

ORIGINAL ARTICLE

Regulation of immunological balance by sustained interferon- γ gene transfer for acute phase of atopic dermatitis in mice

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Interferon (IFN)- γ , a potent T helper 1 (Th1) cell cytokine, is suggested to suppress Th2 cell responses. Here, we aimed to investigate whether pCpG-Mu γ , a plasmid continuously expressing murine IFN- γ , is an effective treatment of atopic dermatitis, a Th2-dominant skin disease. Nishiki-nezumi Cinnamon/Nagoya (NC/Nga) atopic mice with early dermatitis were transfected with pCpG-Mu γ by a hydrodynamic tail vein injection at a dose of 0.05 or 0.2 pmol per mouse. The skin lesions improved only in mice receiving the high dose of pCpG-Mu γ . IFN- γ gene transfer resulted in a high mRNA expression of IFN- γ and interleukin (IL)-12 and regulatory T cell (Treg) related cytokines, such as IL-10 and transforming growth factor- β , in the spleen, whereas it reduced the IL-4 mRNA expression, and serum levels of immunoglobulin (Ig) G1 and IgE. In addition, the gene transfer markedly inhibited the epidermal thickening, infiltration of inflammatory cells into the skin, the occurrence of dry skin and pruritus. No exacerbating effect on the Th1-mediated contact dermatitis was observed after IFN- γ gene transfer. Taken together, these results indicate that sustained IFN- γ gene transfer induced polarized Th1 immunity under Th2-dominant conditions in NC/Nga mice, leading to an improvement in the symptoms of acute atopic dermatitis without adverse side effects.

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Keywords: atopic dermatitis; interferon- γ ; NC/Nga mice; nonviral vector

INTRODUCTION

Atopic dermatitis is a chronic inflammatory skin disorder accompanied by particular skin lesions. The main characteristics of atopic dermatitis are eczematous skin lesions, epidermal hypertrophy, intense pruritus and infiltration by inflammatory cells. Increased serum immunoglobulin (Ig) E and an excessive production of T helper 2 (Th2) cytokines are also frequently observed in patients with atopic dermatitis. The balance of Th1 and Th2 immune responses, which is maintained by their immunoregulatory cytokines under healthy conditions, is considered to be upset in atopic dermatitis. Generally, atopic dermatitis was classified into two phases, acute and chronic. In the acute phase of atopic dermatitis, patients often have raised Th2 cytokines, such as interleukin (IL)-4, IL-5 and IL-13, which induce the class switching of B cells to IgE secretion, but low levels of IFN- γ or IL-12. In contrast, Th1 cytokines, especially IFN- γ , are dominant in chronic phase of atopic dermatitis.^{1–6} Therefore, the administration of interferon (IFN)- γ , a typical Th1 cytokine that shifts the differentiation of naive T cells to the Th1 subtype and suppresses the production of Th2 cytokines, appears to be an attractive option for the treatment of acute phase of atopic dermatitis.^{7,8}

It has recently been shown that regulatory T cells (Tregs), a subset of T cells, are involved in the modulation of allergic diseases. For example, Tregs and their related cytokines, IL-10 and transforming growth factor (TGF)- β , have been reported to suppress Th2 responses and IgE production.^{9–11} Furthermore, IFN- γ as well as the IL-12-specific receptor subunit β 2 has important roles in the production of Tregs.^{12,13}

Nishiki-nezumi Cinnamon/Nagoya (NC/Nga) mice is a mouse model of human atopic dermatitis. These mice spontaneously develop atopic dermatitis-like skin lesions around the age of 6–8 weeks when raised under conventional conditions but not in specific pathogen-free conditions. The skin lesions of conventional NC/Nga mice are characterized by erythema, hemorrhage, edema, erosion, scaling and dryness of the skin. Hyperproduction of IgE and Th2-type chemokines have also been reported in these mice. These clinical features are similar to those of human atopic dermatitis.^{14–17}

We demonstrated in a previous study¹⁸ that sustained expression of IFN- γ is effective in preventing the onset of symptoms of atopic dermatitis in NC/Nga mice. The severity of the atopic lesions and other notable symptoms, such as scratching, dry skin, epidermal thickening, infiltration of inflammatory cells and the raised expression of Th2 cytokines and IgE, were markedly inhibited in mice receiving pCpG-Mu γ , a plasmid expressing murine IFN- γ for a long period of time.¹⁸ To use IFN- γ gene transfer in the treatment of atopic dermatitis patients, it is important to show that such gene transfer is effective not only in preventing the onset of the disease but also in improving the symptoms. IFN- γ gene transfer could counteract the progression of atopic dermatitis through the induction of Treg cells.

