

RESEARCH ARTICLE

In vivo transfection of a cis element ‘decoy’ against signal transducers and activators of transcription 6 (STAT6)-binding site ameliorates IgE-mediated late-phase reaction in an atopic dermatitis mouse model

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Signal transducers and activators of transcription 6 (STAT6) play a crucial role in the transactivation of IL-4 and IL-13, which might be involved in the pathogenesis of atopic dermatitis (AD). We herein reported that the IgE-mediated late-phase reaction significantly decreased in STAT6-deficient (STAT6^{-/-}) mice in AD model mice induced by intravenous injection of monoclonal anti-dinitrophenyl (DNP)-IgE antibody and subsequent skin testing with dinitrofluorobenzene. We therefore hypothesized that synthetic double-stranded DNA with a high affinity for STAT6 could be introduced *in vivo* as decoy cis elements to bind the transcriptional factor and block the gene activation contributing to the onset and progression of AD, thus providing effective therapy for AD. Treatment by the transfection of STAT6 decoy oligodeoxynucleotides (ODNs), but not scramble decoy ODN after sensitization by anti-DNP-IgE antibody,

had a significant inhibitory effect on not only STAT6 binding to nuclei but also on the late-phase response. A histological analysis revealed that both edema and the infiltration of neutrophils and eosinophils significantly decreased in STAT6 decoy ODN-transfected mice. To examine the mechanism of the *in vivo* effect of STAT6 decoy ODN, we employed an *in vitro* mast cells culture system. After IgE receptor engagement, mast cells transfected by STAT6 decoy ODN exhibited normal histamine release, but their cytokine release (TNF- α , IL-6) markedly decreased. We herein report the first successful *in vivo* transfer of STAT6 decoy ODN to reduce the late-phase reaction, thereby providing a new therapeutic strategy for AD.

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Introduction

In atopic dermatitis (AD), the mechanism underlying skin lesion formation has remained unclear except for the delayed hypersensitivity reaction against environmental substances^{1–3} or the IgE-mediated immediate or late hypersensitivity reaction against environmental substances, food or inhalant allergens.^{4–6} To clarify the pathomechanism underlying the development of eczematous skin lesions in AD, we established the eczematous skin reaction induced in mice by the intravenous injection of monoclonal anti-dinitrophenyl (DNP)-IgE antibody and subsequent skin testing with 2,4-dinitrofluorobenzene (DNFB) and analyzed these skin reactions as AD mouse model.^{7–9} We previously demonstrated that mast cells and

inflammatory cells other than T cells are thought to play an important role in these IgE-mediated biophasic reaction,⁹ however, it remains unclear as to whether Th2-type cytokines, IL-4, IL-5 or IL-13, play a role in the induction of the IgE-mediated reaction. Recently, STAT6 signaling has been demonstrated to play an essential role in the induction of contact hypersensitivity.¹⁰

We also recently established signal transducers and activators of transcription 6 (STAT6)-deficient (STAT6^{-/-}) mice¹¹ and demonstrated that STAT6 plays a central role in IL-4- and IL-13-mediated biological responses.^{11,12} In an attempt to clarify the role of Th2 cytokines, especially IL-4 and IL-13, in the IgE-mediated biphasic reaction, we examined the IgE-mediated response in the STAT6^{-/-} mice in which the IL-4 and IL-13 signaling pathways have been completely abolished.

Furthermore, there are currently no effective therapies for severe AD except for the administration of glucocorticoids or immunosuppressants, which have several severe side effects. The transfection of *cis*-element double-stranded oligodeoxynucleotides (ODNs), referred to as

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'decoy', has been reported to be a powerful tool and a new type of antigen treatment strategy for gene therapy and for studying gene transfection. Systemic ODNs act as decoy *cis* elements to block the binding of nuclear factors to the promoter regions of targeted

genes, thus resulting in the inhibition of gene transactivation both *in vitro* and *in vivo*. For example, an NF- κ B decoy has been shown to reduce both myocardial reperfusion injury by inhibiting protein expression (IL-6, IL-8) and adhesion molecules expressed by aortic

