

Transforming growth factor- β_1 suppresses atopic dermatitis-like skin lesions in NC/Nga mice

K. Sumiyoshi*†, A. Nakao*, H. Ushio*, K. Mitsuishi†, K. Okumura*, R. Tsuboi†, C. Ra* and H. Ogawa*†

*Atopy (Allergy) Research Center, and †Department of Dermatology, Juntendo University School of Medicine, Tokyo, Japan

Summary

Background Atopic dermatitis is a chronic, relapsing inflammatory disorder characterized by pruritic and eczematous skin lesions. Transforming growth factor (TGF)- β_1 has been implicated in the suppression of inflammatory responses.

Objective The purpose of this study is to determine whether TGF- β_1 suppresses skin lesions in a mouse model of atopic dermatitis.

Methods We used the NC/Nga strain of mice as an *in vivo* model of atopic dermatitis. The effects of exogenous TGF- β_1 on atopic dermatitis-like skin lesions in NC/Nga mice were evaluated clinically, histologically and immunologically.

Results Subcutaneous injection of recombinant TGF- β_1 macroscopically suppressed eczematous skin lesions in NC/Nga mice associated with reduced serum immunoglobulin E (IgE) levels. Histological analysis showed that TGF- β_1 significantly inhibited the infiltration of inflammatory cells such as mast cells and eosinophils into the skin of NC/Nga mice. Spontaneous interferon (IFN)- γ production from splenocytes of NC/Nga mice was down-regulated by the treatment with TGF- β_1 and neutralizing antibody against IFN- γ inhibited skin lesions in NC/Nga mice. The inhibitory effect of TGF- β_1 on the skin lesions lasted at least 1 week after cessation of the treatment.

Conclusion These findings indicate that TGF- β_1 suppressed atopic dermatitis-like skin lesions in NC/Nga mice at least in part through down-regulation of IFN- γ . These results suggest that TGF- β_1 may have a therapeutic potential for atopic dermatitis.

Keywords atopic dermatitis, TGF- β_1 , NC/Nga mice, inflammation, IFN- γ

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Introduction

Atopic dermatitis is a chronically relapsing inflammatory disorder that is characterized by pruritic and eczematous skin lesions often associated with elevated serum immunoglobulin (Ig)E levels [1–3]. It has been suggested that T lymphocytes, mast cells, monocytes/macrophages, and eosinophils infiltrated into skin lesions of atopic dermatitis are involved in the development of atopic dermatitis through the release of various cytokines/chemokines including interleukin (IL)-4, IL-5, and, interferon (IFN)- γ [4].

Transforming growth factor (TGF)- β_1 is a member of the TGF- β superfamily that exerts a wide range of biological functions [5]. Importantly, TGF- β_1 null mice develop extensive inflammation in various organs and die shortly after birth, suggesting that TGF- β_1 plays a critical role in the suppression of the immune system *in vivo* [6]. In fact, TGF- β_1 has been implicated in the suppression of various inflammatory disorders [7]. However, it remains unclear whether TGF- β_1 suppresses skin lesions of atopic dermatitis.

In this study, we determined the efficacy of exogenous TGF- β_1 to suppress atopic dermatitis-like skin lesions in NC/Nga mice which are a model of atopic dermatitis [8]. We showed that the administration of TGF- β_1 suppressed atopic dermatitis-like skin lesions in NC/Nga mice at least in part through the down-regulation of IFN- γ . The results suggest that TGF- β_1 may have a therapeutic potential for atopic dermatitis.

Materials and methods

Mice

The NC/Nga strain of mice originated from Japanese fancy mice and was established as an inbred strain by K. Kondo [9]. Male 18-weeks-old NC/Nga mice with fully developed skin lesions were purchased from SLC (Tokyo, Japan). These mice were kept in conventional surroundings. As a control for *in vivo* and *in vitro* experiments examining the effects of TGF- β_1 on atopic dermatitis-like skin lesions and on IFN- γ production from splenocytes, male 18-weeks-old NC/Nga mice that were kept in specific pathogen free (SPF) condition and showed no skin lesions were also employed. Animal experiments were approved by the Institutional Review Board of Juntendo University.

