

Gene silencing of STAT6 with siRNA ameliorates contact hypersensitivity and allergic rhinitis

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Abstract

Background: Silencing of genes using small interfering RNA (siRNA) is a recently developed strategy to regulate the synthesis of target molecules. Signal transducer and activator of transcription 6 (STAT6) is a nuclear transcription factor that mediates Th2-type immunity.

Methods: To elucidate the therapeutic potential of using siRNA to inhibit STAT6 in allergic reactions, we determined the nucleotide sequences of siRNA specific for STAT6.

Results: The selected sequences of STAT6 siRNA specifically inhibited the generation of STAT6 synthesis in dermal fibroblasts and eotaxin (CCL11) production in response to IL-4/TNF- α *in vitro*. Local administration of STAT6 siRNA *in vivo* alleviated contact hypersensitivity responses to chemical haptens. This was accompanied by reduced local production of IL-4, IL-13, eotaxin (CCL11), TARC (CCL17) and MDC (CCL22). Similarly, consecutive intranasal instillation of STAT6 siRNA markedly inhibited inflammatory cellular infiltration of mucosal tissues in allergic rhinitis responses in association with reduced IL-4 and IL-5 production from regional lymph node cells. Immediate responses, such as sneezing and nasal rubbing behaviors, were also improved by STAT6 siRNA.

Conclusions: Local administration of STAT6 siRNA is thus a promising therapeutic strategy for both Th2-mediated cutaneous diseases and allergic rhinitis.

Signal transducer and activator of transcription 6 (STAT6) is a transcription regulator and is essential for the induction of a Th2-type immunity. Binding of IL-4 or IL-13 induces phosphorylation of STAT6 via JAK1 and JAK3 kinases, resulting in the homodimer formation and translocation to the nucleus (1). STAT6 regulates Th2-cell development and Ig class switching to IgE in B cells (2). STAT6 mediates IL-4/IL-13-induced eotaxin (CCL11) and eotaxin-3 (CCL26) production from fibroblasts (3, 4). STAT6 activation is also essential for endothelial P-selectin expression (5). Mice deficient in STAT6 failed to develop airway hyper-responsiveness (6).

RNA interference (RNAi) is a mechanism characterized by the cleavage of double-stranded RNA by Dicer, leading to the

generation of small interfering RNAs (siRNAs). siRNAs are subsequently incorporated into RISCs (RNA-induced silencing complexes), which cleave the homologous target mRNAs (7, 8). Recent advances in technologies based on RNAi have provided opportunities to experimentally regulate and silence genes of interest. Thus, the introduction of siRNAs that induce specific post-transcriptional silencing of STAT6 gene may be an ideal approach for inhibiting allergic inflammatory diseases where the contribution of Th2-type immunity is predominant.

Contact hypersensitivity (CHS) is an immune response in the skin to a reactive hapten covalently coupled to a cell surface protein. It is generally considered to be mediated by T cells, although a recent report demonstrated the essential involvement of hepatic NK cells (9). It has been revealed that Th1-type immunity mediated by IFN- γ contributes to the elicitation phase of CHS (10–13). Nevertheless, other findings demonstrated that CD8 (+) cells producing IL-17 exert important effector functions in the elicitation phase of skin responses (14). In addition to these somewhat inconsistent data regarding

Abbreviations

CHS, contact hypersensitivity; DNFB, dinitrofluorobenzene; ODN, oligodeoxynucleotide; Ox, oxazolone; siRNA, small interfering RNA; STAT, signal transducers and activators of transcription; TNCB, trinitrochlorobenzene; TNP-SC, trinitrophenyl-modified spleen cells.

the involvement of Th1 and Th17 immunity, whether Th2-type immunity contributes to skin inflammation in CHS is more controversial. IL-4 production by invariant NKT cells during the early stages of sensitization appears to be an essential step for the development of CHS (15). IL-4 also has been shown to provide positive signals in the efferent phase of CHS (16–18). In contrast, other reports demonstrated negative regulatory roles of IL-4 in CHS elicitation (19, 20).

In this study, we attempted to verify the effects of inhibiting Th2-immunity via siRNA on CHS responses. To this end, we identified nucleotide sequences to produce siRNA that specifically inhibits STAT6 synthesis. We also assessed the therapeutic effects of intranasal instillation of STAT6 siRNA on a model of allergic rhinitis, which is principally mediated by Th2-type immunity (21).