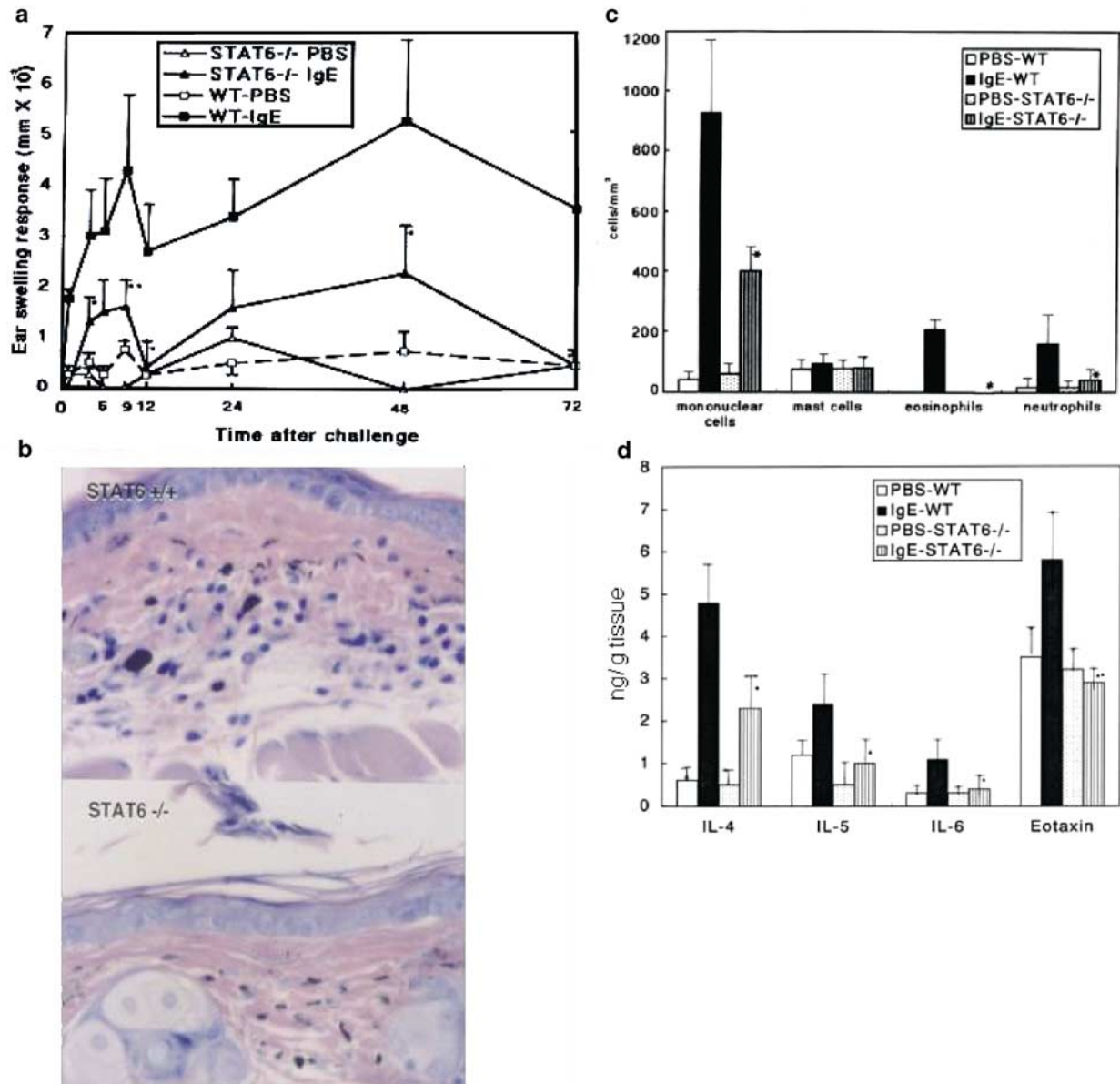


Figure 1 STAT6-deficient (STAT6^{-/-}) mice exhibit a reduced IgE-mediated biphasic response. (a) STAT6^{-/-} mice (Δ-Δ, ▲-▲) and wt mice (□-□, ■-■) were sensitized with anti-DNP-IgE antibody, 1 day after the immunization, and were challenged. Maximal ear swelling was detected at 9 and 48 h for both the wt and STAT6^{-/-} mice. Both the early- and late-response in STAT6^{-/-} mice decreased. The data presented are the mean $\pm$ s.d. of five mice per group and are representative of five independent experiments. * $P < 0.01$, significant compared to the results obtained for the wt mice. ** $P < 0.05$, significant compared to the results obtained for the wt mice. (b) Histopathological findings of the late-phase reaction induced by anti-DNP-IgE antibody in wt or STAT6^{-/-} mice. Wt or STAT6^{-/-} mice were immunized by intravenous injection with anti-DNP antibody. At 1 day after immunization, the mice were challenged by applying 0.2% DNFB solution. The histopathological features of 24-h late-phase reaction challenged by DNFB in wt mice (STAT6^{+/+}) or in STAT6^{-/-} mice (STAT6^{-/-}) stained with Giemsa's solution are shown. An extremely large degree of edema was detected in the DNFB-challenged skin in wt mice (STAT6^{+/+}) but not in STAT6^{-/-} mice (STAT6^{-/-}). A strong infiltration of mononuclear cells, neutrophils and eosinophils was observed in the dermis of wt mice (STAT6^{+/+}); however, a diminished infiltration of neutrophils and mononuclear cells was observed in the dermis of STAT6^{-/-} mice (STAT6^{-/-}). (c) Effect of STAT6 deficiency on the cellular distribution of challenged skin. Challenged skin was obtained from wt and STAT6^{-/-} mice after being challenged with the application of 0.2% DNFB or olive oil (control). The columns represent the number of eosinophils, neutrophils, monocytes/macrophages and mast cells that infiltrated the skin challenged after 48 h. The results shown are from a single experiment representative of three separate experiments. * P -value < 0.01 versus DNFB-challenged wt mice. (d) The cytokine levels in the skin tissue supernatants in olive oil and DNFB-challenged, sensitized STAT6^{-/-} or wt mice. The data represent the mean $\pm$ s.d. for groups of four mice and are representative of three independent experiments. The cytokine levels were measured by comparing them with all other groups. * P -value < 0.01 compared with DNFB-sensitized wt mice groups. ** P -value < 0.005 compared with DNFB-sensitized wt mice groups.

endothelial cells,^{13–15} while also preventing AD¹⁶ and sunburn.¹⁷ Recently, a targeted disruption of the STAT6 DNA-binding activity by a STAT6 decoy has been reported to block IL-4-derived Th2 cell response *in vitro*.¹⁸ We therefore examine the inhibitory effect of a STAT6 decoy ODN on the induction of the IgE-mediated late-phase reaction in AD mouse model *in vivo* system.

Results

Diminished biphasic anti-DNP-IgE antibody-induced response to DNFB in STAT6^{-/-} mice

To evaluate the capacity of STAT6^{-/-} mice to develop an anti-DNP-IgE antibody-induced biphasic response, we examined the ear swelling response induced by anti-DNP-IgE antibody in STAT6^{-/-} mice and wild type (wt) (C57BL/6) mice. As shown in Figure 1a, the ear swelling response to DNFB decreased drastically in both the early-phase and late-phase response in STAT6^{-/-} mice as compared with wt mice (Figure 1a). The peak response of ear swelling was detected at 9 and 48 h after the challenge in both wt mice and STAT6^{-/-} mice.

Histopathology of anti-DNP-IgE antibody-induced late-phase response in STAT6^{-/-} mice

Since the anti-DNP-IgE antibody-induced response to DNFB in STAT6^{-/-} mice decreased at 48 h after challenge, we performed a histopathological analysis in STAT6^{-/-} mice and wt mice sensitized by anti-DNP-IgE antibody. As shown in Figure 1b, a histological examination revealed that edema was detected in the challenged skin of the wt mice in the early-phase (data not shown) and late-phase response (Figure 1b), but not in the STAT6^{-/-} mice (Figure 1b). An inflammatory response with a strong infiltration of eosinophils, neutrophils and mononuclear cells was observed in the wt mice at 48 h after challenge (Figure 1b). In contrast, only a mild inflammatory response with a diminished infiltration of mononuclear cells and neutrophils was observed in the challenged skin in STAT6^{-/-} mice (Figure 1b). The number of infiltrated mononuclear cells, eosinophils and neutrophils into the skin was calculated in both the STAT6^{-/-} mice and wt mice. Anti-DNP-IgE antibody-sensitized wt mice challenged after 48 h had a significantly greater number of eosinophils and neutrophils than the DNFB-challenged STAT6^{-/-} mice ($P < 0.01$) (Figure 1c). The cytokine levels of IL-4, IL-5, IL-6 and eotaxin from the challenged skin specimens obtained from STAT6^{-/-} mice were reduced in comparison with those from wt mice (Figure 1d). IL-13 level was also reduced weakly but not significantly (data not shown).

In vivo transfection of fluorescence isothiocyanate-labeled ODN into skin

In order to evaluate the efficacy and localization of delivered STAT6 decoy ODN or scrambled decoy ODN, we introduced the fluorescence isothiocyanate-labeled decoy ODN against STAT6 or the scrambled decoy ODN into the mouse skin by subcutaneous injection with HVJ-liposomes. It is noteworthy that the binding affinity of the decoy ODN against STAT6 was markedly detected

in the diffuse area of transfected skin and the lower layer of epidermis in comparison to the control skins transfected with FITC-labeled scrambled decoy ODN or the empty liposomes (Figure 2a, b).

In vivo transfection of STAT6 decoy ODN

To examine whether STAT6 decoy ODN inhibits the binding of STAT6, we conducted the gel shift assay (Figure 2c). The gel shift analysis documented that the challenge resulted in an increased STAT6 binding activity as compared with negative control mice challenged with olive oil (Figure 2c, lines 1 and 2). This increased STAT6 binding was abolished by excess quantities of cold STAT6 double-stranded ODN to determine whether this STAT6 binding band is specific or not (Figure 2c, line 8).

