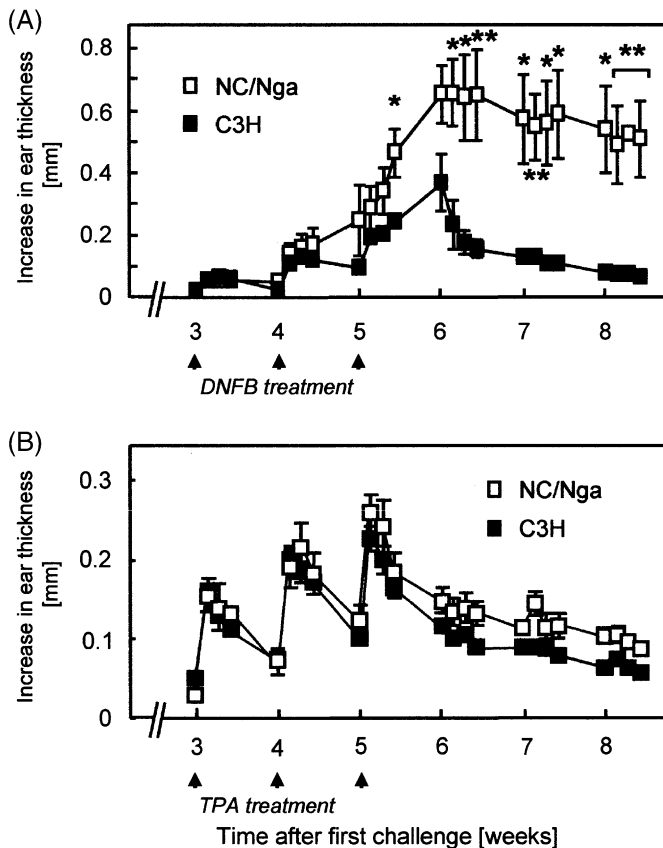


**Figure 1**  
Change in ear thickness by repeated challenge with 2,4-dinitrofluorobenzene (DNFB) or 12-O-tetradecanoylphorbol 13-acetate (TPA) in NC/Nga and C3H mice. Skin inflammation was induced by topical application of DNFB (A) or TPA (B) to ears five times at 7 d intervals and ear thickness was determined as described in Materials and Methods. Data are mean  $\pm$  SEM ( $n=5-7$ ). \* $p<0.05$ , \*\* $p<0.01$  compared with C3H mice (Student's  $t$  test).

(Tomimori *et al*, 2002). Repeating the challenge with DNFB weekly five times not only enhanced the transient response to DNFB, but also increased baseline of skin thickness in C3H as well as NC/Nga mice (Fig 1A). After the application was discontinued following the fifth challenge, the skin response was ameliorated gradually in C3H mice and disappeared completely by 3 wk after the last challenge. In contrast, the skin thickness of NC/Nga mice remained close to that at 6 wk at least for 3 wk. The prolonged skin reaction induced in NC/Nga mice appears to be specific for DNFB, since 12-O-tetradecanoylphorbol 13-acetate (TPA) painting induced a transient skin reaction in C3H and NC/Nga mice, which was improved similarly in the two strains of mice even after the fifth challenge (Fig 1B).

**Change in serum IgE Level of NC/Nga and C3H mice following DNFB treatment** As shown in Fig 2, serum IgE concentration in C3H mice started to increase after the fourth application of DNFB and reached to the maximal level at 7 wk of the experiment. The IgE level was subsequently declined and returned to the basal level by 9 wk. The serum IgE in DNFB-treated NC/Nga mice showed similar kinetics for 6 wk, whereas the level was higher than that in C3H mice at all the time points over the period. The IgE