Material and methods

Mice

BALB/c mice were purchased from Sankyo Labo Service Co. (Tokyo, Japan). The use of animals was in full compliance with the guidelines of the Committee for Animal Experiments of Tokyo Medical and Dental University.

Antibodies

Anti-STAT6 (M20), anti-mouse/human β -actin (C4) and RPE-conjugated anti-vimentin (S-20) antibodies (Abs) were purchased from Sant Cruz Biotechnology Inc. (California, USA). Anti-mouse IL-4 (11B11), IFN- γ (XMG 1.2) and STAT3 (POLY6246) Abs were from BioLegend Inc. (San Diego, CA, USA). Anti-mouse IL-13 (38213) Ab was from R&D systems Inc. (Minneapolis, MN, USA). RPE-conjugated CD117 (c-kit) (2B8) Ab was from BD Biosciences Pharmingen (New Jersey, USA).

Cell culture

NIH3T3 fibroblast cell line and mouse normal dermal fibroblasts obtained from neonatal mouse skin were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Cells were used at 4–10 passages with 60–70% confluency.

Organization of nucleic acid sequences of STAT6 siRNAs

Organization of nucleotide sequences of siRNAs specific for STAT6 was performed based on the previously reported theoretical guidelines for the selection of siRNA sequences (22), followed by screening for specificity and risk of off-target effects by BLAST search. Secondary structures of nucleic acids and RNA folding were predicted according to previously reported protocols (23, 24). The nucleotide sequences for three mouse STAT6 siRNA candidates were as follows:

Mouse STAT6 siRNA 1

5' - GCCGAGGCACCCUGUAUAUCC sense strand

GACGGCUCCGUGGGACAUUAU - 5' anti-sense strand

Mouse STAT6 siRNA 2

5' - CCUGGUUCUGUUAAGGAUUA sense strand

GGGGACCAAGACAAUCCUAA - 5' anti-sense strand

Mouse STAT6 siRNA 3

5' - CGAAUGUGAUACAACUGUAUC sense strand

GAGCUUACACUAUGUUGACAU - 5' anti-sense strand

The following siRNA was used as negative control siRNA: Scramble siRNA

5'-AUCCGCGCGAUAGUACGUATT sense strand

TTUAGGCGCGCUAUGCAU-5' anti-sense strand

Real-time PCR

Total cellular RNA was isolated using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan). Reactions for reverse transcription were carried out at 37°C for 60 min. The samples were stored at -80°C.

Quantitative RT-PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye (Brilliant SYBR Green QPCR Master Mix; Stratagene, La Jolla, CA, USA) with the Mx3000P Real-Time PCR system (Stratagene). The PCR primers were 5'-CATCACCATTGCACACGTCATC-3' and 5'-TGAGCGAATGGACAGGTCTTTG-3' for mouse STAT6 gene and 5'-TGTGTCCGTCTGGATCTG-3' and 5'-TTGCTGTTGAAGTCGCAGC-3' for mouse GAPDH gene.

Microarray analysis

Microarray experiments were performed with Mouse Genome 430A2.0 GeneChip (Affymetrix, Santa Clara, CA, USA) and Expression 3' Amplification One-Cycle Target Labeling and Control Reagents (Affymetrix) according to the manufacturer's instructions. The intensity of each probe set was calculated by the MAS5 method of the GCOS software package (Affymetrix).

MsigDB (25) was used to sort the gene sets with a STAT-family motif. The gene set designated V\$STAT6_02, with promoter regions [-2 kb to 2 kb] around the transcription start site containing the motif NNYTTCY, which matches the annotation for STAT6, was used as STAT6 downstream genes. In addition, the gene sets designated V\$STAT3_01 (NGNNATTTCCSGGAARTGNN) and V\$STAT1_01 (NNNSANTTCCGGGAANTGNSN) were used as STAT3 and STAT1 downstream gene sets, respectively. The log ratio of Lipofectamine/negative control was used as a control data set, and the log ratio of STAT6 siRNA/negative control was used as the siRNA for STAT6, STAT3 and STAT1 data sets. We calculated *P*-values by Wilcoxon signed-rank test.