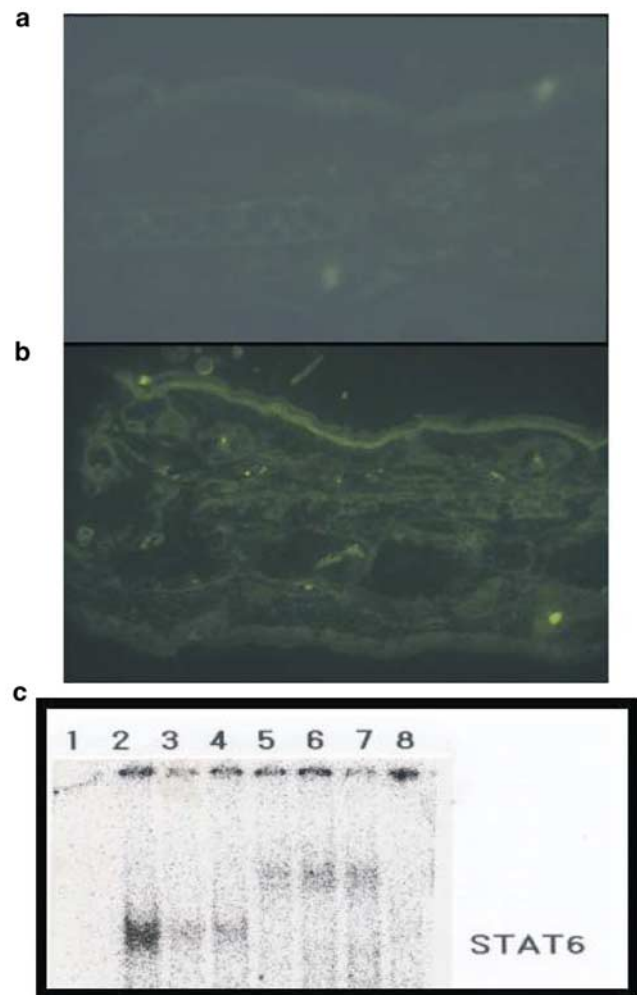


Figure 2 *In vivo* distribution of fluorescence isothiocyanate-labeled ODN delivered via subcutaneous injection into the ear skin by the HVJ-liposome method. The control group was transfected with an empty liposome ($\times 100$) (a) or FITC-labeled STAT6 decoy ODN (b). (c) Effect of the STAT6 decoy ODN on the STAT6 activity in challenged skin. Normal skin without challenge (lane 1), 20 mM scrambled ODN (lanes 2 and 7), 20 mM STAT6 decoy ODN-treated challenged skin (lanes 3 and 5) and 10 mM STAT6 decoy ODN-treated challenged skin (lanes 4 and 6) were harvested 24 h after challenge. Nuclear extracts were prepared and 10 μ g of each extract was subjected to EMSA. Control EMSAs were performed in 100 times excess of cold STAT6 probe (lane 8), or antibodies against STAT6 (lanes 5–7). The results are representative of five experiments. The bars depict mean cpm $\pm$ s.d. for triplicate experiments.

Furthermore, to examine the specificity of this STAT6 band, monoclonal antibody against STAT6 was added to the nuclear extract, and a supershift was detected (Figure 2c, lines 5–7) and the *in vivo* transfection of STAT6 decoy ODN markedly inhibited STAT6 binding in a dose-dependent manner (Figure 2c, lines 3, 4).

Inhibition of the late-phase response but not the early-phase response induced by anti-DNP-IgE antibody

To determine the inhibition of decoy ODN against STAT6 in view of the response induced by anti-DNP-IgE antibody, the ear swelling response challenged by DNFB was examined with or without STAT6 decoy ODN or scrambled decoy ODN. STAT6 decoy ODN weakly inhibited the early-phase response but not significantly, while the late-phase response was inhibited dramatically by STAT6 decoy ODN significantly (Figure 3a). Next we compared the effect of STAT6 decoy ODN in between several routes of the injection. Interestingly, both the subcutaneous and intramuscular injection of STAT6 decoy ODN was effective; however, neither the intraperitoneal injection (Figure 3b) nor intravenous injection (data not shown) of the same was effective. We examined the optimal dose of STAT6 decoy ODN by subcutaneous injection to inhibit the late-phase response. As shown in Figure 3c, 5, 10, 20 μ M but not 1 μ M of STAT6 decoy ODN significantly inhibited the late-phase response induced by anti-DNP-IgE antibody in a dose-dependent manner. To compare the inhibition of the late-phase response by STAT6 decoy ODN between mice strains, BALB/c, C3H/He and C57BL/6 mice were sensitized with anti-DNP-IgE antibody and then subcutaneously injected with STAT6 decoy ODN, then challenged. In BALB/c, C3H/He and C57BL/6 mice, the late-phase response was

inhibited by STAT6 decoy ODN; however, it was inhibited most strongly in BALB/c mice (Figure 3d), since BALB/c mice may be th2-dominant mice. These data are consistent with the response of the late-phase reaction in STAT6^{-/-} mice.

Histopathology of anti-DNP-IgE antibody-induced late-phase response in mice with or without STAT6 decoy ODN–liposomes

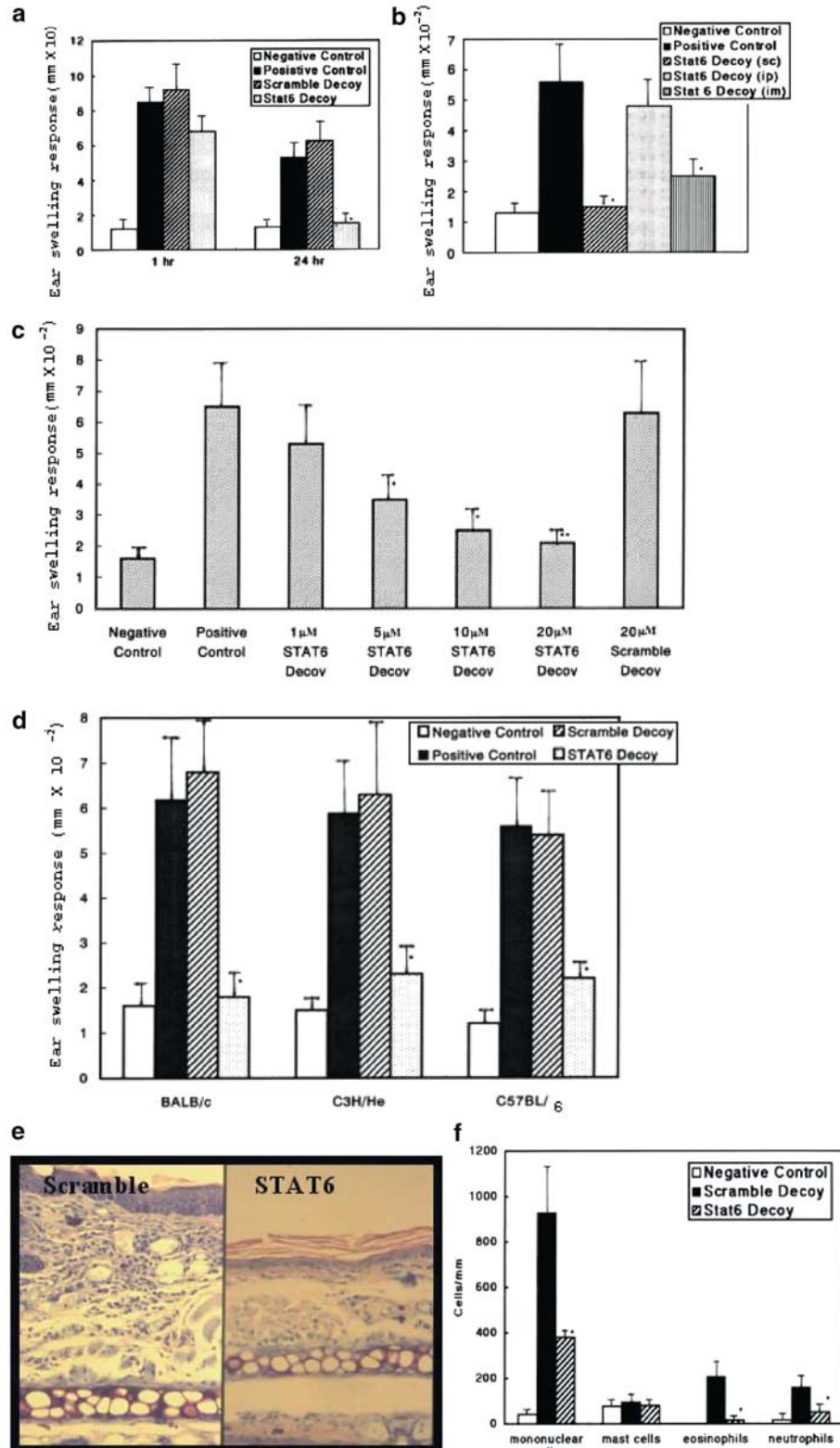
Since the anti-DNP-IgE antibody induced decreased late-phase response to DNFB in mice transfected with STAT6 decoy ODN, we performed a histological analysis in mice injected with or without STAT decoy ODN or scrambled decoy ODN sensitized by anti-DNP-IgE antibody. As shown in Figure 3e, a histological examination revealed that edema was detected in the scrambled decoy ODN-transfected mice in the late-phase response (Figure 3e), but not in the mice transfected with STAT6 decoy ODN (Figure 3e). An inflammatory response with an intense infiltration of eosinophils, neutrophils and mononuclear cells was observed in nontransfected or scrambled decoy ODN-transfected mice at 24 h after challenge (Figure 3e). In contrast, only a mild inflammatory response, with a diminished infiltration of mononuclear cells and neutrophils, was observed in the challenged skin in STAT6 decoy ODN-transfected mice (Figure 3e). The number of infiltrated mononuclear cells, eosinophils and neutrophils into the skin was calculated in scramble or STAT6 decoy ODN-transfected mice. Anti-DNP-IgE antibody-sensitized mice with scrambled decoy ODN transfection challenged after had a significantly greater number of eosinophils and neutrophils than the DNFB-challenged mice with STAT6 decoy ODN transfection ($P < 0.01$) (Figure 3f).