Correspondence: Atsuhito Nakao, Atopy (Allergy) Research Center, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan. E-mail: anakao@med.juntendo.ac.jp

Study design

Mice at the age of 18 weeks with fully developed skin lesions and kept in conventional surroundings were treated with subcutaneous injection of recombinant human TGF- β_1 (1 μ g/mouse) (R & D, Minneapolis, MN, USA) (Group 1) or intraperitoneal injection of water soluble dexamethasone (1 mg/mouse) (Sigma, St Louis, MO, USA) for comparison (Group 2). As a control, mice of the same age with similar lesions were treated with saline alone (Group 3). In each group of mice, injection of TGF- β_1 or dexamethasone was repeated three times at a 1-week interval. 1 week after the last injection, the mice (at the age of 21 weeks) from each group were killed for evaluation of the treatments. To determine the role of endogenous IFN- γ on the development of skin lesions in NC/Nga mice, neutralizing monoclonal antibody against IFN- γ (500 μ g/mouse) (XMG-1.2) [10] was administered once intraperitoneally into the mice at the age of 18 weeks with fully developed skin lesions and that were kept in conventional surroundings. As a control, isotype-matched rat IgG antibody (PharMingen, San Diego, CA, USA) was administered.

Clinical scoring of skin lesions

A total clinical severity score for skin lesions of NC/Nga mice was defined as the sum of the individual score grades as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of five signs and symptoms (itch, erythema/haemorrhage, oedema, excoriation/erosion, and scaling/dryness) as previously described [8]. Itching was evaluated by observing scratching behaviour for >2 min. The clinical scoring was performed in a blinded fashion by one investigator in order to prevent inter-investigator variation.

Skin biopsy

The mice were killed by cervical dislocation, and the hairs of the face were removed. An area of approximately 1.5 $\times$ 1.5 cm² of the skin was excised.

Histological analysis

Skin sections were resected, fixed in 10% paraformaldehyde, embedded into paraffin, sectioned, stained with haematoxylin-eosin solution, and examined by light microscopy for histological evaluation. Mast cells, and eosinophils were stained with toluidine blue or eosin Y methylene blue, respectively. The number of infiltrating mast cells and eosinophils were counted in 5 randomly chosen consecutive fields at $\times$ 400 magnification in sections from two different mice. Skin fibrotic changes were evaluated with Elastica van Gieson staining, a specific histochemical staining for collagen and elastin.

Plasma IgE levels

Blood was collected from the retro-orbital plexus of NC/Nga mice before (at the age of 18 weeks) and after (at the age of 21 weeks) the treatment with TGF- β_1 , as described above, while under ether anaesthesia and heparinized immediately thereafter. Plasma samples were obtained by centrifugation and stored at -20 °C until use. Total IgE levels were measured by a sandwich murine IgE ELISA kit (Yamasa, Japan).

Cytokine ELISA

Spleen cells (5×10^6 cells) isolated from NC/Nga mice which were treated with TGF- β_1 or dexamethasone as described

above were re-stimulated with plate-bound anti-CD3 antibody (2C11) (10 μ g/mL) (PharMingen) 7 days after the last treatment. 72 h after the culture, supernatants were collected for measurement of cytokines. The amount of IL-5, IL-13 and IFN- γ in the culture supernatant of spleen cells was determined using murine IL-5, IL-13, and IFN- γ ELISA kit (ENDOGEN, Woburn, MA, USA).

Data analysis

Data are summarized as mean $\pm$ SD. The statistical analysis of the results was performed by the amount of variance using Fisher's least significant difference test for multiple comparisons. $P < 0.05$ was considered to be significant.

Results

Administration of TGF- β_1 suppresses atopic dermatitis-like skin lesions and serum IgE levels in NC/Nga mice.

The NC/Nga strain of mice has been reported to develop an eczematous condition that is clinically and histologically very similar to atopic dermatitis when kept in conventional surroundings [8]. The eczema develops at the age of 8 weeks, with maximum activity at around 17 weeks with lesions characterized by oedema, haemorrhage, erosion, and dryness typically localized on the ears, back, neck and facial region. Marked hyperkeratosis and infiltration of CD4 T cells, macrophages, eosinophils, and mast cells are observed in the skin lesions.

To determine the efficacy of TGF- β_1 to suppress skin lesions of NC/Nga mice, we treated the NC/Nga mice with fully developed skin lesions at the age of 18 weeks with recombinant TGF- β_1 or dexamethasone for comparison. As a control, the mice with fully developed skin lesions were treated with saline. 3 weeks after the initiation of treatment with TGF- β_1 , the skin lesions on the back, ears and facial regions of the NC/Nga mice showed extensive regression when compared with saline-treated mice (Figs 1a and b). The skin lesions of dexamethasone-treated mice also showed extensive regression (Figs 1c and d). Scoring of the clinical skin severity revealed marked reduction of the score in the TGF- β_1 -treated mice (Fig. 1e). The reduction in score in TGF- β_1 -treated mice was comparable to that in dexamethasone-treated mice (Fig. 1e).

