

IFN- γ secretion by CD8⁺ T cells inhibits allergen-induced airway eosinophilia but not late airway responses

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Background: CD8⁺ T cells can suppress allergen-induced late airway responses (LARs) and airway inflammation.

Objective: To test the hypothesis that the suppression of LARs and airway eosinophilia by CD8⁺ T cells is IFN- γ mediated, we tested the effects of adoptively transferred CD8⁺ T cells, in which IFN- γ synthesis was inhibited by an antisense (AS) oligodeoxynucleotide (ODN), on the airway responses of a rat model of allergic asthma.

Methods: CD8⁺ T cells were harvested from the cervical lymph nodes of ovalbumin (OVA)-sensitized Brown Norway rats for administration to other actively sensitized syngeneic rats. CD8⁺ T cells (2×10^6) were incubated for 6 hours with 2 μ mol/L AS ODN or sense ODN and were injected intraperitoneally into recipients; inhibition of IFN- γ expression in vitro by AS ODN was shown by means of flow cytometry. Two days later, rats were challenged with aerosolized OVA.

Results: OVA-induced LAR and bronchoalveolar lavage (BAL) fluid eosinophilia were suppressed by sense ODN-treated CD8⁺ T cells. IFN- γ expression in BAL cells was elevated in these animals. IFN- γ expression in BAL cells was at control levels in recipients of AS ODN-treated CD8⁺ cells, confirming the success of the AS treatment in vivo. BAL eosinophilia was also largely restored in the AS ODN treatment group. In contrast, the CD8⁺ T cell-induced suppression of the LAR was not significantly affected by AS ODN pretreatment.

Conclusions: These results indicate that CD8⁺ T cells inhibit airway eosinophilia through secretion of IFN- γ but may suppress the LAR by means of other mechanisms. (*J Allergy Clin Immunol* 2002;109:803-9.)

Key words: Asthma, T_H1/T_H2, antisense oligodeoxynucleotides, eosinophils, late responses

CD4⁺ T cells of the T_H2 type are pivotal cells in allergic inflammation.^{1,2} IL-4 and IL-5 are 2 of the T_H2-type cytokines that are characteristically expressed at sites of allergic inflammation and affect the immune response to

Abbreviations used

AHR:	Airway hyperresponsiveness
AS:	Antisense
BAL:	Bronchoalveolar lavage
BN:	Brown Norway
ConA:	Concanavalin A
LAR:	Late airway response
MBP:	Major basic protein
ODN:	Oligodeoxynucleotide
OVA:	Ovalbumin
PMA:	Phorbol myristate acetate
R _L :	Lung resistance

foreign sensitizing proteins by promoting IgE synthesis, favoring lymphocyte differentiation toward the T_H2 subtype in vivo, and enhancing eosinophil recruitment, activation, and survival.³⁻⁷ In atopic asthma, increased expression of T_H2-type cytokines has been shown in lymphocytes within the bronchial mucosa and in bronchoalveolar lavage (BAL) fluid.^{2,8} In animal studies evidence of T-cell involvement has been obtained through the demonstration that adoptive transfer of antigen-primed CD4⁺ T cells can induce late airway responses (LAR) and airway hyperresponsiveness (AHR) in naive rats.^{9,10} Similar experiments have shown that the transfer of T_H2 cell clones in mice induces airway eosinophilia and AHR.¹¹⁻¹³

In contrast to the CD4⁺ T cell, the role of the CD8⁺ T cell in allergic airway responses is less clear. CD4⁺ T cells recognize antigens presented by MHC class II molecules (eg, HLA-DR, HLA-DP, and HLA-DQ in human subjects), whereas CD8⁺ T cells recognize antigens presented by MHC class I molecules (eg, HLA-A, HLA-B, and HLA-C). Responses to exogenous antigenic proteins are usually MHC class II restricted. However, there is recent evidence that class I pathways may also be involved.^{14,15} Depletion of CD8⁺ cells in the rat leads to a marked increase in the LAR and airway eosinophilia after allergen exposure,^{16,17} whereas the administration of CD8⁺ cells from a sensitized donor rat to a sensitized recipient undergoing challenge has the opposite effect.¹⁸ The mechanisms by which CD8⁺ T cells inhibit the allergic airway response are not clear. These cells have both cytotoxic properties, and they are a source of immunomodulatory cytokines.¹⁹ Most CD8⁺ T cells pro-

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duce large amounts of IFN- γ .²⁰ IFN- γ is a plausible candidate for the inhibition of allergen-induced responses because it is a T_H1-type cytokine that can suppress the proliferation of T_H2-type T cells and promote the development of T_H1-type T cells.²¹ Consistent with this idea are the findings that exogenous recombinant IFN- γ inhibits the LAR in the rat²² and suppresses AHR and airway eosinophilia in mice.^{23,24}

Adoptive transfer of CD8⁺ T cells suppressed the LAR and airway eosinophilia in Brown Norway (BN) rats sensitized and challenged with ovalbumin (OVA).¹⁸ This suppression was associated with increased expression of IFN- γ and downregulation of T_H2-type cytokine (IL-4 and IL-5) expression in BAL cells.⁶ The suppressive effect induced by the CD8⁺ T cells was antigen specific, occurring only when the CD8⁺ cells were harvested from OVA-sensitized, and not BSA-sensitized, animals. Although IFN- γ is a plausible mediator of the suppressive effects of the CD8⁺ T cells, there is as yet no proof that this is the mechanism by which CD8⁺ T cells inhibit the LAR. The aim of the current study was to test that hypothesis that IFN- γ mediates the inhibition of allergic airway responses mediated by CD8⁺ T cells. We used a well-characterized rat model of allergic airway responses and used the technique of adoptive transfer of T cells to explore the mechanisms of the CD8⁺ T-cell effects. The adoptive transfer of CD8⁺ T cells provides a tool to explore the mechanisms by which CD8⁺ T cells act in vivo because it permits the manipulation of these cells before their transfer in such a way that their in vivo functions are also altered. Gene silencing with antisense (AS) oligodeoxynucleotides (ODNs) is a useful tool for studying the function of specific genes²⁵ and was the technique that we chose to use.

