

Small Interfering RNA against Transcription Factor STAT6 Inhibits Allergic Airway Inflammation and Hyperreactivity in Mice¹

Yasemin Darcan-Nicolaisen,* Holger Meinicke,* Gabriele Fels,* Olga Hegend,[†]
Annekathrin Haberland,[†] Anja Kühl,[‡] Christoph Loddenkemper,[‡] Martin Witzenrath,[§]
Stefanie Kube,[§] Wolfgang Henke,[†] and Eckard Hamelmann^{2*¶}

In the context of allergic immune responses, activation of STAT6 is pivotal for Th2-mediated IgE production and development of airway inflammation and hyperreactivity. We analyzed whether gene silencing of STAT6 expression by RNA interference was able to suppress allergen-induced immune and airway responses. Knockdown effectiveness of three different STAT6 siRNA molecules was analyzed in murine and human cell cultures. The most potent siRNA was used for further testing in a murine model of allergen-induced airway inflammation and airway hyperreactivity (AHR). BALB/c mice were sensitized with OVA/alum twice i.p. (days 1 and 14), and challenged via the airways with allergen (days 28–30). Intranasal application of STAT6 siRNA before and during airway allergen challenges reduced levels of infiltrating cells, especially of eosinophils, in the bronchoalveolar lavage fluid, compared with GFP siRNA-treated sensitized and challenged controls. Allergen-induced alterations in lung tissues (goblet cell hyperplasia, peribronchial inflammation with eosinophils and CD4 T cells) were significantly reduced after STAT6 siRNA treatment. Associated with decreased inflammation was a significant inhibition of the development of allergen-induced in vivo AHR after STAT6 siRNA treatment, compared with GFP siRNA-treated sensitized and challenged controls. Importantly, mRNA and protein expression levels of IL-4 and IL-13 in lung tissues of STAT6-siRNA treated mice were significantly diminished compared with sensitized and challenged controls. These data show that targeting the key transcription factor STAT6 by siRNA effectively blocks the development of cardinal features of allergic airway disease, like allergen-induced airway inflammation and AHR. It may thus be considered as putative approach for treatment of allergic airway diseases such as asthma. *The Journal of Immunology*, 2009, 182: 7501–7508.

Allergic asthma is a highly complex disease that involves a multitude of single factors leading from a mere genetic predisposition to a sometimes uncontrollable or even fatal disease. Despite major advances in the understanding of immunological mechanisms initiating and mediating allergic airway response, current treatment is still limited to unspecific anti-inflammatory drugs and bronchodilators aiming to relieve symptoms rather than targeting pathogenetically relevant elements of the disease. It seems therefore mandatory to characterize key elements of the asthmatic cascade and define novel effective and safe modes of specific intervention that may lead to curative or even preventive treatment strategies.

In this regard, certain transcription factors are of particular interest because they are activated specifically in response to Th2 cytokines (1, 2), and function as master switches directing pivotal

functions (3) in the initial phase of allergic responses. One of the most prominent transcription factors regulating the production of Th2 cytokines and effector functions mediated by Th2 cytokines is STAT6. STAT6 is activated in response to IL-4 and IL-13 and contributes to various functions including mitogenesis, expression of cell surface markers, Th cell differentiation, and Ig isotype switching (4). A role of STAT6 in allergic diseases was supported by findings of up-regulated expression levels in the airways of asthmatic patients (5). Furthermore, the importance of STAT6 for Th2 cell differentiation was underscored by studies in STAT6-deficient mice (4, 6, 7) showing their inability to mount Th2 responses upon allergen sensitization and airway challenges, leading to abrogation of airway eosinophilia and hyperresponsiveness compared with wild-type animals. It is therefore intriguing to speculate that directly targeting STAT6 may be effective in attenuating the development of allergic asthma.

The idea of direct inhibition of specified target genes in a pathophysiologic cascade has been greatly forwarded by recent findings of novel gene silencing techniques. Originally discovered as an ancient endogenous defense mechanism of plants against viruses (8, 9), detection of RNA interference in the model organism *Caenorhabditis elegans* (10) rendered small interfering RNA (siRNA)³ the current method of choice for effective and specific gene silencing.

*Department of Pediatric Pneumology and Immunology, [†]Department of Otorhinolaryngology, [‡]Department of Pathology, Research Center ImmunoSciences, and [§]Department of Internal Medicine, Infectious and Respiratory Diseases, Charité University Medicine, Berlin, and the [¶]University Children's Hospital, Ruhr-University, Bochum, Germany

Received for publication November 12, 2007. Accepted for publication April 6, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by a grant from the RiNA Network RNA-Technology Project 10, Grant 0313066D-10 from the Federal Ministry of Education and Research (BMBF), Grant 3066D-10 from the Senate of Berlin and Brandenburg (to E.H. and W.H.) and by Grant SFB650 from Deutsche Forschungsgemeinschaft (to A.K. and C.L.).

² Address correspondence and reprint requests to Dr. Eckard Hamelmann, University Children's Hospital, Ruhr-University Bochum, Alexandrinenstrasse 5, 44791 Bochum, Germany. E-mail address: eckard.hamelmann@klinikum-bochum.de

³ Abbreviations used in this paper: siRNA, small interfering RNA; AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; i.n., intranasal; siGFP, GFP-specific siRNA.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/\$2.00

We therefore analyzed effects of siRNA targeting the essential transcription factor STAT6 in a murine model of allergic airway disease. We chose direct intranasal (i.n.) application of siRNA as a specifically promising delivery for gene therapeutic compounds in the treatment of respiratory diseases (11, 12). In this study, we show for the first time that i.n. administration of siRNA against transcription factor STAT6 significantly inhibited the development of allergen-induced airway inflammation and hyperreactivity by down-modulation of key elements of the pathologic cascade.

Materials and Methods

siRNA preparation

Double-stranded 21-nucleotide long siRNA with 3'-desoxy-thymidin overhangs were synthesized by CureVac. Sequence of siRNA was complementary to murine and human STAT6 mRNA to prove efficacy in murine and human cell lines. The following STAT6 siRNA were investigated: ss1, 5'-UGCCUUCUCUGAGAUGGACtt; ss2, 5'-ACGCUGUCUCCGGAGCUACtt; and ss3, 5'-AGACCUGUCCAUUCGCUCAtt. GFP-specific siRNA (siGFP) applied as a control has the sequence 5'-GCAGCACGA CUUCUUAAGtt. Different STAT6 siRNAs (1DS, 2DS, 3DS, 4DS) and small interfering Luc applied as control were provided by Atugen (Silence Therapeutics).

Cell culture and siRNA transfection

Murine (BaF3, L929) and human (HeLa, A549) cell lines were exponentially grown under standard conditions. The growth medium was supplemented with 10% FCS and 1% penicillin/streptomycin. For transfection experiments, L929, HeLa or A549 cells ($2 \times 10^4/\text{cm}^2$) were seeded in a 6-well plate, incubated for 24 h in their standard growth medium and transfected with 100 nM siRNA in presence of Lipofectamine 2000 (Invitrogen) as described by the manufacturer. BaF3 cells ($5 \times 10^5/\text{ml}$) were transfected with 300 nM siRNA using 3.6 μM DOTAP (*N*-1-(2,3-dioleoyloxy)propyl-*N,N,N*-trimethylammonium methylsulfate; Roth). After 2 h of transfection, supernatant was replaced by 2 ml of growth medium, and 48 h later cells were harvested.