In vitro transfection with siRNA

Preparation of siRNA for transfection was performed with cationic liposomes (Lipofectamine 2000; Invitrogen Corp. Carlsbad, CA, USA) according to the manufacturer's instructions. Transfection with siRNA was conducted at a final concentration of 1 nmol/ml diluted with GIBCO®OPTI-

MEM®I (Invitrogen) : Lipofectamine 2000 = 50 : 1 for 4 h. Cells were then washed and incubated for an additional 44 h with DMEM/10% fetal bovine serum. For cell stimulation, cells treated with siRNA were cultured in DMEM containing 2% fetal bovine serum for 20 h (serum starvation), followed by stimulation with recombinant mouse IL-4 (10 ng/ml) (R&D systems Inc.) and TNF- α (50 ng/ml) (R&D Systems Inc.) for an additional 24 h. Supernatants were collected and stored at -80°C until use.

Cutaneous inflammatory reactions

CHS in BALB/c mice was induced by the application of 50 μl of 5% 2,4,6-trinitrochlorobenzene (TNCB) (Nacalai Tesque Inc., Kyoto, Japan) in ethanol : acetone (3 : 1), 0.5% 2,4-dinitrofluorobenzene (DNFB) (Nacalai Tesque Inc.) in acetone : olive oil (4 : 1) or of 5% 4-ethoxymethylene-2-phenyl-2-oxazole-5-one (Ox) (Sigma Aldrich Inc., St. Louis, USA) in acetone : olive oil (4 : 1) onto the ventral skin on day 0. On day 5, each ear lobe was challenged with 20 μl of 1% TNCB in acetone : olive (1 : 4), 0.2% DNFB in acetone : olive oil (4 : 1) or 1% Ox in acetone : olive oil (4 : 1). Ear thickness was determined before and after each challenge. Control ears were challenged with the respective vehicles.

Induction of allergic rhinitis model

Mice were sensitized *i.p.* with 0.1 mg of OVA (albumin from chicken egg white, grade V; Sigma-Aldrich, Inc., St Louis, MI, USA) with 1 mg of aluminum hydroxide (Alum) on days 0, 7, 14 and 21. Mice were challenged with nasal instillation of 0.2 mg of OVA for 7 consecutive days (from days 21 to 27). On day 27, the frequencies of sneezing and nasal rubbing for 5 min after the final challenge were counted.

Measurement of cytokines and chemokines

Punched ear lobes (8 mm in diameter) from challenged mice were homogenized in PBS containing 0.1% Tween 20 (500 μl /tissue) and centrifuged at 15 000 *g* for 10 min. Supernatants were collected and stored at -80°C until use.

Concentrations of cytokines and chemokines in the homogenate supernatants or cell culture supernatants were determined by sandwich ELISA. ELISA kits for murine IL-4, IL-5, IL-6, IFN- γ , IL-13, IL-17, IP-10 (CXCL10), RANTES (CCL5), eotaxin (CCL11), TARC (CCL17) and MDC (CCL22) were purchased from R&D Systems Inc.

Western blotting analysis

Cell lysates were centrifuged and subjected to SDS-PAGE on 7.5% polyacrylamide gels under reducing conditions. Proteins were transferred onto Hybond-P PVDF transfer membrane (Amersham Biosciences, Pittsburgh, PA, USA) and stained with primary Abs. Reactive products were visualized with an HRP-conjugated secondary Ab (DAKO, Glostrup, Denmark) and the ECL system (Amersham Biosciences).

Statistical analyses

Student's *t*-test was used to assess the statistical significance of differences between mean values. For microarray analysis, Wilcoxon signed-rank test was used.

Results

Identification of STAT6 siRNA

Three candidate sequences for STAT6 siRNA were selected according to the steps described in the Material and methods. For the initial screening, STAT6 siRNAs prepared in Lipofectamine were introduced into an unstimulated NIH3T3 fibroblast cells line. STAT6 mRNA expression was weakly reduced by all of these siRNAs (Fig. 1A). The knock-down efficiencies of these siRNAs were then examined by western blotting analysis for the STAT6 protein with normal dermal fibroblasts. STAT6 expression was detected in unstimulated dermal fibroblasts, and three candidate siRNAs clearly inhibited STAT6 protein synthesis without affecting STAT3 (negative control) (Fig. 1B). In subsequent experiments, STAT6 siRNA 3 was used as the STAT6 siRNA.

The physiological function of STAT6 siRNA *in vitro* was further confirmed by its inhibitory effects on IL-4/TNF- α -induced eotaxin (CCL11) production in dermal fibroblasts, in which the STAT6 pathway has been demonstrated to be essential (Fig. 1C) (3).

