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Sustained Exogenous Expression of Therapeutic Levels of IFN- γ Ameliorates Atopic Dermatitis in NC/Nga Mice via Th1 Polarization

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The short in vivo half-life of IFN- γ can prevent the cytokine from inducing immunological changes that are favorable for the treatment of Th2-dominant diseases, such as atopic dermatitis. To examine whether a sustained supply of IFN- γ is effective in regulating the balance of Th lymphocyte subpopulations, plasmid vector encoding mouse IFN- γ , pCpG-Mu γ , or pCMV-Mu γ was injected into the tail vein of NC/Nga mice, a model for human atopic dermatitis. A single hydrodynamic injection of a CpG motif reduced pCpG-Mu γ at a dose of 0.14 μ g/mouse resulted in a sustained concentration of IFN- γ in the serum, and the concentration was maintained at >300 pg/ml over 80 d. The pCpG-Mu γ -mediated IFN- γ gene transfer was associated with an increase in the serum concentration of IL-12, reduced production of IgE, and inhibition of mRNA expression of IL-4, -5, -10, -13, and -17 and thymus and activation-regulated chemokine in the spleen. These immunological changes were not clearly observed in mice receiving two injections of 20 μ g pCMV-Mu γ , a CpG-replete plasmid DNA, because of the transient nature of the expression from the vector. The mice receiving pCpG-Mu γ showed a significant reduction in the severity of skin lesions and in the intensity of their scratching behavior. Furthermore, high transepidermal water loss, epidermal thickening, and infiltration of lymphocytes and eosinophils, all of which were obvious in the untreated mice, were significantly inhibited. These results indicate that an extraordinary sustained IFN- γ expression induces favorable immunological changes, leading to a Th1-dominant state in the atopic dermatitis model. *The Journal of Immunology*, 2010, 184: 2729–2735.

The number of patients with allergies, including those with asthma, pollinosis, and atopic dermatitis, has been increasing in recent decades, especially in developed countries. It is believed that these disorders result from the imbalance of Th lymphocyte subpopulations (Th1 and Th2), which play major roles in the immune response (1). Under normal conditions, the differentiation of naive T cells to Th1 and Th2 lineages is regulated by cytokines that are secreted from various cells, including themselves, and the Th1/Th2 balance is maintained. However, in atopic dermatitis, which is one of the most common type 1 allergic diseases, the balance shifts to Th2 dominance; this eventually leads to excessive Th2 cytokine production. Th2-like immune responses play an important role in the pathogenic mechanism of atopic disorders, because Th2 cytokines mediate excessive IgE production, a major cause of atopic inflammation (2–5).

IFN- γ , a Th1 cytokine, inhibits the differentiation of naive T cells to Th2 cells, as well as the production of Th2 cytokines from Th2 cells. Thus, IFN- γ has been considered to be capable of correcting the Th1/Th2 imbalance and is effective in the treatment of diseases in which the balance is impaired, such as atopic dermatitis (6). Despite such positive features, few attempts have been made to use IFN- γ as a pharmaceutical agent for the treatment of atopic dermatitis (7–10). This is mainly due to the fact that IFN- γ , as well as other IFNs, has a short half-life in vivo, and multiple injections are required to maintain its concentration at levels high enough to prevent dermatitis (11).

Several approaches have been developed to extend the duration of the therapeutic effects of biologically active proteins. Extension of the in vivo half-life of proteins can be achieved by using controlled- or sustained-release systems (12–15) or by chemical modification of proteins (16–18). Pepinsky et al. (19) demonstrated that the high clearance of IFN- β -1a was reduced by PEGylation, and its increased systemic exposure resulted in better antiviral effects. PEGylated IFN- α in combination with an antiviral drug, ribavirin, is now a standard treatment for hepatitis C virus-induced chronic hepatitis. The increased half-life of IFNs obtained by PEGylation has greatly increased their therapeutic efficacy. In addition to these challenges, gene delivery is an option to increase the in vivo half-life of therapeutic proteins, including IFNs. In previous studies, we proved that the depletion of CpG motifs in plasmid vectors is an effective approach for extending the duration of transgene expression (20, 21). We also succeeded in developing a murine IFN- γ -expressing plasmid DNA, pCpG-Mu γ , which contains no CpG motifs except for those in the cDNA region (22). A single i.v. injection of pCpG-Mu γ resulted in a high and sustained IFN- γ concentration in the serum over 1 mo after hydrodynamic injection into healthy ICR mice. However, little is

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Abbreviations used in this paper: SPF, specific pathogen-free; TARC, thymus and activation-regulated chemokine; TEWL, transepidermal water loss; TNCB, 2,4,6-trinitrochlorobenzene.

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known about how such a sustained transgene expression of IFN- γ influences the Th1/Th2 balance under Th2-dominant conditions.