METHODS

Media and reagents

Supplemented RPMI-1640 (penicillin, 100 μ g/mL streptomycin, and 5% or 10% heat-inactivated FCS) and goat anti-mouse FITC-labeled IgG were purchased from GIBCO BRL (Gaithersburg, Md). W3/25 (anti-CD4 mAb), MRC OX8 (anti-CD8 mAb), MRC OX-33 (anti-CD45 mAb), and ED9 (anti-myeloid), were purchased from Cedarlane (Hornby, Canada). MACS microbeads were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Anti-rat IFN- γ mAb was purchased from BioSource, Inc (Camarillo, Calif). BMK13, an anti-human major basic protein (MBP) mAb, was provided by Dr R. Moqbel (Edmonton, Alberta, Canada). Phosphorothioate-ODNs were synthesized²⁶ by the Sheldon Biotechnology Center (Montreal, Canada).

Study protocol

Airway responses to OVA were measured 14 days after sensitization and 2 days after CD8⁺ T-cell transfers. Three groups were studied. The control group consisted of 8 rats administered cell-free medium only because unsensitized CD8⁺ cells have no effect on airway responses to allergen challenge.¹⁸ The AS group consisted of 8 rats transferred with IFN- γ AS ODN-treated CD8⁺ T cells. The sense group consisted of 8 rats transferred with IFN- γ sense ODN-treated CD8⁺ T cells.

Animals and sensitization

Male BN rats (7-9 weeks; Harlan Inc, Bicester, England) were sensitized subcutaneously in the neck with 1 mg of OVA (grade V; Sigma Immunochemicals, St Louis, Mo) and 4.28 mg of Al(OH)₃ gel (Anachemia Chemicals, Montreal, Quebec, Canada) in 1 mL of normal saline and 0.5 mL of *Bordetella pertussis* vaccine (6×10^9 heat-killed bacilli/mL; Institut Armand Frappier, Laval-Des-Rapides, Quebec, Canada) intraperitoneally. Seven days later, rats were intubated while under the effects of pentobarbital anesthesia (35 mg/kg administered intraperitoneally) and given a 5-minute exposure to aerosolized OVA (5%, wt/vol) to boost sensitization.

Treatment of CD8⁺ T cells with ODNs and adoptive transfer

Cervical lymph node cells were harvested from donor rats ($n = 3$ or 4) 14 days after sensitization and purified to greater than 90% by means of negative selection with immunomagnetic cell sorting (MACS)¹⁸ and the following antibodies: W3/25; anti-CD4, OX-33; anti-B cell and ED9; anti-myeloid cell. CD8⁺ T cells were incubated with ODNs (2 μ mol/L) for 6 hours in medium supplemented with 5% FCS, and 2×10^6 cells were transferred intraperitoneally to OVA-sensitized recipients.

AS ODN and IFN- γ expression in lymphocytes

Mononuclear cells isolated by means of Ficoll-Paque from cervical lymph nodes of naive rats were stimulated with 2 μ g/mL concanavalin A (Con A; Sigma Immunochemicals) for 48 hours in medium with 10% FCS to evaluate the effects of AS ODN on IFN- γ expression by T lymphocytes. After stimulation, cells were incubated with either 2 μ mol/L IFN- γ AS ODN, sense ODN, or culture medium for 6 hours in medium with 5% FCS. The ODN concentration and duration of incubation were based on published work.^{26,27} After removal of ODNs, cells were restimulated with 10 ng/mL phorbol myristate acetate (PMA) plus 0.25 μ g/mL ionomycin (Sigma Immunochemicals) in the presence of GolgiStop for 4 hours. The cells were analyzed by means of flow cytometry (FACScan; Becton Dickinson, Mountain View, Calif) with an FITC-labeled anti-IFN- γ antibody (PharMingen, San Diego, Calif).

Measurement of airway responses to antigen challenge

Two days after the administration of CD8⁺ T cells or cell-free medium, animals were anesthetized with urethane (1.25 g/kg administered intraperitoneally), instrumented, and challenged for 5 minutes with aerosolized OVA or BSA (5% wt/vol). Lung resistance (R_L) was measured for 8 hours.^{6,28}

Bronchoalveolar lavage

BAL was performed 8 hours after challenge with 5×5 mL of saline. The total cell count and cell viability were estimated with a hemacytometer and Trypan blue stain. Slides were prepared with a Cytospin model II (Shandon, Pittsburgh, Pa). The differential cell count was assessed with May-Grünwald-Giemsa staining.

MBP and IFN- γ expression in BAL cells determined by means of immunocytochemistry

BAL cytospin preparations were fixed in acetone-methanol (60:40) for 5 minutes, air-dried, and stored at -80°C until analysis. Cells were stained with mouse anti-human MBP mAb or mouse anti-rat IFN- γ mAb by using the alkaline phosphatase-antialkaline phosphatase method. The slides were assessed by a blinded investigator.

Statistical analysis

The data are expressed as means $\pm$ SEM. We used 1-way ANOVA, followed by the Fischer least significant difference test, for comparison of several means. Pairs of means were compared with the Student *t* test. A significant *P* value was set at the 5% level of confidence.