Western blot analysis

For STAT6 immunoblots, cells were homogenized in RIPA lysis buffer. The protein concentration of the lysate was determined by DC protein assay (Bio-Rad). Cell lysates (10 μg protein per lane) were separated by SDS-PAGE and transferred to nitrocellulose membrane. Blots were blocked 1 h with 5% nonfat powdered milk/0.1% Tween 20/PBS, incubated with anti-STAT6 M20 Ab (Santa Cruz Biotechnology) in blocking solution and washed four times with 0.1% Tween 20/PBS. The protein was detected by chemiluminescence using a HRP-conjugated secondary Ab (Promega) and ECL reagent of GE Healthcare.

IL-13/TNF- α -induced eotaxin production

IL-13/TNF- α -induced eotaxin production was investigated in adult human dermal fibroblasts isolated by standard method. Human dermal fibroblast cells ($7 \times 10^3/\text{ml}$) were seeded in a 96-well plate and transfected 24 h later with 100 nM siRNA and Lipofectamine 2000. After 24 h, medium was supplemented with both recombinant human TNF- α (10 ng/ml) and IL-13 (10 ng/ml) purchased from PeproTech. Eotaxin content in the supernatant of triplicates was determined with human eotaxin ELISA Set (BD Biosciences).

Animals

Female 6- to 8-wk-old BALB/c mice (Harlan Winkelmann) were kept on an OVA-free diet under pathogen-free conditions in our animal experimental institute. All experimental procedures were approved by the Animal Care Committee of the Berlin Office of Health and Social Affairs (Berlin, Germany).

Experimental protocol

Allergen sensitization. Mice were systemically sensitized by i.p. injection of OVA (Grade VI, 100 $\mu\text{g}/\text{ml}$; Sigma-Aldrich), or sham-sensitized with PBS (PAA), adsorbed to adjuvant (10 mg/ml, Al(OH)₃; Pierce) in a total volume of 200 μl on day 0 and day 14.

Allergen challenge. On days 28 and 29, all mice were anesthetized with isoflurane and challenged by i.n. application with OVA (50 μg in 40 μl of PBS).

Treatment. At 48 and 5 h before first airway challenge and 5 h before second allergen airway challenge, animals were anesthetized with isoflurane and i.n. treated with 100 μg of ss3 (PBS/ss3 or OVA/ss3 group) or the same amount of siGFP (PBS/siGFP or OVA/siGFP group) in 40 μl of PBS.

In preliminary experiments, volume levels for application had been determined using different amounts of i.n. administered methylene blue stained PBS. Invasion of i.n. applied siRNA to peripheral lung zones had been proven by fluorescence microscopy of lung tissues after single i.n. application of FITC-labeled STAT6 siRNA and FITC cholesterol-labeled STAT6 siRNA. The effective dose of 100 μg of naked siRNA was chosen after preliminary experiments using different amounts of naked or cholesterol-conjugated siRNA (data not shown).

In vivo airway reactivity

On day 31, in vivo airway reactivity was measured by means of whole-body plethysmography (EMKA Technologies) in response to methacholine (6.25–50 mg/ml), as reported previously (13).

Invasive detection of airway responsiveness in isolated perfused mouse lung

Mouse lungs were prepared, ventilated, and perfused as described (14, 15), and airway resistance was calculated by measurement of chamber pressure and airflow velocity as described (16). After a steady state period of 30 min, methacholine was administered to the perfusate for 30 s at 12-min intervals. The change in airway resistance was expressed as fold airway resistance over baseline.

Bronchoalveolar lavage (BAL)

BAL was performed via a tracheal tube with ice-cold PBS ($2 \times 800 \mu\text{l}$) with protease inhibitor cocktail tablets (complete mini; Roche). Total cell count was determined using a hemocytometer. Cells were counted and cytospin slides were stained with DiffQuik (Dade Behring). BAL cells were differentiated due to morphological criteria by counting at least 100 cells by using standard hematological procedures under light microscopy.

Histology

The left lobe of each individual lung was fixed overnight in 4% formalin at room temperature. After embedding in paraffin, 2- μm tissue sections were stained with periodic acid-Schiff using standard protocol or Giemsa using Accustain (Giemsa stain, modified from Sigma-Aldrich) and Alcian blue staining (Merck), respectively, following instructions provided by the manufacturer. Immunohistochemistry was performed with rabbit polyclonal Ab against murine major basic protein. Briefly, lung tissue sections were deparaffinized, followed by quenching of endogenous peroxidase activity, blocking with 1% normal goat serum and sequentially incubated with rabbit anti-major basic protein (1/1000) and then incubated with biotinylated swine anti-rabbit Ig (DAKO Cytomation) diluted 1/500 in TBS, respectively, for 60 min at 25°C. Sections were incubated first with avidin-biotin-peroxidase complex (DAKO Cytomation) and then with diaminobenzidine for the development of a color-coded reaction product before being counterstained with hematoxylin. The stained sections of each mouse were analyzed by light microscopy by two independent observers, and pathologic alterations and eosinophil quantification were graded semiquantitatively like previously described (17). For immunohistochemical staining of CD3, IL-4, and IL-13, 2- to 3- μm sections were cut, deparaffinized, and subjected to a heat-induced epitope retrieval step before incubation with Abs. Sections were immersed in sodium citrate buffer solutions at pH 6.0 and heated in a high-pressure cooker. The slides were rinsed in cool running water, washed in TBS (pH 7.4), and incubated with the primary Ab against CD3 (dilution 1/10, no. N1580; DAKO Cytomation), IL-4 (dilution 1/100, clone BVD6-24G2; eBioscience) or IL-13 (no. AF-413-NA, dilution 1/20; R&D Systems) followed by biotinylated donkey anti-rabbit (Dianova), rabbit anti-rat or rabbit anti-goat (DAKO Cytomation) secondary Ab. For CD4 and CD8 staining, frozen sections were air-dried, fixed in acetone for 10 min, and incubated with primary rat anti-mouse Abs against CD4 (dilution 1/50, RM-4-5; eBioscience) and CD8 (dilution 1/50, Ly-2; eBioscience) followed by biotinylated donkey anti-rat (Dianova) secondary Ab. Finally, the streptavidin AP kit (K5005; DAKO Cytomation) was applied and alkaline phosphatase was revealed by Fast Red as chromogen. Negative controls were performed by omitting primary Abs. For analysis representative high power fields ($1 = 0.237 \text{ mm}^2$) of peribronchial T cell infiltrates in each histological section were chosen.