In this study, we injected pCpG-Mu γ , a murine IFN- γ -expressing plasmid DNA, into a human atopic dermatitis model (NC/Nga mice) (23), to achieve a sustained transgene expression of IFN- γ . A conventional CpG replete plasmid vector expressing IFN- γ , pCMV-Mu γ (21, 24), was also used for comparison to examine the importance of the duration of transgene expression on the immunological changes induced by IFN- γ gene transfer. The expression profile of IFN- γ was first examined in NC/Nga mice, and the effect of the expression on the level of IL-4, -5, -10, -12, -13, -17, and thymus and activation-regulated chemokine (TARC) was evaluated. Then, skin lesions, the intensity of scratching behavior, trans-epidermal water loss (TEWL), the thickness of the epidermis, and the infiltration of the skin by inflammatory cells were evaluated. In this study we showed that sustained, but not transient, gene expression of IFN- γ can induce favorable immunological changes in a human atopic dermatitis model, which allows the prevention of the development of atopic dermatitis-like skin lesions.

Materials and Methods

Animals

Five-week-old male C57BL/6 mice and 6-wk-old male NC/Nga mice that were raised under conventional conditions, but had not developed dermatitis, were purchased from Japan SLC (Hamamatsu, Japan) and maintained on a standard food-and-water diet under conventional housing conditions. The protocol for the animal experiments was approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences, Kyoto University.

Plasmid DNA

Two types of IFN- γ -expressing plasmid vectors developed in our laboratory were used: pCMV-Mu γ , which was constructed by inserting a murine IFN- γ cDNA fragment into the BamHI site of pcDNA3 (Invitrogen, Carlsbad, CA) (24), and pCpG-Mu γ , which was constructed by inserting the BglIII/NheI IFN- γ cDNA fragment amplified by PCR from the pCMV-Mu γ into the BglIII/NheI site of pCpG-mcs (Invivogen, San Diego, CA) (22).

In vivo gene transfer of IFN- γ

Naked plasmid DNA dissolved in isotonic saline solution was injected into the tail vein of mice over 5 s on day 0 (25, 26). To adjust the peak level of the IFN- γ concentration after gene transfer, the plasmid dose was fixed at 0.14 and 20 μ g/mouse for pCpG-Mu γ and pCMV-Mu γ , respectively, based on preliminary experiments. pCMV-Mu γ was injected twice at an interval of 1 wk (days 0 and 7). The body weight and temperature of mice were measured to assess the adverse effects of IFN- γ gene transfer.

Measurement of concentration of IFN- γ , IgE, and IL-4, -12, and -13

Blood samples were obtained from the tail vein at indicated times after gene transfer, incubated at 4°C for 2 h to allow clotting, and centrifuged to obtain serum. Dorsal skin tissue was homogenized in PBS containing protease inhibitors (protease inhibitor mixture; Sigma-Aldrich, Munich, Germany) and then centrifuged for 30 min at 12,000 \times g. The concentration of IFN- γ , IgE, and IL-4, -12, and IL-13 in the serum or supernatant of skin homogenate was measured using ELISA kits (Ready-SET-Go! Mouse IFN- γ and IL-13 ELISA, eBioscience, San Diego, CA; OptEIA set Mouse IL-12, IgE and IL-13, BD Biosciences, San Jose, CA).

mRNA quantification

Total RNA was extracted from ~100 mg spleen or skin sample using Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan). The total RNA was cleaned up using an RNeasy mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed using a SuperScript II (Invitrogen) and oligo (dT) primer, according to the manufacturer's protocol. For a quantitative analysis of mRNA expression, real-time PCR was carried out with total cDNA using a LightCycler instrument (Roche Diagnostics, Basel, Switzerland). The oligonucleotide primers used for amplification are listed in Table I. Amplified products were detected online via intercalation of the fluorescent dye SYBR green (LightCycler-FastStart DNA Master SYBR Green I kit, Roche Diagnostics, Indianapolis, IN). The cycling conditions were as follows: initial enzyme activation at 95°C for 10 min, followed by

55 cycles at 95°C for 10 s, 60°C for 5 s, and 72°C for 20 s. All cycling reactions were performed in the presence of 3.5 mM MgCl₂. Gene-specific fluorescence was measured at 72°C. The mRNA expression of genes of interest was normalized using the mRNA level of β -actin.

Scoring skin lesions

Skin lesions were scored at indicated times after gene transfer, according to the criteria of Matsuda et al. (23). The scoring was based on the severity of eczema, erosion/excoriation, scaling, erythema/hemorrhage, inflammation of the face, and inflammation of the ear. The total clinical skin severity score was defined as the sum of each of the six signs (none = 0; mild = 1; moderate = 2; and severe = 3).

Observation of scratching behavior

On days 7, 14, and 35, scratching behavior was monitored using SCLABA Real (Noveltec, Kobe, Japan), an automated system to analyze the scratching behavior of small animals. Each mouse was put into an acrylic cage, and the behavior of the mice was recorded for 30 min. The number of episodes and the duration of scratching behavior were automatically quantified. In a different set of mice, the scratching behavior was recorded on video for 1 h on days 7, 14, 35, and 84. 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, was counted as one bout of scratching (27).