In this study, we investigated whether IFN- γ gene transfer was able to effectively treat dermatitis induced in NC/Nga mice. To do this, we evaluated the serum levels of IgE and IgG1, the mRNA expression of Th1 and Treg cytokines, transepidermal water loss (TEWL), the number of scratching episodes, the epidermal

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thickening and the infiltration of inflammatory cells into the skin. In addition, the effect of sustained IFN- γ gene transfer on contact dermatitis was also examined to assess the safety of the gene transfer process.

RESULTS

Time-course and pharmacokinetic parameters of IFN- γ after hydrodynamic injection of pCpG-Mu γ

Figure 1 shows the time-course of the concentrations of IFN- γ in the serum after hydrodynamic injection of pCpG-Mu γ . The serum levels of IFN- γ were dependent on the plasmid dose, and the higher dose of 0.2 pmol resulted in significantly higher concentrations for the first 4 days compared with the lower dose. The profiles were analyzed to obtain area under the concentration-time curve and mean residence time values. Table 1 summarizes the C_{max}, area under the concentration-time curve and mean residence time after IFN- γ gene transfer. The C_{max} and area under the concentration-time curve were increased by increasing the plasmid dose, whereas the mean residence time was hardly affected by the dose.

Effects of IFN- γ gene transfer on the skin lesions of NC/Nga mice NC/Nga mice at the age of 7–8 weeks, which already developed atopic dermatitis-like symptoms, were used to assess the severity of skin lesions. Figure 2 shows the time-course of the clinical skin score of NC/Nga mice after injection of pCpG-Mu γ . The score of mice treated with the lower dose pCpG-Mu γ increased with time and was not significantly different from that of the untreated mice for the first 2 weeks after gene transfer. These results contradicted those of our previous study, in which the lower dose of pCpG-Mu γ (0.05 pmol per mouse) was effective in preventing the onset of atopic dermatitis.¹⁸ In contrast, the clinical skin score of mice receiving 0.2 pmol pCpG-Mu γ was significantly lower than those of the other two groups over the 5-week observation period. Based on these findings, the dose of the plasmid was set at 0.2 pmol per mouse in the subsequent experiments to study the therapeutic effects of pCpG-Mu γ in NC/Nga mice with dermatitis. To confirm the effects of IFN- γ , a control plasmid pCpG-mcs (Invivogen, San Diego, CA, USA) was administered to NC/Nga mice. However, the clinical skin score was hardly affected by this treatment (data not shown).

Serum concentrations of IgG1 and IgE in NC/Nga mice after IFN- γ gene transfer

Figure 3 shows the time-course of the concentration of IgG1 (Figure 3a) and IgE (Figure 3b) in the serum of NC/Nga mice after injection of pCpG-Mu γ . Both the serum levels of IgG1 and IgE of the pCpG-Mu γ -treated mice were significantly lower than those of the untreated mice at several time points, suggesting that the production of these Th2-mediated Igs in NC/Nga mice was at least partly inhibited by IFN- γ gene transfer.

mRNA expression of Th1, Th2 and Treg cytokines in the spleen of NC/Nga mice after IFN- γ gene transfer

The high concentrations of IFN- γ in the serum were expected to induce the changes in the immunological status of NC/Nga mice. Therefore, the spleen, a major lymphoid organ, was excised and the mRNA expression of IFN- γ , IL-12, IL-4, IL-10 and TGF- β was measured. Figure 4 shows the x-fold increase in mRNA expression by pCpG-Mu γ -treated mice compared with untreated mice. The mRNA expression of IFN- γ and IL-12 in the pCpG-Mu γ -treated mice was, respectively, 3- and 1.7-fold higher than that in the untreated mice. In addition, the mRNA expression of IL-10 and TGF- β was also significantly higher in the pCpG-Mu γ -treated group. On the other hand, the mRNA expression of IL-4, a Th2 cytokine, was reduced to about half in the pCpG-Mu γ -treated mice.

