

Adoptively transferred late allergic response is inhibited by IL-4, but not IL-5, antisense oligonucleotide

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Background: We have shown previously that the late airways response (LAR) can be transferred by ovalbumin-primed CD4⁺ T lymphocytes in Brown Norway rats. This response is associated with an increase of eosinophils and high expression of T_{H2} cytokines (IL-4 and IL-5) in bronchoalveolar lavage (BAL) fluid.

Objective: In this study we hypothesized that the inhibition of IL-4 or IL-5 production in the CD4⁺ cells transferred to a naive animal could decrease the LAR and prevent airway eosinophilia in response to antigen challenge.

Methods: CD4⁺ cells, purified from the cervical lymph nodes of ovalbumin-sensitized rats, were maintained in culture for 6 hours with medium alone or with 10 µg/mL IL-4 antisense (AS), IL-5 AS, or control AS oligodeoxynucleotide. Then the cells were administrated intraperitoneally to naive rats, which were challenged 2 days later by a 5% ovalbumin aerosol. The lung resistance was measured for 8 hours, and then BAL was performed. Cytospin preparations from BAL cells were assessed for the presence of eosinophils by immunocytochemistry for major basic protein and for IL-4, IL-5, and IFN-γ expression.

Results: In rats injected with IL-4 AS-treated T cells, LAR, eosinophils, and IL-4 and IL-5 expression were significantly decreased compared with the other groups. Only IL-5 expression in BAL fluid was slightly decreased consequent to the transfer of IL-5 AS-treated T cells.

Conclusion: This study demonstrates that, in the CD4⁺ T cell-driven LAR, the early production of IL-4, but not IL-5, by the transferred CD4⁺ cells is essential for the development of the LAR. (*J Allergy Clin Immunol* 1999;104:205-14.)

Key words: Asthma, T_{H2}-type lymphocytes, lung resistance, eosinophils, cytokines, rats

Asthma is a chronic disease characterized by reversible airway obstruction, hyperresponsiveness, and inflammation of airways. Numerous studies have shown

Abbreviations used

APAAP:	Alkaline phosphatase-anti-alkaline phosphatase
AS:	Antisense
AUC:	Area under the curve
BAL:	Bronchoalveolar lavage
BN:	Brown Norway
EAR:	Early airways response
LAR:	Late airways response
MBP:	Major basic protein
NP-Y:	Neuropeptide Y
ODN:	Oligodeoxynucleotide
OVA:	Ovalbumin
R _L :	Lung resistance

that the number of activated CD4⁺ T cells in bronchoalveolar lavage (BAL) fluid is significantly elevated in asthmatic subjects compared with control subjects.^{1,2} Furthermore, the degree of activation of CD4⁺ lymphocytes correlates with the number of eosinophils in BAL fluid, with the degree of airways hyperresponsiveness, and with disease severity.³ CD4⁺ T cells in asthma express high levels of mRNA for IL-4 and IL-5.⁴ These 2 cytokines have been suggested to play an important role in the development of airways hyperresponsiveness and the symptoms of asthma.⁵ IL-4 promotes IgE synthesis by B lymphocytes^{6,7} and plays an essential role in driving lymphocyte differentiation toward the T_{H2} subtype in vivo.⁸⁻¹⁰ IL-4 also enhances expression of the vascular cell adhesion molecule-1¹¹ on the surface of endothelial cells and may account for selective eosinophil recruitment at sites of allergic inflammation by interacting with very late antigen-4. IL-5 plays a critical role in regulating the terminal differentiation of committed eosinophil precursors, in activating mature eosinophils and thus enhancing their adhesion to vascular endothelium and their subsequent migration into tissues, and in prolonging eosinophil survival at sites of allergic inflammation.¹²⁻¹⁵

Studies performed on samples obtained from the airways of asthmatic subjects are most relevant to the pathogenesis of this disease, but manipulations are limited by ethical considerations. Therefore several animal models of human allergic asthma have been developed that have clarified the role of inflammatory cells and cytokines in the events subsequent to allergen exposure. Murine and rodent models have provided strong evidence of T-cell involvement in allergic airway responses.¹⁶⁻²⁰ Extensive

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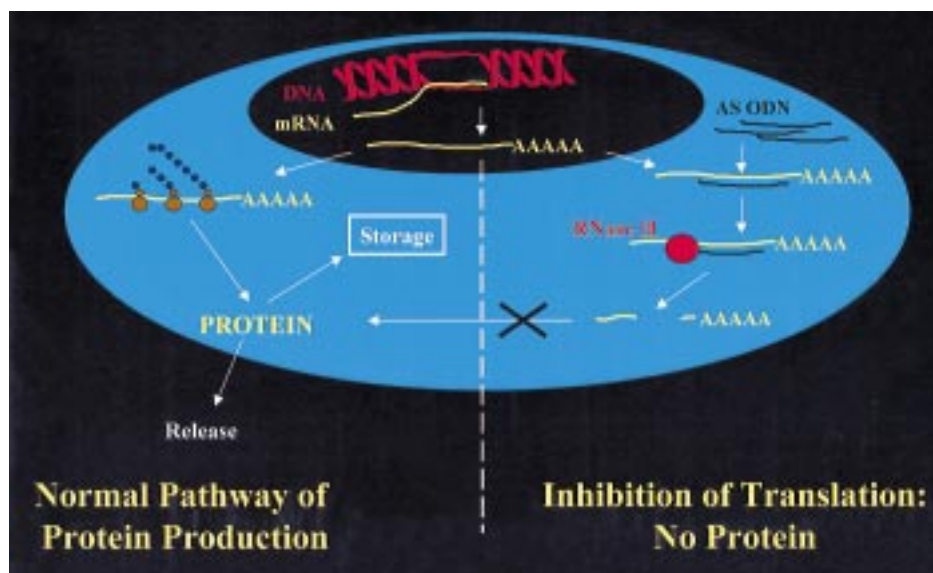


FIG 1. Schematic diagram representing one example of the mechanism by which AS ODNs work. AS ODNs act on the translation stage. Usually designed to hybridize specifically to a particular sequence of mRNA, AS ODNs block the access of mRNA to the ribosomes, thus preventing translation in the protein of interest. Then mRNA degradation is promoted by an RNase H-dependent agent (RNase H specifically degrades the RNA strand of RNA/DNA hybrid). Consequently, the production of a targeted protein is inhibited.

research on the mechanisms underlying allergen-induced changes in lung function and recruitment of inflammatory cells has been conducted with a model of antigen challenge in Brown Norway (BN) rats.²¹⁻²³ In sensitized BN rats the late airway response (LAR) after antigen challenge is associated with airway eosinophilia and increased expression of IL-4 and IL-5 mRNA.²⁴ Moreover, in BN rats antigen primed-CD4⁺ T cells are capable of transferring allergic airway responses to unsensitized animals, which showed airway bronchoconstriction mimicking LAR and BAL eosinophilia,¹⁷ as well as IL-4 and IL-5 mRNA expression in response to antigen challenge.²⁵

The aim of this study was to use antisense (AS) oligodeoxynucleotide (ODN) technology to examine more specifically the participation of T_{H2}-type cytokines (IL-4 and IL-5) in the model of LAR adoptive transfer. Usually designed to specifically hybridize a particular sequence of mRNA, AS ODNs block the access of the mRNA to ribosomes, thus preventing protein translation (Fig 1). As a result, the production of the targeted protein is inhibited without affecting the translation of other proteins. The objectives of this study were to test the hypothesis that, in an adoptive transfer model of LAR in rats, the treatment of CD4⁺ T cells with IL-4 AS ODN and/or IL-5 AS ODN will block the LAR, inhibit airway eosinophilia, and decrease the expression of T_{H2}-type cytokines in the lung.