Histologically, NC/Nga mice with fully developed skin lesions showed hyperkeratosis, partial defect of epidermis and dense infiltration of leucocytes in the dermis (Fig. 2a). After the treatment with TGF- β_1 , the mice showed acanthosis and reduced infiltration of leucocytes in the dermis (Fig. 2e). Dexamethasone-treated mice also showed significant reduction of infiltrating leucocytes in the dermis (Fig. 2i). Toluidine blue staining showed decreased mast cell number in the skin lesions in TGF- β_1 -treated mice (mast cell number: saline 46.7 ± 5.9 vs. TGF- β_1 32.1 ± 9.5 , $n = 5$, mean $\pm$ SD, $P < 0.05$, Figs 2b and f). Eosinophil number was also significantly decreased in the skin lesions of TGF- β_1 -treated mice (eosinophil number: saline 48.3 ± 8.2 vs. TGF- β_1 35.7 ± 5.4 , $n = 5$, mean $\pm$ SD, $P < 0.05$, Figs 2c and g). The reduction of mast cell and eosinophil number in TGF- β_1 -treated mice was also comparable to that in dexamethasone-treated mice (Figs 2j and k). Elastica van Gieson staining of skin tissue sections showed that treatment of skin lesions in NC/Nga mice with recombinant human TGF- β_1 did not induce any significant increase of collagen and elastic fibres in the skin tissue compared with saline-treated or

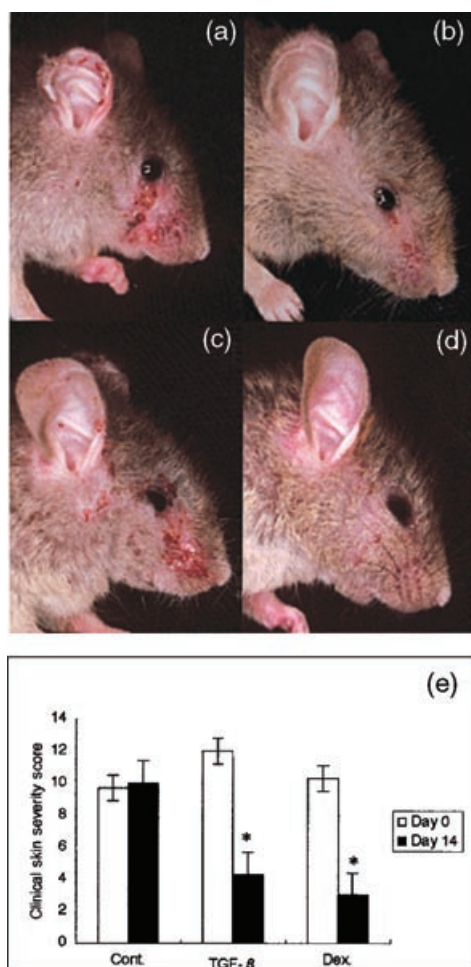


Fig. 1. Inhibition of skin lesions in NC/Nga mice by TGF- β_1 . (a–d) Appearance of skin lesions. NC/Nga mice at the age of 18-weeks-old with fully developed skin lesions were treated with subcutaneous injection of TGF- β_1 (upper panels) or intraperitoneal injection of dexamethasone (lower panels) three times at a 1-week interval. The photograph of mice was taken at the age of 18 weeks (left) or 21 weeks (right). (e) Clinical skin severity scores of NC/Nga mice. Clinical scores were measured 3 weeks after the treatment with TGF- β_1 and dexamethasone (the mice were at the age of 21-weeks-old of age.) Values represent the mean $\pm$ SD ($n = 4$). * $P < 0.05$, significantly different from the mean value of the corresponding control response.

dexamethasone-treated mice although some minor increase of disorganized elastic fibres were observed in TGF- β_1 -treated mice (Figs 2d, h and l).