To more broadly screen the knock-down efficiency and specificity of the identified STAT6 siRNA, microarray analysis was performed. Normal dermal fibroblasts treated with Lipofectamine or STAT6 siRNA were stimulated with IL-4/TNF- α . mRNA expression of genes downstream of STAT6, as well as STAT3 and STAT1, was analyzed. To determine the efficacy of siRNA for STAT families, we compared the log ratio of mRNA expression between Lipofectamine/negative control (medium alone) and STAT6 siRNA/negative control, as described in the Material and methods. A total of 151, 15 and 45 genes that have STAT6-, STAT3- and STAT1-binding motifs in the promoter regions, as sorted by MsigDB (25), were examined, respectively. The numbers of probe sets for assessing gene expression were 246, 20 and 69 for STAT6, STAT3 and STAT1 downstream genes, respectively. Microarray analysis revealed that the inhibitory effects of STAT6 downstream genes were statistically significant (Table 1). On the other hand, downstream genes of STAT3 and/or STAT1 were not affected by STAT6 siRNA.

STAT6 siRNA ameliorates CHS responses

We next sought to verify the *in vivo* effects of STAT6 siRNA. Sensitized mice received local injections of STAT6 siRNA prepared in Lipofectamine into ear lobes (0.5 nmol/30 μl per ear) on day 3, and they were challenged on day 5. CHS responses to TNCB were inhibited by STAT6 siRNA (Fig. 2 A). Similarly, suppression by STAT6 siRNA was also observed in CHS responses to DNFB and oxazolone. The

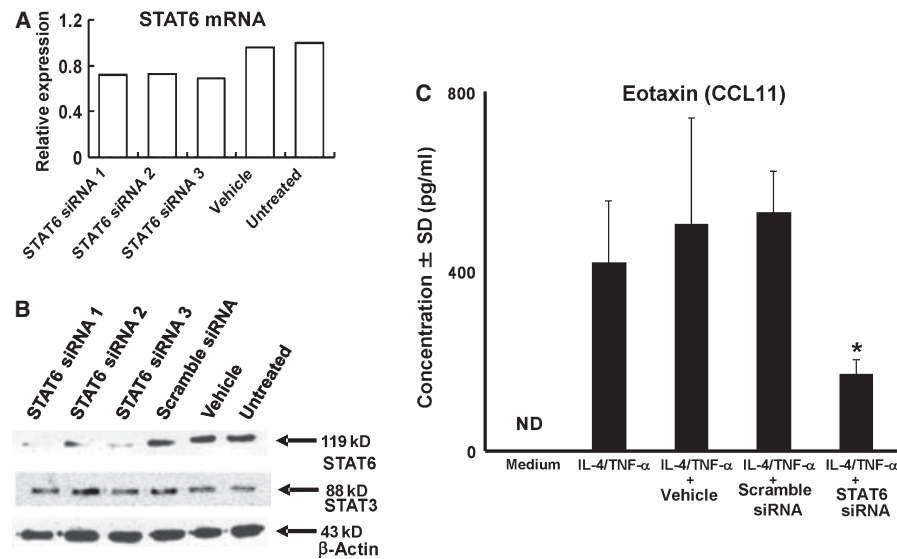


Figure 1 *In vitro* effects of STAT6 siRNAs on fibroblasts. (A) NIH3T3 cells were treated with STAT6 siRNAs in Lipofectamine 2000. After 48 h, total RNA was extracted and subjected to RT-PCR analysis for STAT6 mRNA. Three candidate siRNAs showed moderate inhibitory effects on STAT6 mRNA expression, as detected by real-time PCR analysis. 'Vehicle' indicates treatment with Lipofectamine alone. (B) siRNAs were introduced into unstimulated normal dermal fibroblasts. At 48 h after transfection, proteins were

extracted. STAT6 protein synthesis was markedly suppressed by the introduction of siRNAs. Synthesis of STAT3 was not affected. (C) 48 h after STAT6 siRNA (siRNA3) introduction, dermal fibroblasts were stimulated with IL-4/TNF-α for an additional 24 h. Eotaxin (CCL11) production was markedly suppressed by STAT6 siRNA when compared with vehicle or scrambled siRNA. Representative results of at least two separate experiments are shown. * $P < 0.05$.

Table 1 Effects of STAT6 siRNA on mRNA expression of downstream genes of STAT family

STAT families	Number of downstream genes*	Number of probe sets†	P-value‡
STAT6	151	246	0.0001434
STAT3	15	20	0.7936
STAT1	45	69	0.3992

*Indicated numbers of genes carrying STAT-family binding motif in the promoter region were sorted out by MsigDB as described in the Material and methods.

†Numbers of probe sets used for assessing gene expressions.