METHODS

Rats and sensitization

Male BN rats, 7 to 10 weeks old, were purchased from a commercial source (Harlan Sprague Dawley Inc) and maintained in a standard animal facility. We followed the guidelines from the Canadian Council on Animal Care for the care and use of the animals.

Forty-two rats were actively sensitized by ovalbumin (OVA) subcutaneous injections (1 mg, grade V; Sigma Chemicals, St Louis, Mo) and aluminium hydroxide (4.3 mg; EM Science, Gibbstown, NJ) dissolved in a sterile saline solution. Simultaneously, *Bordetella pertussis* heat-killed bacteria (10¹⁰ organisms per rat, IAF Biovac) was given intraperitoneally as an adjuvant. The sensitized rats were used as donors of the antigen-primed CD4⁺ T lymphocytes to be transferred to unsensitized syngeneic BN rats.

Separation of T-cell subsets

Fourteen days after OVA sensitization, cervical lymph nodes from urethane-anesthetized BN rats were removed and transferred to a Petri dish containing 0.5% BSA-PBS. Mononuclear cells were harvested by mincing and passing the tissue through a stainless steel sieve, and after one wash (400g for 5 minutes at 4°C), cells were passed through a nylon mesh. CD4⁺ cells were purified by negative selection with a magnetic cell sorter (MACS; Miltenyi Biotec, Auburn, Calif). Lymph node cells were first incubated with a mixture of specific mAbs (MCA 620, MCA 340G, and MCA 48G; Cedarlane Laboratories Ltd, Hornby, Ontario, Canada) directed against the undesired cells (ie, myeloid cells, B cells, and CD8⁺ cells, respectively). After a 15-minute incubation at 4°C and a subsequent wash, cells were indirectly labeled with MACS anti-IgG1 microbeads and incubated at 4°C for 30 minutes. Finally, the labeled cells were washed and passed through the MACS column slowly, and we collected the effluent containing the cells of interest, namely the CD4⁺ cells. Cell purity was 94.6% ± 1.6%, and cell viability was 95.8% ± 0.4%.

Synthesis and design of ODNs

ODNs were designed and synthesized at the Sheldon Biotechnology Institute of McGill University. These ODNs were phosphorothioate modified to increase their resistance to nuclease degradation and correspond to 5'-GACTGACATCTCTCGATAACTAC-CCAGAGT-3' for IL-4, 5'-AAAAGAAGTGAAATAACCCCTAATTCAAT-3' for IL-5, and 5'-ACCTGACTGGGAGCGAGAT-

ACTA-3' for neuropeptide Y (NP-Y), an irrelevant protein that is not produced by T cells. The IL-5 AS and IL-4 AS ODNs were tested in vitro to ensure that they could specifically block the translation of the corresponding proteins. Briefly, CD4⁺ T cells were cultured with or without AS ODNs for 48 hours (the period corresponding to the delay between the adoptive transfer and the OVA aerosol challenge) in RPMI culture medium containing 5% heat-inactivated FCS, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 50 μ mol/L β -mercaptoethanol, 50 U/mL penicillin, and 50 μ g/mL streptomycin and then stimulated for 6 hours with concanavalin A (50 μ g/mL final concentration). At the end, cells were recovered and washed, and cytospin preparations were processed for IL-5 and IL-4 immunocytochemistry as described below.

Adoptive transfer

Immediately after immunomagnetic separation, CD4⁺ cells were washed (400g for 7 minutes at 4°C) in sterile RPMI-1640, resuspended at 10⁶ cells/mL in RPMI culture medium (comprised as above), and distributed in a 24-well plate (1 mL/well). Antisense ODNs for IL-4, IL-5, or NP-Y were individually added at a final concentration of 10 μ g/mL, and cells were maintained in culture for 6 hours at 37°C in a 5% CO₂ atmosphere. This time period was determined on the basis of a kinetic study and was considered as optimal for IL-4 and IL-5 AS ODNs to cross the cell membrane, hybridize, and block their specific mRNA. Then oligonucleotide-treated cells were recovered, and the cells were washed to eliminate the excess of AS and injected intraperitoneally into unsensitized syngeneic recipient BN rats (3.0 $\pm$ 0.1 10⁶ cells/rat with a viability of 95.6% $\pm$ 0.3% as assessed by Trypan blue exclusion).

OVA challenge and measurement of airway response

This protocol has been previously described.²⁴ Briefly, 2 days after the adoptive transfer, the recipient rats were administered a steady and long-lasting anesthetic agent (urethane, 1.5 g/kg intraperitoneally), intubated, and challenged with aerosolized OVA (5% wt/vol) for 5 minutes. A commercial software package (RHT Infodat Inc, Montreal, Quebec, Canada) was used to determine the lung resistance (R_L), which was calculated by multiple linear regression by fitting the equation of motion: $P_{tp} = E_L V + R_L \dot{V} + K$ (P_{tp} , transpulmonary pressure; V , volume; $\dot{V}$, flow; E_L , elastance; and K , a constant value). Baseline R_L was analyzed immediately after the challenge and then at 5, 10, and 15 minutes after OVA administration and subsequently every 15 minutes for a period of 8 hours. The early airways response (EAR) was quantified by taking the maximal value of R_L during the first 60 minutes after OVA challenge above the baseline (results expressed as percentage of baseline). We also quantified LAR by calculating the area under the R_L versus time curve (area under the curve [AUC]) above the baseline values from 3 to 8 hours after OVA challenge.

BAL

After the 8 hours of R_L measurements, animals were killed and BAL was performed. Briefly, the lungs were lavaged with 5 $\times$ 5 mL of sterile saline solution. Total BAL cell numbers and cell viability were estimated by using a hemacytometer and Trypan blue stain. Cytospin slides were prepared as follows: one was stained with May Grünwald-Giemsa, whereas the others were fixed for 7 minutes in acetone:methanol (60:40), air-dried, and stored at -20°C until immunocytochemistry was performed.