It was previously shown that the serum IgE levels of NC/Nga mice were markedly elevated from the age of 8 weeks correlating with clinical severity of the dermatitis [8]. We then examined the effect of TGF- β_1 on serum IgE levels of NC/Nga mice. Blood samples were collected from NC/Nga mice with fully developed skin lesions before (at the age of 18 weeks) and after (at the age of 21 weeks) the treatment with TGF- β_1 and serum IgE levels were determined by ELISA. We found that the administration of recombinant TGF- β_1 significantly reduced serum IgE levels of NC/Nga mice after the treatment (serum IgE levels before treatment: 14193 ± 1072 ng/mL vs. after treatment: 8432 ± 590 ng/mL; $n = 4$, mean $\pm$ SD, $P < 0.05$). These results indicated that the administration of recombinant TGF- β_1 macroscopically and histologically suppressed skin lesions of NC/Nga mice associated with reduced serum IgE levels.

Administration of TGF- β_1 suppresses spontaneous IFN- γ production from splenocytes of NC/Nga mice

To explore possible mechanisms for TGF- β_1 -mediated suppression of skin lesions in NC/Nga mice, we examined capacity of splenocytes to produce Th1 or Th2 type cytokines. As shown in Fig. 3(a), spontaneous IFN- γ production was observed in splenocytes from saline-treated mice with fully developed skin lesions whereas the spontaneous IFN- γ production was significantly suppressed in TGF- β_1 or dexamethasone-treated mice. There was no significant difference in IL-5 and IL-13 production from the splenocytes between saline-treated mice and TGF- β_1 -treated mice regardless of the presence or absence of stimulation with anti-CD3 antibody (Fig. 3a). In addition, spontaneous IFN- γ production was not observed in splenocytes isolated from NC/Nga mice that were kept in SPF condition and showed no skin lesions (Fig. 3b). These findings suggested that spontaneous IFN- γ production from the splenocytes of NC/Nga mice was associated with the development of skin lesions in NC/Nga mice.

To further determine whether endogenous IFN- γ contributed to the development of skin lesions in NC/Nga mice, we examined the effect of neutralizing antibody against IFN- γ on the development of skin lesions. As shown in Fig. 4, we found that clinical severity of skin lesions was partially, but significantly reduced 1 week after the treatment with anti-IFN- γ antibody. These findings indicated that TGF- β_1 suppressed IFN- γ production in NC/Nga mice, thereby, at least in part, inhibited the atopic dermatitis-like skin lesions.

Duration of the inhibitory effect of TGF- β_1 on skin lesions of NC/Nga mice

Skin lesions in NC/Nga mice were suppressed at least for 1 week after the last injection of TGF- β_1 (Fig. 5). 2 weeks after the last injection of TGF- β_1 , scoring of clinical severity of skin lesions became worse to the level before the treatment (Fig. 5).

Discussion

In this study, we demonstrated that the systemic administration of recombinant TGF- β_1 suppressed atopic dermatitis-like skin lesions in NC/Nga mice associated with reduced serum IgE levels and down-regulation of IFN- γ production from the splenocytes. Since treatment with anti-IFN- γ antibody partially ameliorated skin lesions in NC/Nga mice, these findings strongly suggested that TGF- β_1 suppressed skin lesions in NC/Nga mice at least in part through down-regulation of IFN- γ .

The pathophysiology of atopic dermatitis-like skin lesions in NC/Nga mice has not been fully understood. Previous studies showed that FK506 and persimmon leaf extract that suppressed mast cell functions improved skin lesions in NC/Nga mice [11,12], suggesting that T cells and mast cells contributed to the development of skin lesions. Our findings suggested that IFN- γ also contributed to the development of skin lesions in NC/Nga mice. Matsuda et al. previously showed that IFN- γ -producing cells were infiltrated in the skin lesions of NC/Nga mice [8]. In addition, Vestergaard et al. recently suggested that IFN- γ induced TARC (thymus- and activation-regulated chemokine) expression in the skin lesions of NC/Nga mice,