Real-time PCR (TaqMan)

Right lung lobes were stored at -80°C in RNAlater (Ambion) until use. Total RNA was isolated from siGFP or ss3 transfected L929 cells or murine

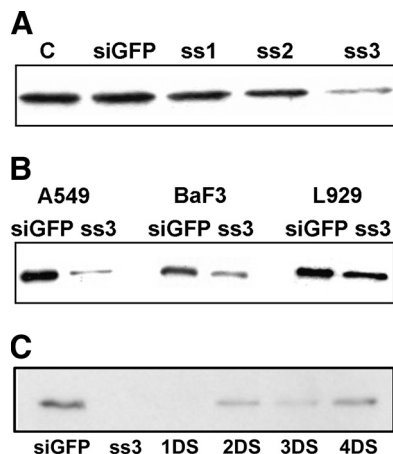


FIGURE 1. Identification of siRNA inhibiting STAT6 expression in human and murine cell lines by immunoblot. *A*, HeLa cells are transfected with Lipofectamine 2000 and 100 nM of the siRNAs, siGFP, ss1, ss2 and ss3. *A* control (C) without siRNA is shown. *B*, Inhibition of STAT6 expression in human lung epithelial cell line A549 and in murine cell lines BaF3 and L929 by 100 nM ss3. *C*, L929 cells transfected with different STAT6 siRNA (1DS, 2DS, 3DS, 4DS, ss3). 1DS showed similar knockdown efficiency to ss3. The siRNA ss3, as the most potent STAT6-silencing siRNA active in both human and murine cell lines, was used for all following experiments.

lung tissue using RNeasy Mini kits (Qiagen) and thereafter reverse-transcribed into cDNA using random hexamers (Invitrogen) and M-MLV Reverse Transcriptase (RNase H Minus, Point Mutant; Promega). Most stable housekeeping genes *Canx*, *CYC1*, and *SDHA* were identified by geNorm (Primer Design) according to the manufacturer's instructions. Primer and probe sequences for amplification of housekeeping genes *STAT1*, *STAT2*, *STAT5a*, *STAT6*, *IL-4*, *IL-5* and *IL-13* were designed by Primer Express Software (v3.0; Applied Biosystems) and were obtained from MWG-Biotech as HPLC grade. Fluorogenic probes contained a reporter dye (FAM) covalently linked at the 5' end and a quencher dye (TAMRA) covalently attached at the 3' end. Quantitative real-time PCR was performed in triplicates on an ABI PRISM 7300 TaqMan (Applied Biosystems) according to the manufacturer's instruction for relative quantification. Relative expression of target genes was determined with reference to negative control groups after normalization against the housekeepers as implemented in ABI PRISM 7300 Sequence Detection System software (Applied Biosystems).

Statistical analysis

Values for all measurements are expressed as mean $\pm$ SEM. Pairs of treatment groups were compared by using Mann-Whitney *U* test. Statistical significance was set at a value of $p < 0.05$.

Results

Identification of STAT6 siRNA in murine and human cell lines

First knockdown efficiency of seven different STAT6 siRNA (ss1, ss2, ss3, 1DS, 2DS, 3DS, 4DS) was investigated in HeLa and A549 cell lines. Second knockdown efficiency of ss3 was analyzed in transfected A549, BaF3, and L929 cell lines. siRNA was transfected by using Lipofectamine 2000. Transfections without siRNA or with siGFP served as control. In Western blot analysis, ss3 is the most potent STAT6-silencing siRNA, but 1DS shows a similarly strong knockdown effect. ss3 is active in both human and murine cell lines (Fig. 1).

To rule out nonspecific inhibition, so called "off-target" effects, of transcription factors by ss3, the expression of *STAT1*, *STAT2*, and *STAT5a* in murine fibroblast cells L929 was investigated by quantitative real-time PCR showing no significant unspecific expression by our construct (Table I).

STAT6 siRNA inhibits eotaxin release in vitro

Functional effects of STAT6 knockdown on STAT6-mediated signal transduction were analyzed in a human skin fibroblast cell line.

Table I. Knockdown of STAT mRNA expression in murine L929 cells

Target	Knockdown by ss3 (%)
STAT1	5.49
STAT2	3.03
STAT5a	3.99
STAT6	60.7

Cells transfected in different concentrations with the most effective siRNA, ss3, or with the GFP siRNA as control, were stimulated with optimal concentrations of IL-13 and TNF- α to induce eotaxin production. As shown in Fig. 2, STAT6 siRNAs treatment before cytokine stimulation resulted in a significant and dose-dependent reduction of eotaxin secretion compared with control (siGFP).

STAT6 siRNA decreases eosinophilic airway infiltration in vivo

First, we analyzed the bioavailability of i.n. delivered siRNA in lung tissues. In preliminary experiments, i.n. application of 50 μ l of solution was shown to result in optimal infusion of whole lung tissues, whereas 25 μ l only reached peribronchial areas, and 75 μ l resulted in unwanted effects. To prove invasion of i.n. administered siRNA to peripheral lung zones, single i.n. application of FITC cholesterol-labeled STAT6 siRNA confirmed in vivo uptake by airway epithelial cells in central and peripheral areas of the organ (data not shown).

Next, we analyzed effects of STAT6 siRNA treatment on the development of airway inflammation and airway hyperreactivity (AHR) in a well-established murine model of allergen-induced airway disease. Accumulation of leukocytes, especially eosinophils, in BAL fluid is one of the cardinal features of allergic asthma. We therefore determined the influx of immune cells in BAL fluid of OVA-sensitized and -challenged mice with and without siRNA treatment. Sensitization and airway challenges resulted in a robust increase in the number of total leukocytes, especially eosinophils, compared with sham-sensitized and -challenged controls. Treatment of sensitized mice with ss3 before and during allergen airway challenges significantly reduced total cell counts, as well as the

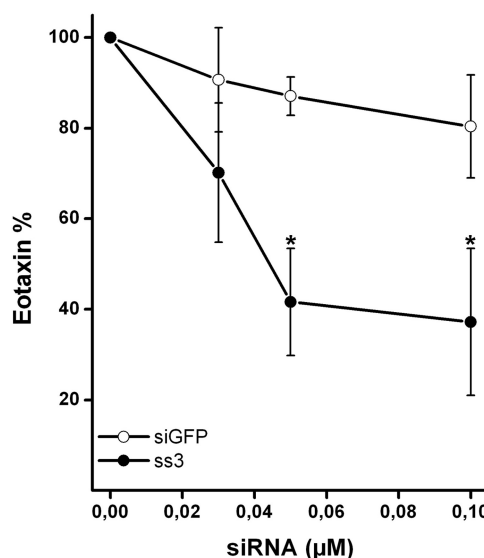


FIGURE 2. STAT6 siRNA reduces IL-13/TNF- α -induced eotaxin production in vitro. Culture of cells with different concentrations of the most effective STAT6 siRNA, ss3, or with the GFP siRNA as control before cytokine stimulation with IL-13 and TNF- α resulted in a significant and dose-dependent reduction of eotaxin secretion compared with control.

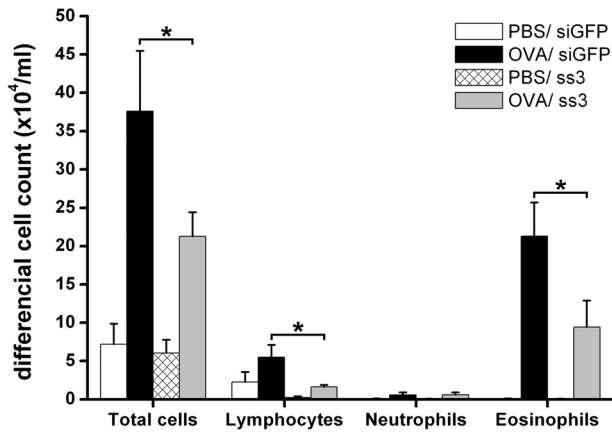


FIGURE 3. STAT6 siRNA reduces leukocyte infiltration in BAL fluid. Mice were sensitized by i.p. injection of OVA/alum on days 1 and 14, and challenged with OVA via the airways on days 28–30. On day 32, 2 days after the last allergen airway challenge, lungs were lavaged, and differential cell counts in BAL fluid were assessed. In contrast to sham-sensitized and -challenged controls, OVA-sensitized and -challenged mice showed strong increase in the number of total leukocytes and especially eosinophils. Treatment of sensitized mice with ss3 before and during allergen airway challenges significantly reduced total cell counts, as well as the number of infiltrating eosinophils in BAL fluid, compared with sensitized and challenged control mice treated with siGFP siRNA. The number of mice are $n = 4$ PBS/siGFP, $n = 10$ OVA/siGFP, $n = 4$ PBS/ss3, and $n = 10$ OVA/ss3. *, $p < 0.05$ for OVA/siGFP vs OVA/ss3.

number of infiltrating eosinophils in BAL fluid, to ~50% compared with sensitized and challenged control mice treated with siGFP siRNA (Fig. 3). This effect was specifically delivered by siRNA against STAT6 because a second construct aiming at the same target gene showed similar reduction in airway inflammation and AHR. Moreover, another negative control using nonspecific siRNA (aimed against luciferase) showed no specific effects but robust development of airway inflammation in sensitized and challenged animals (data not shown).

