

Novel Ribbon-Type Nuclear Factor of Activated T Cells Decoy Oligodeoxynucleotides Preclude Airways Hyperreactivity and Th2 Cytokine Expression in Experimental Asthma

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Key Words

Nuclear factor of activated T cells · Transcription factor · T helper 2 cytokines · Adoptive transfer · Decoy · Gene therapy · Allergic airway inflammation · Experimental asthma

Abstract

Background: Nuclear factor of activated T cells (NFAT) is required for the differentiation of Th2 responses, so we examined its role in mouse experimental asthma and tested the hypothesis that an NFAT blockade with a decoy against NFAT can prevent asthma progression. **Objective:** To determine the effects of the NFAT decoy oligodeoxynucleotides (ODNs) on the development of airway inflammation, we designed a novel ribbon-type ODN containing two binding sites for NFAT in a single decoy molecule without an open end, which is more stable than a conventional decoy, and largely preserved its structural integrity in the presence of nucleases. **Methods:** Ribbon-type NFAT decoy ODNs were transfected into ovalbumin (OVA)-sensitized CD3+ T cells in vitro. OVA-immunized mice received these cells by intraperitoneal in-

jection. Airway hyperreactivity (AHR) was measured and the transfected CD3+ T cells' responses to the airways were characterized. **Results:** Development of AHR after OVA challenge was effectively abolished after adoptive transfer of ribbon-type NFAT decoy ODN transfected CD3+ T cells. Transfer of ribbon-type decoy significantly reduced the number of inflammatory cells and the concentrations of IL-4, IL-5 and IL-13, but not IFN- γ , in the bronchoalveolar lavage of the recipient mice. **Conclusion:** These results suggest the inhibitory effect of ribbon-type decoy ODNs against NFAT on the induction of bronchial asthma. Adoptively transferred CD3+ T cells, which are transfected with NFAT decoy, may be an effective strategy for the treatment of asthma.

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Introduction

Allergic asthma is characterized by episodic airways obstruction, bronchial hyperreactivity and a local accumulation of inflammatory cells, particularly CD3+ T lymphocytes and eosinophils [1]. Colocalization studies

have established that resident T lymphocytes are associated with a specific profile of cytokine expression, including IL-4, IL-5 and IL-13. These Th2-type cytokines are believed to orchestrate local inflammatory response through the recruitment and activation of eosinophils and the prolongation of eosinophil survival within the bronchial mucosa [1]. Implicated in the transcriptional regulation of these cytokine genes is the transcription factor of nuclear factor of activated T cells (NFAT). Binding sites for NFAT have been found in the regulatory domains of many inducible sites for conventional Rel family proteins, or as so-called composite binding sites to which NFAT binds as a complex with AP-1 proteins. Among the genes described to be regulated by NFAT in T cells are IL-4 [2], IL-5 [3], GM-CSF [4], TNF- α [5, 6], IL-13 [7] and CD40 ligand [8]. The family of cytoplasmic components of NFAT consists of NFATc1, NFATc2, NFATc3 and NFATc4 [9–14]. Interestingly, the targeted disruption of individual *Nfat* genes in mice leads to the development of quite disparate phenotypes. NFATc1-deficient T lymphocytes display reduced proliferation and IL-4 production, finally leading to impaired Th2 responses [15, 16]. In contrast, mice deficient for NFATc2 mount enhanced Th2 responses [17, 18]. Therefore, NFAT does not qualify as a bona fide regulatory factor to discriminate Th1- or Th2-specific gene transcription. Nonetheless, Diehl et al. [19] demonstrated that the inhibition of NFAT exclusively in T cells impairs the development of a Th2 immune response in vivo and prevents the lung pathology when using dominant negative NFAT-transgenic mice, which inhibit endogenous NFAT DNA binding and NFAT-mediated transcription. This study suggests that the activation of NFAT in T cells is required for allergic airway inflammation.

It has been shown that short sequences of DNA containing the consensus binding site, even in the absence of surrounding DNA, can bind transcription protein in a highly specific manner. Specific DNA sequences have been used successfully as 'decoys' to bind specific transcription factors in culture cells or in vivo [20, 21], rendering the transcription factors incapable of subsequent binding to the promoter region of the target genes. Therefore, the use of transcription factor decoy as a tool to study gene expression in the asthma model was demonstrated for STAT-1 [22] and AP-1 [23]. These decoy oligonucleotides that were employed for the experiments were phosphorothioate-capped oligonucleotides. These decoy oligonucleotides were not truly stable, possibly explaining the partial and short-term decoy effect. In addition,

the toxicity of phosphorothioated-modified oligodeoxynucleotides (ODNs) may also be relevant. Higher doses over prolonged periods of time may cause kidney damage, as evidenced by the proteinuria and leukocytes in the urine of the animals [24]. In the present study, we devised decoy oligonucleotides with an enhanced stability and decoy effect but without the problems usually associated with modified decoy ODNs. A combination of one identical decoy oligonucleotide molecule to NFAT was adopted to construct a large decoy molecule with a loop and stem. Two of the decoy oligonucleotides were enzymatically joined to form a ribbon-type decoy ODNs to avoid exonuclease digestion. Ribbon-type decoy ODNs were administered to an experimental mice asthma model using the adoptive transfer method. This study explores the stability and sequence-specific inhibition effects of ribbon-type NFAT decoy ODNs on the transactivation of essential Th2-type cytokine regulatory genes, lymphocyte and eosinophil infiltration. We provided a novel potential therapeutic strategy for the prevention of allergic airway inflammation here.

Materials and Methods

Construction of Decoy ODNs

The sequences of phosphorothioate double-stranded linear-form decoy ODNs, ribbon-type decoy ODNs against the NFAT binding site and mutated ODNs are as follows: phosphorothioate double-strand linear-form decoy ODNs (lin), 5'-TTAAATTTTCCAATGTAA-3' and 3'-ATTTTAAAAGGTTACATTT-5'; ribbon-type decoy ODNs (Ri), 5'-TTAAATTTTTC CAATGTAAAAAATTTACATTGGAAAATT-3' and ribbon-type scrambled decoy ODNs (scr), 5'-ATATTCATAAGATAC-ATATTAAAAAATATGTATCTTATGA-3'. Ribbon-type NFAT ODNs were predicted to form a stem-loop structure. The stem is formed by the complementary sequences at both ends of each ODN (fig. 1a–c).