Measurement of TEWL

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

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 made using a microtome and stained with H&E for histological evaluation or with toluidine blue to detect mast cells. The numbers of lymphocytes, eosinophils, and mast cells on H&E-stained sections (lymphocytes and eosinophils) or toluidine blue-stained sections (mast cells) were manually counted under a microscope and expressed as the number per unit length of skin section.

Effect of IFN- γ gene transfer on 2,4,6-trinitrochlorobenzene-induced dermatitis

To assess whether IFN- γ gene transfer increases the risk for contact dermatitis, C57BL/6 mice were sensitized by painting 25 μ l 3% 2,4,6-trinitrochlorobenzene (TNCB) in acetone/olive oil (4:1) on the shaved abdomen (day 0). pCpG-Mu γ was injected by the hydrodynamic injection method to TNCB-treated mice 1 d before or 7 d after the TNCB treatment, at a dose of 0.14 μ g/mouse. On day 5, the thickness of the ear was measured with a Quick Mini thickness gauge (Mitutoyo, Tokyo, Japan). Then, 20 μ l 1.5% TNCB solution was applied to the surface of the ear. The thickness of the ear was measured again 24 h after the second application (challenge), and ear swelling was evaluated by the difference in the thickness before and after the challenge.

Effect of IFN- γ gene transfer on TEWL in tape stripping- and TNCB-induced dermatitis models

C57BL/6 mice were shaved on their dorsal skin on day 0 and injected with pCpG-Mu γ (0.14 μ g/mouse) or saline by the hydrodynamic injection method on the same day. Mice were repeatedly treated with 50 μ l 1% TNCB dissolved in acetone/olive oil (4:1) to the shaved skin on days 1, 8, 11, and 13. In a separate experiment, the dorsal skin of C57BL/6 mice was tape stripped on days 1 and 8 and injected with pCpG-Mu γ (0.14 μ g/mouse) or saline by the hydrodynamic injection method on day 0. TEWL from the stripped or shaved skin was measured as described above.

Statistical analysis

Differences were evaluated by the Student *t* test, and the level of statistical significance was *p* < 0.05.

Results

IFN- γ concentration in the serum of NC/Nga mice after injection of IFN- γ -expressing plasmid DNA

Fig. 1 shows the time courses of the concentration of IFN- γ in the serum after i.v. injection of pCpG-Mu γ or pCMV-Mu γ . A very high and sustained concentration of IFN- γ was detected in the

serum of mice receiving pCpG-Muγ at a dose of 0.14 μg/mouse: >300 pg IFN-γ/ml was detected ~3 mo after a single injection. However, the concentration of IFN-γ had decreased very quickly below the detection limit (25 pg/ml) 3 d after the first injection of pCMV-Muγ (20 μg/mouse). The plasmid was injected again 1 wk later, but the peak level was lower than that after the first injection, and the concentration decreased quickly again.

IL-12 concentration in the serum of NC/Nga mice after injection of IFN-γ-expressing plasmid DNA

It would be expected that a persistent expression of IFN-γ would induce the expression of IL-12, a typical Th1 cytokine that promotes the differentiation of naive T cells into the Th1 phenotype. Therefore, the serum concentration of IL-12 was measured (Fig. 2A). The IL-12 concentration in the untreated group was not constant during the experimental period; it fluctuated, reflecting the acute and chronic phases of the disease (28). The concentration of IL-12 was significantly increased in the pCpG-Muγ-treated group from days 3 to 42 after injection. The concentration in the pCMV-Muγ-treated group was significantly greater 3 d after the first injection compared with that in the untreated group; thereafter, the profile became superimposed on that of the untreated group. The second injection of pCMV-Muγ on day 7 barely affected the serum concentration of IL-12.

IgE concentration in the serum of NC/Nga mice after injection of IFN-γ-expressing plasmid DNA

Next, the concentration of IgE, a diagnostic marker of atopic dermatitis (29), was measured in the serum, because increased IgE production is a hallmark of Th2 immune responses. Fig. 2B shows the concentration of IgE in the serum of the untreated mice or mice treated with pCpG-Muγ or pCMV-Muγ. The concentration at the onset of treatment was ~10 μg/ml, and it increased with time to >100 μg/ml in the untreated group. It also increased in the treated groups, but the increase was significantly inhibited in the pCpG-Muγ-treated group at ≥14 d after the treatment. In addition, we measured the serum concentrations of IL-4 and -13, which play important roles in the regulation of IgE synthesis (30). However, the concentrations of IL-4 and -13 in the serum of all groups were below the detection limit (4 pg/ml) of the analysis.

mRNA expression of cytokine and chemokine in spleen cells of NC/Nga mice after injection of IFN-γ-expressing plasmid DNA

The effect of IFN-γ gene transfer on the expression of cytokines/chemokines was evaluated by measuring the mRNA expression of IL-12, Th2 cytokines (IL-4, -5, -10, and -13), IL-17, and a Th2

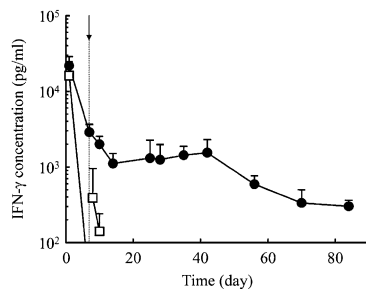


FIGURE 1. Time course of the concentration of IFN-γ in mouse serum after injection of IFN-γ-expressing plasmid DNA. NC/Nga mice were injected i.v. with 0.14 μg pCpG-Muγ (●) or 20 μg pCMV-Muγ (□) by the hydrodynamic injection method. The pCMV-Muγ-injected group received a second injection of 20 μg pCMV-Muγ 7 d after the first injection (indicated by a dashed line and an arrow). The results are expressed as the mean ± SD of five mice.