STAT6 siRNA reduces development of AHR

To determine effects of STAT6 neutralization by siRNA on the development of AHR, OVA-sensitized and nonsensitized, ss3- and siGFP-treated mice were i.n. challenged twice with OVA. At 24 h after last allergen provocation, airway responsiveness to aerosolized methacholine in spontaneously breathing and conscious mice was measured by means of barometric whole-body plethysmography (13). This method was recently shown to correlate very well with invasive measurements of AHR (invasive measurement of airway resistance in anesthetized mice or in isolated perfused and ventilated lungs) in allergic BALB/c mice (15). OVA sensitization and airway allergen challenges (OVA/siGFP) enhanced airway reactivity compared with sham-sensitized, challenged control animals (PBS/siGFP). In contrast to siGFP-treated mice (OVA/siGFP), ss3-treated mice (OVA/ss3) developed reduced levels of AHR (Fig. 4A). Reduction in airway responsiveness after treatment with STAT6 siRNA (OVA/ss3) as compared with siGFP-treated mice (OVA/siGFP) was confirmed by invasive measurement of airway resistance in isolated perfused and ventilated lungs (Fig. 4B).

STAT6 siRNA reduces histological alterations in lung tissues

Lung sections of each mouse were analyzed with regard to pathologic alterations resembling the histopathology of allergic asthma, such as goblet cell hyperplasia and peribronchial infil-

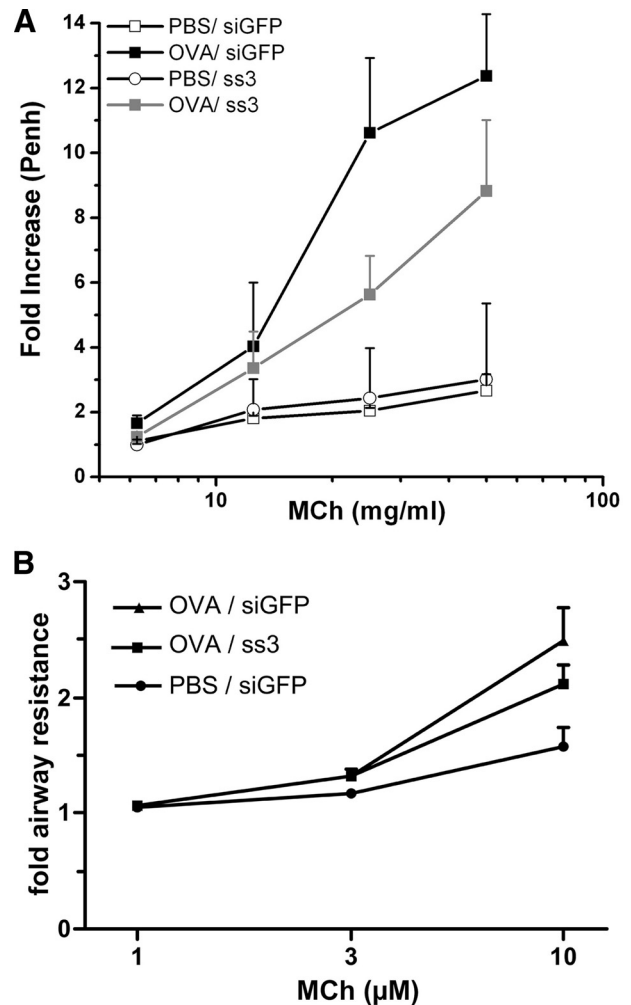


FIGURE 4. STAT6 siRNA reduces development of in vivo AHR. On day 32, in vivo lung function to increasing doses of inhaled methacholine (MCh) was measured by whole-body plethysmography (A) or measurement of airway resistance (B) in isolated perfused and ventilated lungs. Penh represents enhanced pause. OVA sensitization and airway allergen challenges (OVA/siGFP) significantly increased airway reactivity, compared with sham-sensitized, challenged control animals (PBS/siGFP). In contrast to siGFP-treated mice, ss3-treated sensitized and challenged mice (OVA/ss3) developed significantly smaller levels of AHR. The number of mice used were $n = 4$ PBS/siGFP, $n = 10$ OVA/siGFP, $n = 4$ PBS/ss3, $n = 10$ OVA/ss3 (A), and $n = 6$ mice per group (B).

tration. Representative results obtained from these analyses are shown in Fig. 5.

Control mice that were sham-sensitized with PBS and challenged with OVA (PBS/siGFP or PBS/ss3) via the airways did not present obstruction of the bronchoalveolar system or alterations of ciliated epithelium (Fig. 5, A, B, G, and H) or eosinophils in the infiltration (Fig. 5, C and I). OVA-sensitized and -challenged mice treated with siGFP (OVA/siGFP) exhibited a large number of goblet cells, peribronchial infiltrates (Fig. 5, D and E), and eosinophils (Fig. 5F). In contrast, ss3-treated and OVA-sensitized and -challenged mice (OVA/ss3) demonstrated notably reduced levels of goblet cells, peribronchial infiltration (Fig. 5, J and K), and eosinophils (Fig. 5L). Similarly, CD3⁺ peribronchial T cell infiltrates consisting predominantly of CD4⁺ T cells were significantly reduced following ss3 treatment. Scores obtained for histological analysis of pathologic alterations in lungs like peribronchial infiltration or goblet and eosinophil cell count are presented in Fig. 6.