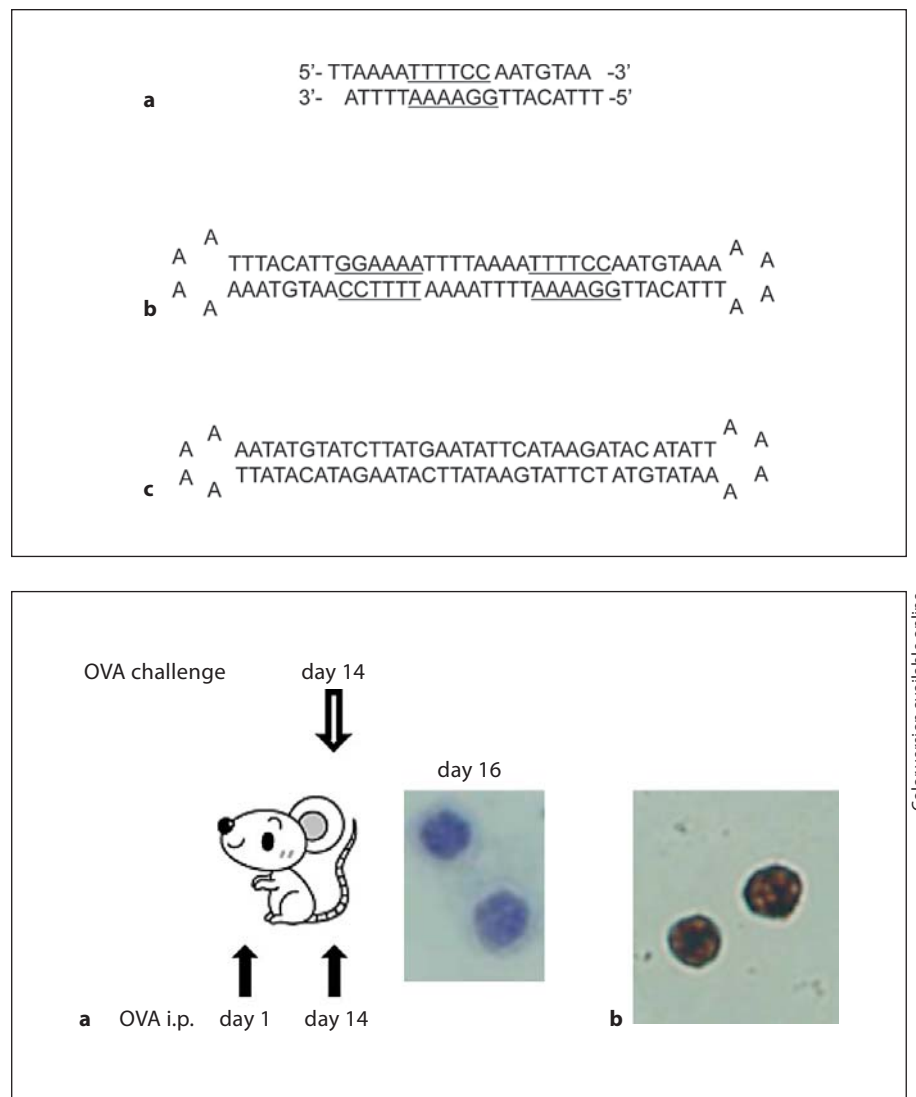
ODNs were ligated for 2 h at a temperature range of 95–23°C. Following the addition of T4 DNA ligase (Promega, Madison, Wisc., USA), the mixture was incubated for 24 h at 16°C to generate an NFAT ODN comprised of two loops and one stem containing two NFAT consensus sequences. Thereafter, NFAT ODNs were labeled by biotin (Vector Laboratories, Burlingame, Calif., USA) to count gene-transferred cells.

Stability Test of Ribbon-Type ODNs

To examine the stability of ribbon-type ODNs, 5 μ g of linear and ribbon-type ODNs were incubated with exonuclease III for 120 h at 37°C. The ODNs were extracted with phenol, chloroform and isoamyl alcohol (Invitrogen, Carlsbad, Calif., USA), then examined on a 10% agarose gel electrophoresis and stained with ethidium bromide.

Fig. 1. Scheme for the construction and sequences of the decoy ODNs used in this study. The NFAT recognition sequences are underlined in each nucleotide. **a** Linear-form decoy ODNs containing the NFAT consensus sequence have been shown to bind the NFAT transcription factor. **b** Ribbon-type decoy ODNs were predicted to form a stem-loop structure. The stem is formed by complementary sequences at both ends of each ODNs. **c** Scrambled decoy ODNs have no NFAT recognition sequences.

Fig. 2. Schematic summary of the experimental protocol of donor mice. **a** Mice were immunized on days 1 and 14 of the protocol by an intraperitoneal (i.p.) injection of 0.5 ml aluminum hydroxide-precipitated antigen containing 8 µg OVA absorbed overnight at 4°C to 4 mg of aluminum hydroxide in 0.9% saline. On day 14, mice were exposed to an aerosol of 5 mg/ml OVA in 0.9% saline for 1 h in a plastic chamber. On day 16, the mice were killed, the spleens were excised, and the splenocytes were disaggregated. The washed splenocytes were cultured in the presence of 200 µg/ml OVA to generate OVA-specific CD3+ T cells. Purified CD3+ T cell populations were also analyzed for OVA-specific cytokine production. The purity of the enriched CD3+ T cell fraction was uniformly >96% (**inset**). **b** CD3+ T cells were transfected with 100 nM of decoy ODNs combined with a hemagglutinating virus of Japan (HVJ) envelope vector. NFAT decoy ODNs-transferred CD3+ T cells were counted as 85–93% of all CD3+ T cells 5 h after transfection.



Generation of Sensitized CD3+ T Cells from Donor Mice

Six- to eight-week-old female BALB/c mice were used in all experiments and were obtained from a special pathogen-free facility (Japan SLC, Shizuoka, Japan). Mice were treated according to the Iwate Medical University Animal Welfare guidelines and housed in an approved containment facility. As donors, the mice were immunized by intraperitoneal injection of 8 µg ovalbumin (OVA; Sigma Chemical Co., St. Louis, Mo., USA) per 4 mg aluminium hydroxide (Wako Chemical Co., Tokyo, Japan) in a 0.9% saline on days 1 and 14. On day 14, the mice were exposed to an aerosol of 5 mg/ml OVA in 0.9% saline for 1 h in a plastic chamber (fig. 2a). On day 16, the mice were killed, the spleens were excised and the splenocytes were disaggregated. Erythrocytes were lysed (R&D Systems, Minneapolis, Minn., USA) and the washed splenocytes were cultured for 5 days at 37°C in the presence of 200 µg/ml OVA to generate OVA-specific CD3+ T cells and were then isolated using high-gradient magnetic separation columns

(Miltenyi Biotec, Gladbach, Germany); 2×10^7 /mouse purified CD3+ T cells were then washed and resuspended in Dulbecco's phosphate-buffered saline (Invitrogen). Purified CD3+ T cell populations were also analyzed for OVA-specific cytokine production. The purity of the enriched CD3+ T cell fraction was uniformly >96% (fig. 2a, inset).