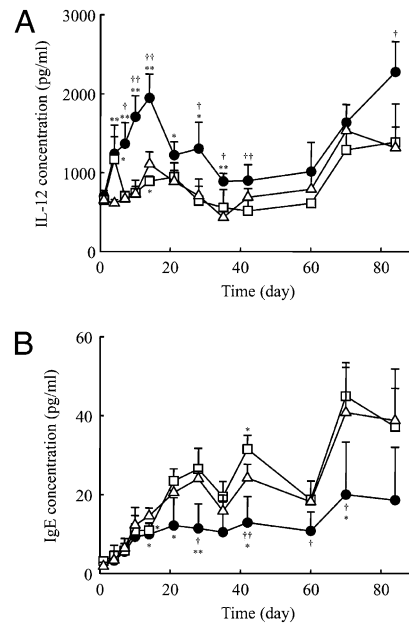


FIGURE 2. Time course of the concentration of IL-12 (A) and IgE (B) in mouse serum after injection of IFN-γ-expressing plasmid DNA. NC/Nga mice were injected i.v. with 0.14 μg pCpG-Muγ or 20 μg pCMV-Muγ, as described in the legend of Fig. 1. Blood samples from untreated (△), pCpG-Muγ-injected (●), or pCMV-Muγ-injected (□) mice were collected from the tail vein at the indicated times after gene transfer. The results are expressed as the mean ± SD of at least three mice. **p* < 0.05 compared with the untreated group; ***p* < 0.01 compared with the untreated group; †*p* < 0.05 compared with the pCMV-Muγ-treated group; ††*p* < 0.01 compared with the pCMV-Muγ-treated group.

chemokine (TARC) in spleen cells collected from mice 14 d after gene transfer using the primers listed in Table I. The mRNA expression of these genes in spleen cells was normalized to that of β-actin, and the ratios were compared between the untreated and the pCpG-Muγ-treated mice (Fig. 3). The differences between the groups were very large for IL-5, -10, -12, and -13 and TARC, although they were not statistically significant because of the limited number of samples. The IL-12 mRNA expression was increased by injection of pCpG-Muγ, which was in good agreement with the serum level of IL-12 (Fig. 2A). The mRNA expression of IL-4, -5, -10, -13, and -17 and TARC in the pCpG-Muγ-treated group was lower than that in the untreated group. The mRNA expression in spleen cells 40 d after gene transfer showed no significant differences (data not shown). In addition, the expression of these cytokines in the skin was examined by ELISA and RT-PCR 40 d after gene transfer. However, no significant differences were detected among the groups (data not shown).

Table I. Primer sequences for quantitative RT-PCR

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
Mouse β-actin	gcaccacacctctacaatgag	ttggcatagaggtctttacgga
Mouse IL-12	catcgatgagctgatgcagt	cagatagcccatcaccctgt
Mouse IL-4	gcttttcgatgcctggattc	gctttccaggaagtctttcagtg
Mouse IL-5	agagaagtgtggcgaggagaga	cattgcccactctgtactcatca
Mouse IL-10	ttgccaaagccttatcggga	ttctctgggcatgctctctct
Mouse IL-13	cagctccctggttctctcac	ccacactccataccatgctg
Mouse IL-17	tccagaagccctcagacta	agcatcttctcgaccctgaa
Mouse TARC	agtggagtgttccagggatg	gtcacaggccgctttatgtt

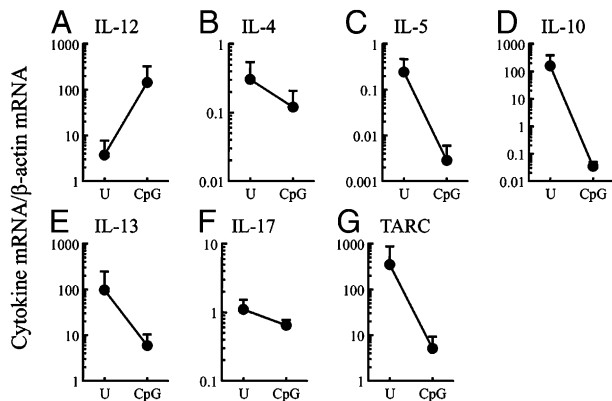


FIGURE 3. mRNA expression of cytokines and TARC in spleen cells. Splens from NC/Nga mice receiving 0.14 μ g pCpG-Mu γ (CpG) were collected 14 d after gene transfer, and the mRNA expression of cytokine and chemokine genes was measured by real-time PCR. The mRNA expression of genes was normalized using the mRNA level of β -actin. In comparison, splens from untreated mice (U) were treated as above. A, IL-12. B, IL-4. C, IL-5. D, IL-10. E, IL-13. F, IL-17. G, TARC. The results are expressed as the mean \pm SD of three mice.