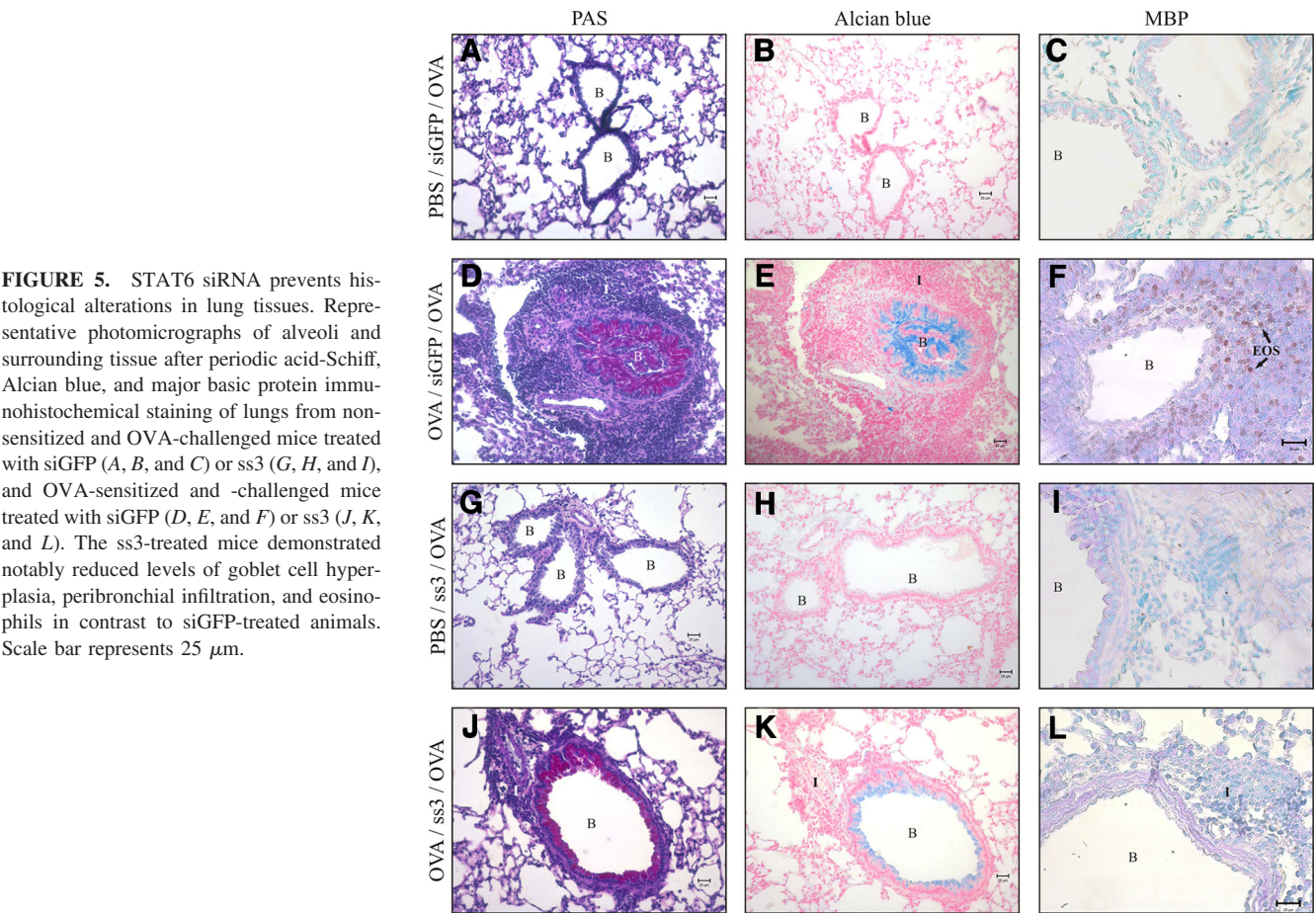


FIGURE 5. STAT6 siRNA prevents histological alterations in lung tissues. Representative photomicrographs of alveoli and surrounding tissue after periodic acid-Schiff, Alcian blue, and major basic protein immunohistochemical staining of lungs from non-sensitized and OVA-challenged mice treated with siGFP (A, B, and C) or ss3 (G, H, and I), and OVA-sensitized and -challenged mice treated with siGFP (D, E, and F) or ss3 (J, K, and L). The ss3-treated mice demonstrated notably reduced levels of goblet cell hyperplasia, peribronchial infiltration, and eosinophils in contrast to siGFP-treated animals. Scale bar represents 25 μ m.

CD3⁺ peribronchial T cell infiltrates following ss3 treatment are shown in Fig. 7.

STAT6 siRNA decreases IL-4, IL-5, and IL-13 production in lung tissue

To better understand the mechanism underlying the inhibitory effects of siRNA, we analyzed expression levels of the main Th2-promoting cytokines in the compartment of interest, the lung tissues. First we analyzed the mRNA production of these cytokines by real-time PCR. Sensitization and airway allergen challenges

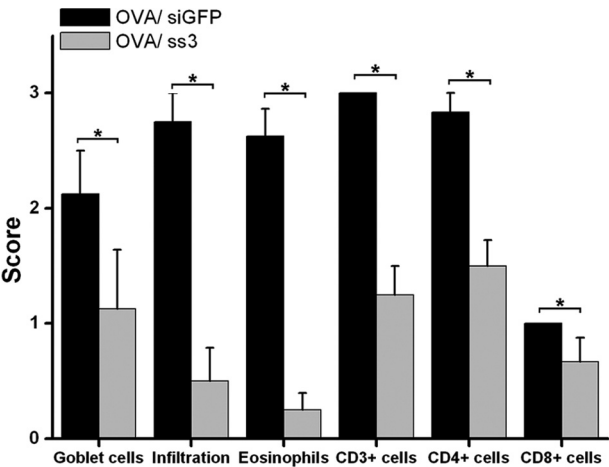


FIGURE 6. Scores obtained for histological analysis of pathologic alterations in lungs, eosinophil count, and CD3⁺, CD4⁺, and CD8⁺ cells. Data were presented as mean $\pm$ SEM. *, $p < 0.05$.

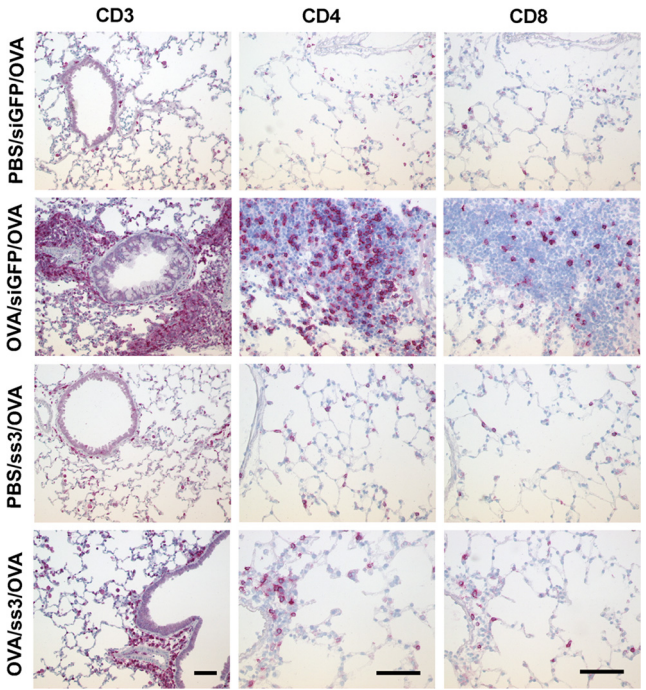


FIGURE 7. STAT6 siRNA reduces T cell infiltrates in the lung. Immunohistochemical stainings of representative lung sections with dense peribronchial infiltrates of CD3⁺ T cells in OVA-sensitized and -challenged mice that show a predominance of CD4⁺ T cells compared with the number of CD8⁺ T cells. Following ss3 treatment, T cell infiltrates are strongly reduced in OVA-sensitized and -challenged mice in contrast to siGFP-treated animals. Scale bar represents 50 μ m.

Table II. Knockdown of mRNA expression in murine lung tissue^a

Target	OVA/siGFP	OVA/ss3
STAT6		
$\Delta C_T \pm SEM$	0.155 ± 0.193	-0.079 ± 0.509
$\Delta\Delta C_T$	0	-0.234
RQ ($2^{-\Delta\Delta C_T}$)	1	1.176
Log ₁₀ RQ	0	0.070
Knockdown		ND
IL-4		
$\Delta C_T \pm SEM$	6.018 ± 0.750	6.840 ± 0.903
$\Delta\Delta C_T$	0	0.823
RQ ($2^{-\Delta\Delta C_T}$)	1	0.565
Log ₁₀ RQ	0	-0.248
Knockdown		43.46%
IL-5		
$\Delta C_T \pm SEM$	6.622 ± 0.416	6.668 ± 1.379
$\Delta\Delta C_T$	0	0.047
RQ ($2^{-\Delta\Delta C_T}$)	1	0.968
Log ₁₀ RQ	0	-0.014
Knockdown		3.12%
IL-13		
$\Delta C_T \pm SEM$	1.588 ± 1.086	2.783 ± 1.035
$\Delta\Delta C_T$	0	1.195
RQ ($2^{-\Delta\Delta C_T}$)	1	0.437
Log ₁₀ RQ	0	-0.360
Knockdown		56.32%

^a mRNA expression of STAT6, IL-4, IL-5, and IL-13 in lysates of murine lung tissue were analyzed by TaqMan. C_T , Threshold cycle; RQ, relative quantification.