In vitro Gene Transfer

CD3+ T cells were fed with fresh culture medium the day before ODNs were added and washed twice with Opti-MEM (Invitrogen), prior to the experiment. Cells were transfected with 100 nM of decoy ODNs combined with a hemagglutinating virus of Japan envelope vector according to the manufacturer's instructions (Ishihara Sangyo Kaisha, Osaka, Japan). To determine the transfection efficiencies, ODNs were labeled with biotin (Vector Laboratories) and then the cells were incubated at 37°C for 5 h. After the addition of fresh medium containing a 10% fetal bovine

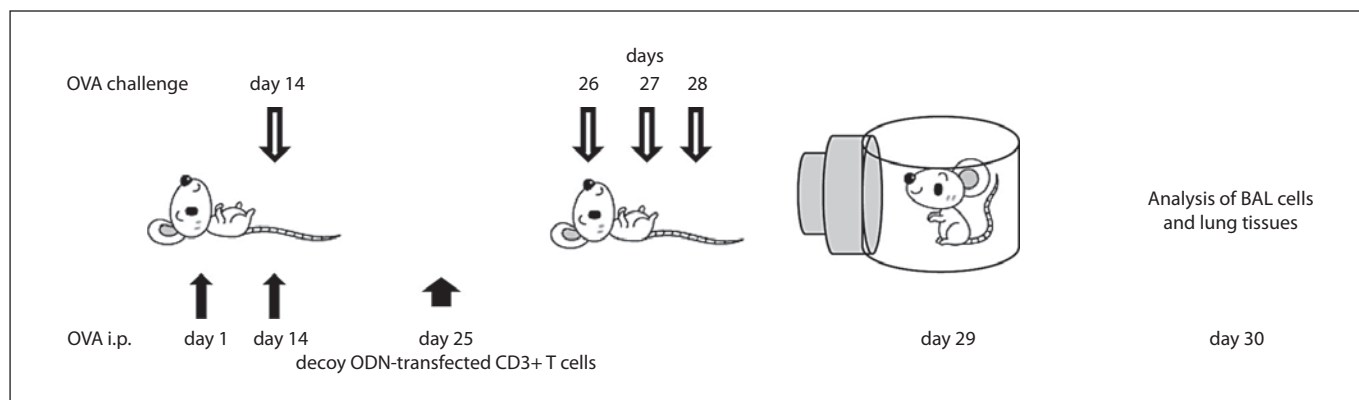


Fig. 3. Schematic summary of the experimental protocol of recipient mice. Administration of NFAT decoy ODNs induction of airway hyperresponsiveness, and analysis of BAL fluid. Recipient mice were immunized on days 1 and 14. On day 14, mice were exposed to an aerosol of OVA as mentioned for the donor mice. On day 25, 1×10^7 or 1×10^8 ODN-transfected CD3+ T cells, or no ODN-transfected CD3+ T cells, which were derived from donor mice, and OVA sensitized strongly in vitro, were injected intraperitoneally (i.p.) to recipient mice. Twenty-four hours after the adoptive transfer, mice were exposed to an aerosol of OVA, which

was repeated every day for 3 days. Airway hyperresponsiveness to methacholine was measured 24 h after the last aeroallergen challenge. Twelve hours after the measurement of airway responsiveness, BAL was performed. One hundred thousand viable BAL cells were cytocentrifuged onto slides and stained with May-Grünwald-Giemsa solution. NFAT decoy ODNs-transferred cells were detected using horseradish peroxidase. Supernatants were analyzed by specific ELISA for concentration of IL-4, IL-5, IL-13 and IFN- γ .

serum, cells were maintained in a CO₂ incubator until use. Gene-transfected CD3+ T cells were counted as 85–93% of all CD3+ T cells 5 h after transfection (fig. 2b).

Adoptive Transfer of NFAT Decoy ODNs and Induction of Airway Hyperreactivity by OVA Challenge

The recipient mice were immunized by an intraperitoneal injection of 8 μ g OVA/4 mg alhydrogel in a 0.9% saline on days 1 and 14. On day 14, mice were exposed to an aerosol of OVA in a 0.9% saline as mentioned previously. On day 25, 1×10^7 or 1×10^8 CD3+ T cells, containing either decoy ODNs or no decoy ODNs, were OVA sensitized strongly in vitro, then injected intraperitoneally into the immunized recipient mice. Twenty-four hours after the adoptive transfer, the mice were exposed to an aerosol of 10 mg/ml OVA in a 0.9% saline for 30 min and then every day for 3 days. Airway hyperreactivity (AHR) to methacholine was measured 24 h after the last aeroallergen challenge (day 29; fig. 3). Airway responsiveness was assessed by methacholine-induced airflow obstruction of conscious mice placed in whole-body plethysmography (Buxco Electronics, New York, N.Y., USA) as previously described [25]. The respiratory parameters were obtained by exposing mice to a 0.9% saline aerosol, followed by incremental doses (10–40 mg/ml) of aerosolized methacholine. Airflow obstruction was monitored and analyzed by system software (Buxco Electronics). Results are expressed as the percentage of the baseline-enhanced pause values after a 0.9% saline exposure.

Collection and Analysis of Bronchoalveolar Lavage Fluid

Twelve hours after the measurement of AHR, bronchoalveolar lavage (BAL) was performed as described (fig. 3) [26]. All BAL fluid (1 ml of saline solution 2 times) was collected, and BAL cells in 100- μ l aliquots were counted. Cell types were identified based

on morphological criteria. The absolute numbers of each cell type were calculated. One hundred thousand viable BAL cells were cytocentrifuged onto slides and stained with May-Grünwald-Giemsa solution as described previously [26]. NFAT decoy ODNs transferred cells were detected using horseradish peroxidase and 3,3'-diaminobenzidine (Dako Japan Inc., Tokyo, Japan). To determine cytokine concentrations, we centrifuged samples at 1,200 rpm for 5 min at 4°C. Supernatants were analyzed by specific ELISA for concentrations of IL-4, IL-5, IL-13 and IFN- γ with commercially available kit systems (RayBiotech Inc., Norcross, Ga., USA).

Lung Histology

Twelve hours after the measurement of AHR, unmanipulated (not subjected to BAL) lungs were fixed in 4% paraformaldehyde-PBS, and processed in a standard manner. Tissue sections were stained with hematoxylin and eosin, and examined for pathological changes under a light microscope at $\times 100$.

Preparation of Cell Lysates and Probe for Electrophoretic Mobility Shift Assay

CD3+ T cells were isolated from BAL using high gradient magnetic separation columns as mentioned above. Nuclear extracts were prepared from CD3+ T cells as described previously [27]. Complementary oligonucleotides 5'-AAATTTTCCAATGTAAAC-3' and 5'-ACATTGGAAATTTTATT-3' containing NFAT binding sites in the mouse IL-4 promoter region with 4-base overhangs were combined, boiled in 0.5 M NaCl and cooled to room temperature. The following primer pairs were used for electrophoretic mobility shift assay (EMSA): IL-5 promoter, 5'-GCACTGGAAACCCTGAGT-3' and 5'-AGGGTTTCCAGTGCCTG-3'; IL-13 promoter, 5'-GGATTTTCCAAAAAGAA-3' and 5'-TTTTTGGAAATCCAGT-3'; IFN- γ promoter, 5'-

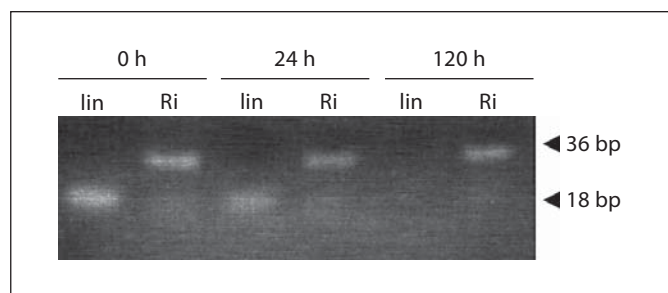


Fig. 4. Stability of linear-form NFAT decoy ODNs and ribbon-type ODNs in the presence of exonuclease III. Stability test of linear-form NFAT decoy ODNs and ribbon-type ODNs after 0, 24 and 120 h at 37°C. Although ribbon-type NFAT decoy ODNs could be detected after 24 h of incubation, the linear-form decoy ODNs diminished. lin = Linear-form decoy ODNs; Ri = ribbon-type decoy ODNs.

