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Long Term Prevention of Allergic Lung Inflammation in a Mouse Model of Asthma by CpG Oligodeoxynucleotides

Sanjiv Sur,*2 James S. Wild,* Barun K. Choudhury,* Nilanjana Sur,* Rafeul Alam,* and Dennis M. Klinman†

Asthma is an inflammatory disease of the airways that is induced by Th2 cytokines and inhibited by Th1 cytokines. Despite a steady increase in the incidence, morbidity, and mortality from asthma, no current treatment can reduce or prevent asthma for a prolonged period. We examined the ability of unmethylated CpG oligodeoxynucleotides (ODN), which are potent inducers of Th1 cytokines, to prevent the inflammatory and physiological manifestations of asthma in mice sensitized to ragweed allergen. Administration of CpG ODN 48 h before allergen challenge increased the ratio of IFN-γ to IL-4 secreting cells, diminished allergen-induced eosinophil recruitment, and decreased the number of ragweed allergen-specific IgE-producing cells. These effects of CpG ODN were sustained for at least 6 wk after its administration. Furthermore, there was a vigorous Th1 memory response to the recall Ag, inhibition of peribronchial and perivascular lung inflammation, and inhibition of bronchial hyperresponsiveness 6 wk after administration of CpG ODN. Administration of CpG ODN in IFN-γ−/− mice failed to inhibit eosinophil recruitment, indicating a critical role of IFN-γ in mediating these effects. This is the first report of a treatment that inhibits allergic lung inflammation in presensitized animals for a prolonged period and thus has relevance to the development of an effective long term treatment for asthma. The Journal of Immunology, 1999, 162: 6284–6293.

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This laboratory has been examining the immunomodulatory activity of synthetic oligodeoxynucleotides (ODN) expressing CpG motifs that consist of a central unmethylated CpG dinucleotide flanked by two 5′-purines and two 3′-pyrimidines. We and others found that CpG ODN rapidly stimulate T, B, NK, and macrophages to proliferate, secrete Abs, and/or produce a variety of Th1-associated cytokines, predominantly IFN-γ and IL-12 (18, 19). Kline et al. (20) demonstrated that systemically administered CpG ODNs could reduce the allergic response of mice sensitized and challenged with Schistosoma eggs. In that study, CpG ODN was administered i.p., and their effect was monitored for 2 wk. Broide et al. (21) recently reported that administration of CpG ODN inhibits allergic responses in mice sensitized and challenged with OVA. In that study, CpG ODNs were administered i.p., intranasally, or intratracheally, and their effects were monitored for 1–6 days.

In this study, we examined whether intratracheal administration of CpG ODN (modeling the effect of nebulizer delivery to humans) could alter the immunological and physiological manifestations of ragweed-induced asthma in mice. These experiments used animals that were presensitized by allergen to mount a pathological Th2 allergic response. We found that CpG ODN administered 2 days before RW challenge converted the predominantly Th2 allergic response to a dominant Th1 response and significantly reduced lung eosinophilia and RW-specific IgE production. The beneficial

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In the RW/CpG-48 h group, CpG ODN was given 48 h before allergen challenge. i.t., intratracheal; CpG i.t., i.t. CpG ODN 35 μg i.t. 200 μg of ragweed.

**Materials and Methods**

**Mice**

Female BALB/c mice, 6–8 wk old, were purchased from the Harlan Laboratories (Indianapolis, IN) to perform all experiments except those requiring IFN-γ −/− and IFN-γ +/+ mice. The latter (IFN-γ −/− and IFN-γ +/+ mice) were 5-wk-old female BALB/c mice that were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a specific pathogen-free environment throughout the experiment.

**Oligonucleotides**

Two immunostimulatory unmethylated CpG-containing ODNs of sequence GCTAGAGCTAGCT and TCAAGCTGTT were synthesized as described (18, 19). Control ODN were synthesized by eliminating the CpG motifs by inversion (GCTAGAGCTAGCT, TCAAGCTGTT) or by methylating the cytosine residues in the CpG motifs. All ODNs were produced on the same synthesizer and were purified by extraction with phenol-chloroform-isoamyl alcohol (25:24:1) followed by ethanol precipitation. These ODN contained undetectable levels of endotoxin (<0.02 UKg, as determined using a Limulus amebocyte lysate analysis kit (QCL-1000 BioWhittaker, Walkersville, MD). All ODN were administered at a dose of 35 μg/100 μl intratracheal instillation.

**Ragweed**

Endotoxin-free ragweed (lot XP56-D10-1320) was purchased from Greer Laboratories (Lenoir, NC). All experiments were performed with ragweed because it is an allergen relevant to human allergic asthma. We previously showed that patients with allergic asthma challenged subseguently with RW and other allergens mount a late phase airway inflammation that is either predominantly neutrophilic or eosinophilic, depending on the quantity of endotoxin in the allergenic extract (22). Because asthma is an eosinophilic disease of the airways, we used endotoxin-free ragweed extract in the current study.