(OVA/siGFP) resulted in increased levels of IL-4, IL-5, and IL-13 mRNA production in lung tissues compared with nonsensitized, challenged control animals (PBS/siGFP). The i.n. application of STAT6-siRNA to sensitized animals before and during airway allergen challenges (OVA/ss3) reduced IL-4 (by 43%) and IL-13 (by 56%) production compared with siGFP control mice (OVA/siGFP) (Table II). Next we analyzed IL-4 and IL-13 protein expression in lung tissues by immunohistochemical staining to confirm biological relevance of mRNA reduction. We observed moderate to strong expression of IL-4 and IL-13 in peribronchial infiltrates of the OVA/siGFP group, whereas IL-4 and IL-13 protein expression in lung tissues from OVA/ss3 treated mice were significantly reduced (Fig. 8).

These data strongly supported that local down-regulation of Th2 cytokine production after treatment with siRNA against STAT6

was responsible for suppression of allergen-induced airway inflammation and AHR.

Discussion

We show in this study using a murine model of allergen-induced airway disease that i.n. application of STAT6 siRNA to sensitized animals right before and during repeated allergen airway challenge significantly reduced two cardinal features of bronchial asthma, airway inflammation and AHR, by abrogation of local Th2 cytokine production and function.

Th2 cytokines IL-4, IL-5, and IL-13 play a pivotal role in induction, regulation, and maintenance of allergic diseases by regulating IgE production, differentiation of naive T cells toward Th2 cells, and activation of mast cells and eosinophils (18–21). The exceptional role of STAT6 for IL-4/IL-13 signaling has been demonstrated in STAT6-deficient mice that did not develop Th2 responses, Ig class switching to IgE (4, 6, 22), AHR, mucus hypersecretion, and airway eosinophilia upon sensitization and airway challenges (23, 24), thus underlining the major role of this transcription factor in the pathogenesis of allergic asthma.

Due to their outstanding position in the pathologic cascade of allergic airway diseases, other transcription factors have already been investigated for therapeutic purposes using i.n. administered gene silencing techniques. We have previously reported from another member of the STAT family, STAT-1, that inhibition of STAT-1 signaling by i.n. application of STAT-1 decoy oligonucleotides reduced the development of allergen-induced eosinophilic airway inflammation and increased airway reactivity in a murine model of allergic asthma (25). This effect was associated with and due to reduced expression of activation markers, CD40, and homing receptors, VCAM-1, in the airways of decoy-treated animals. Finotto et al. (12) successfully used a different technique by administration of antisense oligodeoxynucleotides RNA against transcription factor GATA-3 and demonstrated significant reduction of the asthmatic phenotype in their murine model. Recently, McCusker et al. (26) even targeted STAT6 by inhibitory peptides. They were able to demonstrate a successful i.n. delivery to airway epithelial cells and an inhibition of OVA-induced lung inflammation, mucus production and AHR in a mouse model of allergic airway disease. These three examples together with our own present data offer strong proof-of-principle to target transcription factors for novel treatment strategies against allergic airway diseases.

For our study, we chose siRNA as the most recent technique for powerful and effective gene silencing. The key therapeutic advantage of RNA interference is its ability to specifically knockdown the expression of disease-causing genes. Targeting mRNA by siRNA or antisense oligodeoxynucleotides, rather than the dependent protein itself thus presents a more efficient approach to block a certain biological function because multiple copies of a specific protein are produced by each mRNA molecule. Yet, studies have revealed that siRNAs are more stable in mammalian cells and physiological fluids than antisense oligodeoxynucleotides (27). Although only discovered quite recently, first therapeutic approaches using siRNA are already concentrating on targets in cancer (28), angiogenesis (29, 30), and viral diseases (31). First clinical applications of RNA interference in age-related macular degeneration (32, 33), and respiratory syncytial virus infection (11, 34) show promising results, and preclinical trials in other viral infections (31, 35) and neurodegenerative diseases (36, 37) are ongoing.

The two most important considerations in developing effective RNA interference-based therapy are 1) optimal delivery and 2) optimal sequence of siRNAs. To address the latter, we analyzed a variety of different sequences and identified a specific one, named

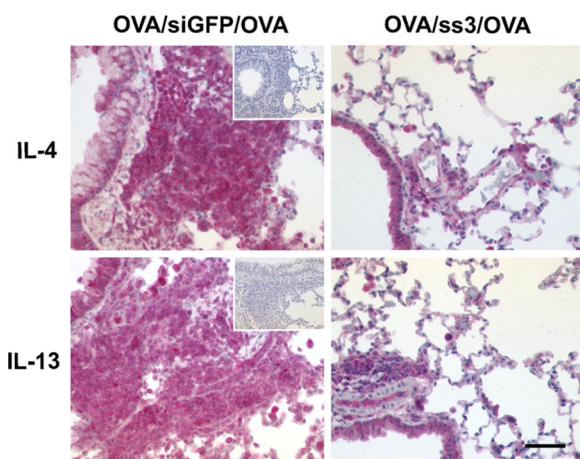


FIGURE 8. STAT6 siRNA reduces IL-4 and IL-13 expression in the lung. In OVA-sensitized and -challenged mice, high expression levels of IL-4 and IL-13 protein can be detected immunohistochemically in siGFP-treated mice, which are strongly reduced in ss3-treated animals. Negative control images are shown (inset). Scale bar represents 50 μ m.

ss3, as the most effective STAT6 siRNA, showing the maximum knockdown of STAT6 and the highest reduction in functional in vitro test of IL-13/TNF- α -induced eotaxin release (38).

Several publications reported on potential off-target effects induced by siRNA-transfection (37, 44). The type I IFN-mediated activation of the JAK/STAT pathway and subsequent up-regulation of IFN-dependent genes like STAT1 and STAT2 seemed to be of special interest. We therefore investigated a possible up-regulation of STAT1 and STAT2 in murine cell line L929 after transfection with our STAT6 siRNA (ss3) and did not find any significant changes in the expression of these genes.

Concerning the choice between local and systemic delivery we decided to administer STAT6 siRNA locally by i.n. application according to a possible clinical viability in the future. Systemic delivery of siRNA may be hampered by rapid filtration through the kidney, serum degradation by RNase activity, and a nonspecific distribution throughout the body decreasing local bioactivity in the compartment of interest. Going for i.n. route gave hope to avoid these hurdles: local delivery may allow using substantially lower doses of siRNA, and a focused administration of the compound will possibly circumvent adverse events. Zhang et al. (39) and Bitko et al. (11) demonstrated specific knockdown of target mRNA by i.n. siRNA delivery without the use of vector or transfection agent. Other authors used complexed siRNA with different vectors for i.n. delivery to increase stability and to solve the most challenging problem: passing siRNA through different tissue barriers to reach target cells. For this regard it was shown that different cationic lipids (40), polymers (41), nanoparticles (42) as well as chemical modifications (43) may enhance stability and cellular uptake of siRNA. However, these modifications might cause off-target effects after distribution to unwanted sites and activation of the IFN pathway (44, 45). Furthermore, viral vectors bear the risk of toxicity limiting their use in humans (46).