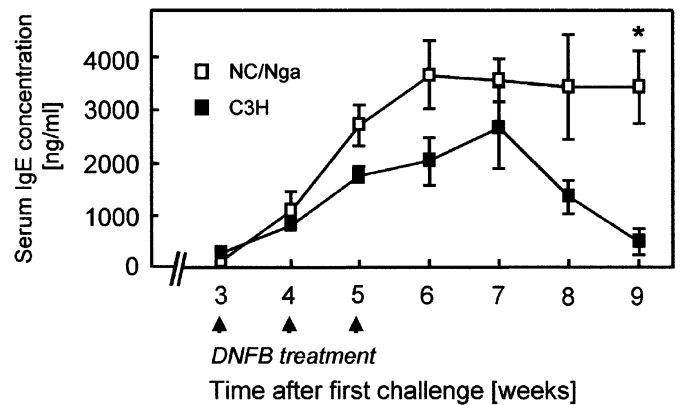


**Figure 2**  
Change in serum immunoglobulin (IgE) level by repeated challenge with 2,4-dinitrofluorobenzene (DNFB) in NC/Nga and C3H mice. Skin inflammation was induced by topical application of DNFB to ears five times at 7 d intervals and serum IgE concentration was measured as described in Materials and Methods. Data are mean  $\pm$  SEM ( $n=3$ ). \* $p<0.05$  compared with C3H mice (Student's  $t$  test).

level in the NC/Nga mice, however, did not decrease even after the challenge was discontinued (Fig 2).

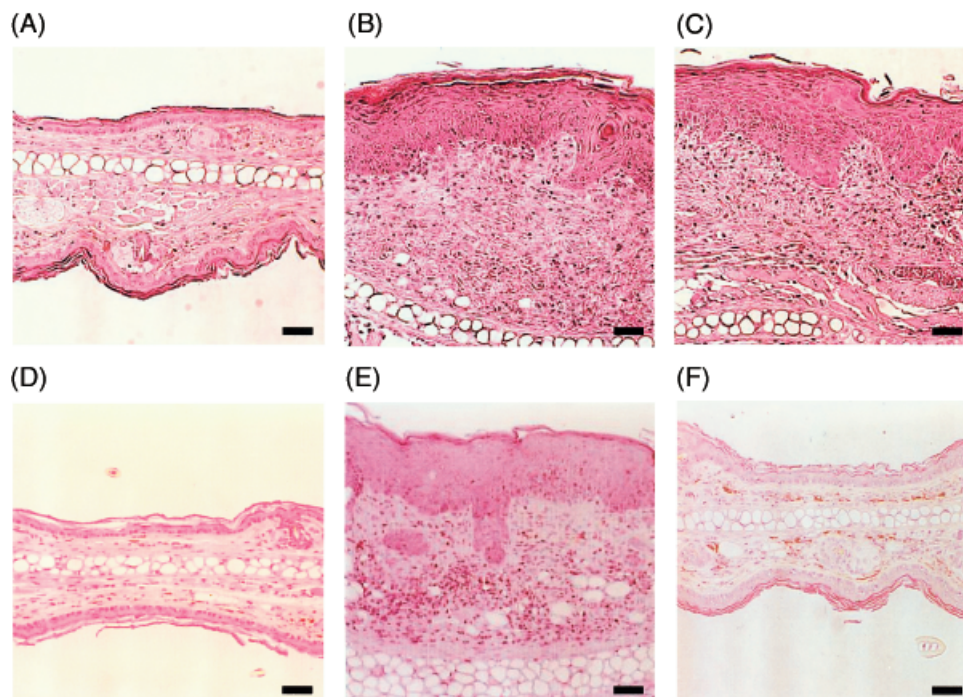
**Histological analysis of DNFB-treated ears in NC/Nga and C3H mice** Figure 3 shows the representative results of histological analyses in the skin lesions of DNFB-treated mice. In the skin lesion at 6 wk, hyperplasia and thickening of epidermis were evident and cell infiltration was marked in NC/Nga (Fig 3B) and C3H mice (Fig 3E) compared with the unaffected control mice (Fig 3A, D). At 9 wk, however, such changes were progressively reduced in C3H mice (Fig 3F). In contrast, the epidermis still remained thick and cell accumulation was observed in the skin lesions of NC/Nga mice even at 9 wk (Fig 3C).