RESULTS

Effect of AS ODN on the level of expression of IFN- γ by activated T cells

To evaluate the efficacy of AS ODN treatment on IFN- γ expression by T cells and to ensure that ODNs were not cytotoxic, we compared the effects of sense and AS ODNs on intracellular IFN- γ expression in ConA-stimulated T-lymphocyte preparations by using flow cytometry. An excess of nonconjugated anti-IFN- γ antibody was used as a control. After incubation with ConA and subsequent stimulation with PMA and ionomycin, 10% to 20% of the gated T lymphocytes identified as probable blasts on the basis of forward and side scatter plots (Fig 1, A) were positive for IFN- γ (Fig 1, B). Only 5% to 10% of T lymphocytes that were incubated with AS ODN for 6 hours before stimulation with PMA and ionomycin expressed IFN- γ (Fig 1, C), representing a reduction of approximately 50% in the level of expression of IFN- γ compared with that seen in control cells, which were incubated without ODN (Fig 2). Pretreatment with sense ODN had a small effect, decreasing by 16% the number of IFN- γ -expressing T cells compared with control cells (Figs 1, D, and 2). The difference between sense and AS treatments was highly significant ($n = 4$, $P < .001$). There were no differences in cell viability, as assessed by means of Trypan blue exclusion, between the AS ODN-treated cells ($83.9\% \pm 2.5\%$), the sense ODN-treated cells ($84.7\% \pm 1.9\%$), and the control cells ($85.9\% \pm 2.4\%$).

Modulation of IFN- γ expression by CD8 $^{+}$ T-cell transfers

The effects of CD8 $^{+}$ T-cell transfers on IFN- γ expression were analyzed in BAL fluid cells. IFN- γ immunoreactivity was identified on the basis of its discrete red cytoplasmic staining. The majority of IFN- γ immunoreactivity was localized to mononuclear cells, with a morphology consistent with T cells and macrophages. OVA-challenged control rats had few IFN- γ -expressing cells in the BAL fluid (Fig 3). CD8 $^{+}$ T cells that were treated with sense ODNs before transfer resulted in a marked increase in the number of IFN- γ^{+} cells compared with both OVA control rats and rats given AS ODN-treated CD8 $^{+}$ T cells ($P < .001$). Rats that received AS ODN-treated CD8 $^{+}$ T cells had comparable IFN- γ expression with that of the control animals. This finding confirms the efficacy of the IFN- γ AS ODN treatment in neutralizing the effects of CD8 $^{+}$ T-cell transfers on IFN- γ expression in vivo.

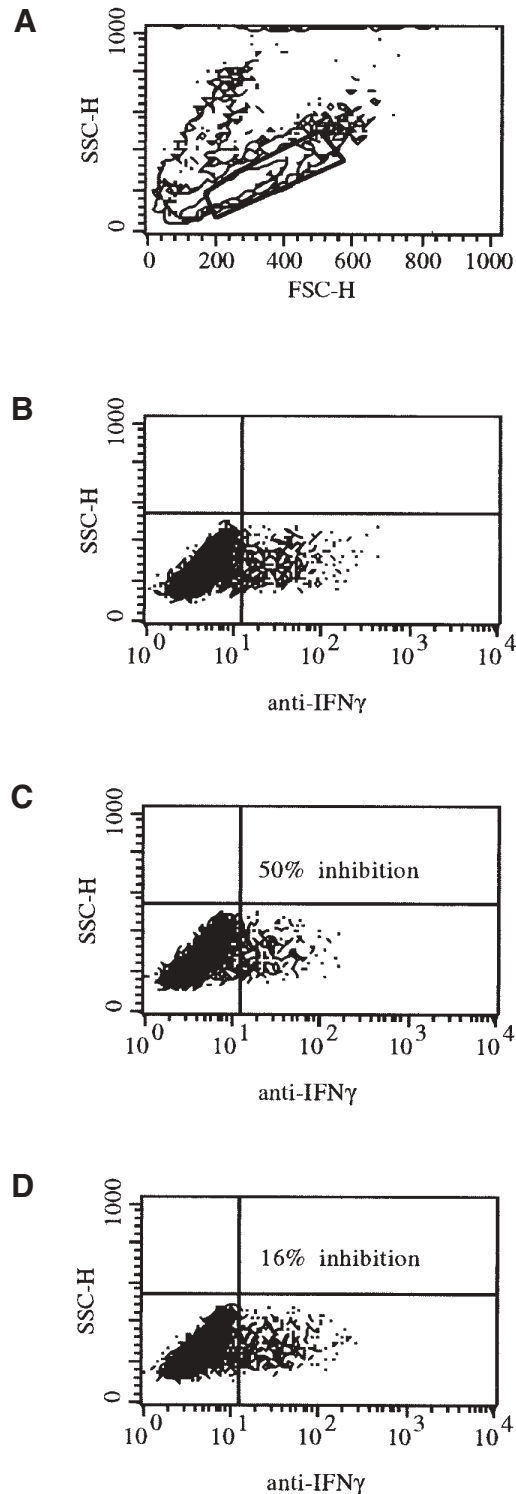


FIG 1. Quantification by means of FACS analysis of IFN- γ intracellular expression in ConA-stimulated T cells. **A** shows the gated population of T cells determined on the basis of their side scatter (SSC) and forward scatter (FSC). **B**, **C**, and **D** show the number of T cells expressing IFN- γ after incubation with culture medium alone, IFN- γ AS ODN ($2 \mu\text{mol/L}$), or IFN- γ sense ODN ($2 \mu\text{mol/L}$) for 6 hours before stimulation with PMA (10 ng/mL) and ionomycin ($0.25 \mu\text{g/mL}$) for 4 hours, respectively. Results correspond to 1 of 4 representative experiments.