**Experimental design**

Two models were used to evaluate the effects of CpG ODN on ragweed asthma, the short term model and the long term model (Tables I and II). In the short term model, BALB/c mice were sensitized by i.p. injection of 150 μg of RW plus alum on days 0 and 4, and as described (17). ODN (35 μg/100 μl/mouse) were administered intratracheally 0–48 h before allergen challenge (200 μg of RW administered intratracheally), which was performed on day 11. Mice were sacrificed and studied on day 14 for bronchoalveolar lavage (BAL) cell counts. In additional animals, the lungs and spleen were dissected for enzyme-linked immunospot (ELISPOT) cytokine analysis 3 days after the final RW challenge.

In the long term model (Table II), mice were sensitized with RW as described above and challenged with RW intratracheally on days 11, 25, and 65 (to mimic repeated seasonal allergen exposure). Groups of animals were treated with PBS or CpG ODN 2 days before each allergen challenge. A control group (RW/PBS) was treated with PBS on days 9, 23, and 63. To evaluate the long term effects of CpG ODN, the second group (RW/CpG-6 wk) received 35 μg of CpG ODN intratracheally on days 9 and 23 and PBS on day 63. A third treatment group (RW/CpG-2 days) received CpG ODN 2 days before each of the three allergen challenges. This group was included to test whether repeated doses of CpG ODN before each of the ragweed challenges was required to maintain its activity. The third group in the long term asthma model was similar to the RW/CpG-48 h group in the short term model in that the last dose of CpG ODN was administered 48 h before the final allergen challenge.

In the long term model, pulmonary function testing was performed with the Buxco system on day 67, 2 days after the final RW challenge. Because pulmonary function testing was performed in unrestrained, conscious animals (to mimic methacholine challenge in patients with asthma), the same animals could be used to collect BAL and to perform histological analysis the following day. In additional animals, the lungs and spleen were dissected for ELISPOT cytokine and Ig analysis, and serum was collected for Ig analysis by ELISA 3 days after the final RW challenge.

**Evaluation of the role of IFN-γ in mediating the rapid effects of CpG ODN**

IFN-γ +/+ and IFN-γ −/− female BALB/c mice, 5 wk old, were purchased from The Jackson Laboratory. A protocol identical with that for the RW/CpG-48 h group in the short term model (Table I) was performed. Seventy-two hours after RW challenge, the mice underwent BAL, and the collected BAL fluid was analyzed for total and differential immune cell counts.

**Sample preparation**

Mice were euthanized with an i.p. injection of ketamine and xylazine to perform BAL as previously described (17). BAL fluids were obtained by cannulating the trachea and lavaging the lungs with two 0.7-ml aliquots of ice-cold Dulbecco's PBS (Sigma Chemical, St. Louis, MO). The BAL cells were pelleted, washed, and stained with Wright-Giemsa. The number of eosinophils, neutrophils, lymphocytes, and macrophages was determined by microscopic examination of a minimum of 200 cells/slide of a cytocentrifuge preparation.

ELISPOT assays and serum IgE assays were performed in parallel experiments as described below. Animals were bled by retroorbital puncture, and serum stored at −20°C until use. Mice were killed by cervical dislocation, and their spleens and lungs were removed aseptically. Mouse spleens and lungs were minced and passed through a wire mesh to generate single-cell suspensions. Where indicated, cells were incubated in complete media (RPMI 1640 plus 10% FBS, 1.5 mM L-glutamine, penicillin, and streptomycin at 100 units/ml).

**Table I. Short term asthma model**

<table>
<thead>
<tr>
<th>Day 9</th>
<th>Day 10</th>
<th>Day 11</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>RW/PBS</td>
<td>PBS i.t.</td>
<td>PBS i.t.</td>
<td>BAL, ELISPOT</td>
</tr>
<tr>
<td>RW/CpG-48 h</td>
<td>CpG i.t.</td>
<td>PBS i.t.</td>
<td>BAL, ELISPOT</td>
</tr>
<tr>
<td>RW/CpG-15 h</td>
<td>PBS i.t.</td>
<td>CpG i.t.</td>
<td>BAL, ELISPOT</td>
</tr>
<tr>
<td>RW/CpG 0 h</td>
<td>PBS i.t.</td>
<td>CpG i.t.</td>
<td>BAL, ELISPOT</td>
</tr>
<tr>
<td>RW/CpG-48 h</td>
<td>GpC i.t.</td>
<td>PBS i.t.</td>
<td>BAL, ELISPOT</td>
</tr>
<tr>
<td>RW/CpG-48 h</td>
<td>mCpG i.t.</td>
<td>PBS i.t.</td>
<td>BAL, ELISPOT</td>
</tr>
</tbody>
</table>

*Mice were sensitized with two doses of 150 μg of RW + alum i.p. given on days 0 and 4. In the RW/CpG-15 h group, CpG ODN was given 15 h before allergen challenge. In the RW/CpG-48 h group, CpG ODN was given 48 h before allergen challenge. i.t., intratracheal; CpG i.t., i.t. CpG ODN 35 μg; RW i.t., i.t. 200 μg of ragweed.