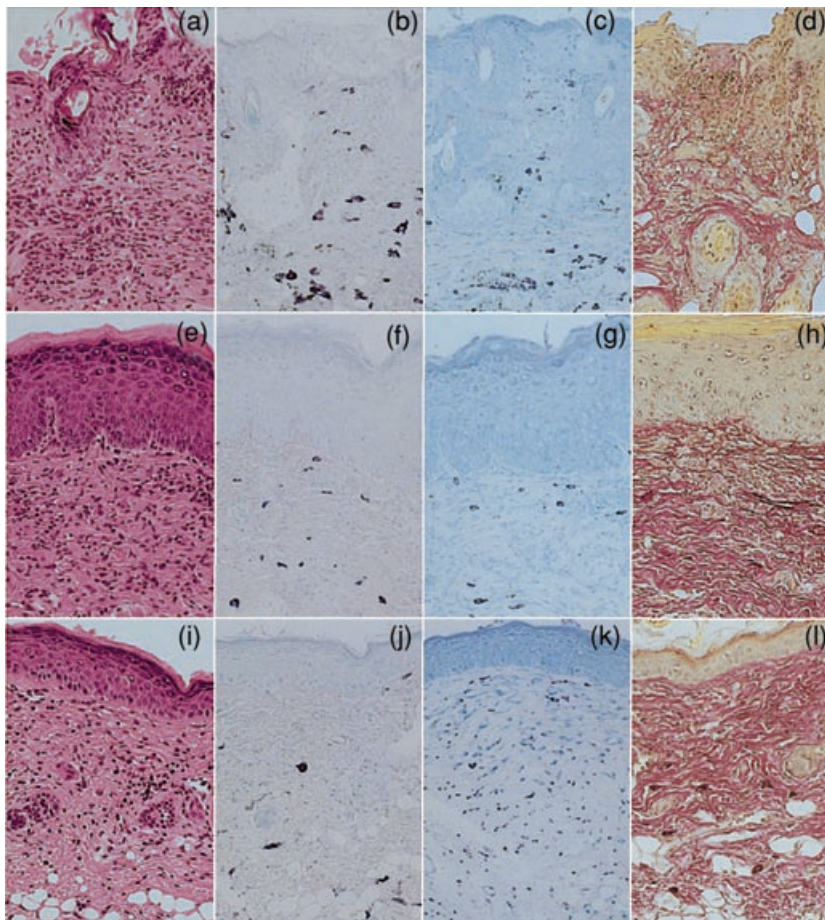


Fig. 2. Reduced skin inflammation in NC/Nga mice by TGF- β_1 . Facial skin sections from saline-treated mice (a–d), TGF- β_1 -treated mice (e–h), and dexamethasone-treated mice (i–l) were stained with haematoxylin and eosin (a, e and i), toluidine blue (b, f and j), eosin Y methylene blue (c, g and k), and Elastica van Gieson (collagen stained red and elastin black) (d, h and l).

thereby contributing to the development of the skin lesions [13]. IFN- γ was also implicated in the pathophysiology of atopic dermatitis [14]. The lymphocytes infiltrating into the skin lesions of atopic dermatitis produced not only Th2 cytokines but also Th1 type cytokines such as IFN- γ in later stages of the disease [4]. Taken together, IFN- γ appeared to play an important role in the pathophysiology of fully developed skin lesions in NC/Nga mice as well as skin lesions in atopic dermatitis.

We found that IFN- γ was spontaneously produced from splenocytes isolated from NC/Nga mice with fully developed skin lesions, but not from splenocytes from NC/Nga mice without skin lesions. The findings suggested that spontaneous IFN- γ production was associated with the development of skin lesions in NC/Nga mice. Our preliminary data showed that depletion of T cells with anti-thy1 antibody did not affect spontaneous IFN- γ production from splenocytes from NC/Nga mice with fully developed skin lesions, suggesting that the major cellular source of IFN- γ was non-T cells (possibly macrophages or dendritic cells). The factors that induce spontaneous IFN- γ production from splenocytes in NC/Nga mice remain to be determined.

Down-regulation of IFN- γ by exogenous TGF- β_1 was previously reported in animal models such as autoimmune insulinitis and experimental colitis [15,16], which was consistent with our results. Treatment with TGF- β_1 inhibited IFN- γ production from lamina propria T cells obtained from a mouse model of colitis [16]. Intracellular mechanisms underlying

TGF- β_1 -mediated suppression of IFN- γ production should be investigated in future.

Since TGF- β_1 acts on multiple targets affecting the immune system [17–19], TGF- β_1 -mediated suppression of skin lesions in NC/Nga mice could not be only attributed to down-regulation of IFN- γ . Because TGF- β_1 suppresses the survival of eosinophils and mast cells [18,19], the decreased number of these cells in the skin lesions might be a result of the suppression of their survival. Alternatively, TGF- β_1 inhibits expression of adhesion molecules involved in leucocyte–endothelial cell interaction [20]. Thus, inhibition of leucocyte infiltration into the skin at the level of leucocyte–endothelial cell interaction by TGF- β_1 could explain the decreased inflammation in the skin lesions of NC/Nga mice. Reduction of serum IgE levels by TGF- β_1 in NC/Nga mice might also contribute to the immunomodulating actions. However, it is most likely that these events occur in concert.