Skin lesions of NC/Nga mice after injection of IFN- γ -expressing plasmid DNA

The results indicated that the Th1/Th2 balance can be shifted to Th1 in the atopic dermatitis model by sustained, but not transient, transgene expression of IFN- γ . The effects of gene transfer on atopic dermatitis were examined in NC/Nga mice. Fig. 4 shows the typical images of the back skin of mice 35 d after the start of treatment. Clinical signs and symptoms were clearly seen on the skin of the untreated mice (Fig. 4A) and the pCMV-Mu γ -treated mice (Fig. 4C), indicating that these mice developed a severe dermatitis. The severity of skin damage was scored using a clinical skin score (23). The untreated and the pCMV-Mu γ -treated groups

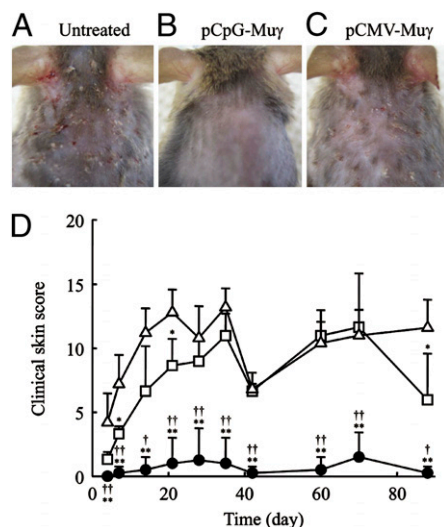


FIGURE 4. Typical images of the back skin of NC/Nga mice (A–C) and the time course of the skin clinical score (D). Photographs were taken 35 d after the start of treatment. A, Untreated mice. B, pCpG-Mu γ -treated mice. C, pCMV-Mu γ -treated mice. D, Clinical features of dermatitis were scored at indicated periods of time after the start of treatment. The results are expressed as the mean \pm SD of at least three mice. * p < 0.05 compared with the untreated group; ** p < 0.01 compared with the untreated group; † p < 0.05 compared with the pCMV-Mu γ -treated group; †† p < 0.01 compared with the pCMV-Mu γ -treated group.

developed dermatitis within a week after the start of the experiment, and the severity of the dermatitis increased with time (Fig. 4D). In contrast, the pCpG-Mu γ -treated mice developed much less severe skin inflammation throughout the experimental period (Fig. 4B), and the clinical skin score of the group was significantly lower than that of the untreated or the pCMV-Mu γ -treated group (Fig. 4D).

Scratching behavior of NC/Nga mice after injection of IFN- γ -expressing plasmid DNA

Fig. 5 shows the number of episodes of scratching and the cumulative time of scratching for a 30-min period. The number and duration of scratching episodes increased with time in the untreated group. The pCpG-Mu γ - and pCMV-Mu γ -treated groups had significantly fewer episodes and shorter duration of scratching than the untreated group, with significantly better results for pCpG-Mu γ -treated mice. Similar results were obtained in a different set of mice whose scratching episodes were counted manually after videotaping (data not shown).

TEWL of NC/Nga mice after injection of IFN- γ -expressing plasmid DNA

Dry skin is a common symptom of atopic dermatitis, which is characterized by extensive water loss through the skin. Thus, the TEWL of the skin was measured on the back (Fig. 6). The TEWL value at day 0 was \sim 10 g/h/m² in all groups. In the untreated mice, the value increased to $>$ 50 g/h/m² by day 14, and an almost constant value was observed in the period that followed. The elevation of TEWL was significantly inhibited in the pCpG-Mu γ -treated group throughout the experimental period. The TEWL of the pCMV-Mu γ -treated mice was as low as that of the pCpG-Mu γ -treated ones for the first 14 d, but it was significantly higher at days 70 and 84.

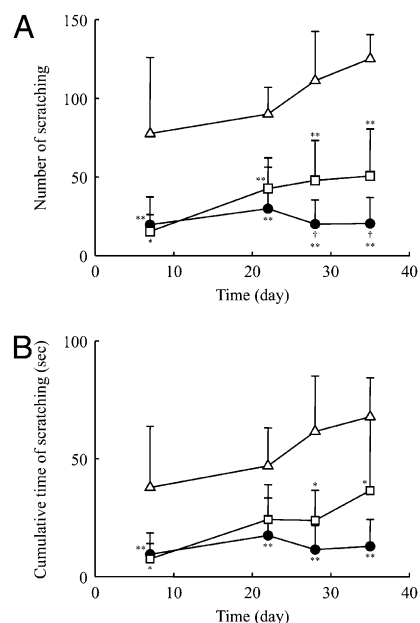


FIGURE 5. Number (A) and cumulative time (B) of scratching episodes. Scratching behavior of NC/Nga mice treated as described in the legend of Fig. 1 were automatically evaluated using SCLABA-Real. Δ , untreated mice; \bullet , pCpG-Mu γ -treated mice; \square , pCMV-Mu γ -treated mice. The results are expressed as the mean \pm SD of at least three mice. * p < 0.05 compared with the untreated group; ** p < 0.01 compared with the untreated group; † p < 0.05 compared with the pCMV-Mu γ -treated group; †† p < 0.01 compared with the pCMV-Mu γ -treated group.