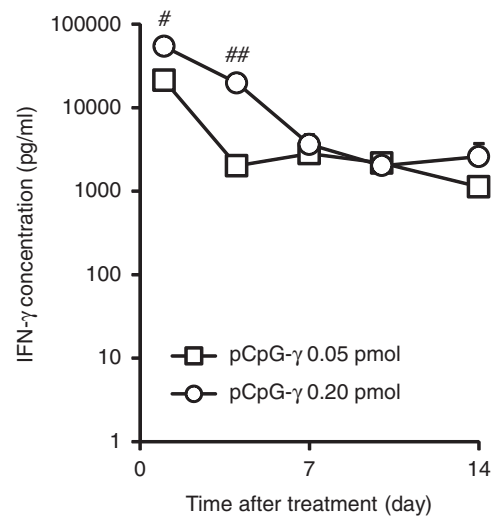


Figure 1. Time-course of the concentration of IFN- γ in serum of NC/Nga mice after hydrodynamic injection of 0.05 or 0.2 pmol pCpG-Mu γ . The results are expressed as the mean $\pm$ s.e.m. of five mice. #, ## P < 0.05, 0.01, compared with the lower dose group.

Table 1. C_{max}, AUC and MRT of serum IFN- γ after hydrodynamic injection of pCpG-Mu γ into mice

Dose (pmol per mouse)	C _{max} (ng ml ⁻¹)	AUC (ng day ml ⁻¹)	MRT (day)
0.05	21.3 $\pm$ 1.5	71.3 $\pm$ 7.9	4.67 $\pm$ 0.78
0.2	54.0 $\pm$ 10 ^a	204 $\pm$ 35 ^b	4.21 $\pm$ 0.70

Abbreviations: AUC, area under the concentration-time curve; IFN, interferon; MRT, mean residence time. The C_{max} values were obtained at 1 day after hydrodynamic injection of pCpG-Mu γ , and are expressed as the mean $\pm$ s.e.m. of five mice. The AUC and MRT were calculated by moment analysis, and are expressed as the calculated mean $\pm$ s.e.m. of five mice. ^a P < 0.05, compared with the lower dose (0.05 pmol per mouse) group. ^b P < 0.01, compared with the lower dose (0.05 pmol per mouse) group.

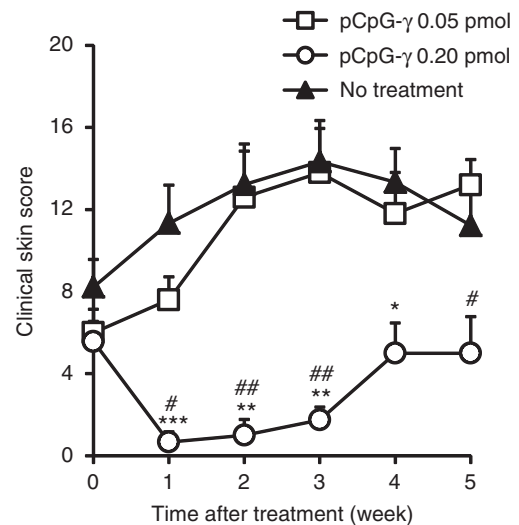


Figure 2. Time-course of the clinical skin score of NC/Nga mice after hydrodynamic injection of pCpG-Mu γ . The results are expressed as the mean $\pm$ s.e.m. of five (0.05 pmol and 0.2 pmol pCpG-Mu γ -treated groups) and nine mice (the untreated group). *, **, *** P < 0.05, 0.01, 0.001, compared with the untreated group; #, ## P < 0.05, 0.01, compared with the lower dose group.