Figure 3 STAT6 decoy ODN exhibits a reduced IgE-mediated late-phase reaction. (a) Naïve BALB/c mice were sensitized with anti-DNP-IgE antibody, and 6 h later injected with STAT6 decoy ODN or scrambled decoy ODN subcutaneously and 1 day later they were challenged. Ear swelling was detected at 1 and 24 h for the positive control mice. STAT6 decoy ODN inhibited the ear swelling at 24 h after challenge but not scrambled decoy; however, neither STAT6 decoy nor the scrambled decoy inhibited significantly the ear swelling at 1 h after challenge. The data presented are the mean $\pm$ s.d. of five mice per group and are representative of five independent experiments. * $P < 0.01$, a significant difference compared to the results obtained with positive control mice. (b) Naïve BALB/c mice were sensitized with anti-DNP-IgE antibody and injected with STAT6 decoy or scrambled ODN either subcutaneously (s.c.), intraperitoneally (i.p.) or into skeletal muscle (i.m.), and 1 day after immunization they were challenged. Maximal ear swelling was detected at 24 h for the positive control mice. Both s.c. and i.m. injections of STAT6 decoy inhibited the ear swelling at 24 h after challenge; however, the i.p. injection of the STAT6 decoy ODN did not inhibit the ear swelling. The data presented are the mean $\pm$ s.d. of five mice per group and are representative of five independent experiments. * $P < 0.01$, significant compared to the results obtained with positive control mice. (c) Dose response of STAT6 decoy ODN in late-phase reaction. Naïve BALB/c mice were sensitized with anti-DNP-IgE antibody and subcutaneously injected with 1, 5, 10 or 20 μ M STAT6 decoy ODN or 20 μ M scrambled ODN, and 1 day after the immunization were challenged. The maximal ear swelling was detected at 24 h for the positive control mice. STAT6 decoy ODN at 5, 10 and 20 μ M inhibited the ear swelling at 24 h after challenge; however, 1 μ M STAT6 decoy ODN did not significantly inhibit the ear swelling. The data presented herein are the mean $\pm$ s.d. of five mice per group and are representative of five independent experiments. * $P < 0.01$, significant compared to the results obtained with the positive control mice. ** $P < 0.05$, significant compared to the results obtained with the positive control mice. (d) Comparison of the inhibitory effect of the STAT6 decoy ODN between the mice strains. To compare the inhibitory effect of the STAT6 decoy ODN between mice strains, the BALB/c mice, C3H/He mice and C57/6 mice were sensitized with anti-DNP-IgE antibody and transfected with STAT6 decoy ODN or scrambled decoy ODN, and 1 day after sensitization, each of their ears was challenged with DNFB. An anti-DNP-IgE antibody-induced late-phase response was observed with a peak response at 24 h after the challenge in both the BALB/c and C3H/He mice; however, the same response was observed in the C57BL/6 mice at 48 h after the challenge. The most inhibitory effect of the STAT6 decoy ODN was detected in the BALB/c mice. The data presented are the mean $\pm$ s.d. of five mice per group and are representative of five independent experiments. * $P < 0.005$, a significant difference compared to the results obtained with the positive control mice. (e) Histopathological findings of the late-phase reaction induced by anti-DNP-IgE antibody in the STAT6 decoy-treated or nontreated mice. BALB/c mice were immunized by the intravenous injection of anti-DNP antibody along the subcutaneous injection of either STAT6 decoy or scrambled decoy ODN. At 1 day after the immunization, the mice were challenged by applying 0.2% DNFB solution. The histological features of 24-h CHS ear skin reaction challenged by DNFB in mice injected with scramble decoy (a,c) or Stat6 decoy (STAT6) stained with Giemsa's solution are shown. An extremely large degree of edema was detected in the DNFB-challenged skin in the mice treated with scrambled decoy (scramble) but not in the mice treated with the STAT6 decoy (STAT6). A strong infiltration of mononuclear cells, neutrophils and eosinophils was observed in the dermis of positive control mice (scramble); however, a diminished infiltration of neutrophils and mononuclear cells was observed in the dermis of STAT6 decoy ODN-transfected mice (STAT6). (f) Effect of STAT6 decoy ODN on the cellular distribution of challenged skin. Challenged skin was obtained from mice pretreated with STAT6 decoy ODN or scrambled decoy ODN after being challenged with the application of 0.2% DNFB or olive oil (negative control). The columns represent the number of eosinophils, neutrophils, monocytes/macrophages and mast cells that infiltrated the challenged skin. The results shown are from a single experiment representative of three separate experiments. * P -value < 0.01 versus DNFB-challenged scrambled decoy-transfected mice.

Local production of cytokines in mice with or without STAT6 decoy ODN

In the supernatant from skin tissue obtained from DNFB-challenged mice after anti-DNP-IgE antibody sensitization, the levels of both IL-4 and IL-5 were significantly

higher than those in the supernatant from olive oil-challenged mice (Figure 4a). In contrast, these Th2 cytokine levels in the supernatant from DNFB-challenged mice with STAT6 decoy ODN transfection decreased (Figure 4a). The level of IFN- γ in the mice



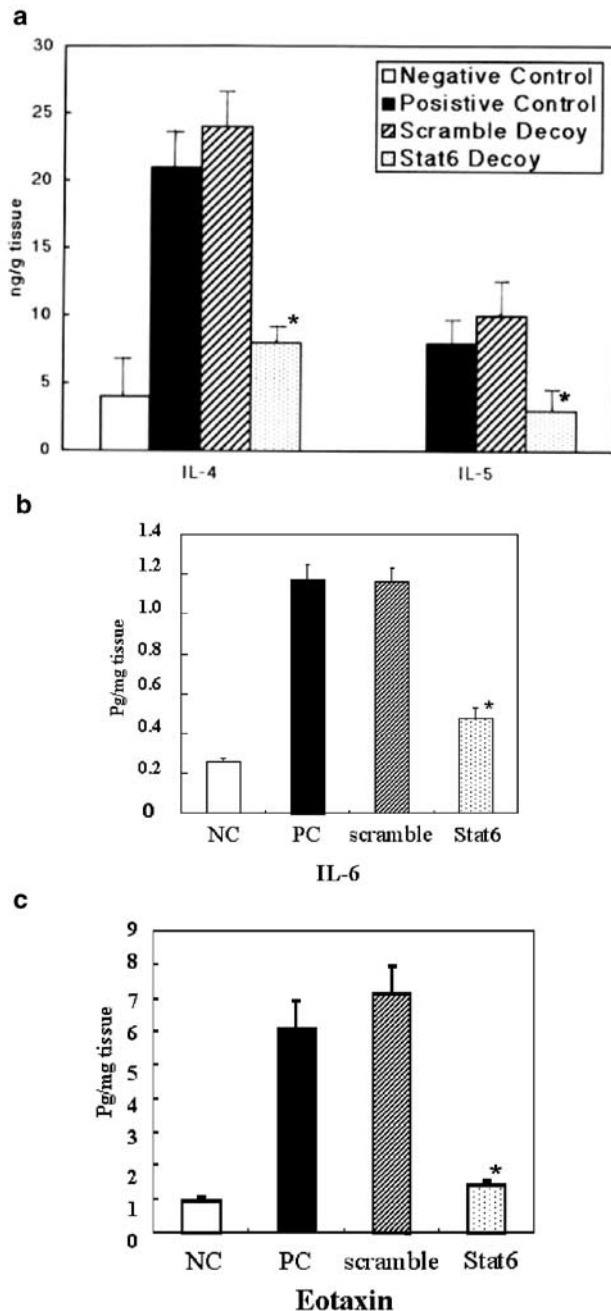


Figure 4 The cytokine levels in the skin tissue supernatants in olive oil and DNFB-challenged, sensitized BALB/c mice pretreated with STAT6 decoy ODN or scrambled decoy ODN. The data represent the mean $\pm$ s.d. for groups of four mice and are representative of three independent experiments. The cytokine levels of IL-4 and IL-5 were measured by comparing them with all other groups (a). The cytokine levels of IL-6 (b) and eotaxin (c) were measured by comparing them with all other groups. *P-value <0.01 compared with positive control groups.