The ability of TGF- β_1 to induce fibrosis is a potential concern. Persistent activity of TGF- β_1 is thought to be important for inducing tissue fibrosis [21]. We did not observe any significant fibrosis in muscles, kidneys, lungs, liver, heart, pancreas, as well as skin in mice treated with TGF- β_1 . We speculate that the activity of TGF- β_1 is not enough to induce tissue fibrosis at the dosage or duration of TGF- β_1 used in the current study. In addition, we do not know about the clinical significance of acanthosis observed in the TGF- β_1 -treated mice and if it is beneficial or not. Acanthosis disappeared at least 4 weeks after

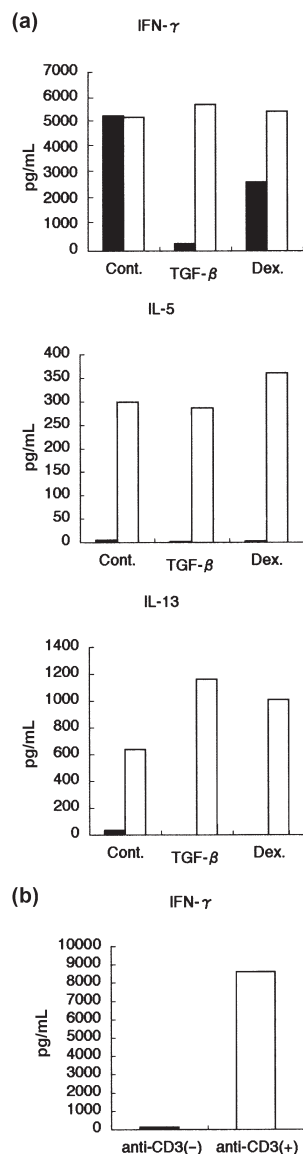


Fig. 3. Suppression of IFN- γ production from splenocytes obtained from NC/Nga mice by TGF- β_1 . (a) Spleen cells from mice with fully developed skin lesions that were non-treated or treated with TGF- β_1 or dexamethasone were cultured in the presence (open column) or absence (closed column) of plate-bound anti-CD3 antibody for 3 days. IFN- γ , IL-5 and IL-13 productions in the culture supernatant were measured by ELISA. Data from one representative experiment are shown. Similar results were obtained from at least other two independent experiments. (b) Spleen cells from mice without skin lesions were cultured in the presence or absence of plate-bound anti-CD3 antibody for 3 days. IFN- γ levels in the culture supernatant were measured by ELISA. Data from one representative experiment are shown. Similar results were obtained from at least two other independent experiments.

the cessation of TGF- β_1 treatment (data not shown). We thus think so far that acantosis would not cause unbeneficial outcome in the TGF- β_1 -treated mice.

In summary, we demonstrated that TGF- β_1 suppressed atopic dermatitis-like skin lesions in NC/Nga mice. These findings suggest that TGF- β_1 may have a therapeutic potential for atopic dermatitis.

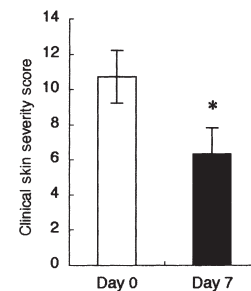


Fig. 4. Suppression of skin lesions in NC/Nga mice by anti-IFN- γ antibody. NC/Nga mice with fully developed skin lesions at the age of 18 weeks were treated with anti-IFN- γ antibody or rat IgG and clinical skin scores were measured 1 week after the treatments. Values represent the mean $\pm$ SD ($n = 4$). * $P < 0.05$, significantly different from the mean value of the corresponding control response.

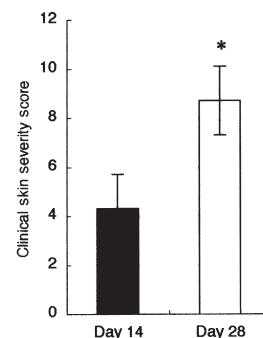


Fig. 5. Duration of TGF- β_1 -mediated suppression of skin lesions in NC/Nga mice. NC/Nga mice with fully developed skin lesions were treated with TGF- β_1 three times at a 1-week interval. Clinical severity of skin lesions was evaluated 1 or 2 weeks after the last injection of TGF- β_1 to determine the duration of the treatment. Values are mean $\pm$ SD for 5 mice in each group. * $P < 0.05$, significantly different from the mean value of the corresponding control response.

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