‡The log ratio of mRNA expression between lipofectamine/control medium and STAT6 siRNA/control medium was calculated. Wilcoxon signed-rank test was used to assess the differences between two groups.

suppressive effects were transient and reversible, as the inhibition was less prominent in mice challenged on day 7 and was not observed for mice challenged on day 10 (data not shown). Histological features of skin responses to TNCB treated with STAT6 siRNA were characterized by decreased cellular infiltrates comprising eosinophils, neutrophils and mononuclear cells (Fig. 2B). Diminished ear swelling responses were associated with reduced local production of IL-4, IL-13, eotaxin (CCL11), TARC (CCL17) and MDC (CCL22) (Fig. 3A). Levels of IL-6 were also reduced by STAT6

siRNA (510.8 ± 134.30 pg/ear and 316.1 ± 119.99 pg/ear for the vehicle- and STAT6 siRNA-treated groups, respectively; not depicted), while IL-17 levels were minimal, and we could not assess the effects of siRNA.

STAT6 siRNA is incorporated into dermal cells

The incorporation of siRNA was visualized by the subcutaneous injection of FITC-labeled STAT6 siRNA. Localization of STAT6 siRNA was detected in a number of dermal cells. These included round mononuclear inflammatory cells, vimentin (+) spindle-shaped dermal fibroblasts and c-kit (+) large cells that appeared to be mast cells (Fig. 3B).

Involvement of Th2-type cytokines in the elicitation phase of CHS

The suppressive effects of STAT6 siRNA suggest the contribution of IL-4- and/or IL-13-signals to the effluent phase of CHS. Indeed, mice receiving blocking Abs against IL-4 and IL-13 during the effluent phase failed to fully develop CHS responses to TNCB. The blocking of IFN-γ also resulted in a mild inhibition (Fig. 4).

STAT6 siRNA alleviates symptoms of allergic rhinitis

As Th2 immunity substantially contributes to the pathogenesis of nasal allergies (21), we next sought to verify the therapeutic effects of STAT6 siRNA in an allergic rhinitis model.

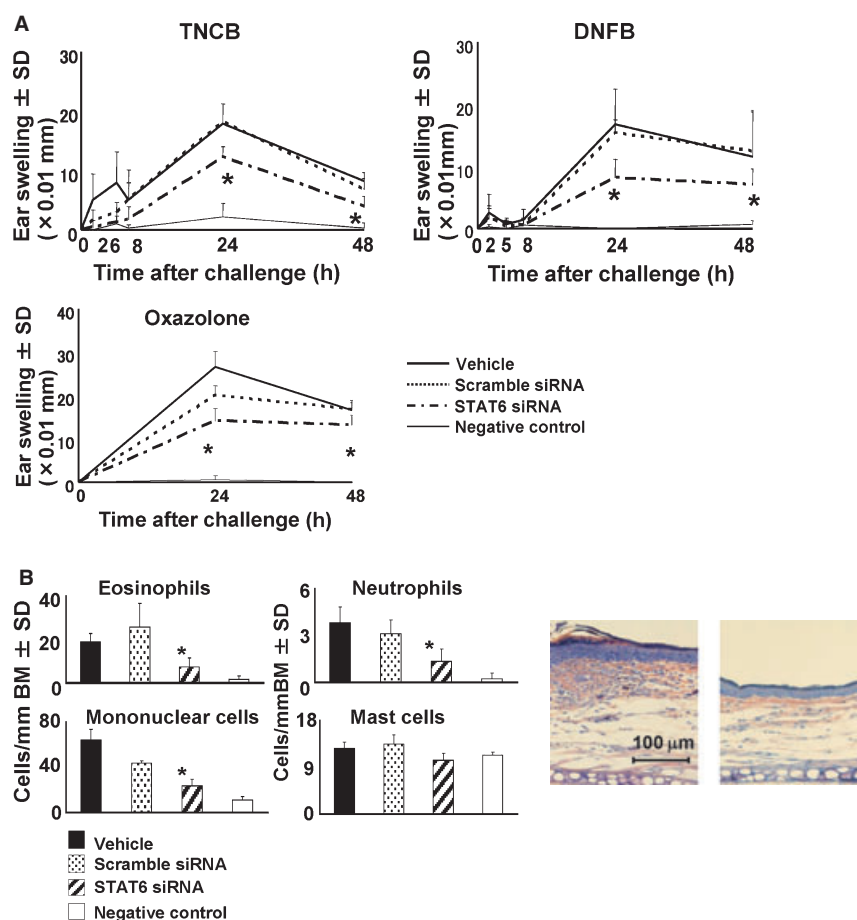


Figure 2 *In vivo* effects of STAT6 siRNA on CHS responses. (A) Sensitized mice (day 0) were administered s.c. into ear lobes with STAT6 siRNA (day 3) and challenged on day 5. Ear-swelling responses to haptens; TNCB, DNFB and oxazolone were inhibited by local introduction of STAT6 siRNA. (B) Changes in cell popula-