with STAT6 transfection was comparable to that in the nontransfected or scrambled decoy ODN-transfected mice (data not shown). Furthermore, not only Th2 cytokines but also the IL-6 and eotaxin levels decreased in the supernatant from skin tissue obtained from STAT6 decoy-transfected mice (Figure 4b and c); however, IL-13 level weakly decreased but not significantly (data not shown).

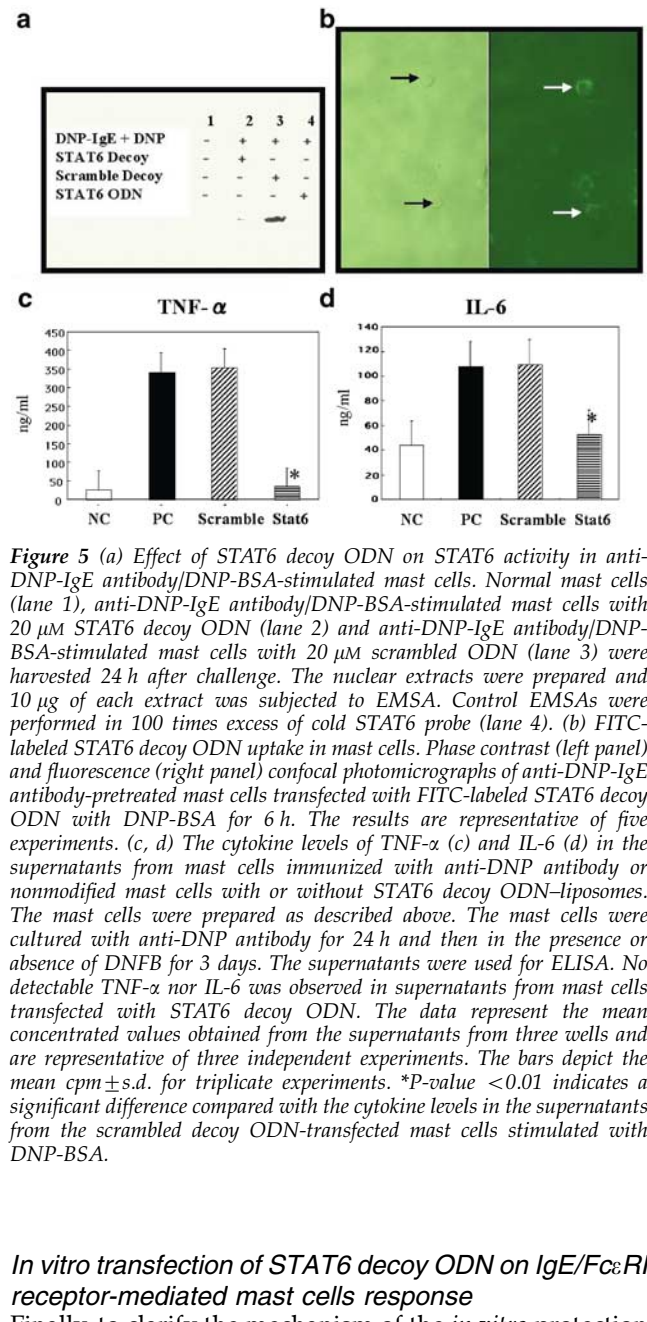


Figure 5 (a) Effect of STAT6 decoy ODN on STAT6 activity in anti-DNP-IgE antibody/DNP-BSA-stimulated mast cells. Normal mast cells (lane 1), anti-DNP-IgE antibody/DNP-BSA-stimulated mast cells with 20 μ M STAT6 decoy ODN (lane 2) and anti-DNP-IgE antibody/DNP-BSA-stimulated mast cells with 20 μ M scrambled ODN (lane 3) were harvested 24 h after challenge. The nuclear extracts were prepared and 10 μ g of each extract was subjected to EMSA. Control EMSAs were performed in 100 times excess of cold STAT6 probe (lane 4). (b) FITC-labeled STAT6 decoy ODN uptake in mast cells. Phase contrast (left panel) and fluorescence (right panel) confocal photomicrographs of anti-DNP-IgE antibody-pretreated mast cells transfected with FITC-labeled STAT6 decoy ODN with DNP-BSA for 6 h. The results are representative of five experiments. (c, d) The cytokine levels of TNF- α (c) and IL-6 (d) in the supernatants from mast cells immunized with anti-DNP antibody or nonmodified mast cells with or without STAT6 decoy ODN-liposomes. The mast cells were prepared as described above. The mast cells were cultured with anti-DNP antibody for 24 h and then in the presence or absence of DNFB for 3 days. The supernatants were used for ELISA. No detectable TNF- α nor IL-6 was observed in supernatants from mast cells transfected with STAT6 decoy ODN. The data represent the mean concentrated values obtained from the supernatants from three wells and are representative of three independent experiments. The bars depict the mean $\pm$ s.d. for triplicate experiments. *P-value <0.01 indicates a significant difference compared with the cytokine levels in the supernatants from the scrambled decoy ODN-transfected mast cells stimulated with DNP-BSA.

In vitro transfection of STAT6 decoy ODN on IgE/Fc ϵ RI receptor-mediated mast cells response

Finally, to clarify the mechanism of the *in vitro* protection effect of STAT6 decoy ODN in the skin sensitized with anti-DNP-IgE antibody and challenged with DNFB, we employed an *in vitro* IgE-Fc ϵ RI receptor-mediated mast cell response. Mast cells were prepared from bone marrow specimens of BALB/c mice after 3 weeks of culture in the medium supplemented with IL-3. Bone marrow cells cultured for 3 weeks with IL-3 displayed typical staining pattern as mast cells. The surface Fc ϵ RI expression level of mast cells was strong enough. STAT6 decoy ODN inhibited anti-DNP-IgE antibody/DNP-BSA-induced STAT6 DNA binding (Figure 5a, line 2) but not scrambled decoy ODN (line 3). After stimulation by anti-DNP-IgE antibody and DNP-BSA, green fluorescence staining was detected both in the nuclei and cytoplasm of mast cells (Figure 5b). After the stimulation by anti-DNP-IgE antibody and DNP-BSA, mast cells cultured without decoy ODN secreted IL-6 and THF- α

(Figure 5c and d: PC). The transfection of STAT6 decoy ODN (10 μ M) markedly inhibited protein production of these cytokines (Figure 5a and b: Stat6). In contrast, the control scrambled decoy ODN failed to inhibit their expression (Figure 5a and b: Scramble). We next examined the release of inflammatory mediators from mast cells. STAT6 decoy ODN did not inhibit either the IgE/hapten-induced histamine release or LTC₄ release from mast cells (data not shown).