AATTTCCAGCCCCCA-3' and 5'-GGCTGGAAATTGCTT-3'. Duplexes were end-labeled with [α - ^{32} P] dCTP with terminal transferase (Promega). Ten micrograms of nuclear extract and ^{32} P-labeled double-strand oligomers (50 fmol) were incubated in binding buffer with or without unlabeled competitors for 30 min at room temperature. After a 30-min prerun of the gel, samples were loaded onto 7.5% native polyacrylamide gels and run at 120 V for 3 h at 4°C. Gels were dried and visualized by autoradiography [28].

RNA Isolation and RT-PCR Analysis

Total RNA was extracted from BAL CD3+ T cells by RNeasy (Qiagen, Valencia, Calif., USA) according to the procedure provided by the vender. An RT-PCR procedure was performed as previously described to determine the relative quantities of mRNA for various cytokines [29]. The following primer pairs were used for amplification: IL-4, forward 5'-ACGGAGATGGATGTGC-CAAACGTC-3' and reverse 5'-CGAGTAATCCATTTGCATG-ATGC-3'; IL-5, forward 5'-CCCACAAAAGTGTGCAATG-3' and reverse 5'-GGTATGTGATCCTCCTGCGT-3'; IL-13, forward 5'-CAGCTCCCTGGTTCTCTCAC-3' and reverse 5'-TG-TGTCCACAGCTGAGATGC-3'; IFN- γ , forward 5'-ACTGGC-AAAAGGATGGTGAC-3' and reverse 5'-TGAGCTCATTGA-ATGCTTGG-3'; β -actin, forward 5'-GTGGGGCGCCCCAG-GCACCA-3' and reverse 5'-CTCCTTAATGTCACGCACGA-TTTC-3'. The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. PCRs without RT samples were performed as a negative control. Signals were quantified by densitometric scanning and normalized against β -actin. Pretreatment for the no ODNs group is standardized to 1.0. The primers for all genes were purchased from Invitrogen.

Statistical Analysis

All data are expressed as means $\pm$ SEM. The one-way ANOVA and post hoc tests were used to assess the statistical significance among the groups. The Student unpaired t test was also used to examine the changes of the mRNA expression of cytokines between the no ODNs treatment group and the ribbon-type decoy ODNs treatment group after testing data for the normality of the population using the Kolmogorov-Smirnov test.

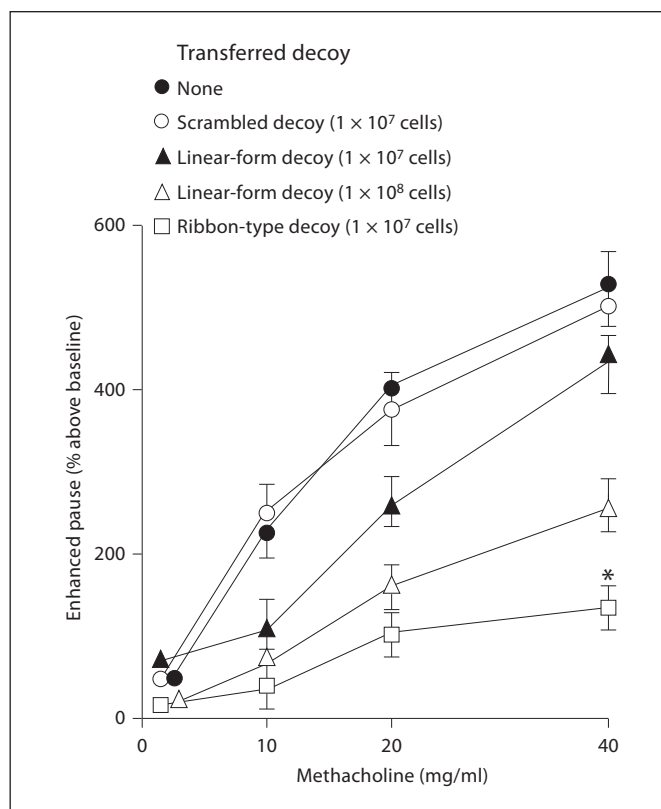


Fig. 5. Ribbon-type NFAT decoy ODNs, but not linear-form ODNs, decrease AHR. BALB/c mice received NFAT decoy ODNs intraperitoneally plus inhalation of OVA. Control mice received either scrambled decoy ODNs or no ODNs. Four days after the adoptive transfer, AHR in response to increasing concentrations of inhaled methacholine was measured in a whole-body plethysmograph. PBS or increasing concentrations of methacholine (10–40 mg/ml) were nebulized for 1.5 min, and specific airway resistance measurements were taken and averaged for 1.5 min after each nebulization. Data is expressed as a percent above baseline (mean $\pm$ SEM); $n > 5$ for each data point. Cell transfer without intranasal administration of OVA had no effect on AHR (not shown).

Results

Comparison of Stability of Linear-Form and Ribbon-Type NFAT Decoy ODNs

We initially examined the molecular stability of synthesized ribbon-type NFAT ODNs in the presence of exonuclease III (fig. 4). Exonuclease constitutes most of the nuclease activity in the cytoplasm and serum. We tested the stability of the decoy ODNs by incubation with exonuclease III. As expected, the linear form of ODNs was significantly hydrolyzed after 24 h of incubation. However, ribbon-type ODNs remained largely intact follow-

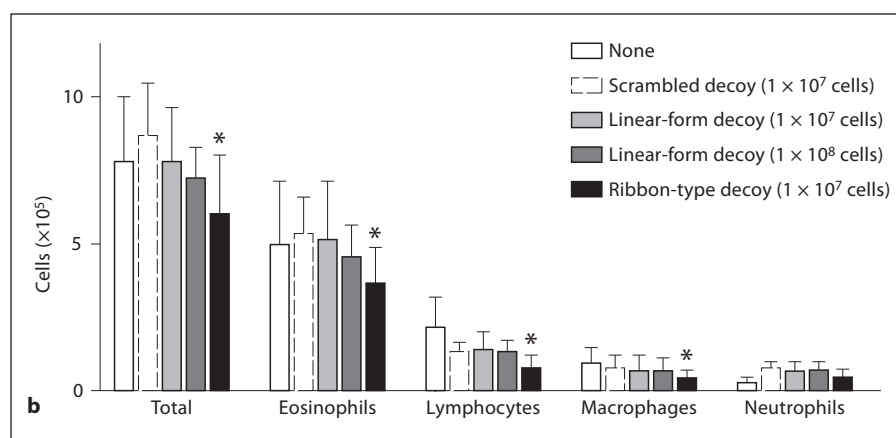
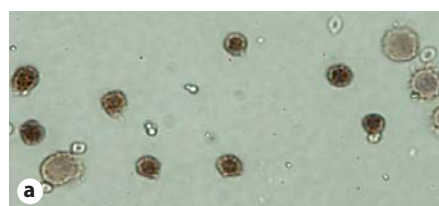