**Synthesis of IL-4 and IFN- $\gamma$  by CD4<sup>+</sup> lymph node cells from NC/Nga and C3H mice** Next, production of Th1 and Th2 cytokines was compared between the two strains of mice. CD4<sup>+</sup> T cells were prepared from cervical lymph nodes and stimulated with anti-T-cell receptor (anti-TCR) plus anti-CD28 antibodies, and their ability to synthesize IL-4 and IFN- $\gamma$  was assessed by ELISA. As shown in Fig 4A, B, the levels of IL-4 and IFN- $\gamma$  produced by CD4<sup>+</sup> cells from C3H mice treated with DNFB five times were significantly higher than those of the unaffected control mice. Similar results were obtained in C3H mice at 9 wk of the experiment (Fig 4C, D). In NC/Nga mice, however, IL-4 production by CD4<sup>+</sup> cells was not significant at 6 and 9 wk (Fig 4A, C), whereas IFN- $\gamma$  production was almost similar to that in C3H (Fig 4B, D).

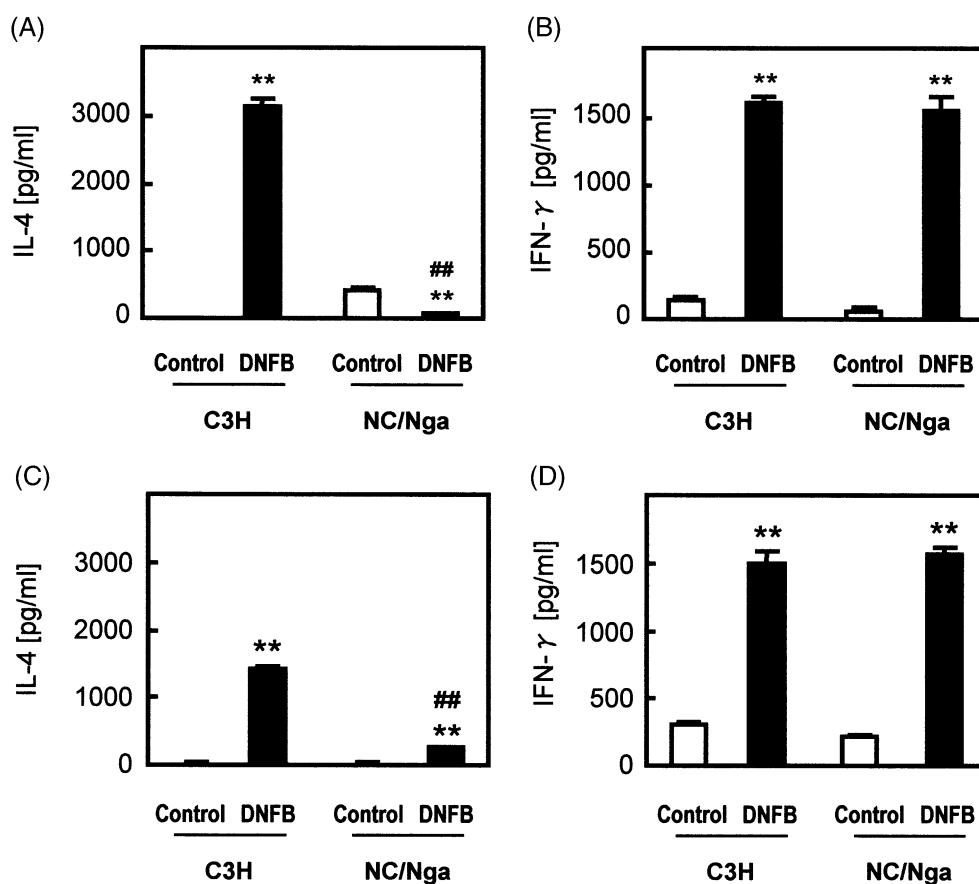
**Content of IL-18 and IL-12 in the skin lesions of NC/Nga and C3H mice** The IL-18 content in the lesions was significantly higher in DNFB-treated NC/Nga and C3H mice compared with their control mice at 6 wk (Fig 5A), whereas that in C3H decreased thereafter (Fig 5C). The content of IL-12 was much higher in NC/Nga mice at 6 and 9 wk (Fig 5B, D), whereas the IL-12 level was little at 3 wk (data not shown). In contrast to NC/Nga mice, the level of IL-12 in the lesions of C3H was little, if any, even at 6 and 9 wk (Fig 5B, D).