Discussion

Although considerable progress has been made in elucidating the mechanism of AD, the cellular and molecular mechanisms regulating AD still remain obscure. Recently, IgE-mediated late-phase reaction is thought to be mainly associated with the pathogenesis of AD.^{4–6} We have established the eczematous skin reaction induced by anti-DNP-IgE antibody and DNFB as an AD model mouse.^{7–9} STAT6 is known as a regulator of IL-4-dependent immune responses. We have also established STAT6-deficient mice and concluded that STAT6 plays a crucial role in exerting IL-4- and IL-13-mediated biological responses.^{11,12} Furthermore, STAT6 has been shown to play an essential role in contact hypersensitivity by regulating Th2-type cytokine production of T cells.¹⁰ Therefore, we utilized the STAT6-deficient mouse model to study the role of Th2 cytokine, especially IL-4, IL-5 and IL-13, in antigen-specific, IgE antibody-induced, late-phase responses.

In comparison with the wt control mice, the ear swelling response in the STAT6^{-/-} mice significantly decreased at both early- and late-phase responses. However, the reduction in ear swelling was not significant at 6 h after the challenge in the anti-DNP-IgE antibody-induced response. The peak response of ear swelling was observed at 9 and 48 h after challenge in both STAT6^{-/-} and wt mice sensitized with anti-DNP-IgE antibody. These data may be consistent with the recent finding that STAT6 plays a major role in the late-phase response induced by antigen-specific IgE antibody.¹⁹ However, our results do not correlate with that report since IgE/Ag-challenged STAT6^{-/-} mice showed normal early-phase response.¹⁹ This discrepancy may be explained by the differences in the assay times after challenge, since they did not measure ear swelling at 9 and 12 h after challenge.

Recently, Yagi *et al*²⁰ demonstrated that the development of AD-like skin lesion in STAT6-deficient NC/Nga mice and Th2-mediated immune response is not necessary for the development of AD-like skin disease.

Their report is not consistent with our results; however, there are several mechanisms except for late-phase reaction in the pathogenesis of AD. A histological analysis revealed a tremendous reduction in the infiltration of both eosinophils and neutrophils in DNFB-challenged skin of STAT6^{-/-} mice. In addition, the antigen-induced edematous changes in the dermis were also completely dependent on STAT6 signaling. So far, the exact roles of these polymorphonuclear cells in the late-phase response are still unclear; however, our data indicate that polymorphonuclear cells, including eosinophils and neutrophils dependent on STAT6 signaling, may play a major role in the induction of the late-phase

response induced by anti-DNP-IgE antibody. STAT6 signaling is essential for hapten-induced late-phase reaction in Th2 cytokines production *in vivo*. The hapten-challenged wt mice demonstrated significant elevations in the Th2 cytokines (ie IL-4 and IL-5). In marked contrast, STAT6^{-/-} mice were unable to produce IL-4 or IL-5 in response to the skin challenge (Figure 1d). Consistent with a recent report,¹⁹ no detectable TNF- α or eotaxin in the supernatants of mast cells upon the crosslinking of their IgE receptor/Fc ϵ RI on their surface in a hapten-specific manner was observed in STAT6^{-/-} mice (data not shown). These data indicated that STAT6 signal is essential for the production of Th2 cytokine, chemokine of neutrophils and eosinophils by mast cells.

The results of the response induced by anti-DNP-IgE antibody and antigen in STAT6^{-/-} mice suggested that blocking of STAT6 signaling can be a therapeutic approach for AD. Next, we examined whether *in vivo* treatment by transfection of STAT6 decoy ODNs with HVJ-liposomes after the injection of anti-DNP-IgE antibody can effectively inhibit STAT6 binding to nuclei or the late-phase response induced by anti-DNP-IgE antibody. Subcutaneous and intramuscular but not intraperitoneal injection of STAT6 decoy ODN with liposomes after sensitization with anti-DNP-IgE antibody has also shown significant inhibitory effect on both STAT6 binding to nuclei and the late-phase response in line with Kaneda *et al*'s²¹ report. A histological analysis revealed a tremendous reduction in the infiltration of both eosinophils and neutrophils in DNFB-challenged skin of STAT6 decoy ODN-transfected mice. STAT6 signaling is essential for hapten-induced increase in Th2 cytokines production *in vivo*. The ELISA technique revealed that expression of protein of the Th2 cytokines (ie IL-4 and IL-5 but not IL-13), IL-6 and eotaxin decreased in STAT6 decoy ODN-transfected mice but not scrambled decoy ODN-transfected mice in line with *in vitro* data.¹⁸ Recently, eotaxin has been reported to be induced by fibroblasts stimulated with IL-4 through the STAT6 pathway.^{22,23} To examine the mechanism of the *in vivo* effect of STAT6 decoy ODN, we employed an *in vitro* mast cell culture system. Immunofluorescence staining and electrophoretic mobility shift assays (EMSA) techniques revealed that transfected STAT6 decoy ODN inhibited STAT6 binding on the nuclei of IgE/hapten-stimulated mast cells. In line with a recent report,¹⁹ no detectable IL-6 and a small amount of TNF- α in the supernatants of mast cells upon crosslinking of their IgE receptor/Fc ϵ RI on their surface in a hapten-specific manner was observed in STAT6 decoy ODN-transfected mast cells. These data supported the hypothesis that mast cell-derived TNF- α plays a critical role in the late-phase reaction of hypersensitivity,²⁴ although a recent paper demonstrated the involvement of Akt in Fc ϵ RI-induced production of TNF- α through NF- κ B activation.²⁵ One possible mechanism of these results should be a crosstalk response between NF- κ B and STAT6 in the production of TNF- α in mast cells. These data indicated that STAT6 decoy ODN inhibited IgE-induced late-phase response by inhibiting the release of IL-6 and TNF- α from mast cells, which induced mainly the late-phase response. However, since STAT6 also affects the cytokine response of other inflammatory cells, such as macrophages, T cells or fibroblasts, there was a significant contribution of cells other than mast cells in

the late-phase allergic response inhibited by STAT6 decoy ODN. These data demonstrated that the STAT6 signal is essential for the production of Th2 cytokine, chemokine for neutrophils and eosinophils and the activation of mast cells.

To summarize, (1) STAT6 plays an essential role in the induction of the biphasic response induced by antigen-specific IgE. (2) STAT6 mediates the biphasic response induced by IgE via its pivotal role in Th2 differentiation resulting in the subsequent stimulation of IgE synthesis and eosinophil or neutrophil infiltration in the skin. (3) *In vitro* experiments have documented that STAT6 decoy ODN selectively inhibited the expression of targeted cytokine genes (IL-4, IL-5, IL-6, eotaxin). (4) The administration of STAT6 decoy ODN markedly inhibited the antigen-specific, IgE-mediated, late-phase reaction. (5) The inhibition of late-phase reaction by STAT6 decoy ODN, delivered by subcutaneous administration either before or after sensitization, was confirmed. (6) The decrease in the number of eosinophils, neutrophils and mononuclear cells was associated with the decreased amount of IL-4, IL-5, IL-6 and TNF- α . Additionally, this study demonstrated that the transcriptional factor STAT6 is one of the key regulators promoting IgE-induced late-phase reaction. Although a number of important issues, such as the safety and side effects, have not yet been addressed in this study, the decoy strategy against STAT6 may provide a new therapeutic modality as gene therapy against AD. Since STAT6 has been postulated to play an important role in the pathogenesis of numerous diseases, for example, contact dermatitis, atopic asthma and allergic rhinitis, the development of STAT6 decoy strategy may also provide a useful therapeutic tool for treating these diseases.

Materials and methods

Animals

C57/BL6 mice with a targeted disruption of the gene encoding STAT6 (STAT6^{-/-}) were generated in the Department of Host Defense, Research Institute for Microbial Disease, Osaka University (Osaka, Japan), as previously reported¹¹ and age- and sex-matched wt littermate controls (C57BL/6 mice) and C3H/He and BALB/c mice were purchased from the Sankyo Co., Tokyo, Japan. All animals were housed under specific pathogen-free conditions, and had free access to a commercial diet and water. They were used at 8–15 weeks of age. Each experimental group consisted of at least five mice.