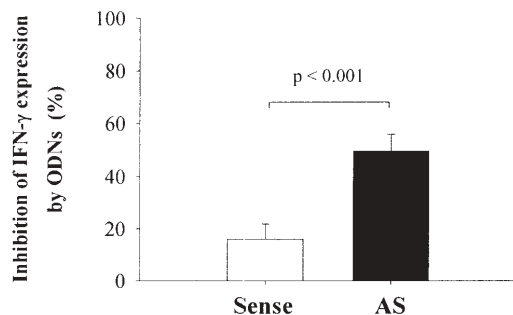


FIG 2. Suppression of IFN- γ expression in ConA-stimulated blasts. Intracellular IFN- γ was assessed by means of flow cytometry after T cells have been incubated 6 hours with culture medium (no ODN), IFN- γ AS ODN (2 μ mol/L), or IFN- γ sense ODN (2 μ mol/L) before stimulation with PMA (10 ng/mL) and ionomycin (0.25 μ g/mL) for 4 hours. The percentage of suppression of IFN- γ was calculated as followed: percentage suppression = (no ODN – AS or sense ODN)/no ODN $\times$ 100. The means and SEs are shown for 4 experiments.

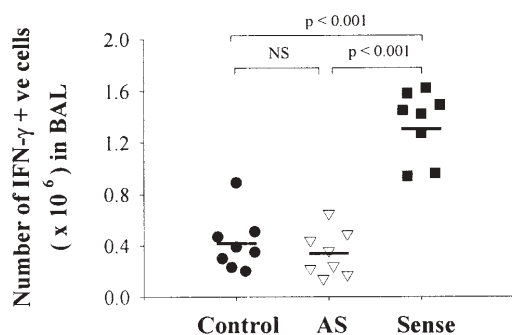


FIG 3. IFN- γ expression in BAL fluid investigated by means of immunocytochemistry. BAL fluid was recovered at the end of the experiment (8 hours after OVA challenge) from OVA-sensitized animals that were injected intraperitoneally with culture medium (control, $n = 8$, filled circles), CD8 $^+$ T cells treated with IFN- γ sense ODN (sense, $n = 8$, filled squares), or CD8 $^+$ T cells treated with IFN- γ AS ODN (AS, $n = 8$, open triangles). IFN- γ was detected by using the alkaline phosphatase–antialkaline phosphatase method with a mouse anti-rat IFN- γ mAb. Results are expressed as the total number of IFN- γ +ve cells in the BAL fluid. P values were determined by means of ANOVA, followed by the Fischer least significant difference test.

Airway responses to OVA challenge in sensitized animals after transfer of CD8 $^+$ T cells

Basal airway caliber was unaffected by the transfer of CD8 $^+$ cells, as indicated by the lack of a significant difference in mean baseline R_L among the 3 groups (recipients of AS ODN-treated CD8 $^+$ T cells, sense ODN-treated CD8 $^+$ T cells, and control animals that received only medium). Fig 4 shows the mean R_L at each time point after OVA challenge for each group. The early airway response was defined as the maximal value of R_L , expressed as a percentage of baseline R_L , in the first 30 minutes after challenge, and there was no significant difference among groups (Fig 5).

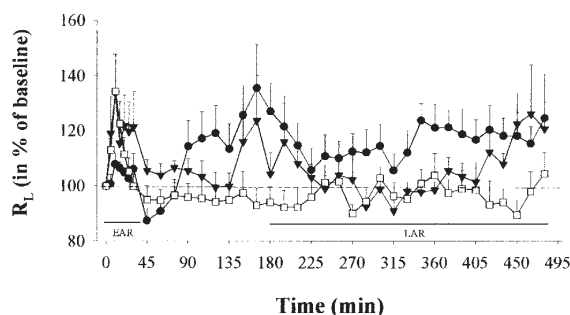


FIG 4. Time course of R_L expressed as percentage of baseline value after aerosol challenge. OVA-sensitized animals were injected intraperitoneally with culture medium (control, $n = 8$, filled circles), CD8 $^+$ T cells treated with AS ODN (AS, $n = 8$, filled triangles), or CD8 $^+$ T cells treated with sense ODN (sense, $n = 8$, open squares). The animals were challenged with a 5% OVA aerosol. Results are expressed as means $\pm$ SEM.

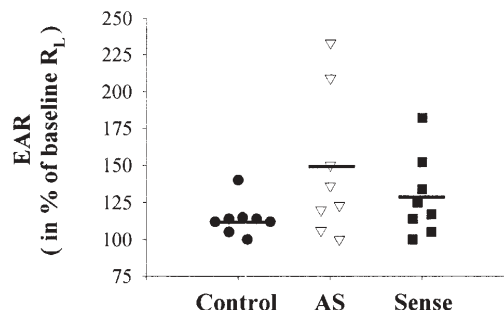


FIG 5. Early airway responses (EAR) in BN rats. OVA-sensitized animals were injected intraperitoneally with culture medium (control, $n = 8$, filled circles), CD8 $^+$ T cells treated with sense ODN (sense, $n = 8$, filled squares), or CD8 $^+$ T cells treated with AS ODN (AS, $n = 8$, open triangles). The early airway responses were calculated as the largest increase in R_L within the 30 minutes after a 5% OVA aerosol. Results are expressed as percentage of baseline for each rat.