**Table II. Long term asthma model**

<table>
<thead>
<tr>
<th>Day 9</th>
<th>Day 11</th>
<th>Day 23</th>
<th>Day 25</th>
<th>Day 63</th>
<th>Day 65</th>
<th>Day 68</th>
</tr>
</thead>
<tbody>
<tr>
<td>RW/PBS</td>
<td>PBS i.t.</td>
<td>RW i.t.</td>
<td>PBS i.t.</td>
<td>RW i.t.</td>
<td>PBS i.t.</td>
<td>BAL, ELISPOT etc.</td>
</tr>
<tr>
<td>RW/CpG-6 wk</td>
<td>CpG i.t.</td>
<td>RW i.t.</td>
<td>PBS i.t.</td>
<td>RW i.t.</td>
<td>PBS i.t.</td>
<td>BAL, ELISPOT etc.</td>
</tr>
<tr>
<td>RW/CpG-2 days</td>
<td>CpG i.t.</td>
<td>RW i.t.</td>
<td>CpG i.t.</td>
<td>RW i.t.</td>
<td>CpG i.t.</td>
<td>BAL, ELISPOT etc.</td>
</tr>
</tbody>
</table>

*Mice were sensitized with two doses of 150 μg of RW + alum i.p. given on days 0 and 4. In the RW/CpG-15 h group, CpG ODN was given 15 h before allergen challenge. In the RW/CpG-48 h group, CpG ODN was given 48 h before allergen challenge. i.t., intratracheal; CpG i.t., i.t. CpG ODN 35 μg; RW i.t., i.t. 200 μg of ragweed.*
Splenic recall response

Splenocytes were incubated at $5 \times 10^6$ cells/ml with either diluent or 100 
$\mu$g/ml ragweed for 4 days. The cells were incubated in complete medium 
at 37°C in a humidified 5% CO$_2$ incubator. The cell supernatants were 
examined for IFN-γ levels with a two-site immunoenzymetric assay using 
anti-IFN-γ Abs (clones R4-6A2 and XMGI2.1, PharMingen, San Diego, CA).

Cytokine ELISPOT assays

Immulon 2 microtiter plates (96-well) were coated with 10 $\mu$g/ml anti- 
IFN-γ (clone RA6a2, Lee Biomolecular, San Diego, CA) or anti-IL-4 
(clone BV4D-1D11, Endogen, Woburn, MA) in 0.1 M carbonate buffer 
(pH 9.6) for 3 h at room temperature (19). The plates were blocked with 
PBS-5% BSA for 1 h and washed with PBS-0.025% Tween 20. Serial 
serial dilutions of single spleen or lung cell suspensions, ranging from 1 to 10 $\times 
10^6$ cells/well, were incubated on anti-cytokine-coated plates in complete 
medium for 8–10 h at 37°C in a humidified 5% CO$_2$ incubator. Plates were 
then washed with PBS-Tween 20 and overlaid with 1 $\mu$g/ml biotinylated 
anti-IFN-γ (clone XMGI 1.2, PharMingen) or anti-IL-4 (clone BV6D-
24G2, Endogen), washed, and treated with a 1:2000 dilution of avidin-
conjugated alkaline phosphatase (Vector Laboratories, Burlingame, CA) 
and Perry, Gaithersburg, MD). ELISPOTs were counted with the aid of a 
dissecting microscope and expressed as ELISPOTs per million cells.

Ragweed-specific ELISA and ELISPOT assays

Immulon 1 microtiter plates (96 wells) were coated with 10 $\mu$g/ml ragweed 
protein and blocked for 1 h with PBS-1% BSA. Plates were overlaid with 
serially diluted sera or cells, incubated as described above, washed, and 
reacted with phosphate-conjugated anti-mouse IgG1, IgG2a (Southern 
Biotechnology, Birmingham, AL) or IgE (a generous gift from Dr. Clifford 
Snapper). Serum Ab concentrations were determined by comparison to 
single spleen or lung cell suspensions, ranging from 1 to 10 $\times 
10^6$ cells/ml with either diluent or 100 $\mu$g/ml ragweed (RW) and alum on days 0 and 4 and challenged intratracheally 
with RW on day 11. CpG ODN was administered intratracheally simultane-
ously with (RW/CpG 0 h, $n = 8$), 15 h before (RW/CpG-15 h, $n = 9$) 
or 48 h before (RW/CpG-48 h, $n = 11$) RW challenge. Three control 
groups received either intratracheal PBS (RW/PBS, $n = 18$) or a control 
ODN in which the critical CpG motif was reversed to GpC (RW/GpC-48 
h, $n = 9$), or methylated CpG ODN (RW/mCpG-48 h, $n = 4$) 48 h before 
RW challenge. Three days after RW challenge BALs were performed on 
each group of mice, and total and differential BAL immune cell counts 
were determined. A. BAL eosinophil cell numbers $\times 10^6$ in the different 
groups; B. BAL total cells $\times 10^6$ in the different groups. Values are 
expressed as mean ± SEM. $p < 0.001$ compared with naive 
animals; *** $p < 0.001$; **** $p < 0.0001$ compared with RW/PBS 
treated group.