tions and histological features of the inflammatory skin induced by TNCB (Giemsa staining). Data are expressed as cell numbers per 1 mm length of basement membrane (BM). Experiments were repeated two or three times. * $P < 0.05$, vs vehicle.

STAT6 siRNA was prepared in Lipofectamine and instilled into the nasal cavity. Administration of STAT6 siRNA solution to the nasal cavity (1.5 nmol/day for three days, i.e., days 22, 23 and 24) during challenge reaction to OVA markedly reduced the frequencies of both sneezing and nasal rubbing (Fig. 5A). The therapeutic effects were also confirmed by the histological features demonstrating the reduced cellular infiltrates, including mononuclear cells and eosinophils (Fig. 5B). Regional submandibular lymph node cells at 24 h after the final challenge were then collected and stimulated with OVA (20 ng/ml) for a further 24 h *in vitro*. Lymph node cells from STAT6 siRNA-treated mice failed to produce IL-4 and IL-5, while levels of IFN- γ were comparable to those from scrambled siRNA-treated mice (Fig. 5C).

Discussion

We determined the nucleotide sequence of a STAT6 siRNA that efficiently and specifically knocks down mouse STAT6

protein. It inhibited IL-4/TNF- α -induced eotaxin (CCL11) production by dermal fibroblasts *in vitro* and successfully alleviated CHS responses *in vivo*. The diminished ear-swelling responses and reduced cellular infiltrates induced by local STAT6 siRNA treatment are in agreement with prior evidence that the STAT6 signaling in resident cells, rather than in Th2-cell differentiation, is primarily responsible for Th2-cell recruitment and production of Th2-type chemokines, such as eotaxin, MDC and TARC, in airway inflammation (26).

The therapeutic effects of locally administered STAT6 siRNA presented in this study confirmed that Th2-type immunity contributes to the pathogenesis of the efferent phase of CHS. We further confirmed this by showing partial reductions in skin responses with IL-4- and/or IL-13-blocking Abs administered in the elicitation phase, which was in striking contrast to prior observations that administration of anti-IL-4 Ab resulted in the enhancement of skin reactions (19, 20). Currently, we have no explanation for this discrep-