Immunocytochemistry

Cytospin BAL cell slides were immunostained with BMK-13 mAb, a mouse anti-human major basic protein (MBP) antibody (a

TABLE I. Effects of the AS ODNs on CD4⁺ T-cell viability

Viability of CD4 ⁺ T cells before cell culture	Culture medium	Viability of CD4 ⁺ T cells after 6 hours of culture with:		
		NP-Y AS	IL-5 AS	IL-4 AS
95.8 $\pm$ 0.4	95.0 $\pm$ 0.7	95.9 $\pm$ 1.2	95.1 $\pm$ 1.3	95.5 $\pm$ 1.0

Total cell counts were assessed by May Grünwald-Giemsa staining. Cell viability, expressed as percent, was performed by the Trypan blue exclusion method. Data are expressed as means $\pm$ SEM.

generous gift from Dr R. Moqbel, University of Alberta); with a biotinylated goat anti-IL-4 antibody (R&D systems); and with monoclonal anti-IL-5 (R&D systems) or anti-IFN- γ antibodies (BIOSOURCE). The incubation with the first antibody was followed in all cases by amplification steps with either the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method²⁶ (MBP, IL-5, and IFN- γ) or with biotinylated rabbit anti-goat and alkaline phosphatase-conjugated streptavidin (IL-4). Positive cells were quantified by microscopy by an investigator blinded to group status, and results were expressed as the number of immunoreactive cells in 1000 cells from BAL fluid.

Statistical analysis

Data were expressed as means $\pm$ SEM. Significant differences among groups were determined by using the nonparametric Kruskal-Wallis test (for comparison of airway responses) and ANOVA (for immunohistochemical results). The Fisher least-significant difference test was applied post hoc to test for differences between pairs of means. Correlation coefficients were obtained by Pearson's rank method with Bonferroni correction. In all cases a P value of less than .05 was considered statistically significant.

RESULTS

Cell viability and cytokine specificity of AS ODNs

To ensure that the AS ODNs were not cytotoxic and had specific actions, the viability and cytokine expression of the CD4⁺ T cells were investigated after different periods of incubation with the AS. Table I shows that, for 6 hours of culture, no significant difference in viability among cells that had been cultured with medium alone, NP-Y AS, IL-5 AS, or IL-4 AS was present. The cell viability was similar to that of cells before culture (Table I). Similar results were observed at up to 48 hours of culture with the AS ODNs. As described in the Methods section, the efficacy and the cytokine specificity of IL-5 AS and IL-4 AS were tested by immunocytochemistry with anti-IL-5 and anti-IL-4 antibodies, respectively. IL-5 AS induced a 30% inhibition of IL-5 synthesis by CD4⁺ T cells compared with cells that had not been incubated with AS ($P < .05$). Similar results were shown with IL-4 immunoreactivity in cells incubated with IL-4 AS ($P < .005$). In both cases the irrelevant NP-Y AS caused results similar to those obtained for the cells that were not incubated with AS ODNs (data not shown). The synthesis of IL-4 by CD4⁺ T cells was not significantly affected by IL-5 AS ODNs, and IL-4 AS ODNs did not significantly decrease IL-5 synthesis (data not shown).

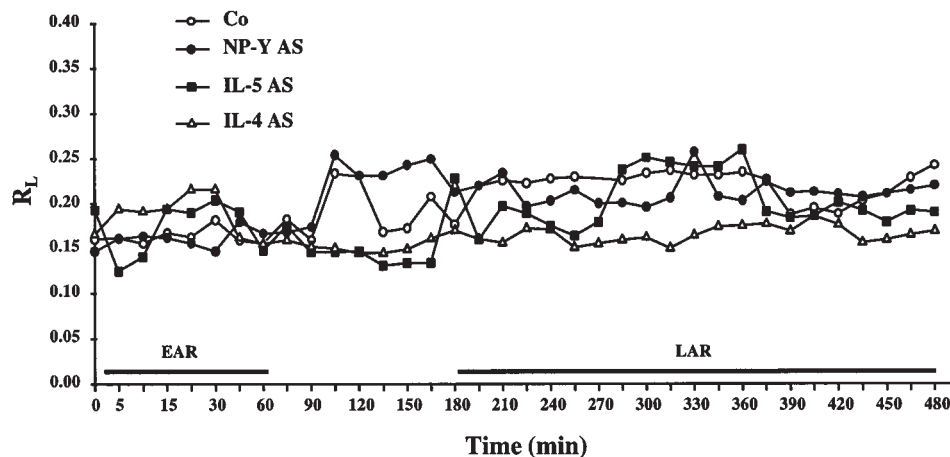


FIG 2. Time course of R_L after OVA challenge in BN rats. Unsensitized animals were injected intraperitoneally with CD4⁺ T cells treated with culture medium (controls [Co], $n = 9$, open circles) or NP-Y AS (irrelevant AS, filled circles, $n = 13$), IL-5 AS (filled squares, $n = 8$), or IL-4 AS (open triangles, $n = 9$) ODNs. Two days later, the animals were challenged with a 5% OVA aerosol. R_L was measured every 5 minutes during a 20-minute period and subsequently every 15 minutes over a period of 8 hours.

TABLE II. Total cell counts in BAL.

Group	Treatment of the CD4 ⁺ transferred T cells	BAL total cell counts (10 ⁶ cells)	Viability (%)
1 ($n = 9$)	Culture medium	1.62 ± 0.41	93.0 ± 3.9
2 ($n = 9$)	NP-Y AS	1.48 ± 0.47	95.8 ± 3.1
3 ($n = 8$)	IL-5 AS	1.55 ± 0.44	95.5 ± 2.1
4 ($n = 9$)	IL-4 AS	1.53 ± 0.65	95.5 ± 2.4

Total cell counts were assessed by May Grünwald-Giemsa staining. Cell viability was performed by the Trypan blue exclusion method. Data are expressed as means $\pm$ SEM.

Airway response to OVA challenge

Fig 2 is an example of the time course of R_L after OVA challenge for one representative animal in each group (NP-Y AS-, IL-4 AS-, and IL-5 AS-treated rats and controls). R_L was not significantly different for the 4 rats during the early period after challenge. In contrast, in the late period after allergen challenge, there was an increase in R_L as early as 105 minutes for the control and NP-Y AS-treated rats and later at 180 minutes for the IL-5 AS-treated rats. Only R_L of the IL-4 AS-treated rats was not increased over the baseline. To compare the results for the different groups, we expressed the R_L during the first hour after challenge in percentage of baseline and the R_L during the following hours as the AUC.

As shown in Fig 3, A, the results were similar for the 4 groups of rats during the first hour after OVA challenge. In fact, none of them showed an EAR as defined by a 200% R_L increase over the baseline. In contrast, when the AUC from 3 to 8 hours after challenge was analyzed (Fig 3, C), only the R_L of recipients of IL-4 AS-treated CD4⁺ cells was decreased in comparison with controls ($P < .001$). This effect was specific because the irrelevant AS (NP-Y AS) did not show any significant effect when com-

pared with controls. Similarly, the recipients of IL-5 AS treated-CD4⁺ cells did not demonstrate changes in LAR compared with controls. To examine the effect of interleukin AS ODNs on R_L between the period of EAR and LAR, we measured R_L from 1 to 3 hours after OVA challenge. IL-4 AS treatment was effective in reducing the AUC at this time (Fig 3, B) in comparison with controls ($P < .005$). However, NP-Y AS and IL-5 AS treatments did not have any effect when compared with controls.