**Figure 3**

**Histological analyses of skin lesions in 2,4-dinitrofluorobenzene (DNFB)-treated NC/Nga and C3H/HeN mice.** Skin inflammation was induced by topical application of DNFB to ears five times at 7 d intervals as described in Materials and Methods. Ears were excised at 6 (A, B, D, and E) and 9 (C, F) wk, and the ear sections were stained with hematoxylin and eosin. (A, D) Unaffected control; (A–C) NC/Nga mice; (D–F) C3H mice. Original magnification  $\times 100$ , Scale bar = 50  $\mu\text{m}$  for all pictures.

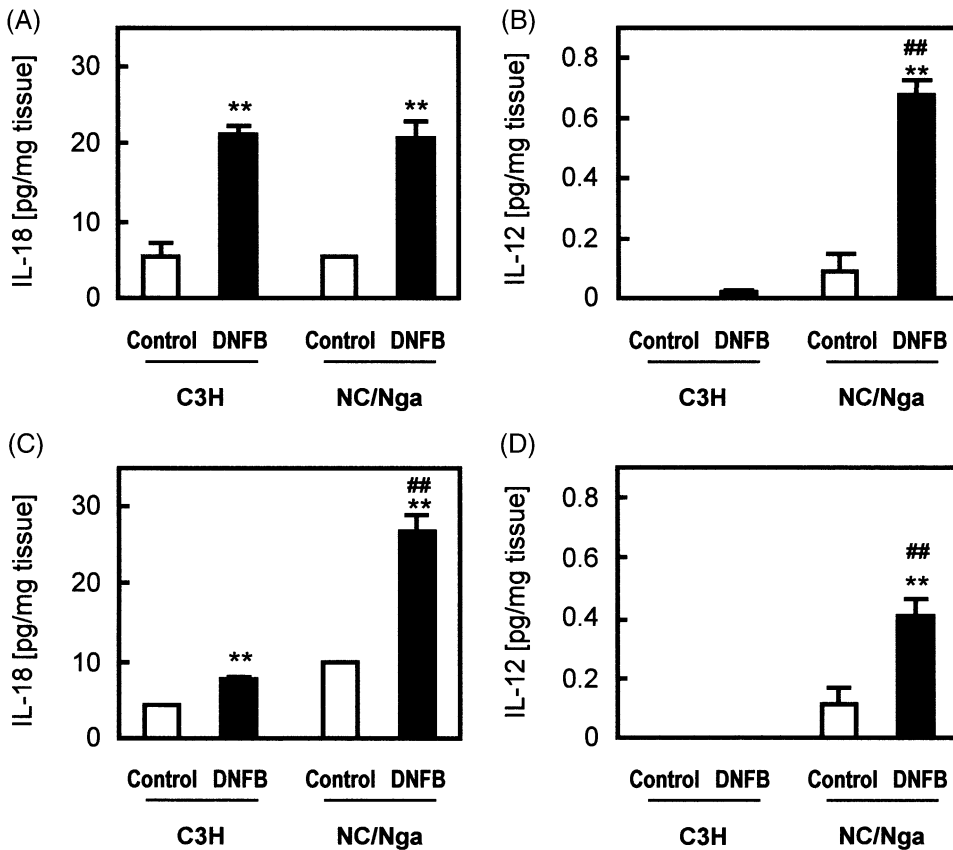
**Figure 4**

**Production of interleukin (IL)-4 and interferon (IFN)- $\gamma$  by CD4<sup>+</sup> cervical lymph node cells from 2,4-dinitrofluorobenzene (DNFB)-treated NC/Nga and C3H mice.** Skin inflammation was induced by topical application of DNFB to ears as described in Materials and Methods. CD4<sup>+</sup> cells were prepared from cervical lymph node of the mice at 6 (A, B) and 9 (C, D) wk and stimulated with TCR and CD28 for 48 h. IL-4 and IFN- $\gamma$  concentrations in the culture medium were measured by ELISA. (A, C) IL-4; (B, D) IFN- $\gamma$ . Data are mean  $\pm$  SEM (n=4). \*\*p<0.01 compared with unaffected control group, ##p<0.01 compared with DNFB-treated C3H mice (Student's *t* test).

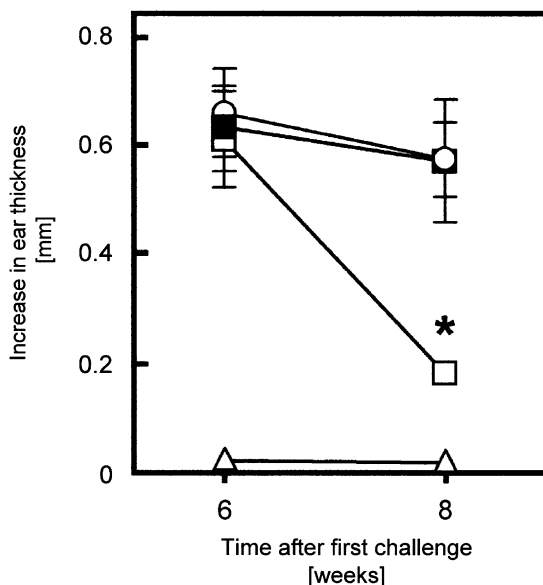