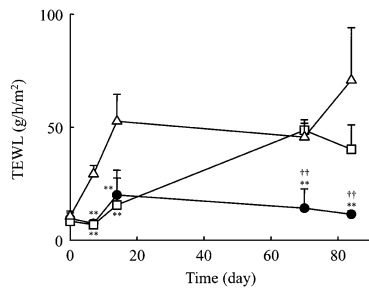


FIGURE 6. Time course of the TEWL of the back skin of mice. TEWL was measured on the back of untreated mice (Δ), pCpG-Mu γ -treated mice (\bullet), and pCMV-Mu γ -treated mice (\square). The results are expressed as the mean \pm SD of at least three mice. $**p < 0.01$ compared with the untreated group; $††p < 0.01$ compared with the pCMV-Mu γ -treated group.

Histological examination of skin sections of NC/Nga mice after injection of IFN- γ -expressing plasmid DNA

Fig. 7 shows the H&E sections of the back skin of treated and untreated mice at day 14 after the start of treatment. NC/Nga mice maintained under specific pathogen-free (SPF) conditions were used as control mice with healthy skin; the skin sections from the SPF control mice showed no pathological features (Fig. 7A). In marked contrast, there was clear hyperplasia of the epidermis (acanthosis) in the untreated group (Fig. 7B). The sections from the untreated mice also showed an extensive infiltration of lymphocytes and eosinophils. These characteristic features of inflamed skin tissues were not as apparent in the skin sections from the pCpG-Mu γ -treated mice (Fig. 7C), which were indistinguishable from the skin sections from the SPF control mice. Compared with the sections from the pCpG-Mu γ -treated mice, the ones from pCMV-Mu γ -treated mice showed less significant improvement (Fig. 7D). Fig. 8 shows the skin sections in which mast cells were stained with toluidine blue. Again, a significant infiltration of mast cells was observed in the untreated mice (Fig. 8B), but not in the SPF control (Fig. 8A) or the pCpG-Mu γ -treated mice (Fig. 8C). The numbers of lymphocytes, eosinophils, and mast cells in skin sections were counted (Fig. 9). The numbers of these cells were significantly less in mice receiving IFN- γ -expressing plasmid DNA compared with untreated mice.

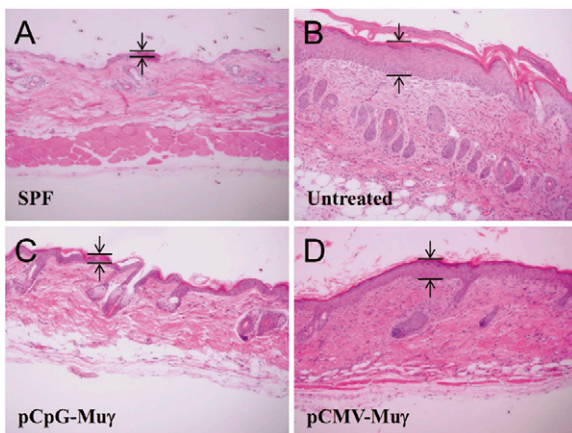


FIGURE 7. H&E sections of the back skin of NC/Nga. Dorsal skin of mice maintained under SPF conditions (A), untreated mice (B), pCpG-Mu γ -treated mice (C), and pCMV-Mu γ -treated mice (D) were collected at day 14. Skin sections were stained with H&E for histological evaluation and detection of inflammatory cells. Arrows and bars indicate the thickness of the epidermis. Original magnification $\times 400$.

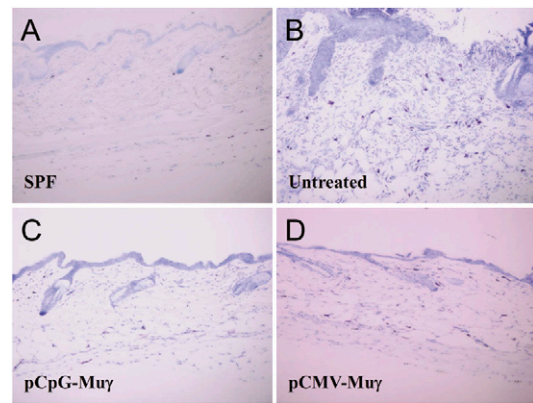


FIGURE 8. Toluidine blue sections of the back skin of NC/Nga mice. Dorsal skin of mice maintained under SPF conditions (A), untreated mice (B), pCpG-Mu γ -treated mice (C), and pCMV-Mu γ -treated mice (D) were collected at day 14. Skin sections were stained with toluidine blue to detect mast cells. Original magnification $\times 400$.