Immunocytochemistry of cells recovered in BAL fluid

Examples of immunocytochemical staining of BAL cells for MBP and IL-4 immunoreactivity are shown in Fig 4. Cells positive for MBP or cytokine immunoreactivity were identified on the basis of their discrete red cytoplasmic staining. There were no significant differences observed in total cell counts or in cell viability in the BAL for the 4 groups of rats (Table II). The differential cell count of NP-Y AS-treated CD4⁺ cell recipients was similar to that of medium-treated CD4⁺ cell recipients (data not shown). Because eosinophils are a hallmark of allergic inflammation, we examined their presence specifically by MBP staining. As shown in Fig 5, eosinophilia was significantly reduced in the BAL fluid of rats that received IL-4 AS-treated CD4⁺ cells in comparison with controls ($P < .001$). In animals that received NP-Y AS- or IL-5 AS-treated CD4⁺ cells, no significant change in BAL eosinophilia was demonstrated compared with controls.

Cytokine immunoreactivity in BAL fluid

Fig 6 shows that in comparison with controls IL-4 expression was significantly decreased in BAL recovered from rats that received IL-4 AS-treated CD4⁺ cells ($P < .001$). In rats that received irrelevant AS- or IL-5 AS-treated CD4⁺ cells, IL-4 expression was not significantly different from that found in controls. Although IL-5

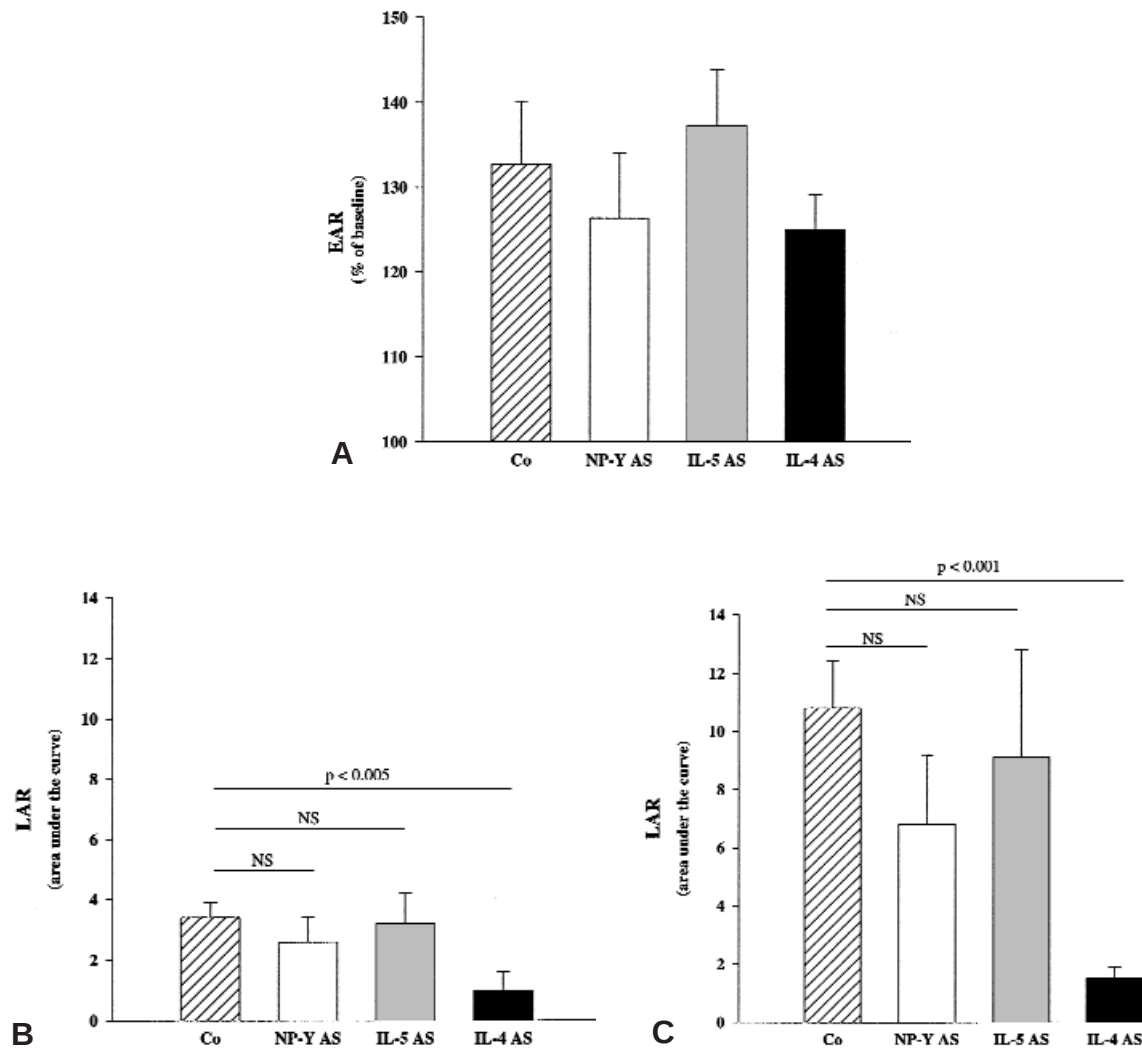


FIG 3. Airway response to OVA in BN rats. Unsensitized animals were injected intraperitoneally with CD4⁺ T cells treated with culture medium (controls [Co], n = 9, hatched bar) or NP-Y AS (irrelevant AS, open bar, n = 13), IL-5 AS (shaded bar, n = 8), or IL-4 AS (filled bar, n = 9) ODNs. Two days later, the animals were challenged with a 5% OVA aerosol. R_L was measured for 8 hours and analyzed during the first hour for the development of an EAR (**A**) and from 1 to 3 hours (**B**) and 3 to 8 hours for the measurement of the LAR (**C**). EAR was quantified by taking the maximal value of R_L during the first 60 minutes after OVA challenge above the baseline (results expressed as percentage of baseline). LAR was calculated as the area under the R_L versus time curve above the baseline values from 3 to 8 hours (results expressed as AUC). Results are expressed as means $\pm$ SEM. Probability (P) values were determined by using the nonparametric Kruskal-Wallis test.

immunoreactivity in rats that received IL-5 AS-treated CD4⁺ cells was decreased in comparison with controls ($P < .01$), this reduction was less than that seen in the IL-4 AS-treated group ($P < .001$; Fig 7). Moreover, this decrease was not significant if compared with that found in the rats that received irrelevant AS ($P = .54$). The majority of IL-4 and IL-5 immunoreactivities were localized to mononuclear cells with an apparent T-cell morphology. However, 10% to 20% of IL-4 and IL-5 immunoreactivities were localized to granulocytes, including eosinophils. Immunoreactivity of the T_H1-type cytokine IFN- γ was also investigated in BAL. Fig 8 shows that IFN- γ expression was increased in the BAL fluid of rats that received IL-4

AS-treated CD4⁺ cells in comparison with controls ($P < .001$), whereas in the BAL fluid of the NP-Y AS- or IL-5 AS-treated groups, IFN- γ expression did not significantly differ from that found in controls.