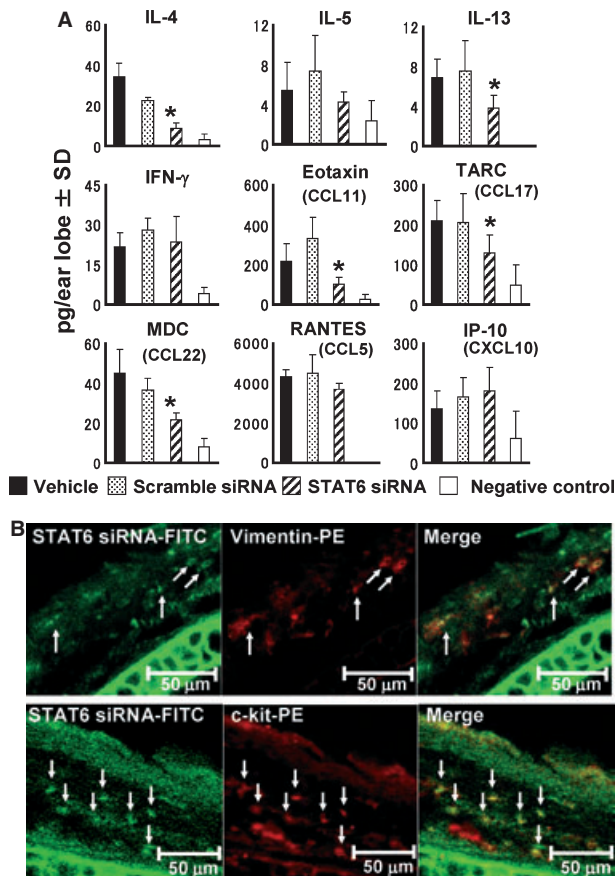


Figure 3 Local production of cytokines/chemokines and localization of introduced siRNA. (A) Ear lobes 24 h after challenge were removed and homogenized. Supernatants were subjected to the measurement of cytokines/chemokines by ELISA. Levels of IL-4, IL-13, eotaxin (CCL11), TARC (CCL17) and MDC (CCL22) were lower in mice treated with STAT6 siRNA. * $P < 0.05$, vs vehicle and/or scramble siRNA. (B) Localization of FITC-labeled STAT6 siRNA was examined by laser scanning microscopy (LSM 510, Carl Zeiss, Oberkochen, Germany). STAT6 siRNA was co-localized with vimentin (+) cells (upper panel) and with c-kit (+) cells (lower panel) (arrows).

ancy, but it may be attributable to differences in either dose of IL-4 Abs (100 μ g/mouse vs 5 mg/mouse) or mouse strain (BALB/c vs C3H/HeN). It was also interesting to note that, in addition to Th2-type cytokines, blocking of the Th1-type cytokine, IFN- γ , exerted a mild but significant inhibition of CHS responses. Th1- and Th2-type responses may not necessarily act competitively but may function together in the development of CHS.

In this study, we performed additional experiments in determining the therapeutic potential of STAT6 siRNA on the mouse model of allergic rhinitis. This was a relevant objective for two reasons. First, it was anticipated that STAT6 siRNA (MW $\approx$ 12,000) would be more efficiently absorbed through nasal mucosa than through skin when applied externally, as skin is covered with cornified layers, while nasal mucosa is not. Second, unlike CHS responses,

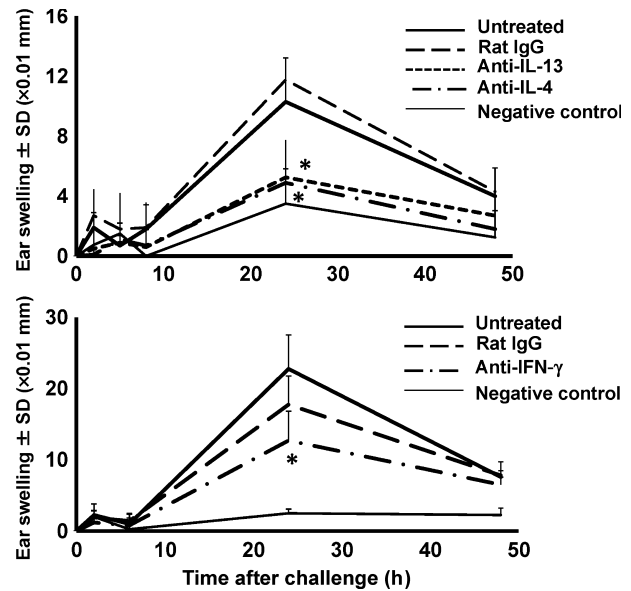


Figure 4 Effects of cytokine blocking on efferent phase of CHS to TNCB. TNCB-sensitized mice received *i.p.* administration of neutralizing antibody against cytokines (100 μ g/mouse) on day 4 and challenge on day 5. * $P < 0.05$.

allergic rhinitis and/or pollen allergy are exclusively mediated by Th2-type immunity (21), and STAT6 expression is elevated in nasal mucosa in atopic allergic rhinitis (27). As expected, intranasal instillation of STAT6 siRNA effectively inhibited cellular infiltrates comprising mononuclear cells and eosinophils to mucosal tissues evoked by OVA challenge. The production of IL-4 and IL-5 by lymph node cells was suppressed. These results indicate that administration of STAT6 siRNA by the use of a nasal spray would have great therapeutic potential in clinical settings.

The fact that STAT6 siRNA markedly improved nasal allergic symptoms, such as sneezing and nasal rubbing provoked within 5 min after challenge, was surprising. Sneezing and nasal rubbing are immediate-type responses induced by mast cell mediators, including histamine (21). In a prior study, STAT6 inhibition in mast cells did not affect IgE-mediated histamine release (28). Thus, STAT6 siRNA was not expected to ameliorate the immediate clinical symptoms, such as sneezing/nasal rubbing. Nevertheless, ongoing allergic rhinitis has been considered to be a continuous late-phase response characterized by marked eosinophil infiltration and the release of various inflammatory mediators, including cysteinyl leukotrienes, eosinophil cationic proteins, eosinophil peroxidase and eosinophil major basic protein, following the immediate-type responses (29). These events cause an increased hyper-reactivity of nasal mucosa to histamine, as well as to other nonspecific stimuli (29, 30). STAT6 siRNA-induced suppression of inflammatory cellular infiltrates, in particular, of eosinophils during the repeated elicitation of nasal responses probably led to the prevention of increased nasal hyper-reactivity, resulting in the improvement in sneez-