**Effect of anti-IL-12 antibody on prolongation of dermatitis in NC/Nga mice** When anti-IL-12 antibody was administered to the DNFB-treated NC/Nga mice intravenously twice a week starting at 6 wk of the experiment, the estab-

lished ear edema was dramatically ameliorated (Fig 6). In contrast, the isotype-matched normal IgG showed little effect. These results suggest that IL-12 might participate in the prolongation of skin reaction in NC/Nga mice.

**Figure 5**

**Expression of interleukin (IL)-18 and IL-12 in the skin lesions of 2,4-dinitrofluorobenzene (DNFB)-treated NC/Nga and C3H mice.** Skin inflammation was induced by topical application of DNFB to ears as described in Materials and methods. IL-18 and IL-12 content in the skin lesions was determined at 6 (A, B) and 9 (C, D) wk by measuring their concentration in the supernatant of homogenate in the lesions by ELISA. (A, C) IL-18; (B, D) IL-12. Data are mean  $\pm$  SEM (n=5). \*\*p<0.01 compared with unaffected control group, ##p<0.01 compared with DNFB-treated C3H mice (Student's *t* test).

**Figure 6**

**Effect of anti-interleukin (IL)-12 antibody on the prolongation of the dermatitis.** Skin inflammation was induced by topical application of 2,4-dinitrofluorobenzene (DNFB) to ears as described in Materials and Methods. Rat anti-mouse IL-12 (open square), rat immunoglobulin (Ig)G1 (closed square), and saline (open circle) were administered intravenously twice a week, starting at 6 wk of the experiment. Open triangle, unaffected control group. Data are mean  $\pm$  SEM (n=3). \*p<0.01 compared with rat IgG-treated group (Dunnett's *t* test).