Relationships between LAR, BAL eosinophilia, and cytokine expression in BAL fluid

Correlational analyses were performed between the LAR and MBP, IL-4, IL-5, IFN- γ BAL immunoreactivities for rats belonging to the control group and rats that had been administered IL-4 AS-treated CD4⁺ T cells. These results indicated that the LAR positively correlated with MBP-, IL-4-, and IL-5-immunoreactive protein expres-

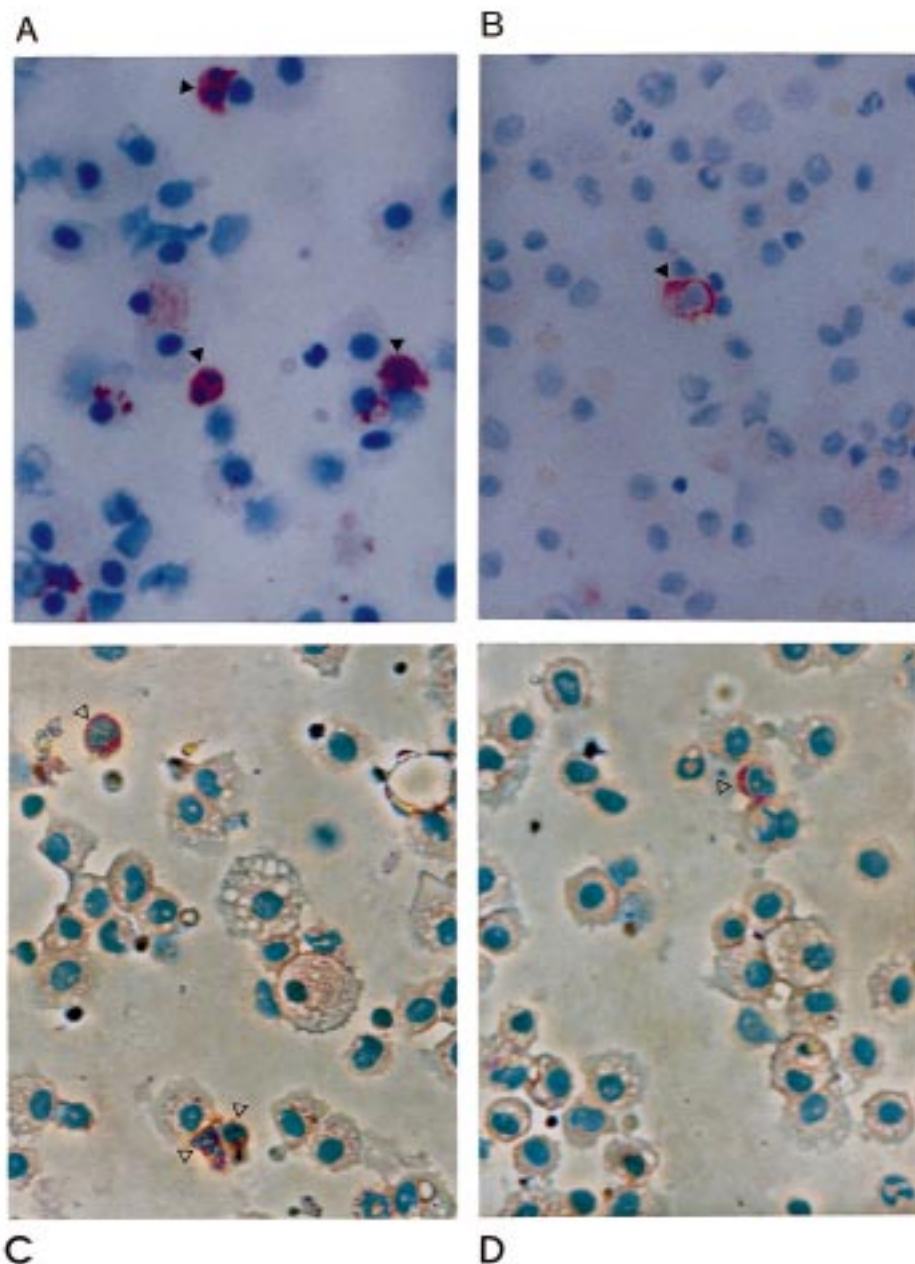


FIG 4. Representative photomicrographs of BAL cells recovered from control rats (**A** and **C**) and from rats that received IL-4 AS-treated CD4⁺ T cells (**B** and **D**). Specific staining shown is for MBP-positive eosinophils (**A** and **B**; black arrows) and IL-4-immunoreactive cells (**C** and **D**; white arrows).

sion in BAL fluid ($r = 0.74$, $P = .001$; $r = 0.62$, $P = .008$; and $r = 0.76$, $P < .001$; respectively), whereas numbers of IFN- γ -immunoreactive cells in BAL fluid inversely correlated to the LAR ($r = -0.65$, $P = .004$). Moreover, the number of eosinophils was found to correlate with IL-4- and IL-5-immunoreactive protein expression in BAL fluid ($r = 0.68$, $P = .003$; and $r = 0.74$, $P = .001$; respectively) but inversely correlated with IFN- γ immunoreactivity ($r = -0.67$, $P = .003$). IL-4 and IL-5 immunoreactivity were linked, with a strong coefficient of correlation ($r = 0.84$, $P < .001$). Although there was a trend for IL-4 and IL-5

expression to be negatively correlated with IFN- γ expression ($r = -0.45$ and -0.59 , respectively), these associations were not found to be significant.

DISCUSSION

In this study we used the BN rat to evaluate the role of the T_H2-type cytokines IL-4 and IL-5 in allergen-induced airway narrowing and inflammation. This model has several features in common with human allergic asthma, including high levels of specific IgE after allergen sensi-

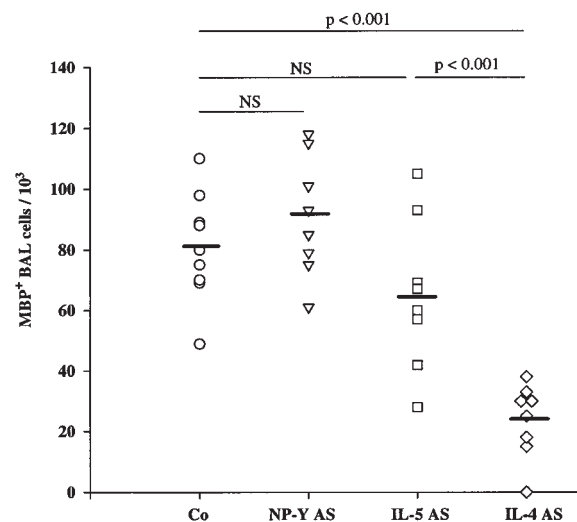


FIG 5. Eosinophilia in BAL fluid investigated by immunocytochemistry. BAL fluid was recovered at the end of the experiment (8 hours after OVA challenge) from the recipients of culture medium (controls [Co], $n = 9$, circles) or NP-Y AS- (irrelevant AS, triangles, $n = 8$), IL-5 AS- (squares, $n = 8$), or IL-4 AS- (diamonds, $n = 8$) treated CD4⁺ T cells. Eosinophils were detected by the APAAP method by using the BMK-13 antibody, a mouse anti-human MBP mAb. Results are expressed as the number of MBP⁺ cells per 10³ BAL cells counted for each rat. Probability (P) values were determined by using ANOVA, and the Fisher test was applied to test for differences.