The LAR was calculated as the area under the curve of R_L against time (in centimeters of water per milliliter per second $\times$ minutes) from 3 to 8 hours after challenge after correction of R_L for the baseline value. As shown in Fig 6, OVA-sensitized rats that received cell-free medium (control group) had LARs comparable in magnitude with those previously described in this animal model.¹⁸ The transfer of sense ODN-treated CD8 $^+$ T cells inhibited the LAR, an inhibitory effect that was comparable with the transfer of untreated OVA-primed CD8 $^+$ T cells.¹⁸ Interestingly, the AS-treated CD8 $^+$ T cells also inhibited the LAR to a degree that did not differ significantly from that seen with the sense-treated CD8 $^+$ T cells (Fig 6).

CD8 $^+$ T cell-mediated changes in airway inflammation: Relationship to IFN- γ synthesis

To evaluate the effects of the transferred CD8 $^+$ T cells on airway inflammation, we analyzed changes in leukocyte numbers in BAL fluid. Approximately 22 mL of fluid was recovered per BAL, and this did not differ significant-

TABLE I. Total and differential cell counts in BAL

Group	CD8 ⁺ T-cell treatments	Total cell count (10 ⁶)	Macrophages (%)	Lymphocytes (%)	Granulocytes (%)
I (n = 8)	IFN- γ AS	4.4 $\pm$ 0.6	78.5 $\pm$ 3.3	1.9 $\pm$ 0.3	19.8 $\pm$ 3.2
II (n = 8)	IFN- γ sense	3.2 $\pm$ 0.3*	78.6 $\pm$ 4.1	1.7 $\pm$ 0.4	20.0 $\pm$ 4.1
III (n = 8)	Culture medium	5.0 $\pm$ 0.7	75.0 $\pm$ 3.5	1.2 $\pm$ 0.2	23.8 $\pm$ 3.5

Cell counts were assessed by means of May Grünwald-Giemsa staining. Data are expressed as means $\pm$ SEM.

* $P < .05$ (ANOVA and Fischer least significant difference test) in comparison with group III.

ly among groups. The total cell count was significantly lower only in the group of animals treated with sense ODN in comparison with that seen in the nontreated animals (Table I). There were no significant differences in the absolute numbers of macrophages, granulocytes, and lymphocytes among the 3 groups (Table I). The contribution of the eosinophils to airway inflammation was assessed by means of MBP immunostaining because of the previous finding that the conventional May-Grünwald-Giemsa stain lacked sensitivity.⁹ As shown in Fig 7, eosinophilia was significantly reduced in the BAL fluid of rats that received either sense ODN- or AS ODN-treated CD8⁺ T cells in comparison with that seen in the control group ($P < .01$ and $P < .05$, respectively). However, the CD8⁺ cell-mediated inhibition of eosinophilia was less in the AS ODN- than in the sense ODN-treated group ($P < .05$).

DISCUSSION

The mobilization of CD8⁺ T cells into the lungs of human subjects after an allergen-induced isolated early airway response in asthma has led to the suggestion that CD8⁺ T cells might have a suppressor effect.²⁹ Animal models have also implicated CD8⁺ T cells in a number of aspects of allergic responses, including AHR and IgE synthesis.³⁰⁻³² Given the difficulty in directly testing hypotheses in relationship to the role of the CD8⁺ T-cell cytokines in the modulation of the LAR, we have used the technique of adoptive transfer in a model of allergic asthma, the BN rat.¹⁸ In this rat antigen-primed CD8⁺ T cells have a potent suppressive effect on the LAR, inhibit BAL eosinophilia, and reduce the expression of T_H2-type cytokines in BAL fluid of recipient sensitized rats undergoing allergen challenge. The inhibition of the LAR and of airway eosinophilia is associated with an increase in IFN- γ mRNA expression in BAL cells.¹⁸ The reported inhibitory effects of IFN- γ in vitro on T_H2-type cytokine expression suggested a possible mechanism by which CD8⁺ T cells might act to inhibit allergic airway responses. The current study with AS oligonucleotides for IFN- γ provides evidence that IFN- γ synthesis by CD8⁺ T cells inhibits allergen-induced eosinophilic inflammation but not the LAR. The latter must be inhibited by other mechanisms.

It was necessary to effect a significant alteration in the synthesis of IFN- γ production by airway CD8⁺ T cells to test its importance in the modulation of allergic airway responses. We targeted the synthesis of IFN- γ by the exposure of the CD8⁺ T cells to AS ODNs directed against IFN- γ . By using specific AS ODNs previously tested for effica-

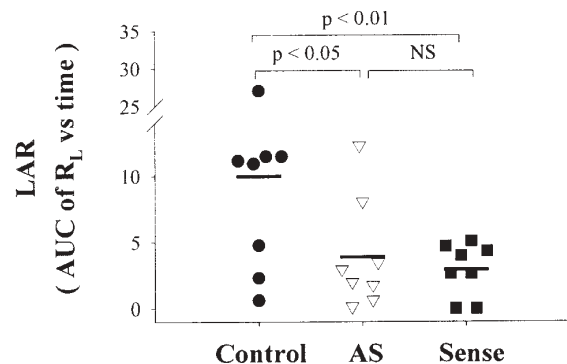


FIG 6. LARs in BN rats. OVA-sensitized animals were injected intraperitoneally with culture medium (control, n = 8, filled circles), CD8⁺ T cells treated with sense ODN (sense, n = 8, filled squares), or CD8⁺ T cells treated with AS ODN (AS, n = 8, open triangles). The magnitude of the LARs was calculated as the area under the curve (AUC) of R_L versus time curve from 3 to 8 hours after a 5% OVA aerosol.