Fig. 6. Effect of NFAT decoy ODNs inhibition on inflammatory cell infiltration in the murine model of asthma. BALB/c mice were immunized to OVA (8 μ g) in alhydrogel on days 1 and 14, and recipient mice were transferred with CD3+ T cells (1×10^7 or 1×10^8) containing NFAT decoy ODNs intraperitoneally on day 25, then subjected to challenge with OVA in saline on days 26, 27

and 28. BAL was performed on day 30 with two aliquots of 1 ml PBS per mouse. **a** Biotinylated NFAT-positive cells in BAL fluid were detected with diaminobenzidine. **b** Inflammatory cells in BAL fluid were stained with May-Grünwald-Giemsa and counted with a hemocytometer. The data are expressed as means $\pm$ SEM of values from 6 mice per group.

ing incubation with exonuclease III, indicating greatly improved stability compared to linear-form NFAT decoy ODNs.

Treatment with NFAT Decoy ODNs Abrogates AHR in OVA-Challenged Mice

We next analyzed whether treatment with NFAT decoy ODNs would affect AHR in OVA-challenged mice. AHR was assessed by measuring the methacholine-induced airflow obstruction in a whole-body plethysmograph, which involves having an animal roam freely in a closed container while only the pressure inside the container is measured. Cyclic variations in container pressure occurs as gas is inspired and expired, and changes in the nature of these pressure variations are taken as indicatives of changes in lung function. However, these variations in container pressure have an indeterminate, and possibly little, bearing on mechanical lung function, and in fact are much more relevant to the control of breathing as reflected in the respiratory pattern (that is, breathing frequency and tidal volume). These factors severely limit the specificity and precision of unrestrained plethysmography as a means of assessing lung function [30]. Nevertheless, we did not input impedance in anesthetized, paralyzed mice, but used unrestrained plethysmography in freely moving animals because BAL cells were analyzed by opening the tracheas and lungs 12 h following the measurement of airway resistance. Baseline

levels of airway reactivity were not significantly different among the no decoy, scrambled decoy, linear-form decoy and ribbon-type decoy ODNs transfected CD3+ T cells adoptively transferred to recipient mice. However, OVA inhalation induced a marked AHR in the no decoy and scrambled decoy adoptively transferred mice (fig. 5). In contrast, in the presence of ribbon-type NFAT decoy ODNs, AHR was abrogated, suggesting that ribbon-type NFAT decoy ODNs act to regulate enhanced reactivity.

Adoptive Transfer of Biotinylated NFAT Decoy ODNs into Airways Decreased OVA-Induced Allergic Inflammatory Cell Infiltration in the Airway of Ribbon-Type Decoy ODN-Administered Mice

BAL fluid was examined for inflammatory cell infiltration. Biotinylated NFAT decoy ODNs were only found in the nucleus of lymphocytes in BAL cells (fig. 6a).

As can be seen in figure 6b, the majority of infiltrated cells were eosinophils in OVA-challenged mice. The absolute numbers of eosinophils, macrophages and neutrophils were calculated as described in the Materials and Methods section. The number of leukocytes in the BAL fluid was significantly reduced in ribbon-type decoy ODN-administered mice. Eosinophilia was strongly decreased in mice treated with NFAT decoy ODNs compared to the other groups. In addition to a blunted eosinophil recruitment, lymphocyte and macrophage recruitment were also significantly attenuated in the

Fig. 7. Histological analysis of lung sections from the murine model of asthma. BALB/c mice were immunized with OVA and transferred with CD3+ T cells (1×10^7 or 1×10^8) containing NFAT decoy ODNs intraperitoneally, and then exposed to an OVA mist as in figure 6. The lungs were fixed and stained with hematoxylin and eosin in a standard manner. Perivascular and peribronchial inflammatory cells were assessed (original magnification, $\times 100$). **a** Adoptive transfer of 1×10^7 CD3+ T cells, which were transfected without ODNs. **b** Adoptive transfer of 1×10^7 CD3+ T cells, which were transfected with scrambled ODNs. **c** Adoptive transfer of 1×10^8 CD3+ T cells, which were transfected with linear-form NFAT decoy ODNs. **d** Adoptive transfer of 1×10^7 CD3+ T cells, which were transfected with ribbon-type NFAT decoy ODNs. Figures highlight the presence (**a, b**) or absence (**c, d**) of perivascular and peribronchial inflammation.

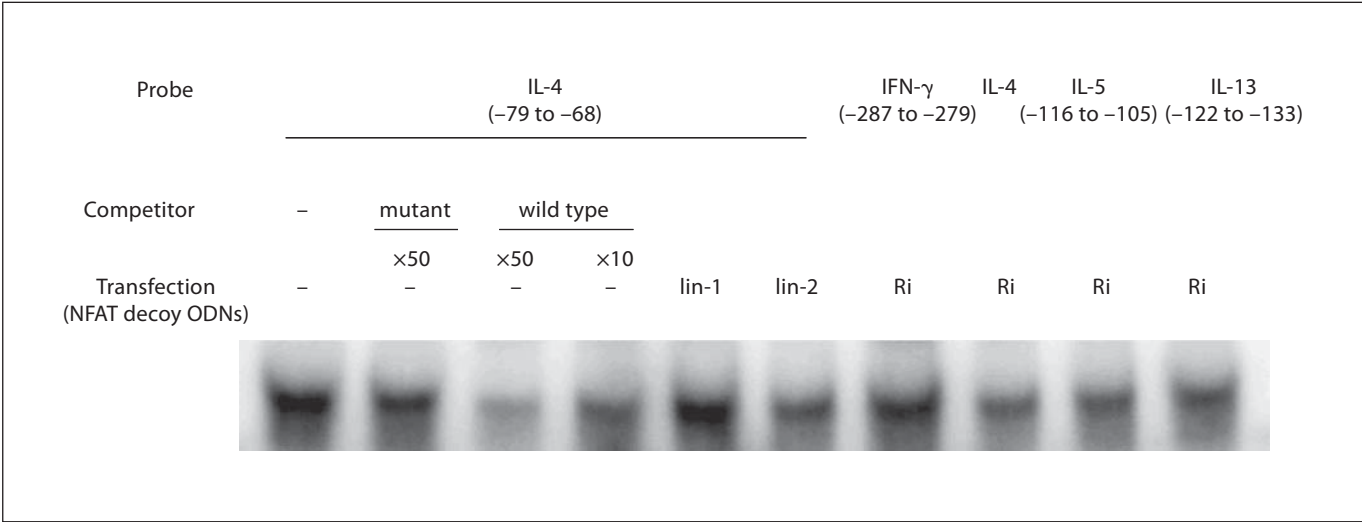
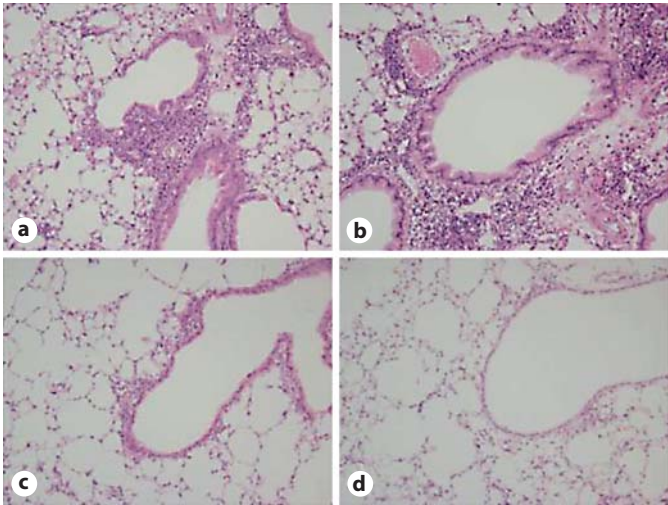