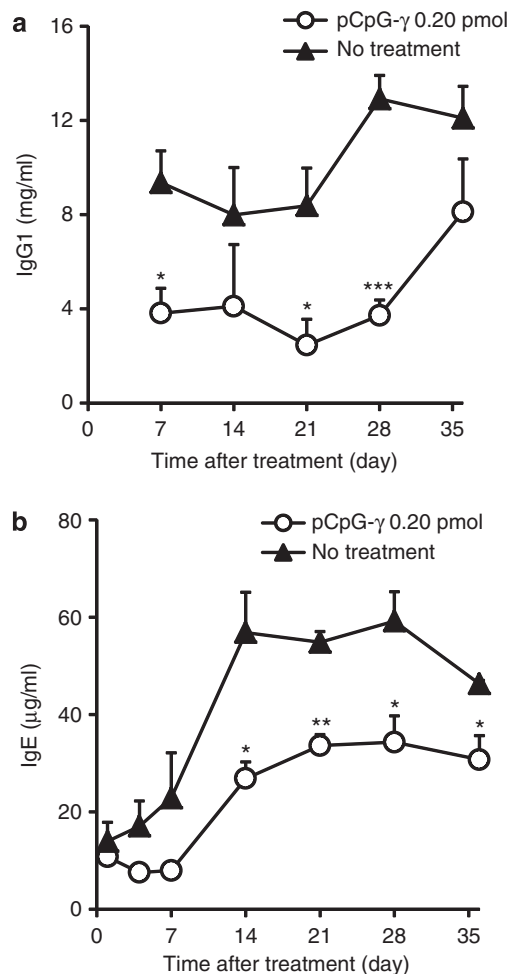


Figure 3. Time-course of the concentration of IgG1 (**a**) and IgE (**b**) in serum of NC/Nga mice after hydrodynamic injection of 0.05 or 0.2 pmol pCpG-Mu γ . The results are expressed as the mean $\pm$ s.e.m. of four mice. * **P < 0.05, 0.01, 0.001, compared with the untreated group.

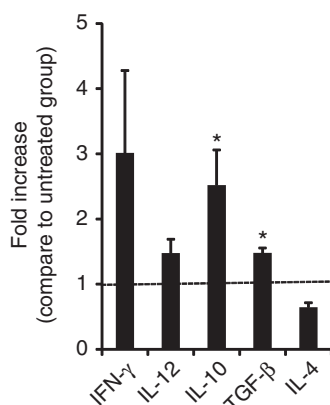


Figure 4. mRNA expression of cytokines in spleen cells of NC/Nga mice. Spleens of untreated mice and pCpG-Mu γ -treated mice were excised at day 36 after treatment. mRNA expression was normalized with that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are expressed as the mean $\pm$ s.e.m. of three (pCpG-Mu γ -treated group) or four mice (the untreated group). *P < 0.05 compared with the untreated group.

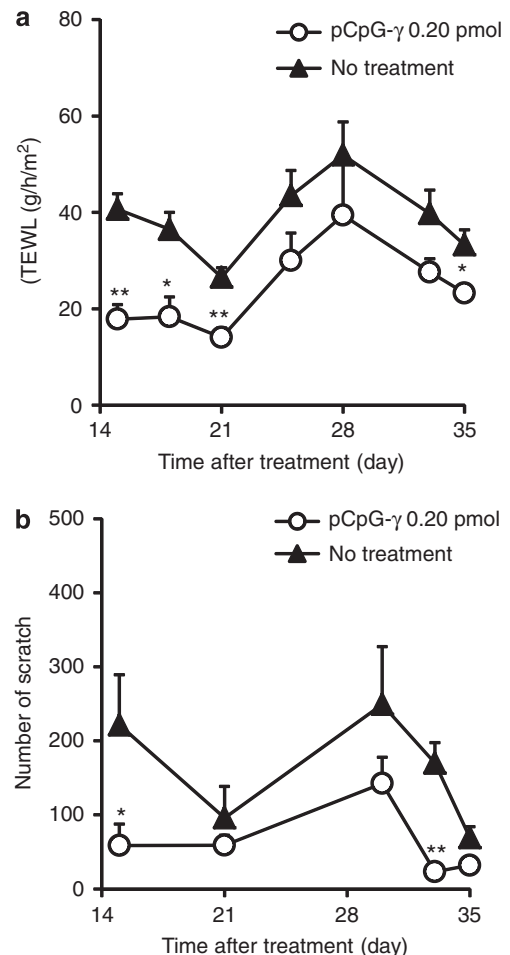


Figure 5. Time-course of the TEWL of the dorsal skin (**a**) and number of scratching episodes (**b**) of NC/Nga mice untreated or after hydrodynamic injection of 0.2 pmol pCpG-Mu γ . The results are expressed as the mean $\pm$ s.e.m. of four mice. * **P < 0.05, 0.01, compared with the untreated group.