Bonferroni/Dunn’s post hoc test. All ELISPOT results were analyzed by one-way ANOVA using SigmaStat (Jandel Scientific, San Rafael, CA), and significant ANOVAs were checked by Student-Newman-Keuls post hoc test to establish normality and significance. The limited number of lung cells that could be derived from each animal required performing ELIS-
spot assays on pooled lung cells from 6 animals/group. Thus, statistically 
significant differences between groups cannot be established for these 
samples. The comparison of histology scores was analyzed by the Mann-Whit-
ney test. The PENH data were analyzed by Student’s t test.

Results

Rapid effects of CpG ODN on allergic lung inflammation and 
IgE-secreting cells

When BALB/c mice were sensitized and challenged with RW, they rapidly developed the immunological abnormalities charac-
teristic of an allergic response. Thus, the total number of BAL cells 
in the RW/PBS group rose 5-fold ($p < 0.0001$), whereas the total 
number of eosinophils rose 400-fold ($p < 0.0001$) when compared 
with naive animals (Fig. 1). We examined whether the timing of 
intratracheal administration of CpG ODN with respect to allergen 
challenge influenced the development of allergic lung inflamma-
tion. Compared with the RW/PBS group, administration of CpG 
ODN 48 and 15 h before RW challenge inhibited BAL eosinophil 
numbers by 70% ($p < 0.0001$) and 55% (Fig. 1A, $p < 0.001$),
respectively. Administration of CpG ODN 48 h before RW challenge also reduced total immune cell counts in the BAL fluids by 50% (Fig. 1B, p < 0.0001). In contrast, total BAL immune cell counts and eosinophil numbers were not significantly reduced by the administration of negative control ODN in which the critical CpG dinucleotide was inverted or the cytosine bases were methylated (RW/GpC-48 h and RW/mCpG-48 h groups, respectively).

Compared with naive animals, there was a 9-fold increase in IgE producing spleen cells (p < 0.05) and >18-fold increase in IgE-producing lung cells (Table III) in allergen-sensitized, PBS-treated animals. Administration of CpG ODN 48 h before allergen challenge reduced the number of IgE-secreting cells in the spleen by 44% (p < 0.05) and the lungs by 83%. These results indicate that CpG ODN rapidly inhibits eosinophilic lung inflammation and IgE production in a ragweed model of allergic asthma.

**CpG ODN increase the ratio of cells secreting IFN-γ-IL-4**

Cytokine ELISPOT assays were used to monitor the number of cells actively secreting IFN-γ and IL-4 in the lungs and spleen of mice sensitized and challenged with RW. The number of spleen cells producing either of these cytokines was significantly higher in the RW/PBS-treated mice than in naive controls (p < 0.05; Fig. 2A). When administered 48 h before allergen challenge, CpG ODN significantly increased the number of IFN-γ-producing cells in the spleen (p < 0.05; Fig. 2A). This altered the cytokine milieu by increasing the ratio of IFN-γ-IL-4-secreting cells 3.5-fold when compared with the RW/PBS group. This effect required administration of the CpG motif, because no change was observed in the RW/GpC-48 h group. Similarly, administration of CpG ODN 48 h before allergen challenge increased the number of IFN-γ-producing cells in the lungs (Fig. 2B) and increased the ratio of IFN-γ-IL-4-secreting cells 3.5-fold relative to the RW/PBS group. Thus, intratracheal administration of CpG ODN in the short term asthma model increases the number of cells producing Th1 cytokines in the lungs and systemically in the spleen.

**CpG ODN have long term immunological effects**

We next examined the duration of CpG ODN activity by utilizing a long term asthma model. This model produces a greater magnitude of lung inflammation than the short term model because each group received three intratracheal ragweed challenges over an 8-wk period (mimicking repeated seasonal allergen exposure). The RW/PBS and RW/CpG-2 days groups received three doses of PBS or CpG ODN, respectively, by intratracheal administration 2 days before each of the three subsequent RW challenges. The RW/CpG-6 wk group was designed to examine the long term effects of CpG ODN and was administered CpG ODN before the first two RW challenges but not before the final RW challenge 6 wk later.