Reagents

The following reagents were obtained from commercial sources: DNFB from the Sigma Chemical Co., St Louis, Mo, USA and trypsin, 1:250, from Difco. The following mAb were used: anti-DNP-IgE mAb (ICN Biomedicals Inc., Auora, USA), FITC-conjugated anti-IgE mAb (PharMingen, San Diego, CA, USA; hamster), FITC-conjugated Fc ϵ RI mAb (PharMingen, San Diego, CA, USA; hamster), PE-conjugated anti-Ly2 (PharMingen, San Diego, CA, USA; hamster), PE-conjugated anti-mouse CD4 (L3T4) mAb (PharMingen, San Diego, CA, USA), FITC-conjugated anti-mouse I-A^d (Cedar lane, Ontario, Canada; mouse). IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-13, IFN- γ and

eotaxin ELISA kits were purchased from Genzyme Techne (Minneapolis, MN, USA). Histamine and LTC₄ ELISA kits were purchased from Immunotech (Immunotech, France). The preparation of DNP-BSA was purchased from Molecular Probes Inc. (Leiden, Netherlands). Mouse r-IL-3, IL-4 and SCF were purchased from Genzyme/Techne (USA).

Synthesis of ODN and selection of sequence targets

ODN was synthesized and the sequence targets were selected. The STAT6 decoy ODN is double-stranded phosphorothioate 28-mer that exhibits a high sequence-specific binding affinity to the transcription factor STAT6. The sequences utilized were as follows: STAT6 decoy ODN, 5'-GAT CAA GAC CTT TTC CCA AGA AAT CTA T-3' and 3'-CTA GTT CTG GAA AAG GGT TCT TTA GAT A-5'; scrambled decoy ODN, 5'-CGA AAA TTC GTT AAA TCA CTA GCT TAC C-3' and 3'-GCT TTT AAG CAA TTT AGT GAT CGA ATG G-5'.¹⁸ Synthetic ODNs were dissolved in sterile TE buffer (10 mmol/l Tris, 1 mmol/EDTAm pH 8.0), purified by high-performance liquid chromatography and quantitated by spectrophotometry. Each pair of single-stranded ODN was annealed for 3 h, during which time the temperature decreased from 90 to 25°C.

Preparation of HVJ-liposome complex

Phosphatidylcholine, dioleoyophosphatidylethanolamine, sphingomyelin, phosphatidylserine and cholesterol were mixed in a molar ratio of 13.3:13.3:13.3:10:50 for anionic-type HVJ-liposome. A dried lipid mixture was prepared and then hydrated in 200 μ l of balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6) containing 200 μ g of plasmid DNA. Liposomes were prepared by vortexing and extraction. Purified HVJ (Zstrain) was inactivated by UV irradiation (198 mJ/mm²) just before use. The liposome suspension (2 ml, containing 9.75 mg lipid) was incubated with HVJ (15 000 hemagglutinating units) for 10 min on ice and then for 1 h at 37°C with gentle shaking. Free HVJ was removed from the HVJ-liposomes by sucrose density gradient centrifugation. HVJ-liposome was collected from the top of a 30% sucrose layer, filled up to 1 ml by BSS and then stored at 4°C.²⁶

Electrophoretic mobility shift assays

Nuclear extracts were prepared from the skin as described previously.¹³ In brief, mouse skin were homogenized in 4 volumes of ice-cold homogenization buffer containing 10 mM HEPES, pH 7.5, 500 mM sucrose, 0.5 mM spermidine, 0.15 mM spermine, 5 mM EDTA, 0.25 mM EGTA, 7 mM β -mercaptoethanol and 1 mM PMSF and were centrifuged at 2000 g for 30 min at 4°C. After centrifugation, each pellet was lysed in 1 volume of ice-cold homogenizing buffer containing 0.1% NP-40 by homogenized in a Dounce homogenizer. The samples were then centrifuged at 12 000 g for 30 min at 4°C, and the pellet nucleus was washed twice with ice-cold buffer containing 0.35 M sucrose. After washing, the nuclei were extracted with 1 volume of ice-cold homogenizing buffer containing 0.005 M NaCl and 10% glycerol for 15 min at 4°C. The nuclei were then extracted with the homogenization buffer containing 0.3 M NaCl and 10% glycerol for 1 h at 4°C, and the concentration of DNA was adjusted to 1 mg/ml. After pelleting the extracted

nuclei at 12 000 g for 30 min at 4°C, the supernatant was added to 45% NH₄SO₄ and stirred for 30 min at 4°C. The precipitated protein was collected at 17 000 g for 30 min, resuspended in homogenization buffer containing 0.35 M sucrose and the nuclear extract was precipitated and stored at -80°C.²⁷ After preincubation with 2 µg poly(dI-dC) as nonspecific competitor DNA, 15 µg of the crude nuclear extract was incubated in a binding buffer containing 10 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 2 mM DTT, 1 mM PMSF, 5% glycerol and 100 mM KCl with approximately 10 000 cpm of annealed ³²P-labeled probe for 30 min at room temperature. The sequences of single-stranded oligonucleotides for STAT6 were 5'-AGT CAA GAC CTT TTC CCA AGA AAT CTA TC-3' (Santa Cruz Biotechnology Inc.). The binding reaction mixtures were separated from free oligonucleotide probes by electrophoresis on a native 5% (w/v) polyacrylamide gel as described.²⁸ For supershift assay, 4 µg of anti-STAT6 antibody or nonimmune rabbit serum was added to the reaction mixture simultaneously with the nuclear extract. The reaction mixture was subjected to electrophoresis as previously described and thereafter the gels were dried and autoradiographed.

Induction of skin reaction and in vivo and in vitro transfer of ODN

The mice (5 mice/group) were given an intravenous application of anti-DNP-IgE antibody 1 day before a skin test with 0.2% DNFB in 4:1 acetone olive oil applied to both sides of the right pinna. The left pinna was treated as a control. Skin specimens were obtained from skin-tested sites at various times after the skin test for the histopathological analysis. A 1–20 µM portion of STAT6 decoy ODN or scrambled decoy ODN with HVJ-liposomes was administered subcutaneously, intravenously or intraperitoneally, 6 h after the anti-DNP-IgE antibody injection and 1 day before the challenge. FITC-liposome complex was also given subcutaneously, intravenously, intramuscularly or intraperitoneally 1 day before the challenge. For *in vitro* transfection of decoy ODN, mast cells were cultured with 1, 5, 10 or 20 µM of STAT6 decoy ODN or scrambled decoy ODN at 37°C for 6 h. Quantitative measurement of fluorescence was also performed to compare the efficiency of FITC-labeled ODN delivery by HVJ-liposome method and empty HVJ-liposomes without ODN. To measure fluorescence, the skin specimen was homogenized in 1 ml PBS buffer, centrifuged, and then 400 µl of the supernatant was used for measurement using a fluorescence method (Model 2550 spectrofluorophotometer, Bio-Rad, USA).

Quantification of cytokine levels in skin tissue extracts

Samples of ear tissue extracts for ELISA were prepared as described by Ferguson *et al.*²⁹ Briefly, at 24 h after the application of DNFB, the ears were excised and immediately homogenized with 10-fold volume of 0.1% Tween-20 in PBS. The samples were quickly frozen in liquid nitrogen, thawed in a 37°C water bath, sonicated for 15 s, and centrifuged for 5 min at 13 000 g, and the supernatants were used for ELISA. The supernatants were stored at -80°C. The ELISA for IL-2, IL-4, IL-5, IL-13, IL-6 and IFN-γ were conducted by using ELISA kits (Endogen Inc., MA, USA) according to the manufacturer's instructions.

Histopathological examination

The ear skin specimens were excised and fixed in 10% formalin. Thereafter, they were processed and stained with hematoxylin and eosin (HE), May-Grunwald-Giemsa or toluidine blue. The number of mononuclear cells, mast cells and granulocytes, such as neutrophils and eosinophils, infiltrating into the dermis was evaluated by staining the tissue specimens with Giemsa solution. The section was examined at a magnification of ×400. At least 10 fields were examined for each lobe. The number of cells was counted and expressed as the number of cells per mm².