## Discussion

NC/Nga mice spontaneously develop eczematous skin lesions when maintained in conventional but not in SPF

condition, indicating that the conventional NC/Nga mice are a useful animal model for AD. Under conventional environment, however, microbial and pathogen statuses vary between animal facilities, which could affect the incidence and severity of the disease of the mice. The data in this study showed that NC/Nga mice in SPF condition develop dermatitis with IgE hyperproduction following repeated painting with DNFB, and that the reaction in NC/Nga but not in C3H mice continues even after the painting is stopped. The mouse model presented in this study is valuable for studying pathogenesis of AD under SPF condition.

Repeated painting with DNFB increased expression of IFN- $\gamma$  and IL-4 by CD4<sup>+</sup> T cells from cervical lymph node in C3H mice (Fig 4). In contrast, the level of IFN- $\gamma$  produced by CD4<sup>+</sup> cells from NC/Nga was higher than that from the control mice, but that of IL-4 was not. IFN- $\gamma$  is known to be associated with chronicity of AD lesions (Grewe *et al*, 1994), and IL-4 antagonizes the function of IFN- $\gamma$  (Paludan, 1998). It is therefore possible that the deficiency in IL-4 expression may be involved in the sustained skin reaction in DNFB-treated NC/Nga mice.

The failure of NC/Nga mice to express IL-4 after repeated DNFB challenge raises the idea that the mechanism underlying the hyperproduction of IgE and its continuation in the mice is not necessarily dependent on the level of IL-4, which is the major cytokine for IgE synthesis (Kuhn *et al*, 1991). Recently, Matsumoto *et al* (1999) have shown that hyperproduction of IgE by conventional NC/Nga mice is attributed to constitutive phosphorylation of Janus kinase 3 (JAK3), a non-receptor protein tyrosine kinase involved in IL-4 signaling (Witthuhn *et al*, 1994). Thus it is also possible that repeated painting with DNFB in SPF condition induces

continuous JAK3 phosphorylation exclusively in NC/Nga mice. If so, SPF NC/Nga mice could produce excessive IgE either in response to the marginal level of or without IL-4. An alternative explanation for IgE hyperproduction in DNFB-treated NC/Nga mice would be a participation of IL-13, which induces IL-4-independent IgE synthesis (Punnonen *et al*, 1993).

The expression of IL-18 and IL-12 increased in the skin lesions of DNFB-treated NC/Nga, whereas C3H mice expressed high level of IL-18 but not IL-12 (Fig 5), suggesting that the IL-12 is also involved in the difference in kinetics of DNFB-induced dermatitis between NC/Nga and C3H mice. In fact, administration of anti-IL-12 antibody ameliorated the established dermatitis in NC/Nga mice (Fig 6). Thus, these findings suggest that IL-12 may participate in the chronicity of skin inflammation, in agreement with the finding that the IL-12-positive cells increase in chronic skin lesions compared with acute lesions and uninvolved skin from AD patients (Hamid *et al*, 1996). Since it is known that IL-12 favors Th1 differentiation (Trinchieri, 2003), the ability of IL-12 to prolong skin reaction could be mediated by IL-4 deficiency.

The role of IL-18 in pathogenesis of AD patients and NC/Nga mice has been controversial. It has been shown that IL-18 is elevated in the sera from patients with AD and from NC/Nga mice (Tanaka *et al*, 2001), and that KIL-18Tg, the transgenic mice that overexpress IL-18 in their keratinocytes, spontaneously develop AD-like skin disease (Kobayashi *et al*, 2002). On the contrary, Habu *et al* (2001) have reported the data conflicting with this idea, i.e., treatment of NC/Nga mice with IL-18 substantially inhibited elevation of the serum IgE levels, serum IL-4 levels and dermatitis under conventional condition. More recently, in addition, it has been reported that continuous injections of anti-IL-18 antibody do not inhibit the onset and development of dermatitis and IgE elevation in conventional NC/Nga mice, and that the treatment rather leads to an exacerbation of dermatitis and scratching behavior of the mice (Higa *et al*, 2003). The mechanism for the induction of dermatitis might differ between conventional and SPF conditions.