Compared with naive animals, repeated challenge with RW resulted in a substantial rise in the number of cells secreting IL-4 in both lungs and spleen (Fig. 3, A and B). Consistent with results from the short term model, treating mice with CpG ODN 2 days before all of the RW challenges (RW/CpG-2 days group) increased the ratio of IFN-γ-IL-4-secreting cells in the lungs and spleen by 4–5-fold. Among mice treated with CpG ODN and then challenged 6 wk later with RW (RW/CpG-6 wk group), the ratio of IFN-γ-IL-4-secreting cells in the lungs increased 3–4-fold when compared with the RW/PBS group. In contrast, there was no difference in this ratio among spleen cells from this group compared with the RW/PBS group. These results suggest that RW-specific Th1 cells are localized in the lungs 6 wk after intratracheal administration of CpG ODN.

To identify RW-specific Th1 memory cells, splenocytes from all three groups were restimulated in vitro with the recall Ag (RW). As seen in Fig. 3C, splenocytes derived from both the RW/CpG-2 days and RW/CpG-6 wk groups responded vigorously to the recall Ag with IFN-γ production. Compared with the RW/PBS group, the RW/CpG-6 wk group demonstrated a 4.7-fold increase (p < 0.001) and the RW/CpG-2 days group demonstrated a 5.5-fold increase (p < 0.01) production of IFN-γ. Thus, in addition to increasing the ratio of IFN-γ-IL-4-secreting cells, intratracheal administration of CpG-ODN stimulated an Ag-specific Th1 memory response that persisted for at least 6 wk.

We then evaluated the production of anti-RW Abs between treatment groups. Repeated challenge with RW (RW/PBS group) resulted in a 17-fold elevation in serum ragweed-specific IgE levels compared with naive animals (Fig. 4A). Compared with RW/PBS group, the fold increase in serum ragweed-specific IgE was 73% lower in the RW/CpG-2 days group (p < 0.05) and 43% lower in the RW/CpG-6 wk group (p < 0.05). These results suggest that administration of CpG ODN before the initial challenge had a long term impact on the development of RW-specific IgE production.

### Table III. Rapid effects of CpG ODN on IgE-producing cells in the spleen and lung

<table>
<thead>
<tr>
<th></th>
<th>Spleen</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>2 ± 0.6</td>
<td>0</td>
</tr>
<tr>
<td>RW/PBS</td>
<td>18 ± 1.2</td>
<td>20</td>
</tr>
<tr>
<td>RW/CpG-48 h</td>
<td>10 ± 1.2*</td>
<td>3</td>
</tr>
<tr>
<td>RW/CpG-48 h</td>
<td>24 ± 2.3</td>
<td>18</td>
</tr>
</tbody>
</table>

*p < 0.05 compared with naive animals; *p < 0.05 compared with the RW/PBS group.

### FIGURE 2. Rapid effects of CpG ODN on IFN-γ- and IL-4-secreting cells in the lungs and spleen

ELISPOT assays were performed on single-cell suspensions of spleen and lung cells obtained from the animal groups described in Fig. 1. Shown are the number of IFN-γ- and IL-4-secreting cells per million spleen (A) or lung (B) cells. For A (spleen), each bar represents the mean ± SEM values for three animals. Because only a limited number of lung cells could be derived from each animal, each bar in B (lung) represents the ELISPOT results of pooled lung cells from three animals. +, p < 0.05 compared with naive animals; *, p < 0.05 compared with the RW/PBS group.
anti-RW Ig secreting B-cells mature, and this effect was longer lasting in the lungs than spleen.

To pursue this observation, we analyzed the frequency of B cells secreting RW-specific IgG1 and IgG2a. Consistent with the ability of IFN-γ to induce isotype switching from IgG1 to IgG2a, CpG ODN decreased the number of IgG1-producing cells (Fig. 4C) and increased the number of IgG2a-secreting cells in the lungs (Fig. 4D). Compared with PBS-treated animals (RW/PBS group), the number of IgG1-secreting cells in the RW/CpG-2 days group were 77% fewer in the spleen (p < 0.05) and 38% fewer in the lungs. Similarly, in the RW/CpG-6 wk group, there were 57% fewer cells in the spleen (p < 0.05) and 36% fewer in the lungs. Compared with the RW/PBS group, the RW/CpG-2 days group and the RW/CpG-6 wk group demonstrated 6.3-fold and 5-fold more IgG2a-producing lung cells, respectively. Although far fewer IgG2a-producing cells were present in the spleen, a similar pattern of effects of CpG ODN administration on IgG2a secretion was observed in this organ. The RW/CpG-2 days and RW/CpG-6 wk groups demonstrated significantly more IgG2a-producing splenocytes compared with the RW/PBS group (p < 0.05). These results provide further evidence that the increased ratio of IFN-γ-IL-4 secreting cells resulting from CpG ODN administration altered the milieu for the maturation of B cells secreting anti-RW Igs from a Th2 bias to a Th1 bias.