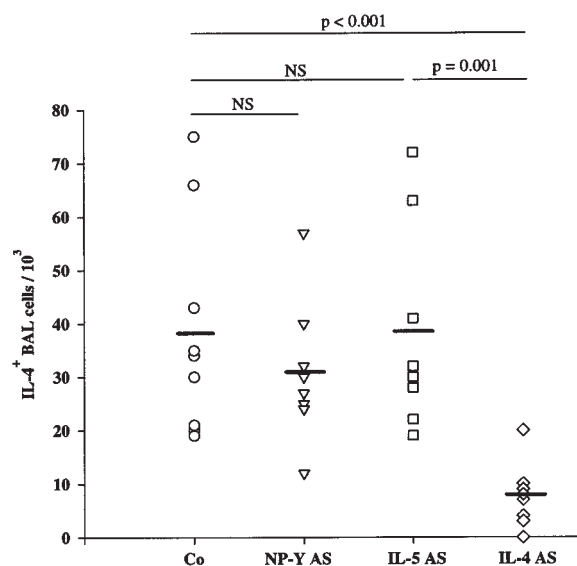


FIG 6. IL-4 expression in BAL fluid investigated by immunocytochemistry. BAL fluid was recovered at the end of the experiment (8 hours after OVA challenge) from the recipients of culture medium (controls [Co], $n = 9$, circles) or NP-Y AS- (irrelevant AS, triangles, $n = 8$), IL-5 AS- (squares, $n = 8$) or IL-4 AS- (diamonds, $n = 8$) treated CD4⁺ T cells. IL-4 was detected by the streptavidin/biotin-alkaline phosphatase conjugated method by using a biotinylated goat anti-mouse IL-4 antibody. Results are expressed as the number of IL-4⁺ cells per 10³ BAL cells counted for each rat. Probability (P) values were determined by using ANOVA, and the Fisher test was applied to test for differences.

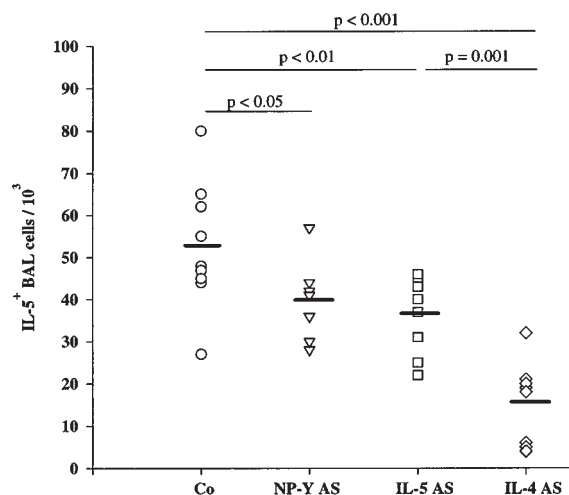


FIG 7. IL-5 expression in BAL fluid investigated by immunocytochemistry. BAL fluid was recovered at the end of the experiment (8 hours after OVA challenge) from the recipients of culture medium (controls [Co], $n = 9$, circles) or NP-Y AS- (irrelevant AS, triangles, $n = 7$), IL-5 AS- (squares, $n = 8$) or IL-4 AS- (diamonds, $n = 8$) treated CD4⁺ T cells. IL-5 was detected by the APAAP method by using a mouse anti-rat IL-5 mAb. Results are expressed as the number of IL-5⁺ cells per 10³ BAL cells counted for each rat. Probability (P) values were determined by using ANOVA, and the Fisher test was applied to test for differences.

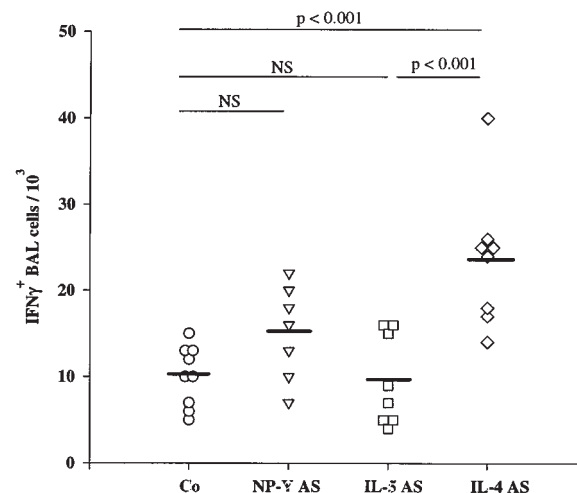


FIG 8. IFN- γ expression in BAL fluid investigated by immunocytochemistry. BAL fluid was recovered at the end of the experiment (8 hours after OVA challenge) from the recipients of culture medium (controls [Co], $n = 9$, circles) or NP-Y AS- (irrelevant AS, triangles, $n = 7$), IL-5 AS- (squares, $n = 8$) or IL-4 AS- (diamonds, $n = 8$) treated CD4⁺ T cells. IFN- γ was detected by the APAAP method by using a mouse anti-rat IFN- γ mAb. Results are expressed as the number of IFN- γ ⁺ cells per 10³ BAL cells counted for each rat. Probability (P) values were determined by using ANOVA, and the Fisher test was applied to test for differences.

tization with OVA, a high prevalence of both EARs and LARs, and increases in bronchial responsiveness.^{21,27} T lymphocytes and their cytokines appear to be the principal cells responsible for orchestrating the inflammatory

response to antigen. We have recently shown that LARs can be transferred by OVA-primed CD4⁺ T cells to unsensitized rats and that this response is associated with the accumulation of eosinophils and high expression of IL-4 and IL-5.^{17,25} However, formal proof of direct involvement of IL-4 and IL-5 in the LAR is lacking.

We used AS ODNs to block specifically IL-4 and IL-5 protein translation in CD4⁺ cells before their transfer. We demonstrated that an irrelevant AS targeting NP-Y, a neuropeptide that has never been demonstrated to be produced by T lymphocytes or to be involved in the development of airway responses, gave similar results as the control group, ruling out the possibility that AS oligo-treated CD4⁺ T cells nonspecifically modulated airway responses. The blockade of IL-4 production with specific AS ODNs in the CD4⁺ T cells before transfer to a naive animal decreased the LAR and airway eosinophilia in response to antigen challenge. IL-4 protein expression was also significantly decreased in the BAL fluid of animals that received IL-4 AS oligo-treated CD4⁺ cells, confirming the efficacy of IL-4 mRNA blockade by AS. These data also suggest that IL-4 production and release by the transferred CD4⁺ T cells is involved in the amplification of the allergic inflammatory response in the recipient rats. Indeed, the IL-4-immunoreactive cells in the BAL fluid are likely to represent not only the transferred cells but also a significant number of resident cells. IL-5 immunoreactivity in the BAL fluid of rats that received IL-4 AS-treated CD4⁺ T cells was also significantly reduced. This was not surprising because IL-5 production by lymphocytes requires the presence of IL-4 to shift the T cells toward a T_{H2}-type phenotype.²⁸

In the same experimental conditions the treatment of CD4⁺ cells with IL-5 AS ODNs before the adoptive transfer did not reduce the LAR, eosinophilia, or IL-4 production. Although the level of IL-5 protein was reduced, the effect of IL-5 AS on IL-5 expression in the BAL fluid was less than that induced by IL-4 AS ODN. This apparent lack of IL-5 AS effect cannot be attributed to a lack of functional activity because IL-5 AS was shown to reduce IL-5 production *in vitro*. When compared with the control group, IFN- γ expression was shown to be increased in the BAL fluid of rats that received IL-4 AS-treated CD4⁺ T cells only.