## Materials and Methods

**Mice** SPF NC/Nga and C3H/HeN (C3H) mice were purchased from Charles River Japan (Kanagawa, Japan) and CLEA Japan (Tokyo, Japan), respectively, and maintained in SPF condition. All animal experiments were approved by Institutional Review Board for animal studies of Daiichi Suntory Biomedical Research Co. Ltd.

**DNFB-induced dermatitis** Allergic dermatitis was induced by repeated topical application with DNFB as described (Tomimori *et al*, 2002). Briefly, 25  $\mu$ L of 0.15% DNFB in acetone/olive oil (3:1) was applied to each side of right ears of mice five times at 7 d intervals. Ear thickness was measured 0, 24, 48, and 72 h after each painting using a thickness gauge (Digimatic Indicator, Mitsutoyo, Tokyo, Japan), and increase in ear thickness was determined by subtracting ear thickness before the first painting from that of each time point. To examine continuation of the skin inflammation, measurement of ear thickness was similarly performed for additional 3 wk after the challenge was stopped. To examine the effect of anti-IL-12 antibody, in addition, either 150  $\mu$ g of rat anti-mouse IL-12 (C15.6, Biosource, Camarillo, California), the same amount of rat IgG1 (BD Pharmingen, San Diego, California) or saline was administered intravenously twice a week starting at 6 wk of the

experiment, and ear thickness was measured at 6 and 8 wk. In some experiments, the same volume of solvent for DNFB was painted instead of DNFB solution as unaffected control. Furthermore, 20  $\mu$ L of 0.005% TPA in acetone was applied instead of DNFB so as to examine the specificity of the effect of DNFB.

**Serum IgE measurement** Blood samples were collected once a week and IgE concentration in the serum was quantified using mouse IgE EIA kit (Yamasa Corp., Tokyo, Japan).

**Histological analysis** Ear samples were fixed in 10% (v/v) buffered formalin, embedded in paraffin, sectioned at 4  $\mu$ m, and then stained with hematoxylin and eosin.

**Measurement of cytokine production *in vitro*** Measurement of cytokine production was carried out as described (Nakanishi *et al*, 2001) with a slight modification. Briefly, the cervical lymph node was excised from mice and CD4<sup>+</sup> cells were purified using MACS separation column (Daiichi Pure Chemicals, Tokyo, Japan) according to the manufacturer's procedure. The purified CD4<sup>+</sup> cells ( $5 \times 10^5$  cells per mL) were stimulated with plate-bound hamster anti-mouse TCR (H57-597, Beckman Coulter K.K., Tokyo, Japan) plus soluble form of hamster anti-mouse CD28 (PV-1, Southern Biotech, Birmingham, Alabama) for 48 h in RPMI-1640 supplemented with 10% fetal bovine serum, 50  $\mu$ M of  $\beta$ -mercaptoethanol (Wako Pure Chemical, Osaka, Japan), and 2 mM glutamine (2 mM, GIBCO, Invitrogen Corp., Carlsbad, California). Then IL-4 and IFN- $\gamma$  in the supernatant were quantified by ELISA kit (Biosource).

**Measurement of cytokine contents in the skin lesions** Ear samples were excised from mice and homogenized in 20 mM Tris-HCL buffer, pH7.5 (10 times volume of the tissue weight), and their homogenate was centrifuged at  $10,000 \times g$  for 10 min. IL-12 and IL-18 concentration in the supernatant of the homogenate was quantified by ELISA kit for IL-12 (Biosource) and IL-18 (MBL, Nagoya, Japan), respectively.

**Statistical analysis** The statistical analysis was performed with Dunnett's multiple comparison test or Student's *t* test using Super ANOVA (Abacus Concepts, Berkeley, California) or Statview (SAS Institute Inc., Cary, North Carolina), respectively. A *p*-value less than 0.05 was considered significant.

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