TEWL and scratching behavior of NC/Nga mice after IFN- γ gene transfer

To evaluate the severity of skin dryness and itchiness, the TEWL¹⁹ and the number of scratching episodes were measured. Figure 5a shows the time-course of the TEWL of NC/Nga mice from day 15 to day 35 after injection of pCpG-Mu γ . The TEWL value of the pCpG-Mu γ -treated mice was significantly lower than that of the untreated mice on days 15, 18, 21 and 35 after gene transfer. Figure 5b shows the number of scratching episodes during a 30-min period of observation. The number of scratching episodes exhibited by the mice receiving pCpG-Mu γ was fewer than that of the untreated mice at all-time points examined and the difference was significant on days 15 and 33 after injection of pCpG-Mu γ . These results indicate that IFN- γ gene transfer relieves the pruritus, a common symptom of atopic dermatitis that may lead to destruction of the skin barrier and is related to the increased TEWL of NC/Nga mice.^{20,21}

Histopathological examination of skin sections of NC/Nga mice

Figure 6 shows the hematoxylin and eosin sections (Figures 6a and b) and toluidine blue-stained sections (Figures 6c and d) of the dorsal skin of NC/Nga mice at 36 days after treatment. The sections of the untreated mice showed obvious thickening of the epidermis (Figures 6a and c). However, these characteristic

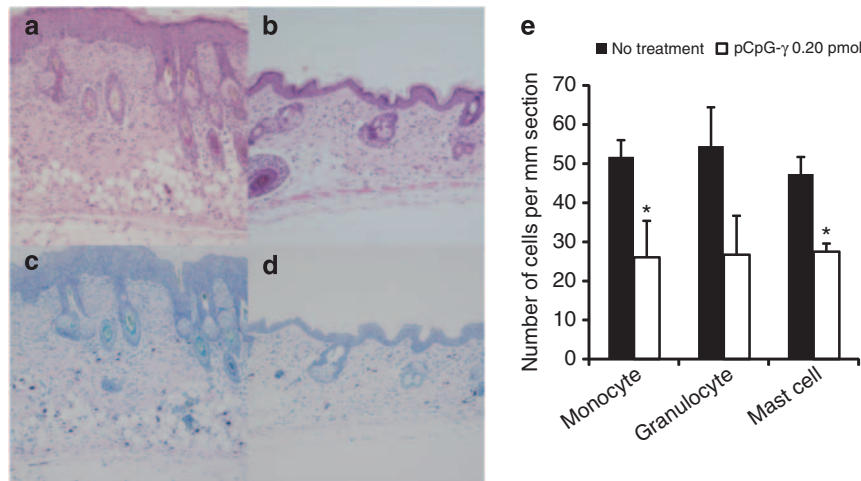


Figure 6. Hematoxylin and eosin (H&E) (a, b) and toluidine blue (c, d) sections of the dorsal skin of NC/Nga mice untreated (a, c) or pCpG-Mu γ -treated (b, d). Numbers of inflammatory cells in skin sections (e). The numbers of cells were expressed as the mean $\pm$ s.e.m. of three (pCpG-Mu γ -treated group) or four mice (the untreated group). * $P < 0.05$ compared with the untreated group.

features of skin inflammation were hardly seen in the sections from the pCpG-Mu γ -treated mice (Figures 6b and d). Furthermore, the numbers of monocytes, granulocytes and mast cells in the skin sections were counted (Figure 6e). The numbers of these cells infiltrating into the skin of the pCpG-Mu γ -treated animals were about a half those in the untreated mice, and there were significant differences between the groups with regard to the number of monocytes and mast cells.

Effect of IFN- γ gene transfer on body temperature and liver injury
The body temperature of the mice was measured to monitor the side effects of the high-dose pCpG-Mu γ . No significant difference was observed in body temperature between the treated and untreated groups (data not shown). In addition, there were no significant differences in the serum aspartate aminotransferase and alanine aminotransferase levels between the pCpG-Mu γ -treated and the untreated groups (data not shown).

Effect of IFN- γ gene transfer on contact dermatitis

There are some concerns that IFN- γ gene transfer might increase the risk of Th1-associated diseases. To clarify this issue, the ear swelling response in the oxazolone (OX)-induced contact dermatitis model was used to evaluate the contact hypersensitivity response in mice. As shown in Figure 7, OX treatment induced ear swelling but this response was not exacerbated by IFN- γ gene transfer.