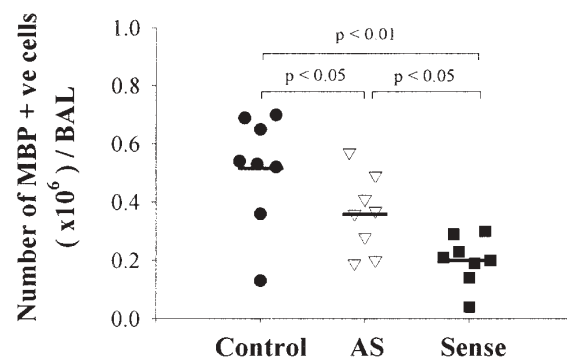


FIG 7. Eosinophilia in BAL fluid investigated by means of immunocytochemistry. BAL fluid was recovered at the end of the experiment (8 hours after OVA challenge) from OVA-sensitized animals that were injected intraperitoneally with culture medium (control, n = 8, filled circles), CD8⁺ T cells treated with sense ODN (sense, n = 8, filled squares), or CD8⁺ T cells treated with AS ODN (AS, n = 8, open triangles). Eosinophils were detected by using the alkaline phosphatase-antialkaline phosphatase method with the BMK-13 mAb. Results are expressed as the number of MBP⁺ cells in the total volume of BAL fluid.

cy in inhibiting IFN- γ synthesis by cultured hepatocytes stimulated with IL-13,²⁶ we produced about 50% inhibition of IFN- γ expression in ConA-, PMA-, and ionomycin-stimulated T cells. Although this degree of inhibition ex vivo is relatively modest, the AS treatment appears to have been much more effective against the stimulus of allergen chal-

lenge in vivo. The IFN- γ AS ODN treatment of the CD8 $^{+}$ T cells ablated their enhancement of IFN- γ expression in the BAL after allergen challenge. Indeed, rats transferred with AS-pretreated CD8 $^{+}$ T cells had as few IFN- γ -expressing cells in the BAL as the rats that were not given CD8 $^{+}$ T cells.

It seems unlikely that the increases in the number of IFN- γ^{+} cells in the BAL fluid are attributable to the appearance of the transferred CD8 $^{+}$ T cells. Presumably, IFN- γ produced and released by transferred CD8 $^{+}$ T cells leads to local amplification of IFN- γ synthesis by resident cells in the airways of recipient animals or to the recruitment of other IFN- γ -producing cells. This is consistent with a previous study suggesting that IFN- γ may favor the expansion of T lymphocytes of the T $_{H}1$ -type phenotype.³³ We do not know the phenotype of the T cells in the BAL that produce IFN- γ , but both CD8 $^{+}$ T cells and CD4 $^{+}$ T $_{H}1$ cells are potential sources. Additionally, and based on morphological criteria, it appears that IFN- γ expression by alveolar macrophages was stimulated also.

Several studies have reported an alternate mechanism of action of synthetic ODNs caused by the presence of a particular motif (CpG) within the nucleotide sequence, leading to an increased production of IFN- γ by T cells.³⁴ Airway eosinophilia and induction of T $_{H}2$ -type cytokine expression were prevented by such treatment.³⁵ We were therefore concerned that ODN treatments might alter the suppressive properties of the CD8 $^{+}$ T cells through the stimulation of IFN- γ production in a nonspecific manner. Even though the AS and sense ODNs we used contained 1 and 2 CpG motifs, respectively, FACS analysis of the ODN-treated CD8 $^{+}$ T cells did not show such an effect. The lack of nonspecific effects likely resides in the fact that we selectively treated the targeted T cells before the injection rather than injecting the ODNs intraperitoneally during the process of sensitization.³⁵

The successful abrogation of IFN- γ expression in BAL fluid by means of specific AS treatment of the CD8 $^{+}$ T cells was associated with a partial restoration of allergen-induced BAL eosinophilia. This regulatory role of IFN- γ is likely mediated, at least in part, by effects on IL-5 whose expression in previous experiments was substantially reduced by CD8 $^{+}$ T-cell transfers.¹⁸ Such observations are consistent with the reported inhibitory effect of IFN- γ on T $_{H}2$ -type cytokine expression by CD4 $^{+}$ T cells.³³ Despite the striking effects on IFN- γ expression and eosinophilia in the BAL fluid, there was no observable alteration of the LAR; an equivalent suppression of the LAR was seen in the recipients of both sense ODN- and AS ODN-treated CD8 $^{+}$ cells. This lack of effect of IFN- γ AS treatment on the LAR seems unlikely to be caused by a failure to inhibit IFN- γ synthesis for the reasons discussed above. Therefore IFN- γ is unlikely to be the only CD8 $^{+}$ T cell-derived mediator that is responsible for the inhibition of OVA-induced LARs. The results also suggest that the eosinophil is not the effector cell responsible for the LAR.

The mechanism of involvement of CD8 $^{+}$ T cells in OVA-induced airway responses is not entirely clear. Activated B cells have been shown to present peptides to CD8 $^{+}$ T cells and to trigger their suppressive activity for

CD4 $^{+}$ cells.³⁶ There is increasing evidence of MHC class I-restricted activation of CD8 $^{+}$ T cells by OVA, resulting in cytokine expression (IFN- γ and IL-2) and cytolytic activity.³⁰ Fas-mediated apoptosis has been induced in antigen-activated CD4 $^{+}$ cells by superantigen-activated CD8 $^{+}$ cells.³⁷ Moreover, the preferential expression of Fas ligand by activated CD8 $^{+}$ T cells may play a role in the ability of CD8 $^{+}$ T cells to suppress immune responses by induction of CD4 $^{+}$ T-cell apoptosis.