**CpG ODN has long term antiallergic effects**

We sought to determine whether CpG ODN have long term anti-allergic effects. In the RW/CpG-6 wk group, the BAL eosinophil and total immune cell counts were inhibited 66% (p < 0.01) and 48% (p < 0.01), respectively (Fig. 5, A and B) compared with the RW/PBS group. Similarly, in the RW/CpG-2 days group, the BAL eosinophil and total immune cell recruitment were inhibited 76% (p < 0.001) and 53% (p < 0.01), respectively. These results were similar in magnitude to the inhibition of BAL eosinophils produced by CpG ODN administered 48 h before challenge (70%) in the short term asthma model described above. Furthermore, there was no significant difference between the reduction in eosinophils and total cells demonstrated by the RW/CpG-2 days and the RW/CpG-6 wk groups, indicating that the inhibitory effects of CpG ODN on allergic lung inflammation are sustained for at least 6 wk.

Following BAL, the lungs were formalin fixed, embedded in paraffin, sectioned at 4 μm thickness, and stained with hematoxylin and eosin. Total lung inflammation was determined histologically and defined as the sum of peribronchial and perivascular inflammation scores. Fig. 6A (naive), 6B (RW/PBS) and 6C (RW/CpG-6 wk) show representative hematoxylin and eosin-stained peribronchial and perivascular areas of the lungs (×40). Naive animals had no detectable lung inflammation, whereas the RW/PBS group had significant peribronchial and perivascular inflammation. Compared with the RW/PBS group, the peribronchial (56% inhibition, p < 0.01), the perivascular (47% inhibition, p < 0.01), and the total (51% inhibition, p < 0.001) lung inflammation were inhibited in the RW/CpG-6 wk group (Table IV).

Airway responsiveness was measured in unrestrained animals using whole body plethysmography (BUXCO, Troy, NY) (23). Mice were placed in the main chamber of the plethysmograph, and baseline readings were taken and averaged for 5 min. Airway reactivity was expressed as fold increase in enhanced pause (PENH) for each concentration of methacholine relative to the PENH values produced by PBS exposure for individual mice. Compared with the RW/PBS group, the RW/CpG-6 wk group demonstrated a 32% reduction in the PENH index at 50 mg/ml dose of methacholine (p < 0.01, Fig. 7).
Inhibition of eosinophil recruitment by CpG ODN requires IFN-γ

Because administration of CpG ODN dramatically increased the ratio of IFN-γ-IL-4 cells in the lungs, we hypothesized that IFN-γ played a critical role in mediating the effects of CpG ODN in allergic lung inflammation. To test this hypothesis, CpG ODN or PBS was administered 48 h before RW challenge in allergen-sensitized IFN-γ1/1 and IFN-γ2/2 BALB/c mice in the short term asthma model. Administration of CpG ODN resulted in a 78% reduction in BAL eosinophil numbers compared with the RW/PBS group in the IFN-γ1/1 mice (p, 0.001, Fig. 8). The degree of inhibition was similar to that observed in the RW/CpG-48 h group (70%, Fig. 1). In contrast, CpG ODN failed to inhibit eosinophil recruitment in IFN-γ2/2 mice, indicating that IFN-γ is an essential mediator of the CpG ODN antiinflammatory effect.

Discussion

There has been a steady increase in the incidence, morbidity, and mortality caused by allergic asthma. Thus, a form of therapy that could suppress for long periods the lung inflammation found in this disease would be of considerable public health benefit. This study demonstrates for the first time a prolonged effect abrogating allergic lung inflammation in presensitized mice. The intratracheal administration of synthetic oligonucleotides carrying immunostimulatory CpG motifs converted the Th2 cell-mediated allergic response to a dominant Th1 phenotype and significantly reduced eosinophilic lung inflammation, RW-specific IgE production, and bronchial hyperresponsiveness 6 wk after the last dose of CpG ODN.

Inhibition of eosinophil recruitment by CpG ODN requires IFN-γ

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There are two previous reports (Kline et al. (20); Broide et al. (21)) of CpG ODN having antiallergic properties. Kline et al. administered ODNs along with Schistosoma eggs i.p. in the Th1-prone mouse strain C57BL/6. Six hours after Schistosoma egg Ag challenge, BAL eosinophil were reduced in mice treated with CpG ODN. Broide et al. evaluated the effects of CpG ODN in two models of OVA-sensitized and -challenged BALB/c mice. In the
first model, i.p. administration of three 50-μg doses of CpG ODNs 24 h before each of the three OVA challenges inhibited bronchial hyperresponsiveness. In the second model, administration of 100-μg dose(s) of CpG i.p., intranasally, or intratracheally 1 or 6 days before the final OVA challenge inhibited eosinophil recruitment. Thus, both Kline et al. and Broide et al. have demonstrated short term effects of CpG ODN on allergic lung inflammation. As in our study, both groups used nuclease-resistant phosphorothioate ODN, given that their greater half-life is expected to improve activity in vivo. Our study represents an important extension of these reports in that it is the first study demonstrating that CpG ODN reduces immunological and physiological manifestations of allergic asthma for a prolonged period. Our study was conducted in a Th2-prone mouse strain (BALB/c) using an allergen (RW) that is relevant to human allergic asthma. Like Broide et al., we directly delivered CpG ODN to the lungs mimicking nebulizer delivery. Finally, ours is the first study to document that CpG ODNs require IFN-γ to inhibit eosinophilic lung inflammation.