Fig. 8. Effects of NFAT decoy ODNs on DNA binding activity in CD3+ T cells in BAL fluid. EMSA with nuclear extracts from CD3+ T cells in BAL of mice pretreated with NFAT decoy ODNs at varying numbers and forms of decoy ODNs were assessed. The nuclear extracts from CD3+ T cells in BAL were incubated with ^{32}P -labeled, double-stranded oligomers containing the NFAT binding sites in the IL-4 (lanes 1–6, and 8), IFN- γ (lane 7), IL-5 (lane 9), IL-13 (lane 10) promoter regions. Lanes 1–4: competition assays with unlabelled homologues or mutant oligonucleotides (lane 1, no competitor; lane 2, 50-fold excess mutant competitor; lane 3, 50-fold excess unlabelled specific competitor; lane 4, 10-fold excess unlabelled specific competitor). Lanes 5 and 6: NFAT binding activity in CD3+ T cells in BAL, when CD3+ T cells,

which were transfected with linear-form NFAT decoy ODNs, were intraperitoneally transferred to recipient mice (lane 5, 1×10^7 ; lane 6, 1×10^8 CD3+ T cells were transferred). Lanes 7–10: radio-labeled oligonucleotides containing NFAT binding sites in promoter regions (lane 7, IFN- γ promoter region; lane 8, IL-4 promoter region; lane 9, IL-5 promoter region; lane 10, IL-13 promoter region). lin-1 = Adoptive transfer of 1×10^7 CD3+ T cells, which were transfected with linear-form NFAT decoy ODNs; lin-2 = adoptive transfer of 1×10^8 CD3+ T cells, which were transfected with linear-form NFAT decoy ODNs; Ri = adoptive transfer of 1×10^7 CD3+ T cells, which were transfected with ribbon-type NFAT decoy ODNs.

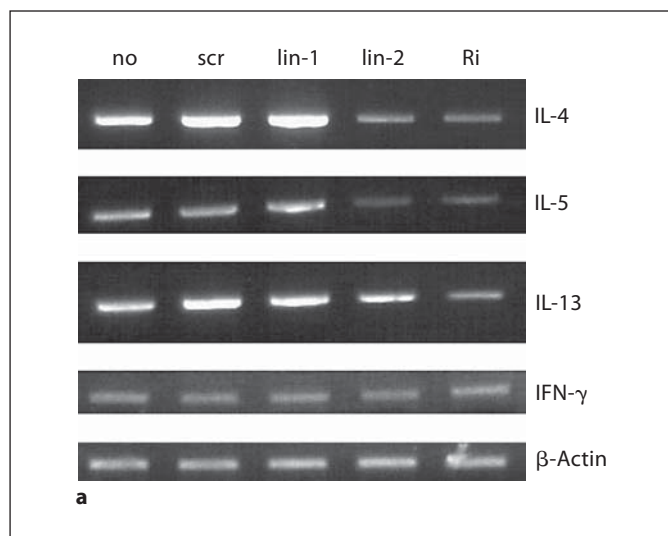
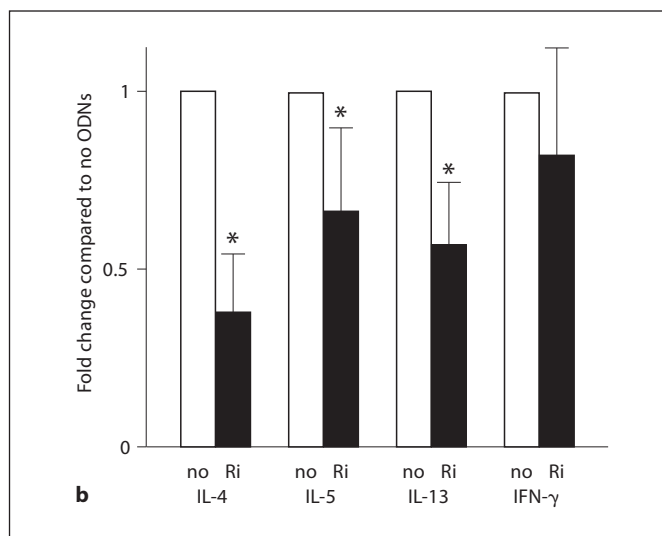


Fig. 9. Ribbon-type NFAT decoy ODNs impair Th2 cytokines mRNA expressions. NFAT decoy ODN-transfected CD3+ T cells were adoptively transferred into recipient mice (1×10^7 or 1×10^8 per mouse) peritoneally. CD3+ T cells from BAL of recipient mice were submitted to analysis of mRNA expression of indicated cytokines. Expression of IL-4, IL-5 and IL-13 mRNA in CD3+ T cells was decreased when ribbon-type NFAT decoy ODNs were adoptively transferred. CD3+ T cell cytokine levels were determined by mRNA expressions. **a** Representative of 6 separate experiments. **b** Summary of data (means $\pm$ SEM) from 6 experiments.



* $p < 0.05$ between adoptive transfer of no ODNs and ribbon-type NFAT decoy ODNs groups. no = Adoptive transfer of 1×10^7 CD3+ T cells, which were transfected without ODNs; scr = adoptive transfer of 1×10^7 CD3+ T cells, which were transfected with scrambled ODNs; lin-1 = adoptive transfer of 1×10^7 CD3+ T cells, which were transfected with linear-form NFAT decoy ODNs; lin-2 = adoptive transfer of 1×10^8 CD3+ T cells, which were transfected with linear-form NFAT decoy ODNs; Ri = adoptive transfer of 1×10^7 CD3+ T cells, which were transfected with ribbon-type NFAT decoy ODNs.

airways of ribbon-type decoy ODN-treated mice. By contrast, treatment with scrambled and linear-form decoy ODNs had no significant effect on eosinophil, lymphocyte and macrophage influx. These findings were substantiated by evaluation of the lung histology of animals from each group and confirming the appearance of peribronchial and perivascular inflammation in recipient mice (fig. 7).