It has been demonstrated that the severity of asthma correlates with the presence of activated T lymphocytes and eosinophils in BAL fluid,³ and it has been suggested that T cells initiate and augment responses by secreting inflammatory cytokines, including IL-4 and IL-5. In this study, using an adoptive transfer model, we demonstrated that IL-4, and not IL-5, synthesized by OVA-primed CD4⁺ T cells is the main cytokine involved in the generation of the LAR and eosinophilia. Our results also suggest that IL-4 production, during the period of T-cell priming, is critically associated with the later manifestations of airway bronchoconstriction. These results are in agreement with other studies. The existence of an IL-4-dependent mechanism leading to airway inflammation was demonstrated by the failure of IL-4-deficient

mice to exhibit airway eosinophil infiltration and airways hyperresponsiveness in response to antigen challenge.²⁹ IL-4 is essential both for the induction of a local T_{H2} response and for the development of eosinophilic inflammation of the lung. Coyle et al¹⁰ reported that the local T_{H2} response (IL-4 and IL-5), the development of eosinophilic inflammation of the lung, and IgE production were significantly inhibited only when anti-IL-4 antibody was administered during immunization. Anti-IL-4 administration before antigen challenge had only little effect.¹⁰ This confirmed a previous *in vitro* study demonstrating that IL-4 was required for the priming of T_{H2} cells early in the response to antigen, but once a T_{H2} pattern was established, IL-4 was not required to maintain T_{H2} cytokine production.⁹ Our study showed that by transferring IL-4 AS ODN-treated CD4⁺ T cells to naive rats, which therefore cannot produce IL-4 on antigen challenge, it is possible to inhibit the T_{H2} commitment in recipient animals (ie, IL-4 and IL-5 production) and, as a consequence, LAR and lung eosinophilia. This is supported by the strong correlations observed between the magnitude of the late airway bronchoconstriction, eosinophilia, and T_{H2}-type cytokine expression in BAL fluid.

The inhibition of late allergic responses may not be entirely caused by a decline in IL-4, but the effect may have been reinforced by IFN- γ because its expression was increased in the BAL fluid of rats that received IL-4 AS-treated CD4⁺ cells. Indeed, IL-4 and IFN- γ have been demonstrated to have reciprocal relationships with endogenously produced IL-4 preventing IFN- γ release, so that the T_{H2} cell phenotype is stabilized. However, if IL-4 is neutralized, IFN- γ can prime T_{H2} cells and produce IFN- γ , generating a T_{H1} cell phenotype.³⁰ An alternate mechanism of action of AS ODN treatment is suggested by recent studies in which an increase in IFN- γ secretion by T lymphocytes was induced by synthetic ODNs containing a particular motif (CpG).³¹ This treatment prevented airway eosinophilia and T_{H2}-type cytokine induction.³² However, the route and the timing of ODN administration in that study was totally different from that found in our protocol. Even though the IL-4 AS ODN that we used contained this motif, the NP-Y AS ODN did also and yet gave results similar to those found in the control group; the slightly greater IFN- γ expression in BAL cells in the NP-Y AS group compared with that found in the control group was not significant. It seems more likely that the inhibition of LAR, BAL eosinophilia, and IL-4 and IL-5 immunoreactivities in BAL fluid, as well as the increase of IFN- γ , is the consequence of the absence of IL-4 itself rather than a nonspecific effect of the ODN. Furthermore, we can conclude that because the treatment of primed CD4⁺ T cells with IL-4 AS before their transfer inhibited the adoptive transfer of airway physiologic and inflammatory responses, it appears that the initiation and the propagation of these responses are a donor cell-derived event.

Several animal models have shown IL-5 to be critical

for the allergic airway response, particularly eosinophil influx into tissues and their survival at sites of inflammation.^{13,33} Studies in mice have suggested that IL-5 may play a more important role in allergen-induced airways hyperresponsiveness.^{18,34} Therefore we expected the treatment of CD4⁺ T cells with IL-5 AS ODN to be effective also in reducing LAR and airway eosinophilia in our model. However, because resident cells in the recipients have the ability to produce IL-5 (on IL-4 stimulation), the IL-5 blockade in the transferred cells might not be sufficient to inhibit the eosinophil infiltration into tissues. Because we did not investigate the responsiveness of the airways in our model, we cannot exclude that IL-5 AS treatment of CD4⁺ T cells may influence this parameter in rats that received these cells. However, recent studies have demonstrated that IL-5 is not always required for airways hyperreactivity.^{35,36}

In summary, our results suggest a critical role for IL-4 during antigen priming in establishing the cascade required for allergic airway inflammation, and therefore events capable of reprogramming CD4⁺ T cell-subset development to respiratory allergens might suggest a possible tool to design new therapies for the control of asthma. Our model provides useful information concerning the role of IL-4 and IL-5 synthesis by the CD4⁺ T cells because the cell population targeted by the AS is well defined, and therefore we can attribute the observed modifications to these AS-modified cells exclusively. This is the major difference with most of the studies in which intravenous or intraperitoneal antibody administration has been used to neutralize cytokine action. Although the antibody usually acts on one specific molecule, it is shared with different cell types, making it difficult to pinpoint the exact participation of each cell type. Knock-out animals also have limitations because we do not know to what extent the animals adapt to the gene deletion and bypass the mutation across the generations. Several clinical trials are already in progress for the treatment of acute myelogenous leukemia, HIV, cytomegalovirus, and human papilloma virus infection using AS technology.³⁷ Recently, AS ODNs were successfully used in a rabbit model of allergic asthma. The administration of an aerosolized AS ODN targeting the adenosine A₁ receptor desensitized the animals to subsequent challenge with either adenosine or dust mite allergen.³⁸

Our results need to be extended to the use of AS in vivo in animal models and in humans. The lung represents an ideal organ, given that its large surface area and the mucus secretion could facilitate the absorption of ODNs, which could be administered by aerosol.