Our experiments indicate that CpG ODN suppress allergic lung inflammation optimally if delivered 2 days before allergen challenge. Indeed, no benefit was observed when the ODN were co-administered with the allergen. These findings are consistent with other evidence that CpG ODN require 2–3 days to induce an optimal Th1-mediated immune response in vivo and support the hypothesis that CpG ODN trigger an immunomodulatory cascade that matures over a period of several days (25). Of interest, once CpG ODN established a RW-specific Th1 bias in the lungs, the preferential induction of a Th1 response persisted for at least 6 wk. This long term preferential induction of Th1 response was associated with a reduction in the number of cells secreting IL-4, suggesting inhibition of Th2 response. In keeping with the known

**FIGURE 6.** Long term effects of CpG ODN on peribronchial and perivascular lung inflammation. The lungs of a naive group and the RW/PBS and RW/CpG-6 wk long term groups were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. A pathologist blinded to the treatment groups evaluated the degree of peribronchial and perivascular inflammation on a scale of 0 to 4 with an increment of 0.5 if the inflammation fell between two integers. The total lung inflammation was defined as the sum of peribronchial and perivascular inflammation scores. The values are expressed as mean ± SEM for six animals. ***, p < 0.001 compared with the RW/PBS group.

**FIGURE 7.** Prolonged effects of CpG ODN on bronchial hyperresponsiveness. The PENH index was used to measure bronchial hyperresponsiveness in the RW/PBS and RW/CpG-6 wk long term groups. ***, p < 0.01 compared with the RW/PBS group. Values represent the mean ± SEM for six animals.

Table IV. Long-term effects of CpG ODN on peribronchial and perivascular lung inflammation

<table>
<thead>
<tr>
<th></th>
<th>Lung Inflammation Score</th>
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<tr>
<td></td>
<td>RW/PBS</td>
</tr>
<tr>
<td>Peribronchial</td>
<td>2.67 ± 0.33</td>
</tr>
<tr>
<td>Perivascular</td>
<td>3.17 ± 0.31</td>
</tr>
<tr>
<td>Total</td>
<td>5.83 ± 0.54</td>
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*Inflammation score was determined by a pathologist blinded to the treatment groups. Peribronchial and perivascular inflammation were graded on a subjective scale of 0, 1, 2, 3, and 4 corresponding to mild, moderate, marked, or severe inflammation respectively with an increment of 0.5 if the inflammation fell between two integers. The total lung inflammation was defined as the sum of peribronchial and perivascular inflammation scores. The values are expressed as mean ± SEM for six animals. ***, p < 0.001 compared with the RW/PBS group.
FIGURE 8. Role of IFN-γ in mediating the rapid effects of CpG ODN on eosinophil recruitment. IFN-γ+/+ (WT) or IFN-γ−/− (KO) BALB/c mice (The Jackson Laboratory) were first sensitized with two doses of ragweed and alum on days 0 and 4. CpG ODN or PBS was administered intratracheally 48 h before RW challenge to maximize the inhibitory effects of CpG ODN on allergic lung inflammation. Three days after RW challenge, BALs were performed on each group of mice. Shown are BAL eosinophil cell numbers × 10⁴/ml in the four treatment groups, n = 4 or 5 per group. All values are expressed as mean ± SEM. ***, p < 0.001 compared with the corresponding RW/PBS group.

opposing effects of Th1 and Th2 cytokines on isotype switching by B cells, the reduction in IL-4 and increase in IFN-γ was associated with a reduction in IgE- and IgG1-secreting cells and an increase in the number of IgG2a-secreting cells in the spleen and lungs. These effects on Ig production may have relevance to the prolonged inhibitory effects of CpG ODN on allergic lung inflammation because prior studies indicate that allergen-specific IgE and IgG1, but not IgG2a, augments allergic lung inflammation (10, 26). The long term effect on the IFN-γ/IL-4 ratio, combined with the increased Th1 memory response to allergen and the absence of an effect in IFN-γ−/− mice, indicates that the stimulation of Th1 cells producing IFN-γ plays a key role in maintaining the anti-inflammatory effects of CpG ODN in the lung.

In the present study, CpG ODN increased the number of IFN-γ-producing cells in the lungs 6 wk after the last dose of CpG ODN. This could reflect the generation of resident memory cells in the lungs or the recruitment of Th1 cells from a systemic reservoir to the lung following each intratracheal RW challenge. It is likely that after allergen challenge, Th1 memory cells are recruited in greater numbers to the lungs from systemic “reservoir” organs such as spleen. We and others have shown that the CC chemokines, RANTES and macrophage-inflammatory protein 1α, are produced in asthma and allergic inflammation (27–29). These CC chemokines are also efficient chemoattractants for Th1 cells and have been shown to induce a dose-dependent transmigration of Th1 but not of Th2 cells (30, 31). Thus, in the long term asthma model in our study, the final intratracheal RW allergen challenge may have initiated the recruitment of Th1 cells to the lung by increasing the intrapulmonary levels of RANTES and macrophage-inflammatory protein 1α.