Adverse effects of IFN- γ gene transfer

There were no significant differences in the body weight or temperature between the saline-injected mice and the pCpG-Mu γ -treated mice. To examine whether IFN- γ gene transfer increases the risk for contact dermatitis, the ear thickness was measured in a mouse model of TNCB-induced contact dermatitis. The challenge with TNCB significantly increased the thickness, but the injection of pCpG-Mu γ had no significant effects on the change (data not shown). Fig. 10 shows the time courses of the TEWL from the dorsal skin of tape stripped (Fig. 10A) or TNCB-treated mice (Fig. 10B). Again, no IFN- γ gene transfer-induced increase was observed in any case examined.

Discussion

Because of the multiple functions of cytokines and their complicated network, the effects of externally administered cytokines, including IFN- γ , depend on their pharmacokinetics. Atopic dermatitis, a chronic inflammatory disease with skin inflammation, is characterized by Th2-dominant immunity; therefore, any treatment that normalizes the Th1/Th2 balance can be useful for treatment of the disease. IFN- γ , a typical Th1 cytokine, has been considered to induce a variety of immunological changes, leading to a Th1-dominant state, but its effects on the Th1/Th2 balance in patients with Th2 predominance is not fully understood. The outcome from the previous clinical experiments in which IFN- γ was administered to patients with atopic dermatitis suggested that

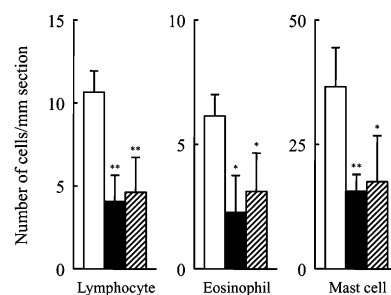


FIGURE 9. Number of lymphocytes, eosinophils, and mast cells in skin sections. Lymphocytes and eosinophils in H&E-stained sections were counted under a microscope. The toluidine blue sections were used for the counting of mast cells. The number of cells was expressed as the mean \pm SD of three sections: untreated mice (open bars), pCpG-Mu γ -treated mice (filled bars), and pCMV-Mu γ -treated mice (striped bars).

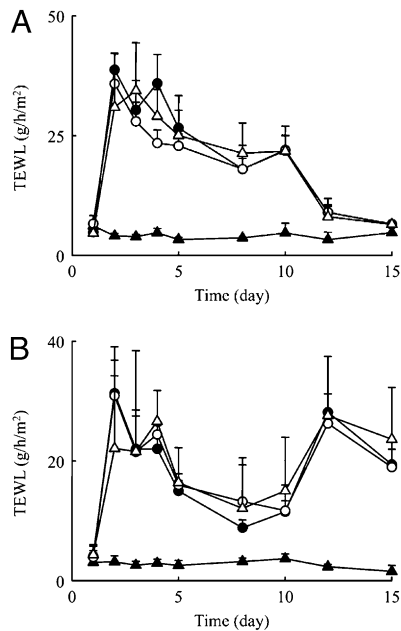


FIGURE 10. Time course of the TEWL of the back skin of mice in a stripped skin and a TNCB-induced dermatitis model. *A*, The back skin of C57BL/6 mice was shaved and tape stripped on days 1 and 8. ▲, untreated mice; △, tape-stripped mice; ○, tape-stripped, saline-treated mice; ●, tape-stripped, pCpG-Mu γ -treated mice. *B*, The back skin of C57BL/6 mice was shaved, and TNCB was applied on days 1, 8, 11, and 13. ▲, untreated mice; △, TNCB-treated mice; ○, TNCB-treated, saline-treated mice; ●, TNCB-treated, pCpG-Mu γ -treated mice. Results in *A* and *B* are expressed as the mean \pm SD of four mice.

there would be few significant changes in the immune system of the patients receiving such treatments (7–10). This can primarily be attributed to the unfavorable pharmacokinetic properties of IFN- γ , which easily passes through the glomerulus of the kidney because it is smaller than the threshold of glomerular filtration, and it disappears from the systemic circulation very quickly (11).

In the current study, a sustained level of IFN- γ in the serum was successfully achieved by injecting pCpG-Mu γ . An IFN- γ concentration >300 pg/ml, which is greater than the half maximal effective concentration of the protein needed to inhibit virus replication (100 pg/ml), was obtained throughout the experimental period of 84 d. This profile of IFN- γ in the serum was in marked contrast to that obtained by the injection of pCMV-Mu γ , which resulted in a very transient IFN- γ concentration in the serum. Such a large difference in the duration of transgene expression is most likely due to the number of CpG motifs in plasmid DNA (21, 31). A detailed discussion on this sustained expression of IFN- γ from pCpG-Mu γ was presented elsewhere (22).