Mast cell cultures and stimulation of mast cells

Mast cells were cultured from the bone marrow specimens of STAT6^{-/-}, wt control mice or BALB/c mice in a medium with 10 U/ml r-IL-3 for 3 weeks as previously described.³⁰ The cell density was adjusted to 2 × 10⁵ cells/ml on a weekly basis. After 3 weeks, the mast cells were characterized by staining with toluidine blue and Alcian blue.

Bone marrow mast cells (BMMCs) cultured from the bone marrow cells of STAT6^{-/-}, wt mice and BALB/c mice were sensitized with monoclonal anti-DNP IgE antibody for 1 h at 37°C. Unbound IgE was removed by washing the cells with PBS. After washing, the BMMCs were resuspended in RPMI-HEPES buffer at a cell density of 1 × 10⁶ cells/ml. The cells were challenged with 20 ng/ml DNP-BSA at 37°C.

Chemical mediator and cytokine release assays

Histamine or cytokine content in cell-free supernatants and in the solubilized cell pellets was established using a commercially available enzyme immunoassay. LTC₄ levels were estimated in cell-free supernatants by immunoassay.³¹

IgE binding assay

BMMCs were incubated with 0.24 µg/ml monoclonal anti-DNP IgE antibodies for 1 h at 37°C. The cells were washed thoroughly three times with saline containing 1% BSA and labeled with 10 µg/ml anti-IgE-FITC (BD PharMingen) for 30 min at 4°C. After washing, the cells were analyzed by flow cytometry for FcεRI receptor expression.

Statistical analysis

The experimental data are expressed as the mean ± s.d. (standard deviation). The statistical analysis was performed using Student's *t*-test.

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References

- 1 Michell EB *et al.* Basophils in allergen-induced patch test sites in atopic dermatitis. *Lancet* 1982; **1**: 127–130.

- 2 Uehara M, Ofuji S. Patch test reactions to human skin dander in atopic dermatitis. *Arch Dermatol* 1976; **112**: 951.
- 3 Adinoff AD, Tellez P, Clark AF. Atopic dermatitis and aeroallergen in contact sensitivity. *J Allergy Clin Immunol* 1988; **81**: 736–742.
- 4 Bruijzeel PL et al. The involvement of eosinophils in the patch test reaction to aeroallergens in atopic dermatitis: its relevance for the pathogenesis of atopic dermatitis. *Clin Exp Allergy* 1993; **23**: 97–109.
- 5 Sampson HA. The role of food allergy and mediator release in atopic dermatitis. *J Allergy Clin Immunol* 1988; **81**: 635–645.
- 6 Pathomechanism RG. Genetic and immunological factors. In: *Essential Aspects of Atopic Dermatitis*. Springer-Verlag: Tokyo, 1988, pp 108–111.
- 7 Katayama I, Tanei R, Nishioka K, Dohi Y. Induction of eczematous skin reaction in experimentally induced hyperplastic skin of BALB/c mice by monoclonal anti-DNP-IgE antibody: possible implication for skin lesion formation in atopic dermatitis. *Int Arch Allergy Appl Immunol* 1990; **93**: 148–154.
- 8 Katayama I, Minatohara K, Yokozeki H, Nishioka K. Topical vitamin D3 downregulates IgE-mediated murine biphasic cutaneous reaction. *Int Arch Allergy Immunol* 1996; **111**: 71–76.
- 9 Katayama I, Otoyama K, Yokozeki H, Nishioka K. Effect of mast cell modulators on IgE-mediated murine biphasic cutaneous reaction. *Int Arch Allergy Immunol* 1996; **109**: 390–397.
- 10 Yokozeki H et al. Signal transducer and activator of Transcription 6 is essential in the induction of contact hypersensitivity. *J Exp Med* 2000; **191**: 995–1004.
- 11 Takeda K et al. Essential role of Stat6 in IL-4 signaling. *Nature* 1996; **380**: 627–630.
- 12 Takeda K et al. Impaired IL-13-mediated functions of macrophages in STAT6-deficient mice. *J Immunol* 1996; **157**: 3220–3222.
- 13 Morishita R et al. A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth proliferation *in vivo*. *Proc Natl Acad Sci USA* 1995; **92**: 5855–5859.
- 14 Morishita R et al. Role of transcriptional *cis*-elements, angiotensinogen gene-activating element, of angiotensinogen gene in blood pressure regulation. *Hypertension* 1996; **27**: 502–507.
- 15 Morishita R et al. *In vivo* transfection of *cis* element 'decoy' against nuclear factor- κ B binding site prevents myocardial infection. *Nature Med* 1997; **3**: 894–899.
- 16 Nakamura H et al. Prevention and regulation of atopic dermatitis by ointment containing NF- κ B decoy oligodeoxynucleotides in NC/Nga atopic mouse model. *Gene Therapy* 2002; **9**: 1221–1229.
- 17 Abeyama K, Eng W, Jester JV, Vink AA. A role for NF- κ B-dependent gene transaction in sunburn. *J Clin Invest* 2000; **105**: 1751–1759.
- 18 Wang LH et al. Targeted disruption of Stat6 DNA binding activity by an oligonucleotide decoy blocks IL-4-derived Th2 cell response. *Blood* 2000; **95**: 1249–1257.
- 19 Malaviya R, Uckun MF. Role of STAT6 in IgE receptor/Fc ϵ R1-mediated late phase allergic response of mast cells. *J Immunol* 2002; **168**: 421–426.
- 20 Yagi R et al. Development of atopic dermatitis-like lesions in STAT6-deficient NC/Nga mice. *J Immunol* 2002; **168**: 2020–2027.
- 21 Kaneda Y, Iwaki K, Uchida T. Introduction of the human insulin gene in adult rat liver. *J Biol Chem* 1989; **264**: 12126–12129.
- 22 Hoeck J, Woisetschlager M. Activation of Eotaxin-3/CCL26 gene expression in human dermal fibroblast is mediated by stat6. *J Immunol* 2001; **167**: 3216–3222.
- 23 Hoeck J, Woisetschlager M. STAT6 mediates eotaxin-1 expression in IL-4 or TNF- α -induced fibroblasts. *J Immunol* 2001; **166**: 4507–4515.
- 24 Wershil BK, Wang ZS, Gordon JR, Gall J. Recruitment of neutrophils during IgE-dependent cutaneous late phase reactions in the mouse is mast cell-dependent: partial inhibition of the reaction with antiserum against tumor necrosis factor- α . *J Clin Invest* 2001; **87**: 446.
- 25 Kitauro J et al. Akt-dependent cytokine production in mast cells. *J Exp Med* 2000; **192**: 729–739.
- 26 Saeki Y et al. Development and characterization of cationic liposomes conjugated with HVJ (Sendai virus): reciprocal effect of cationic lipid for *in vitro* and *in vivo* gene transfer. *Hum Gene Ther* 1997; **8**: 2133–2141.
- 27 Molitor JA et al. NF- κ B: a family of inducible and differentially expressed enhancer-binding proteins in human T cells. *Proc Natl Acad Sci USA* 1990; **87**: 10028–10032.
- 28 Schreiber EP, Matthais MM, Schaffner W. Rapid detection of octameric binding proteins with 'mini-extracts' prepared from a small number of cells. *Nucleic Acids Res* 1989; **17**: 6419.
- 29 Ferguson T, Dube P, Griffith TS. Regulation of contact hypersensitivity by interleukin 10. *J Exp Med* 1994; **179**: 1597–1674.
- 30 Malaviya R, Chen CL, Dibirdik I, Uckun FM. Targeting Janus kinase 3 in mast cells prevents immediate hypersensitivity reaction and anaphylaxis. *J Biol Chem* 1999; **274**: 27028–27038.
- 31 Malaviya R, Abraham SN. Interaction of bacteria with mast cells. *Methods Enzymol* 1995; **253**: 27–43.