The ability of CpG ODN to stimulate IL-12 production may be a key factor mediating the prolonged effects of CpG ODN. We and others have recently shown that IL-12, a cytokine that promotes Th1 differentiation and production of IFN-γ, inhibits eosinophil recruitment, decreases IgE levels, and suppresses BHR in murine models of allergic asthma when it is given systemically within 4–72 h of allergen challenge (17, 32–35). More recently, we have found that intratracheal administration of IL-12 with ragweed in the mouse model of asthma has long term effects that inhibit eosinophil recruitment (36). Prior studies indicate that systemic administration of IL-12 at the time of live parasite egg inoculation or at the time of parasite Ag injections leads to long term vaccine adjuvant effects that decrease footpad swelling and pathology induced by a parasite challenge (37–39). Some of these studies reported persistence of Ag-specific Th1 memory a few weeks after immunization with Ag and IL-12 (37–39). In one of these long term studies, Ag-specific Th1 memory persisted for 8 wk (38). Because our study indicates that CpG ODNs stimulate long term Ag-specific Th1 memory, it is possible that this is mediated by induction of IL-12 production (20).

In the current study, CpG ODN was shown to augment IFN-γ production and, in the long term protocol, reduce the number of cells producing IL-4. In mouse models of asthma, individual treatment with anti-IL-4 and intratracheal administration of IFN-γ and IL-12 have been shown to inhibit allergic lung inflammation (14, 33, 40). Treatment with exogenous Th1-promoting or Th2-limiting agents, however, may not be sufficiently efficacious in the treatment of human asthma or may pose toxic consequences. Even though animal studies of IFN-γ have been encouraging, clinical trials of IFN-γ in patients with allergic rhinitis and asthma have not yielded promising results (41–43). Use of exogenous IFN-γ may also be limited by its side effects, which include influenza-like symptoms including fever and fatigue (43). Systemically administered IL-12 has also been reported to produce toxicity (44), although these effects may be avoidable by the use of low dose IL-12 administered intratracheally or via other non-systemic routes of administration. The ability of CpG ODN to stimulate the combination of Th1-promoting and Th2-limiting effects suggests a potent therapeutic potential in the treatment of allergic diseases. Also, because endogenously produced cytokines are likely to be homeostatically regulated, CpG ODN may produce less toxicity than administration of exogenous cytokines. However, because of the limited data from clinical trials and the potential differences between effects in animal studies and in patients with asthma, the efficacy of anti-IL-4, IL-12, and CpG ODN as therapeutic agents in asthma remains to be determined.

The ability of intratracheally administered CpG ODN to provide long term protection against allergic lung inflammation and alter the balance between IL-4 and IFN-γ suggest that intratracheally administered CpG ODN may have therapeutic benefit in asthma. Many current asthma therapies utilize inhalation administration of medications to maximize patient compliance and minimize systemic toxicity. Pulmonary administration of CpG ODN may provide more effective inhibition of allergic lung inflammation than systemic administration. In a recent study by Erb et al. (45), intranasal infection with bacillus Calmette-Guérin (BCG) produced significantly greater inhibition of allergic airway eosinophilia than i.p. or s.c. infection. Studies in our laboratory indicate that intratracheal administration of recombinant IL-12 is 100-fold more effective in suppressing allergic lung inflammation than the same dose of IL-12 delivered systemically.4 If CpG ODN is also more efficacious when administered intratracheally, then CpG ODN delivery by this route might minimize the dose of CpG ODN required for treatment, thereby reducing potential side effects. Moreover, since delivery of CpG ODN results in long term reduction in allergic asthma, this method of administration might require infrequent dosing. With regard to potential toxicity, we found that 35 μg of CpG ODN administered intratracheally to naive BALB/c mice did not induce pulmonary inflammation as determined in BAL fluids 4 and 48 h later. This is in contrast to the results of

4 S. Sur et al. Submitted for publication.
Serves further study as a potential treatment for asthma. Prolonged period. Thus, local administration of CpG ODN decreases allergen-specific IgE production, and bronchial hyperresponsiveness for a prolonged period. CpG ODN preferentially stimulated the production of Th1 cytokines in the presensitized mouse model of asthma. Intratracheally administered CpG ODN preferentially stimulated the production of Th1 cytokines and suppressed eosinophilic airway inflammation, allergen-specific IgE production, and bronchial hyperresponsiveness for a prolonged period. Thus, local administration of CpG ODN serves further study as a potential treatment for asthma.

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**References**