DISCUSSION

NC/Nga mice spontaneously develop atopic dermatitis-like skin lesions around the age of 6–8 weeks when raised under conventional condition. After that, the severity of the skin lesions and the parameters reflecting the severity, such as serum IgE level, increases with age. A previous study reported that the serum IgE levels of conventional NC/Nga mice increased markedly from 6 weeks of age to around 10 weeks and reached plateau at 17 weeks.¹⁶ Thus, the mice with atopic dermatitis at the age of 7–8 weeks, which were used in this study, represent a suitable model to evaluate the therapeutic effect of IFN- γ in the acute phase of atopic dermatitis. In this study, we demonstrated that pCpG-Mu γ is effective not only in preventing the onset of this condition but also in alleviating the symptoms of the disease, although a higher dose of pCpG-Mu γ was required to treat the symptoms of NC/Nga

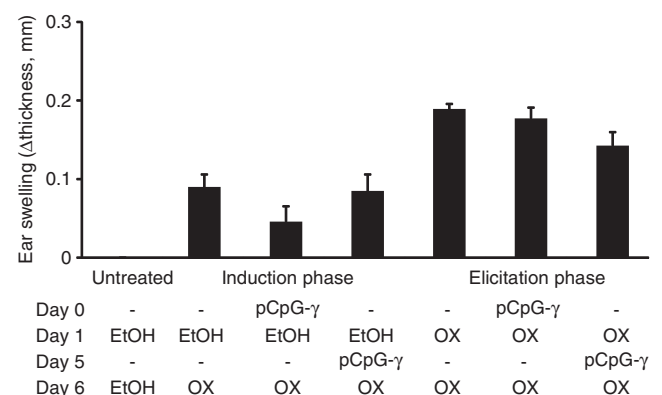


Figure 7. Ear swelling response in OX-treated mice. pCpG-Mu γ was injected on day 0 or day 5. Mice were sensitized by OX or ethanol on the shaved abdomen on day 1 and on the right ear on day 6. Ear thickness was measured 24 h after the last OX application. Data are expressed as the mean $\pm$ s.e.m. of four mice.

mice with dermatitis. The mRNA expression of IFN- γ and IL-12, Th1 cytokines, in the spleen of mice receiving pCpG-Mu γ was increased and accompanied with a reduced expression of IL-4 mRNA in the spleen and reduced levels of serum IgG1 and IgE (Figure 3). IFN- γ acts in conjunction with IL-12 by mediating a positive feedback loop to drive the Th1 response and inhibits IL-4 production by Th2 cells, resulting in the suppression of IgE and IgG1 secretion by B cells.^{22–24} The mRNA expression of IL-10 and TGF- β , two representative Treg cytokines, in the spleen of mice receiving pCpG-Mu γ were also increased. It has been reported that Tregs inhibit Th2 cell function in allergic diseases by releasing IL-10 and TGF- β .^{11,25} The development of atopic dermatitis-like skin lesions in NC/Nga mice was suppressed by IL-10 expressing plasmid DNA.²⁶ It is possible that the increased expression of IFN- γ and IL-12 might induce Treg differentiation^{12,13} and partially contributed to these immunological changes by suppressing Th2 cell response. Taking all these findings into consideration, it is likely that IFN- γ gene transfer stimulates IL-12 production and these two Th1 cytokines act synergistically to polarize the T-cell response toward Th1 subset and inhibit IgG1 and IgE production by suppressing the Th2 pathway. Further investigation is needed

to prove whether the induction of Treg cytokines is related to these changes.

Scratching destroys skin barriers and worsens skin lesions,^{20,21} which is closely linked to an increased TEWL. Histological examination of the skin sections clearly showed that IFN- γ gene transfer markedly suppressed skin inflammation and the infiltration of monocytes, granulocytes and mast cells. Mast cells can be sensitized by IgE and regulate the secretion of cytokines, such as IL-4, which subsequently mediates the recruitment of leukocytes,²⁷ so that the reduced infiltration of these inflammatory cells into the skin could be due to the reduced production of IgE and IL-4. This suppression will prevent mice from scratching, which will accelerate skin repair and reduce TEWL. The number of scratching episodes of mice receiving pCpG-Mu γ was lower than that of the untreated mice at all-time points, although it was fluctuated in the both groups. This could be explained by the difference in the time for measurement in each day,²⁸ which was not be controlled in this study.