Sustained expression of IFN- γ from pCpG-Mu γ induced a variety of immunological changes in NC/Nga mice. In summary, the level of Th1 cytokines IFN- γ and IL-12 (Fig. 2A) was increased and that of IL-4, -5, -10, -13, and -17 and TARC tended to be decreased (Fig. 3); thus, the Th1/Th2 balance shifted toward Th1 dominance. In addition, the level of IgE, a hallmark of allergic reactions, was also significantly decreased. These changes can be considered to be due to the biological activity of IFN- γ , which promotes the production of IL-12, a typical Th1 cytokine important for differentiation to the Th1 subset. IL-12 is produced from macrophages or NK cells stimulated with IFN- γ . The reduced expression of the Th2 cytokines and TARC also resulted from the expression of IFN- γ , as reported using PBMCs, keratinocytes, or Langerhans cells (32–34).

A major finding of the current study is that the immunological changes induced by IFN- γ gene transfer differ, depending on the vector used (i.e., on the pharmacokinetics of IFN- γ). A variety of changes, including the increase in the IL-12 concentration and the decrease in the IgE level, were observed only in the mice receiving pCpG-Mu γ . These results clearly indicate the importance of a sustained concentration of IFN- γ at a level high enough to allow it to modulate the Th1/Th2 balance. In addition, these results may explain the fact that most challenges using IFN- γ protein for the treatment of atopic dermatitis are not very effective, because IFN- γ protein quickly disappears from the systemic circulation (11).

The modulation of Th1/Th2 imbalance by sustained expression of IFN- γ was significantly effective in preventing the onset of symptoms of atopic dermatitis in NC/Nga mice. Rash, scratching, bleeding on the back or ear, acanthosis, and infiltration of lymphocytes, eosinophils, and mast cells were not very apparent in the pCpG-Mu γ -treated mice. Inhibition of cellular infiltration into skin would be the consequence of the changes induced by the sustained concentration of IFN- γ ; this directly inhibits the infiltration of eosinophils (35, 36), and it could inhibit Th2 cell migration into skin promoted by Th2 chemokines (37, 38). Because Th2 lymphocytes are the cells that release Th2 cytokines (39), our findings strongly suggest that IFN- γ gene transfer results in the inhibition of the differentiation of Th2 lymphocytes. Based on the results obtained, a mechanism of inhibition of atopic dermatitis by sustained expression of IFN- γ is proposed as follows. A sustained concentration of IFN- γ promotes the production of IL-12 from macrophages and NK cells, and these two Th1 cytokines inhibit the production of the Th2 cytokines/chemokines. These changes inhibit the recruitment of immune cells to the skin and prevent the onset of symptoms typical of atopic dermatitis. It is important to prove whether sustained IFN- γ gene transfer is also effective in individuals with dermatitis. A few NC/Nga mice that developed dermatitis were injected with pCpG-Mu γ , and the clinical skin score and TEWL were measured. These parameters were reduced by IFN- γ gene transfer, although the reduction was less significant than in mice that did not develop dermatitis. Further studies are needed to conclude that IFN- γ gene transfer provides efficacy for patients with atopic dermatitis.

There would be concerns about sustained delivery of IFN- γ , because it is a highly potent Th1 cytokine. However, we observed no significant adverse effects of IFN- γ gene transfer on physiological parameters, such as body weight and body temperature. Also, contact dermatitis, a Th1-associated allergic dermatitis, was not aggravated by the injection of pCpG-Mu γ . In addition, the improvement in TEWL observed in NC/Nga mice receiving pCpG-Mu γ would not be due to the direct effects of IFN- γ gene transfer, because TEWL was minimally affected by IFN- γ gene transfer in other models (Fig. 10).

The hydrodynamic injection method used for gene transfer in the current study is one of the most efficient methods; using this method, $>99\%$ of transgenes were expressed in the liver, followed by kidneys, spleen, lung, and other internal organs (25, 40). Although there is concern about the toxicity related to the mode of gene delivery (25, 26, 41, 42), a recent report showed that this method of gene delivery can be applied to humans with few toxic effects when plasmid DNA is delivered to a lobe using a balloon catheter (43). Computer-assisted hydrodynamic gene delivery would also be a less invasive method (44). Other modes for gene delivery could be used to achieve sustained transgene expression of IFN- γ at a relatively high level, because the dose of pCpG-Mu γ used was as low as 0.14 μ g/mouse (i.e., ~ 7 μ g/kg body weight). In general, greater amounts of plasmid DNA up to 100 μ g/mouse (5 mg/kg) have been administered, and an increase in the dose

may compensate for the low efficiency of other gene-delivery methods, such as intradermal injection of naked plasmid DNA.

In conclusion, it was proved that an extraordinarily sustained IFN- γ expression induces a variety of immunological changes, leading to a Th1-dominant state in the atopic dermatitis model. A short expression of IFN- γ was not effective in inducing such changes, indicating the importance of a sustained concentration of IFN- γ . These results provide a novel strategy for the treatment of atopic dermatitis, in which biologically active IFN- γ protein is supplied to patients from cells transduced with plasmid vector expressing the protein for a long period of time.

Disclosures

The authors have no financial conflicts of interest.

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