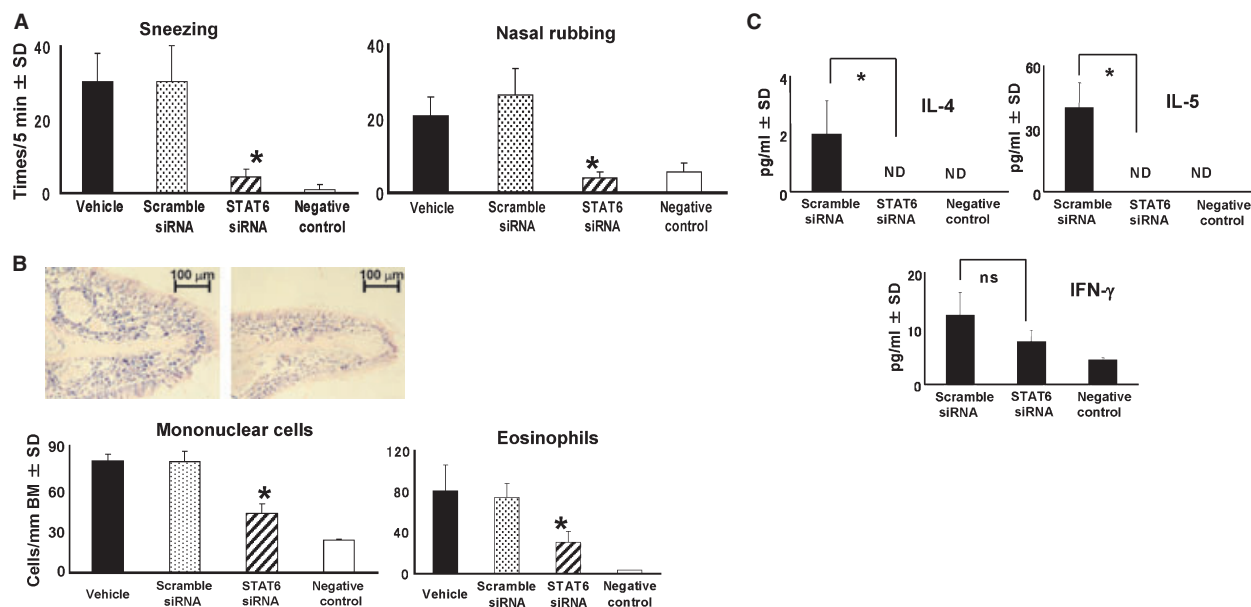


Figure 5 Allergic nasal responses in STAT6 siRNA-treated mice. (A) Symptoms of sneezing and nasal rubbing during 5 min after challenge were markedly improved by STAT6 siRNA treatment. Negative controls received subcutaneous sensitization with OVA + Alum and challenge to nasal mucosa with PBS. (B) Histolog-

ical changes in nasal mucosa (Giemsa staining). (C) Submandibular lymph node cells from challenged mice were stimulated with OVA *in vitro* for 24 h. Cytokine levels in the supernatants were measured. N.D.: not detected. Representative data of two separate experiments are shown. **P* < 0.05.

ing/nasal rubbing. This explanation could be further supported by the prior observation that both sneezing frequencies and mucosal eosinophil levels were reduced in mice lacking the STAT6 gene (31).

Recently, we observed a moderate improvement in clinical skin symptoms in patients with atopic dermatitis following external application of STAT6 'decoy' oligodeoxynucleotides (32). In contrast to 'decoy' oligodeoxynucleotides against nuclear transcription factors (33), siRNA does not require delivery into the nucleus, as it exerts biological functions in the cytoplasm. In these studies in mice and humans, we were not able to detect any apparent adverse effects. However, we may need to consider some clinical adverse effects that are related to impaired STAT6 functions, i.e., the imbalance of

Th1/Th2 immunity, leading to increased risk of exacerbated Th1-mediated diseases.

After completion of this work, another study using STAT6 siRNA reported the therapeutic effects of STAT6 inhibition on asthmatic airway responses in mice (34). Thus, therapies using STAT6 siRNA may be promising for eczematous skin disease and upper and lower airway tract inflammations that involve Th2-immune responses.

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