The use of IFN- γ as a therapeutic agent would be a double-edged sword. The need for a high dose of 0.2 pmol per mouse to treat, not to prevent, atopic dermatitis symptoms increases the chance of adverse reactions. The most common adverse events of IFN- γ therapy, which have been evidenced in several clinical trials, are 'flu-like' symptoms, including fever, headache, fatigue and myalgia. Diarrhea, erythema at the injection site and the elevation of liver transaminase levels have also been reported in patients receiving the therapy. These common adverse events are generally transient and well tolerated.^{29–34} In this study, we measured the body temperature and the liver transaminase levels in serum of mice for monitoring the signs of adverse events and demonstrated that IFN- γ gene transfer is safe for use in mice as long as the dose of the plasmid is 0.2 pmol per mouse or lower. However, the clinical skin score seems to return to baseline level at 4 weeks after treatment with this dose of plasmid (Figure 2). This result may be explained by the reduction of IFN- γ levels in the serum of mice with time, thus increasing the dose of pCpG-Mu γ within the safety range could be an option to extend the duration of therapeutic effects. Recently, we have established a plasmid DNA expressing IFN γ in a constant manner with no initial high concentration of IFN- γ that could cause the unwanted effects.³⁵ This novel IFN- γ expressing plasmid would also be useful to enhance the therapeutic effect of IFN- γ . Another interesting point is that IFN- γ has been reported to be dominant in the skin of patients with chronic atopic dermatitis.⁵ Further investigation is required to prove whether IFN- γ gene transfer is suitable for the treatment of chronic phase of atopic dermatitis.

In conclusion, we have demonstrated that sustained transgene expression of IFN- γ is effective in treating the atopic dermatitis in NC/Nga mice without any apparent adverse effects. These results raise a possibility that IFN- γ gene transfer can be a therapeutic option for patients with acute-phase atopic dermatitis.

MATERIALS AND METHODS

Animals

In all, 7- to 8-week-old male NC/Nga mice and 7-week-old female C57BL/6 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were raised and maintained on a standard food and water diet under conventional housing conditions. The NC/Nga group spontaneously developing dermatitis was used as a model of atopic dermatitis. The protocol for the animal experiments was approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences of Kyoto University.

In vivo gene transfer of IFN- γ

The plasmid pCpG-Mu γ constructed previously³⁶ was used for the treatment. The naked plasmid pCpG-Mu γ was dissolved in normal saline

solution and injected into the tail vein of the mice by the hydrodynamic injection method^{37,38} at a dose of 0.05 or 0.2 pmol per mouse on day 0.

Measurement of the concentrations of IFN- γ , IgG1 and IgE

Blood samples were obtained from the tail vein at indicated times after gene transfer, kept at 4 °C for 2 h to allow clotting, and then centrifuged to obtain serum. The concentration of IFN- γ , IgG1 and IgE in the serum was measured using ELISA kits (Ready-SET-Go! Mouse IFN- γ ELISA, eBioscience, San Diego, CA, USA; Mouse IgG1 ELISA Quantitation Set, Bethyl Laboratories, Inc., Montgomery, TX, USA; and Mouse IgE ELISA Set, BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

Calculation of the pharmacokinetics parameters of IFN- γ gene transfer

The area under the concentration-time curve and the mean residence time of IFN- γ after gene transfer were calculated from the concentrations of IFN- γ in the serum of NC/Nga mice using moment analysis.³⁹