In preliminary experiments, we tested cholesterol-conjugation of siRNA to improve invasion of lipophilic membranes (47, 48). Although this compound significantly inhibited eosinophilia in BAL fluid and reduced AHR after allergen sensitization and airway challenges, cholesterol-siRNA-treated mice also showed a robust increase in lung neutrophil numbers independent of siRNA sequence (as demonstrated by use of cholesterol-conjugated control siRNA), most probably due to off-target effects of conjugation (data not shown).

We therefore went on and delivered naked siRNA to already sensitized mice before and during allergen airway challenges to more closely mimic the clinical situation of atopic patients. Using different doses of siRNA (40–100 μ g) we found that augmented siRNA concentrations of 100 μ g were able to significantly reduce total cell counts and eosinophilia in BAL fluid and inhibit development of in vivo AHR. This effect was specific for the interaction with the STAT6 pathway because a second STAT6 siRNA showed similar reduction in airway inflammation and AHR. As IL-4 and IL-13 together with the most potent chemoattractant IL-5 (49) mediate eosinophil activation and recruitment to the lung (50), blocking of IL-4/IL-13 signaling confirmed the critical role of STAT6 in these responses, supporting similar results obtained by use of STAT6-deficient mice (23, 24). Direct proof for this mechanism of STAT6-siRNA was obtained by analysis of Th2 cytokine mRNA and protein expression in lung tissues. We examined IL-4 and IL-13 cytokine levels in lungs by means of quantitative real-time PCR and immunohistological staining and found notably reduced levels of these cardinal cytokines in STAT6 siRNA-treated group compared with sensitized and challenged animals treated with control siRNA, which again showed enhanced levels of Th2 cytokines in comparison to negative controls. These changes in cytokine pro-

duction were associated with a significantly reduced number of CD4⁺ T cells, supporting the role of STAT6-mediated IL-4 production not only for the induction but also for the maintenance of allergic airway inflammation.

Taken together, our results indicate that siRNA against STAT6 presents an effective new method to inhibit the production of several Th2 cytokines in allergen-mediated immune and airway reactions, thus leading to significant reduction of allergic airway inflammation and AHR. Furthermore, our data indicate that local i.n. delivery of uncoupled gene silencing compounds may be an appropriate approach to modulate gene expression in the respiratory tract. To establish STAT6 -siRNA as a possible new strategy for treatment of allergic asthma, additional experiments in experimental models are required. This additional treatment will help to delineate optimal dosing and application kinetics of siRNA therapy to gain strongest possible and long lasting knockdown of pro-allergic target genes and thus allergic symptoms without risking major adverse events. Once all questions of safety and efficacy in this regard are solved, the use of siRNA will be an intriguing new way of target-specific treatment of allergic airway diseases such as asthma or chronic obstructive pulmonary disease.

Acknowledgments

We thank Christine Seib, Martin Krokowski, and Simone Spieckermann for excellent technical assistance as well as Dr. Martin Klein supporting intensively the preparation of human adult dermal fibroblasts and especially Dr. J. Kaufmann (Silence Therapeutics) for providing different STAT6 siRNAs.

Disclosures

The authors have no financial conflict of interest.

References

- Hou, J., U. Schindler, W. J. Henzel, T. C. Ho, M. Brasseur, and S. L. McKnight. 1994. An interleukin-4-induced transcription factor: IL-4 Stat. *Science* 265: 1701–1706.
- Quelle, F. W., K. Shimoda, W. Thierfelder, C. Fischer, A. Kim, S. M. Ruben, J. L. Cleveland, J. H. Pierce, A. D. Keegan, and K. Nelms. 1995. Cloning of murine Stat6 and human Stat6, Stat proteins that are tyrosine phosphorylated in responses to IL-4 and IL-3 but are not required for mitogenesis. *Mol. Cell. Biol.* 15: 3336–3343.
- Ortmann, R. A., T. Cheng, R. Visconti, D. M. Frucht, and J. J. O'Shea. 2000. Janus kinases and signal transducers and activators of transcription: their roles in cytokine signaling, development and immunoregulation. *Arthritis Res.* 2: 16–32.
- Takeda, K., T. Tanaka, W. Shi, M. Matsumoto, M. Minami, S. Kashiwamura, K. Nakanishi, N. Yoshida, T. Kishimoto, and S. Akira. 1996. Essential role of Stat6 in IL-4 signalling. *Nature* 380: 627–630.
- Christodouloulopoulos, P., L. Cameron, Y. Nakamura, C. Lemiere, S. Muro, M. Dugas, L. P. Boulet, M. Laviolette, R. Olivenstein, and Q. Hamid. 2001. TH2 cytokine-associated transcription factors in atopic and nonatopic asthma: evidence for differential signal transducer and activator of transcription 6 expression. *J. Allergy Clin. Immunol.* 107: 586–591.
- Shimoda, K., J. van Deursen, M. Y. Sangster, S. R. Sarawar, R. T. Carson, R. A. Tripp, C. Chu, F. W. Quelle, T. Nosaka, D. A. Vignali, et al. 1996. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature* 380: 630–633.
- Tomkinson, A., A. Kanehiro, N. Rabinovitch, A. Joetham, G. Cieslewicz, and E. W. Gelfand. 1999. The failure of STAT6-deficient mice to develop airway eosinophilia and airway hyperresponsiveness is overcome by interleukin-5. *Am. J. Respir. Crit. Care Med.* 160: 1283–1291.
- Napoli, C., C. Lemieux, and R. Jorgensen. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in *trans*. *Plant Cell* 2: 279–289.
- van Der Krol, A. R., L. A. Mur, M. Beld, J. N. Mol, and A. R. Stuitje. 1990. Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* 2: 291–299.
- Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391: 806–811.
- Bitko, V., A. Musiyenko, O. Shulyayeva, and S. Barik. 2005. Inhibition of respiratory viruses by nasally administered siRNA. *Nat. Med.* 11: 50–55.
- Finotto, S., G. T. De Sanctis, H. A. Lehr, U. Herz, M. Buerke, M. Schipp, B. Bartsch, R. Atreya, E. Schmitt, P. R. Galle, et al. 2001. Treatment of allergic airway inflammation and hyperresponsiveness by antisense-induced local blockade of GATA-3 expression. *J. Exp. Med.* 193: 1247–1260.