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REFERENCES

1. Gerblach AA, Salik H, Schuyler MR. Dynamic T-cell changes in peripheral blood and bronchoalveolar lavage after antigen bronchoprovocation in asthmatics. *Am Rev Respir Dis* 1991;143:533-7.
2. Bentley AM, Meng Q, Robinson DS, Hamid Q, Kay AB, Durham SR. Increases in activated T lymphocytes, eosinophils, and cytokine mRNA expression for interleukin-5 and granulocyte/macrophage colony-stimulating factor in bronchial biopsies after allergen inhalation challenge in atopic asthmatics. *Am J Respir Cell Mol Biol* 1993;8:35-42.
3. Walker C, Kaegi MK, Braun P, Blaser K. Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J Allergy Clin Immunol* 1991;88:935-42.
4. Robinson DS, Hamid Q, Ying S, Tscopoulos A, Barkans J, Bentley AM, et al. Predominant Th2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992;326:298-304.
5. Kay AB, Ying S, Varney V, Gaga M, Durham SR, Moqbel R, et al. Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL)-3, IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects. *J Exp Med* 1991;173:775-8.
6. Pène J, Rousset F, Brière F, Chrétien I, Bonnefoy J-Y, Spits H, et al. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons gamma and alpha and prostaglandin E₂. *Proc Natl Acad Sci USA* 1988;85:6880-4.
7. Del Prete G, Maggi E, Parronchi P, Chrétien I, Tiri A, Macchia D, et al. IL-4 is an essential factor for the IgE synthesis induced in vitro by human T cell clones and their supernatants. *J Immunol* 1988;140:4193-8.
8. Finkelman FD, Katona IM, Urban JF, Holmes J, Ohara J, Tung AS, et al. IL-4 is required to generate and sustain in vivo IgE responses. *J Immunol* 1988;141:2335-41.
9. Swain SL, Weinberg AD, English M, Hutson G. IL-4 directs the development of Th2-like helper effectors. *J Immunol* 1990;145:3796-806.
10. Coyle AJ, Le Gros G, Bertrand C, Tsuyuki S, Heusser CH, Kopf M, et al. Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *Am J Respir Cell Mol Biol* 1995;13:54-9.
11. Thornhill MH, Wellicome SM, Mahiouz DL, Lanchbury JS, Kyan-Aung U, Haskard DO. Tumor necrosis factor combines with IL-4 or IFN-γ to selectively enhance endothelial cell adhesiveness for T cells. The contribution of vascular cell adhesion molecule-1-dependent and -independent binding mechanisms. *J Immunol* 1991;146:592-8.
12. Yamaguchi Y, Hayashi Y, Sugama Y, Miura Y, Kasahara T, Kitamura S, et al. Highly purified murine interleukin-5 (IL-5) stimulates eosinophil function and prolongs in vitro survival. *J Exp Med* 1988;167:1737-42.
13. Lopez AF, Sanderson CJ, Gamble JR, Campbell HD, Young IG, Vadas MA. Recombinant human interleukin 5 is a selective activator of human eosinophil function. *J Exp Med* 1988;167:219-24.
14. Fujisawa T, Abu-Ghazaleh R, Kita H, Sanderson CJ, Gleich GJ. Regulatory effect of cytokines on eosinophil degranulation. *J Immunol* 1990;144:642-6.
15. Walsh GM, Wardlaw AJ, Hartnell A, Sanderson CJ, Kay AB. Interleukin-5 enhances the in vitro adhesion of human eosinophils, but not neutrophils, in a leucocyte integrin (CD11/18)-dependent manner. *Int Arch Allergy Appl Immunol* 1991;94:174-8.
16. Gavett SH, Chen X, Finkelman F, Wills-Karp M. Depletion of murine CD4⁺ T lymphocytes prevents antigen-induced airway hyperactivity and pulmonary eosinophilia. *Am J Respir Cell Mol Biol* 1994;10:587-93.
17. Watanabe A, Mishima H, Renzi PM, Xu L-J, Hamid Q, Martin JG. Transfer of allergic airway responses with antigen-primed CD4⁺ T cells but not CD8⁺ T cells in Brown Norway rats. *J Clin Invest* 1995;96:1303-10.
18. Hogan SP, Koskinen A, Matthaie KI, Young IG, Foster PS. Interleukin-5-producing CD4⁺ T cells play a pivotal role in aeroallergen-induced eosinophilia, bronchial hyperactivity, and lung damage in mice. *Am J Respir Crit Care Med* 1998;157:210-8.
19. Kaminuma O, Mori A, Ogawa K, Nakata A, Hikkawa H, Naito K, et al. Successful transfer of late phase eosinophil infiltration in the lung by infusion of helper T cell clones. *Am J Respir Cell Mol Biol* 1997;16:448-54.
20. Cohn L, Homer RJ, Marinov A, Rankin J, Bottomly K. Induction of airway mucus production by T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J Exp Med* 1997;186:1737-47.
21. Eidelman DH, Bellofiore S, Martin JG. Late airway responses to antigen challenge in sensitized inbred rats. *Am Rev Respir Dis* 1988;137:1033-7.
22. Sapienza S, Eidelman DH, Renzi PM, Martin JG. Role of leucotriene D₄ in the early and late pulmonary responses of rats to allergen challenge. *Am Rev Respir Dis* 1990;142:353-8.
23. Renzi PM, Olivenstein R, Martin JG. Inflammatory cell populations in the airways and parenchyma after antigen challenge in the rat. *Am Rev Respir Dis* 1993;147:967-74.

24. Renzi PM, Al Assaad A, Yang J, Yasruei Z, Hamid Q. Cytokine expression in the presence or absence of late airway responses after antigen challenge of sensitized rats. *Am J Respir Cell Mol Biol* 1996;15:367-73.
25. Watanabe A, Mishima H, Kotsimbos TC, Hojo M, Renzi PM, Martin JG, et al. Adoptively transferred late allergic airway responses are associated with Th2-type cytokines in the rat. *Am J Respir Cell Mol Biol* 1997;16:69-74.
26. Cordell JL, Falini B, Erber WN, Ghosh AK, Abdulaziz Z, MacDonald S, et al. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J Histochem Cytochem* 1984;32:219-29.
27. Sapienza S, Du T, Eidelman DH, Wang NS, Martin JG. Structural changes in the airways of sensitized Brown Norway rats after antigen challenge. *Am Rev Respir Dis* 1991;144:423-7.
28. Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Köhler G. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* 1993;362:245-8.
29. Brusselle G, Kips J, Joos G, Bluethmann H, Pauwels R. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am J Respir Cell Mol Biol* 1995;12:254-9.
30. Nakamura T, Lee RK, Nam SY, Podack ER, Bottomly K, Flavell RA. Roles of IL-4 and IFN- γ in stabilizing the T helper cell type 1 and 2 phenotype. *J Immunol* 1997;158:2648-53.
31. Klinman DM, Yi A-K, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon γ . *Proc Natl Acad Sci USA* 1996;93:2879-83.
32. Kline JN, Waldschmidt TJ, Businga TR, Lemish JE, Weinstock JV, Thorne PS, et al. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J Immunol* 1998;160:2555-9.
33. Kung TT, Stelts DM, Zurcher JA, Adams GK, Egan RW, Kreutner W, et al. Involvement of IL-5 in a murine model of allergic pulmonary inflammation: prophylactic and therapeutic effect of an anti-IL-5 antibody. *Am J Respir Cell Mol Biol* 1995;13:360-5.
34. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 1996;183:195-201.
35. Corry DB, Folkesson HG, Warnock ML, Erle DJ, Matthay MA, Wiener-Kronish JP, et al. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J Exp Med* 1996;183:109-17.
36. Coyle AJ, Kohler G, Tsuyuki S, Brombacher F, Kopf M. Eosinophils are not required to induce airway hyperresponsiveness after nematode infection. *Eur J Immunol* 1998;28:2640-7.
37. Wagner RW. Gene inhibition using antisense oligodeoxynucleotides. *Nature* 1994;372:333-5.
38. Nyce JW, Metzger WJ. DNA antisense therapy for asthma in an animal model. *Nature* 1997;385:721-5.