Other studies with animal models of asthma demonstrated that IFN- γ may play an important role in the immunoglobulin synthesis and allergic inflammation. The immunoregulatory action of the CD8 $^{+}$ T cells on IgE synthesis in vivo and in vitro appears to be mediated by IFN- γ ,³⁸ and both the $\alpha\beta^{+}$ and $\gamma\delta^{+}$ CD8 $^{+}$ T cells have been implicated in the observed effects on IgE synthesis.^{38,39} Mice lacking the IFN- γ receptor have higher levels of IgE and IgG1, lower levels of IgG2a, and a sustained lung eosinophilic inflammation in the lungs associated with a prolonged capacity of lung T cells to maintain a T $_{H}2$ profile.⁴⁰ Hessel et al⁴¹ demonstrated that IFN- γ is essential to the development of AHR because IFN- γ antibodies administered during the challenge period completely inhibit the induction of AHR in OVA-challenged mice. A recent study, also with the BN rat, showed that administration of IFN- γ attenuated AHR and allergen-induced airway inflammation.⁴² There was also evidence of inhibition of IL-4, IL-5, and IL-10 expression by IFN- γ , whereas the converse occurred after anti-IFN- γ treatment. Perhaps the discrepancy between the effects of exogenous IFN- γ and antibodies directed against IFN- γ on allergen-induced AHR and the results of the current study reflects the fact that only the CD8 $^{+}$ T-cell production of IFN- γ was targeted by the AS ODN pretreatment of these cells. It is also possible that the factors that cause AHR are not the same as those responsible for the LAR. One additional caveat is that it is possible that the inhibition of IFN- γ synthesis by the AS ODN treatment was not complete and that the thresholds for alterations in eosinophilia and the LAR differ, with the latter requiring more complete inhibition of IFN- γ than eosinophilia.

In conclusion, we have shown that CD8 $^{+}$ T cells transferred from sensitized donors can potentially inhibit LAR responses and airway eosinophilia in sensitized recipients undergoing allergen challenge. The inhibitory effect of the CD8 $^{+}$ cells is associated with a reduction in eosinophilia and enhanced expression of IFN- γ in the BAL. The pretreatment of the CD8 $^{+}$ cells before transfer with an AS ODN directed against IFN- γ abrogated the enhanced expression of IFN- γ in the BAL, suggesting successful targeting of this cytokine expression by the CD8 $^{+}$ cells. The results of our experiments indicate a role for IFN- γ in the modulation of eosinophil recruitment into the BAL but not as a determinant of the suppressive effects of CD8 $^{+}$ cells on the LAR. It also seems that the eosinophil may not be the effector cell of the LAR.