13. Hamelmann, E., J. Schwarze, K. Takeda, A. Oshiba, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156: 766–775.
14. Held, H. D., C. Martin, and S. Uhlig. 1999. Characterization of airway and vascular responses in murine lungs. *Br. J. Pharmacol.* 126: 1191–1199.
15. Witzensrath, M., B. Ahrens, S. M. Kube, A. Braun, H. G. Hoymann, A. C. Hocke, S. Rousseau, N. Suttrop, E. Hamelmann, and H. Schutte. 2006. Detection of allergen-induced airway hyperresponsiveness in isolated mouse lungs. *Am. J. Physiol.* 291: L466–L472.
16. Held, H. D., and S. Uhlig. 2000. Basal lung mechanics and airway and pulmonary vascular responsiveness in different inbred mouse strains. *J. Appl. Physiol.* 88: 2192–2198.
17. Darcan, Y., A. Petersen, W. M. Becker, J. Galle, M. Ernst, J. Ahmed, and U. Seitzer. 2005. Immunoprophylaxis with DNA vaccination inhibits Th2-mediated responses and airway inflammation in mice sensitized with the major timothy grass pollen allergen Phl p 5b. *Vaccine* 23: 4203–4211.
18. Corry, D. B., G. Grunig, H. Hadeiba, V. P. Kurup, M. L. Warnock, D. Sheppard, D. M. Rennick, and R. M. Locksley. 1998. Requirements for allergen-induced airway hyperactivity in T and B cell-deficient mice. *Mol. Med.* 4: 344–355.
19. Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282: 2258–2261.
20. Finkelman, F. D., I. M. Katona, J. F. Urban, Jr., J. Holmes, J. Ohara, A. S. Tung, J. V. Sample, and W. E. Paul. 1988. IL-4 is required to generate and sustain in vivo IgE responses. *J. Immunol.* 141: 2335–2341.
21. Nilsson, G., and K. Nilsson. 1995. Effects of interleukin (IL)-13 on immediate-early response gene expression, phenotype and differentiation of human mast cells: comparison with IL-4. *Eur. J. Immunol.* 25: 870–873.
22. Kaplan, M. H., U. Schindler, S. T. Smiley, and M. J. Grusby. 1996. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 4: 313–319.
23. Kuperman, D., B. Schofield, M. Wills-Karp, and M. J. Grusby. 1998. Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J. Exp. Med.* 187: 939–948.
24. Akimoto, T., F. Numata, M. Tamura, Y. Takata, N. Higashida, T. Takashi, K. Takeda, and S. Akira. 1998. Abrogation of bronchial eosinophilic inflammation and airway hyperactivity in signal transducers and activators of transcription (STAT)6-deficient mice. *J. Exp. Med.* 187: 1537–1542.
25. Quarcoo, D., S. Weixler, D. Groneberg, R. Joachim, B. Ahrens, A. H. Wagner, M. Hecker, and E. Hamelmann. 2004. Inhibition of signal transducer and activator of transcription 1 attenuates allergen-induced airway inflammation and hyperactivity. *J. Allergy Clin. Immunol.* 114: 288–295.
26. McCusker, C. T., Y. Wang, J. Shan, M. W. Kinyanjui, A. Villeneuve, H. Michael, and E. D. Fixman. 2007. Inhibition of experimental allergic airways disease by local application of a cell-penetrating dominant-negative STAT-6 peptide. *J. Immunol.* 179: 2556–2564.
27. Bertrand, J. R., M. Pottier, A. Vekris, P. Opolon, A. Maksimenko, and C. Malvy. 2002. Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo. *Biochem. Biophys. Res. Commun.* 296: 1000–1004.
28. Pai, S. I., Y. Y. Lin, B. Macaes, A. Meneshian, C. F. Hung, and T. C. Wu. 2006. Prospects of RNA interference therapy for cancer. *Gene Ther.* 13: 464–477.
29. Lu, P. Y., F. Y. Xie, and M. C. Woodle. 2003. siRNA-mediated antitumorogenesis for drug target validation and therapeutics. *Curr. Opin. Mol. Ther.* 5: 225–234.
30. Reich, S. J., J. Fosnot, A. Kuroki, W. Tang, X. Yang, A. M. Maguire, J. Bennett, and M. J. Tolentino. 2003. Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. *Mol. Vis.* 9: 210–216.
31. Dykxhoorn, D. M., and J. Lieberman. 2006. Silencing viral infection. *PLoS Med.* 3: e242.
32. Check, E. 2005. A crucial test. *Nat. Med.* 11: 243–244.
33. McFarland, T. J., Y. Zhang, B. Appukuttan, and J. T. Stout. 2004. Gene therapy for proliferative ocular diseases. *Expert Opin. Biol. Ther.* 4: 1053–1058.
34. Zhang, W., H. Yang, X. Kong, S. Mohapatra, H. San Juan-Vergara, G. Hellermann, S. Behera, R. Singam, R. F. Lockey, and S. S. Mohapatra. 2005. Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. *Nat. Med.* 11: 56–62.
35. Rossi, J. J. 2006. RNAi as a treatment for HIV-1 infection. *BioTechniques* Suppl: 25–29.
36. Raoul, C., S. D. Barker, and P. Aebischer. 2006. Viral-based modelling and correction of neurodegenerative diseases by RNA interference. *Gene Ther.* 13: 487–495.
37. Wood, M. J., B. Trulzsch, A. Abdelgany, and D. Beeson. 2003. Therapeutic gene silencing in the nervous system. *Hum. Mol. Genet.* 12(Spec. No. 2): R279–R284.
38. Teran, L. M., M. Mochizuki, J. Bartels, E. L. Valencia, T. Nakajima, K. Hirai, and J. M. Schroder. 1999. Th1- and Th2-type cytokines regulate the expression and production of eotaxin and RANTES by human lung fibroblasts. *Am. J. Respir. Cell. Mol. Biol.* 20: 777–786.
39. Zhang, X., P. Shan, D. Jiang, P. W. Noble, N. G. Abraham, A. Kappas, and P. J. Lee. 2004. Small interfering RNA targeting heme oxygenase-1 enhances ischemia-reperfusion-induced lung apoptosis. *J. Biol. Chem.* 279: 10677–10684.
40. Chien, P. Y., J. Wang, D. Carbonaro, S. Lei, B. Miller, S. Sheikh, S. M. Ali, M. U. Ahmad, and I. Ahmad. 2005. Novel cationic cardiolipin analogue-based liposome for efficient DNA and small interfering RNA delivery in vitro and in vivo. *Cancer Gene Ther.* 12: 321–328.
41. Leng, Q., P. Scaria, J. Zhu, N. Ambulos, P. Campbell, and A. J. Mixson. 2005. Highly branched HK peptides are effective carriers of siRNA. *J. Gene Med.* 7: 977–986.
42. Hu-Lieskova, S., J. D. Heidel, D. W. Bartlett, M. E. Davis, and T. J. Triche. 2005. Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. *Cancer Res.* 65: 8984–8992.
43. Czauderna, F., M. Fechtner, S. Dames, H. Aygun, A. Klippel, G. J. Pronk, K. Giese, and J. Kaufmann. 2003. Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. *Nucleic Acids Res.* 31: 2705–2716.
44. Jackson, A. L., S. R. Bartz, J. Schelter, S. V. Kobayashi, J. Burchard, M. Mao, B. Li, G. Cavet, and P. S. Linsley. 2003. Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* 21: 635–637.
45. Sledz, C. A., M. Holko, M. J. de Veer, R. H. Silverman, and B. R. Williams. 2003. Activation of the interferon system by short-interfering RNAs. *Nat. Cell. Biol.* 5: 834–839.
46. Reid, T., R. Warren, and D. Kirn. 2002. Intravascular adenoviral agents in cancer patients: lessons from clinical trials. *Cancer Gene Ther.* 9: 979–986.
47. Soutschek, J., A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, et al. 2004. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 432: 173–178.
48. Lorenz, C., P. Hadwiger, M. John, H. P. Vornlocher, and C. Unverzag. 2004. Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells. *Bioorg. Med. Chem. Lett.* 14: 4975–4977.
49. Sanderson, C. J. 1992. Interleukin-5, eosinophils, and disease. *Blood* 79: 3101–3109.
50. Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, D. M. Rennick, D. Sheppard, M. Mohrs, D. D. Donaldson, R. M. Locksley, and D. B. Corry. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282: 2261–2263.