Ribbon-Type NFAT Decoy ODNs Reduce IL-4, IL-5 and IL-13 DNA Binding Activity

To determine whether ribbon-type decoy ODNs effectively blocked the NFAT binding activity in CD3+ T cells, we performed EMSA. Incubation of nuclear extracts from CD3+ T cells in BAL with double-strand oligonucleotides containing the NFAT binding site in the mouse IL-4, IL-5, IL-13 and IFN- γ promoter regions showed mobility-shifted complexes. NFAT decoy ODNs, which were adoptively transferred, decreased NFAT binding activity to the IL-4, IL-5 and IL-13 but not to the IFN- γ promoter region oligonucleotides. In both 10- and 50-fold molar excess of unlabeled specific competitor, IL-4

promoter oligonucleotides abolished binding to labeled NFAT doses dependently, whereas 50-fold molar excesses of mutant competitor oligonucleotides had no effect on NFAT binding activity (fig. 8). The same results were obtained with the IL-5 and IL-13 promoter oligonucleotides (not shown). NFAT is inactivated in nuclear extracts from ribbon-type NFAT decoy ODNs transfected CD3+ T cells and is incapable of interacting with the fragments of the IL-4, IL-5 and IL-13 promoters. These data suggested that the NFAT decoy ODNs may contribute to IL-4, IL-5 and IL-13, but not IFN- γ , downregulation in CD3+ T cells exposed to allergen- or antigen-presenting cells in airways.

Ribbon-Type NFAT Decoy ODNs Control Th2 Cytokines

We have demonstrated that the nuclear proteins of CD3+ T cells taken from BAL were attenuated in the binding activity with promoter regions of Th2 cytokines. To further confirm the critical requirement of NFAT decoy ODNs for interference of Th2 cytokines transcription, Th2 cytokine mRNA were assessed. When the rib-

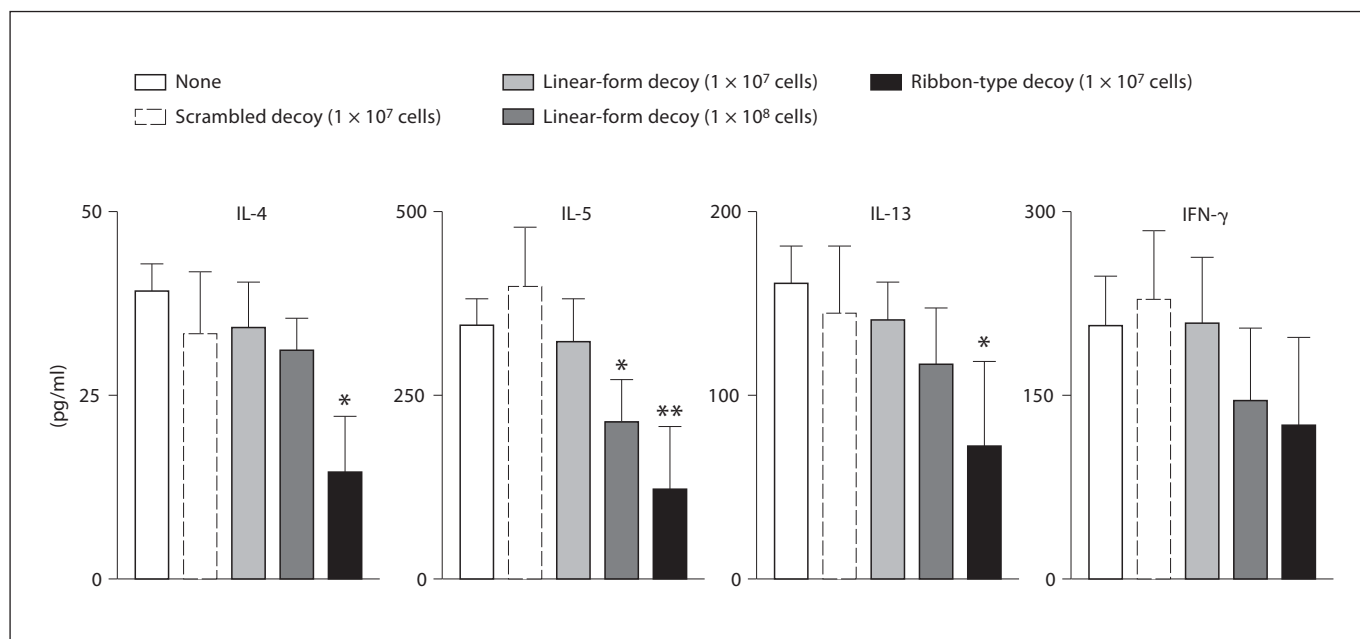


Fig. 10. Treatment with ribbon-type NFAT decoy ODNs suppresses IL-4, IL-5 and IL-13, but not IFN- γ , production in the airways on allergen challenge. NFAT decoy ODN-transfected CD3+ T cells were adoptively transferred into recipient mice (1×10^7 or 1×10^8 per mouse) peritoneally. BAL fluids of recipient mice were submitted to analysis for the expression of indicated cytokines. Concentrations of IL-4, IL-5 and IL-13 in BAL fluid were de-

creased when ribbon-type NFAT decoy ODNs were adoptively transferred. Again, concentration of IL-5 in BAL fluid was decreased when linear-form NFAT decoy ODNs were adoptively transferred using 1×10^8 CD3+ T cells. Data are expressed from 6 experiments. * $p < 0.05$ versus no ODN treatment; ** $p < 0.01$ versus no ODN treatment.

bon-type NFAT decoy ODNs are adoptively transferred, IL-4, IL-5 and IL-13 mRNA expression of CD3+ T cells of BAL were reduced, whereas scramble and linear-form decoy ODNs did not decrease IL-4, IL-5 and IL-13 mRNA expression (fig. 9). In contrast, the level of IFN- γ was not significantly lower than the control's when NFAT decoy ODNs were adoptively transferred. These data indicate that NFAT decoy ODNs potentially have the ability to interfere with the synthesizing of Th2 cytokines, which in turn could influence allergic inflammation.

Treatment with NFAT Decoy ODNs Reduces IL-4, IL-5 and IL-13, but Not IFN- γ , Production in the Airways of OVA-Challenged Mice

We next measured IL-4, IL-5, IL-13 and IFN- γ concentrations in BAL fluid recovered from the mice. As expected in this model, BAL fluid levels of IL-4, IL-5 and IL-13, but not IFN- γ , were markedly decreased in ribbon-type decoy ODN-treated OVA-challenged mice. Treatment with linear-form decoy ODNs affected BAL fluid levels of IL-5. No changes were seen after administration in the scrambled or no ODN groups. This shows that

adoptive transfer of NFAT decoys significantly reduces IL-4, IL-5 and IL-13, but not IFN- γ , production in the lungs of OVA-challenged mice (fig. 10).

Discussion

Several new findings have emanated from this study. First, NFAT decoy ODNs transfected-CD3+ T cells, which were injected peritoneally to donor mice, kept ODNs in the nucleus and assembled in the airways after antigen inhalation. Second, we have shown that the administration of NFAT decoy ODNs markedly inhibited the expression of targeted cytokine genes (IL-4, IL-5 and IL-13). The decrease in the number of eosinophils, lymphocytes and macrophages in the airways was associated with the decreased amount of these cytokines and AHR. Finally, we have demonstrated that the inhibitory effect of ribbon-type NFAT decoy ODNs on allergic airway inflammation was 10 times more potent than that of linear-type phosphorothioated decoy ODNs.