Real-time PCR analysis of cytokine expression

RNA was extracted from approximately 100 mg of spleen samples using Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan). A mixture of recombinant DNase I-RNase-free (TAKARA, Shiga, Japan) and RNase OUT recombinant ribonuclease inhibitor (Invitrogen, Carlsbad, CA, USA) was used for DNase treatment. Reverse transcription was performed using a ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). For a quantitative analysis of mRNA expression, reverse transcribed samples were amplified by PCR using the primers listed below, and a LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics GmbH, Mannheim, Germany) in a LightCycler instrument (Roche Diagnostics GmbH) according to the manufacturer's protocols. The sequences of primers used were as follows (forward and reverse, respectively): IFN- γ (5'-ATGAACGCTACACA CTGCATC-3' and 5'-CCATCCTTTTGGCCAGTTCCTC-3'), IL-12 (5'-CATCGATGAG CTGATGCAGT-3' and 5'-CAGATAGCCCATCACCCTGT-3'), IL-4 (5'-ACAGGAG AAGGGACGCCAT-3' and 5'-GAAGCCCTACAGACGAGCTCA-3'), IL-10 (5'-CCA AGCCTTATCGGAAATGA-3' and 5'-TTTTCACAGGGGAGAAATCG-3'), TGF- β (5'-TTGCTTCAGCTCCACAGAGA-3' and 5'-TGGTTGTAGAGGGCAAGGAC-3') and glyceraldehyde 3-phosphate dehydrogenase (5'-CTGCCAAGTATGATG ACATCAAGAA-3' and 5'-ACCAGGAAATGAGCTTGACA-3'). Individual PCR products were analyzed by melting curve analysis and the length of the products was determined by agarose gel electrophoresis. The mRNA expression of each gene was normalized using the mRNA level of glyceraldehyde 3-phosphate dehydrogenase.

Scoring skin lesions

Skin lesions were scored at indicated times after gene transfer according to the criteria previously reported.¹⁸ In brief, the scoring was based on (i) the eczema severity, (ii) erosion/excoriation, (iii) scale formation, (iv) erythema/hemorrhage, (v) inflammation of the face and (vi) inflammation of the ear. The total clinical skin severity score was defined as the sum of the each of the six signs (none = 0; mild = 1; moderate = 2; and severe = 3).

Measurement of TEWL

TEWL was measured using an evaporimeter (VAPO SCAN, AS-VT 100RS, Asahi Biomed, Yokohama, Japan) applied to the shaved back of the mice.

Observation of scratching behavior

The scratching behavior was recorded on video for 30 min on days 15 and 21. The videotape was played back at a later time and the number of scratching episodes was counted manually. A series of scratching behaviors, starting with the stretching of the hind paws to the head, face or back and ending with the set-back of the paws, were counted as one bout of scratching. On days 30, 33 and 35 after gene transfer, the scratching behavior was monitored using a SCLABA Real (Noveltec Inc., Kobe, Japan), an automated system to analyze the scratching behavior of small animals. Each mouse was put into an acrylic cage, and its behavior was recorded for 30 min. The number of scratching episodes was automatically quantified.¹⁸

Analysis of skin sections

The dorsal skin of the mice was excised, fixed in 4% paraformaldehyde and embedded in paraffin. Then, 4 μ m-sections were obtained using a microtome and stained with hematoxylin and eosin for histological evaluation and quantification of the numbers of monocytes and granulocytes or with toluidine blue for the detection of mast cells. The number of monocytes, granulocytes and mast cells were manually counted under a microscope, and expressed as the number per unit length of the skin section.

Monitoring of adverse effects of IFN- γ gene transfer

The body temperature of the mice was monitored using a digital rectal thermometer (Physitemp Instruments Inc., Clifton, NJ, USA). To assess liver damage, the serum alanine aminotransferase and aspartate aminotransferase levels of the mice were assayed using commercial test reagents (Transaminase CII-Test Wako, Wako Pure Chemical Industries, Osaka, Japan).

OX-induced contact hypersensitivity

The plasmid pCpG-Mu γ was injected into the tail vein of C57BL/6 mice (female, 7-week-old) by the hydrodynamic injection method at a dose of 0.2 pmol per mouse on day 0 or day 5. Mice were sensitized by the application of 100 μ l 2% OX in ethanol (elicitation phase group) or ethanol alone (induction phase-group) to the shaved abdominal skin on day 1, followed by an application of 10 μ l 1% OX to the right ear on day 6. Ethanol was applied to the left ear. For the untreated group, ethanol was applied instead of OX to the abdomen and right ear on either day 1 or 6. At 24 h after the second application, the thickness of both ears was measured using a digital thickness gauge (Quick Mini, Mitutoyo Co., Kawasaki, Japan). Ear swelling was calculated from the difference in ear thickness between the hapten- and vehicle-treated ears.

Statistical analysis

Statistical significance was evaluated by analysis of variance with a *post-hoc* Tukey–Kramer test for multiple comparisons (clinical skin scores) and Student's *t*-test for comparisons between two given groups. The level of statistical significance was set at $P < 0.05$.

ABBREVIATIONS

IFN- γ , interferon γ ; Ig, immunoglobulin; IL, interleukin; Th, T helper.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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