REFERENCES

1. Bentley AM, Menz G, Storz CHR, Robinson DS, Bradley B, Jeffery PK,

- et al. Identification of T lymphocytes, macrophages, and activated eosinophils in the bronchial mucosa in intrinsic asthma. *Am Rev Respir Dis* 1992;146:500-6.
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. Pene J, Rousset F, Briere F, Chretien I, Bonnefoy JY, 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* 1988;85:6880-4.
4. Del Prete G, Maggi E, Parronchi P, Chretien 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.
5. Swain SL, Weinberg AD, English M, Huston G. IL-4 directs the development of Th2-like helper effectors. *J Immunol* 1990;145:3796-806.
6. 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. IL-5 as an eosinophil chemotactic factor. *J Exp Med* 1988;167:1737-42.
7. 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.
8. Robinson DS, Hamid Q, Ying S, Tscipoulos 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.
9. Watanabe A, Rossi P, Renzi PM, Xu LJ, Guttman RD, Martin JG. Adoptive transfer of allergic airway responses with sensitized lymphocytes in BN rats. *Am J Respir Crit Care Med* 1995;152:64-70.
10. Mishima H, Hojo M, Watanabe A, Hamid QA, Martin JG. CD4+ T cells can induce airway hyperresponsiveness to allergen challenge in the Brown Norway rat. *Am J Respir Crit Care Med* 1998;158:1863-70.
11. Hogan SP, Koskinen A, Matthei KI, Young IG, Foster PS. Interleukin-5-producing CD4+ T cells play a pivotal role in aeroallergen-induced eosinophilia, bronchial hyperreactivity, and lung damage in mice. *Am J Respir Crit Care Med* 1998;157:210-8.
12. Kaminuma O, Mori A, Ogawa K, Nakata A, Kikkawa 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.
13. 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.
14. Rock KL, Gamble S, Rothstein L. Presentation of exogenous antigen with class I major histocompatibility complex molecules. *Science* 1990;249:918-21.
15. McMenamin C, Holt PG. The natural immune response to inhaled soluble protein antigens involves major histocompatibility complex (MHC) class I-restricted CD8+ T cell-mediated but MHC class II-restricted CD4+ T cell-dependent immune deviation resulting in selective suppression of immunoglobulin E production. *J Exp Med* 1993;178:889-99.
16. Olivenstein R, Renzi PM, Yang JP, Rossi P, Laberge S, Wasserman S, et al. Depletion of OX-8 lymphocytes from the blood and airways using monoclonal antibodies enhances the late airway response in rats. *J Clin Invest* 1993;92:1477-82.
17. Allakhverdi Z, Lamkhieou B, Olivenstein R, Hamid Q, Renzi PM. CD8 depletion-induced late airway response is characterized by eosinophilia, increased eotaxin, and decreased IFN-gamma expression in rats. *Am J Respir Crit Care Med* 2000;162:1123-31.
18. Suzuki M, Taha R, Ihaku D, Hamid QA, Martin JG. CD8+ T cells modulate late allergic airway responses in Brown Norway rats. *J Immunol* 1999;163:5574-81.
19. Kelso A, Troutt AB, Maraskovsky E, Gough NM, Morris L, Pech MH, et al. Heterogeneity in lymphokine profiles of CD4+ and CD8+ T cells and clones activated in vivo and in vitro. *Immunol Rev* 1991;123:85-114.
20. Mueller R, Chanez P, Campbell AM, Bousquet J, Heusser C, Bullock GR. Different cytokine patterns in bronchial biopsies in asthma and chronic bronchitis. *Respir Med* 1996;90:79-85.
21. Nakamura T, Lee RK, Nam SY, Podack ER, Bottomly K, Flavell RA. Roles of IL-4 and IFN-gamma in stabilizing the T helper cell type 1 and 2 phenotype. *J Immunol* 1997;158:2648-53.
22. Isogai S, Hamid Q, Minshall E, Miyake S, Yoshizawa Y, Taha R, et al. Interferon-gamma increases IL-12 mRNA expression and attenuates allergic late-onset airway responses in the Brown Norway rat. *Eur Respir J* 2001;16:22-9.
23. Hofstra CL, Van Ark I, Hofman G, Nijkamp FP, Jardieu PM, Van Oosterhout AJ. Differential effects of endogenous and exogenous interferon-gamma on immunoglobulin E, cellular infiltration, and airway responsiveness in a murine model of allergic asthma. *Am J Respir Cell Mol Biol* 1998;19:826-35.
24. Iwamoto I, Nakajima H, Endo H, Yoshida S. Interferon gamma regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4+ T cells. *J Exp Med* 1993;177:573-6.
25. Wagner RW. Gene inhibition using antisense oligodeoxynucleotides. *Nature* 1994;372:333-5.
26. Schroeder RA, Gu JS, Kuo PC. Interleukin 1beta-stimulated production of nitric oxide in rat hepatocytes is mediated through endogenous synthesis of interferon gamma. *Hepatology* 1998;27:711-9.
27. Molet S, Ramos-Barbon D, Martin JG, Hamid Q. Adoptively transferred late allergic response is inhibited by IL-4, but not IL-5, antisense oligonucleotide. *J Allergy Clin Immunol* 1999;104:205-14.
28. Bates JHT, Shardonofsky F, Stewart DE. The low frequency dependence of respiratory system resistance and elastance in normal dogs. *Respir Physiol* 1989;78:369-82.
29. Gonzalez MC, Diaz P, Galleguillos FR, Ancic P, Cromwell O, Kay AB. Allergen-induced recruitment of bronchoalveolar helper (OKT4) and suppressor (OKT8) T-cells in asthma. Relative increases in OKT8 cells in single early responders compared with those in late-phase responders. *Am Rev Respir Dis* 1987;136:600-4.
30. MacAry PA, Holmes BJ, Kemeny DM. Ovalbumin-specific, MHC class I-restricted, alpha beta-positive, Tc1 and Tc0 CD8+ T cell clones mediate the in vivo inhibition of rat IgE. *J Immunol* 1998;160:580-7.
31. Hamelmann E, Oshiba A, Paluh J, Bradley K, Loader J, Potter TA, et al. Requirement for CD8+ T cells in the development of airway hyperresponsiveness in a murine model of airway sensitization. *J Exp Med* 1996;183:1719-29.
32. Renz H, Lack G, Saloga J, Schwinzer R, Bradley K, Loader J, et al. Inhibition of IgE production and normalization of airways responsiveness by sensitized CD8 T cells in a mouse model of allergen-induced sensitization. *J Immunol* 1994;152:351-60.
33. Gajewski TF, Joyce J, Fitch FW. Antiproliferative effect of IFN-gamma in immune regulation. III. Differential selection of TH1 and TH2 murine helper T lymphocyte clones using recombinant IL-2 and recombinant IFN-gamma. *J Immunol* 1989;143:15-22.
34. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci* 1996;93:2879-83.
35. 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.
36. Noble A, Pestano GA, Cantor H. Suppression of immune responses by CD8 cells. I. Superantigen-activated CD8 cells induce unidirectional Fas-mediated apoptosis of antigen-activated CD4 cells. *J Immunol* 1998;160:559-65.
37. Noble A, Zhao ZS, Cantor H. Suppression of immune responses by CD8 cells. II. Qa-1 on activated B cells stimulates CD8 cell suppression of T helper 2 responses. *J Immunol* 1998;160:566-71.
38. Holmes BJ, MacAry PA, Noble A, Kemeny DM. Antigen-specific CD8+ T cells inhibit IgE responses and interleukin-4 production by CD4+ T cells. *Eur J Immunol* 1997;27:2657-65.
39. McMenamin C, McKersy M, Kuhnlein P, Hunig T, Holt PG. Gamma delta T cells down-regulate primary IgE responses in rats to inhaled soluble protein antigens. *J Immunol* 1995;154:4390-4.
40. Coyle AJ, Tsuyuki S, Bertrand C, Huang S, Aguet M, Alkan SS, et al. Mice lacking the IFN-gamma receptor have impaired ability to resolve a lung eosinophilic inflammatory response associated with a prolonged capacity of T cells to exhibit a Th2 cytokine profile. *J Immunol* 1996;156:2680-5.
41. Hessel EM, Van Oosterhout AJ, Van Ark I, Van Esch B, Hofman G, Van Loveren H, et al. Development of airway hyperresponsiveness is dependent on interferon-gamma and independent of eosinophil infiltration. *Am J Respir Cell Mol Biol* 1997;16:325-34.
42. Huang TJ, MacAry PA, Wilke T, Kemeny DM, Chung KF. Inhibitory effects of endogenous and exogenous interferon-gamma on bronchial hyperresponsiveness, allergic inflammation and T-helper 2 cytokines in Brown-Norway rats. *Immunology* 1999;98:280-8.