There has been an explosion in the use of transcription factor decoy as a tool for studying gene regulation and as an experimental therapy to treat a variety of pathological conditions [31]. Improvement of this technology by addressing the method and timing of delivery, ODN stability and specificity of actions will facilitate the development of a transcription factor decoy as an important therapeutic modality for airway diseases such as bronchial asthma. Unfortunately, progression of airway inflammation could not be completely inhibited by decoy ODN inhalation, as large amounts of the administered ODNs are eliminated by the lung tissues within a short period of time, thus resulting in a short half-life and low decoy ODN concentration in the target inflammatory cells. To overcome the lack of specificity with regard to the target cells, the main features of appropriate decoy ODN carriers are high accumulation in the target tissue, low uptake rates in normal tissue, low toxicity, the biochemical potential to be linked to decoy ODNs, the release of the drug in the target tissue as well as general availability and affordability. In this study, we have shown that an adoptive transfer system fulfills these requirements for the delivery of decoy ODNs into bronchial mucosa, which is then exposed to antigen. Our preclinical study demonstrates the favorable properties of ribbon-type decoy ODNs and antigen-sensitized lymphocytes to target inflamed airways.

Bronchial asthma is a chronic disease associated with reversible airway obstruction, bronchial hyperreactivity and an abundance of inflammatory cells within the bronchial mucosa, predominantly T cells and eosinophils. In resting T cells, NFAT is highly phosphorylated and resides mainly in the cytosol. A potential ODN-based approach is the use of synthetic double-strand decoy ODNs, which bind to the NFAT in the cytosol, to prevent binding with promoter gene. The therapeutic effectiveness of synthetic double-strand ODNs in modulating specific gene expression largely depends on several factors, including stability, specificity and efficient cellular and tissue uptake of ODNs. One of the obstacles in using the decoy ODN strategy as a pharmaceutical drug is related to the stability of ODNs in cells. Since phosphodiester ODNs and nonphosphorothioate ODNs are precluded because of their instability under physiologic conditions, chemical modifications such as phosphorothioation and methylphosphonation of ODNs have been employed in order to decrease their susceptibility to degradation by exo- and endonucleases. Although the efficacy in inhibiting a large variety of transcription factors has been reported, the use of the phosphorothioated ODNs has brought other problems, such as safety and the cost of production resulting

from that chemical modification. To overcome this, the laboratory of Morishita developed a nonchemical modified decoy ODN targeting NF- κ B by the ligation of the extremities of two single phosphodiester strands that resulted in a dumbbell-shaped structure [32]. Such construction significantly increases the stability of the phosphodiester backbone against nucleases compared to nonphosphorothioated ODNs and has shown to be as efficient as phosphorothioated ODNs in inhibiting the transcription factor NF- κ B in vascular smooth muscle cells. In this study, we have confirmed the inhibitory effect of nonchemically modified ribbon-type decoy ODNs, which could be detected in CD3+ T cells and prevent the expression of Th2 cytokine mRNA and protein in BAL after 4 days of transfection, while the effect of linear ODNs was diminished.

We previously evaluated that the expression of the Th2 cytokine-associated transcription factors, c-Maf and GATA-3-positive T cells, is increased in the bronchial mucosa of the patients with asthma when compared to controls through endobronchial biopsy [33]. c-Maf, the cellular homolog of the avian viral oncogene v-maf, belongs to the AP-1 family of basic region/leucine zipper factors and binds to the consensus site in the proximal IL-4 promoter. GATA-3, a member of the GATA family of zinc finger-type transcription factors, is both necessary and sufficient in initiating Th2 cell differentiation. GATA-3 binds to sites of 3' of *Il4* (DNase I hypersensitivity site VA) and in the *Il4-Il13* intergenic region (conserved noncoding sequence 1) in Th2 cells [34]. GATA-3 binding sites have also been identified in the promoters of *Il5* and *Il13* [35, 36]. This led us to hypothesize that c-Maf or GATA-3 decoy ODNs would contribute to the suppression of allergic airway inflammation in experimental asthma. In the preliminary studies, we could not demonstrate that there was a significant reduction of IL-5 and IL-13, or IL-4 expression in BAL and airway resistance in the mouse model after the administration of c-Maf or GATA-3 decoy ODNs, respectively. While a direct inhibitory transcriptional effect of c-Maf decoy ODNs could easily account for the suppression of only IL-4 transcription, this does not readily explain why the GATA-3 decoy ODNs fail to inhibit IL-4 expression in developing Th2 inflammation. One possibility is that the requirement of GATA-3 for inducing and sustaining IL-4-producing capacity differs for naive T cells undergoing Th2 differentiation and established Th2 cells. Since GATA-3 is involved in chromatin remodeling of the *Il4-Il13* locus, early deletion of *Gata3* could inhibit the opening of the *Il4-Il13* locus and thus limit IL-4 transcription. Again,

deletion of *Gata3* from established Th2 cells has been shown to abolish IL-5 and IL-13, but not IL-4, production. In this study, the expression of Th2 cytokines and AHR was decreased when adoptive transfer of NFAT decoy ODNs was used, providing a possible mechanism for NFAT-dependent maintenance of Th2 cytokine-producing capacity.

The IFN- γ is a Th1 cytokine that generally counteracts the Th2 response. It also has an NFAT binding site in the promoter region; however, NFAT decoy ODNs exhibited a modest impairment in OVA-induced IFN- γ expression in BAL. This discrepancy between IL-4, IL-5, IL-13 and IFN- γ levels appears to be explained by the failure of decoy ODNs to prevent the binding of NFAT with the promoter region of IFN- γ . NFAT binding site 5'ATTCCAGC: positions -287 are defined as important in the regulation of the IFN- γ gene expression and as a critical independent inducer. These NFAT consensus sequence-protein complexes are seen in EMSA and indicate that the decoy ODNs failed to compete for binding to the NFAT protein. This can be explained by the differences of binding affinity of transfected decoy ODNs and promoter regions of IFN- γ .

Taken together, these data suggest that ribbon-type decoy ODN treatment against NFAT is a novel selective

way to simultaneously suppress the production of several Th2 cytokines in asthmatic reactions in vivo. Furthermore, we have demonstrated here that NFAT decoy ODNs' effectiveness is sustained for 5 days, suggesting that NFAT decoy ODNs may be a novel molecular approach for the treatment of inflammatory disorders such as allergic asthma and rhinitis in humans.

Acknowledgements

The present authors would like to acknowledge M. Shibana and M. Niisato (Iwate Medical University School of Medicine) for their help in performing the study. We would also like to thank Prof. R. Morishita and Y. Kaneda (Division of Clinical Gene Therapy, Osaka University Graduate School of Medicine), N.N. Jarjour, W.W. Busse and J.E. Gern (Allergy, Pulmonary and Critical Care Section, University of Wisconsin, Madison, Wisc., USA), and J.S. Malter (Department of Pathology and Laboratory Medicine, University of Wisconsin, Wisc., USA) for their helpful comments.

Disclosure Statement

This study was funded by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

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