

Immunostimulatory CpG Oligonucleotides Abrogate Allergic Susceptibility in a Murine Model of Maternal Asthma Transmission¹

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We tested the potential of CpG oligodeoxynucleotides (ODN) to reverse the increased susceptibility to allergic airways disease in neonatal mice in a model of maternal transmission of asthma risk. Offspring of OVA-sensitized and challenged BALB/c mother mice were subjected to an intentionally suboptimal sensitization protocol that has minimal effects on normal mice, but results in airway hyperresponsiveness (AHR) and airway inflammation (AI) in babies of asthmatic mother mice. We evaluated pulmonary function and AI in CpG- or control ODN-treated offspring. CpG treatment of neonates on day 4 of life prevents the AHR otherwise seen in this model (enhanced pause at 100 mg/ml methacholine: CpG, 0.9 ± 0.1 ; ODN control, 3.8 ± 0.6 ; $n = 62$; $p < 0.005$). It also prevented the development of AI, as evident in decreased bronchoalveolar lavage eosinophilia (CpG, $1.2 \pm 0.3\%$; ODN, $31.4 \pm 4.1\%$; $n = 56$; $p < 0.005$), diminished the severity of AI on histopathology, and resulted in lower IL-5 levels in bronchoalveolar lavage fluid. The effect of CpG persisted for at least 4–6 wk and was allergen independent. Treatment with CpG just before OVA aerosol challenge also prevented allergic responses. The data support the potential for immunomodulatory therapy with CpG in early life to reduce susceptibility to asthma. *The Journal of Immunology*, 2005, 175: 4292–4300.

Maternal asthma is a risk factor for the development of asthma in children (1–5). To allow experimental analysis of this process, we have developed a mouse model of maternal transmission of asthma risk. In this model, offspring of OVA-sensitized and challenged mother mice develop airway hyperresponsiveness (AHR)³ and airway inflammation (AI) if subjected to an intentionally suboptimal OVA sensitization protocol that does not cause AHR or AI in the offspring of normal mothers. Identical BALB/c background and housing in a pathogen-free facility allow exclusion of genetic and environmental influences. Our finding of maternal influence on susceptibility to an asthma phenotype is consistent with other models of in utero influences on allergy in mice (6) and dogs (7). This model allows investigating possible early life interventions that might prevent development of asthma in at-risk or susceptible children of asthmatic mothers. One type of such therapeutic agents may be CpG oligodeoxynucleotides (ODN).

CpG ODNs are synthetic sequences containing unmethylated cytosine-phosphate-guanosine dinucleotides that mimic immunostimulatory properties of bacterial DNA (8). The mechanisms of CpG action were reviewed previously (9) and include prevention (10) or reversal of existing (11–13) Th2 polarization in vivo (14) by creating a Th1 cytokine milieu or by restoring normal Th1–Th2 balance, as demonstrated in several conventional, nonmaternal,

OVA allergy models (15–18). The importance of Th2 polarization in our maternal model of transmission of asthma risk is supported specifically by the blocking effects of anti-IL-4 (19) and more generally by the extensive literature implicating Th2-dependent mechanisms in asthma (reviewed in Refs. 20 and 21). Hence, we hypothesized that the CpG might be effective in preventing or reversing the increased susceptibility of neonates in our model of maternal asthma transmission.

Materials and Methods

Animals

Newborn BALB/c mice were obtained commercially from Charles River Laboratories as litters with their mothers on day 2 of age or by in-house breeding, as described below. Each mother and litter was housed separately, fed commercial pelleted mouse feed, and given water ad libitum. The mice were housed in an animal facility that was maintained at 22–24°C with a 12-h dark, 12-h light cycle. All experimentation was conducted under a protocol approved by our institutional review board. All reagents not otherwise specified were obtained from Sigma-Aldrich.

Allergen sensitization and exposure, and CpG treatment

A detailed description of maternal transmission of asthma model was previously reported (19). The experimental protocols used in our studies are summarized in Fig. 1. Briefly, maternal sensitization was achieved by initial i.p. injections of 5 μ g of OVA with 1 mg of alum in 0.1 ml of PBS into mice at 3 and 7 days of age. After weaning, female mice were exposed to aerosols of allergen (3% (w/v) OVA (grade III; Sigma-Aldrich) in PBS, pH 7.4) for 10 min on 3 consecutive days at 4, 8, and 12 wk of age. The aerosol exposure was performed within individual compartments of a mouse pie chamber (Braintree Scientific) using a Pari IS2 nebulizer (Sun Medical Supply) connected to an air compressor (PulmoAID; DeVilbiss). Immediately after the last aerosol exposure, the female mice were placed in cages with normal male mice to allow mating. On day 4 after birth, babies received a single i.p. injection of 5 μ g of OVA with 1 mg of alum (an intentionally suboptimal protocol). On days 12–14 of life, these baby mice were exposed to aerosolized OVA as described above. Physiologic and pathologic analyses were performed the next day (age, 15 days) for the regular suboptimal protocol. Coding for these animals was AsAs; the first letters designate the sensitized mother, and the last two letters indicate the status of the offspring. Controls for the major variables of the protocol included omission of treatment for the mothers (maternal sensitization and OVA aerosol exposure), resulting in newborns treated in the suboptimal

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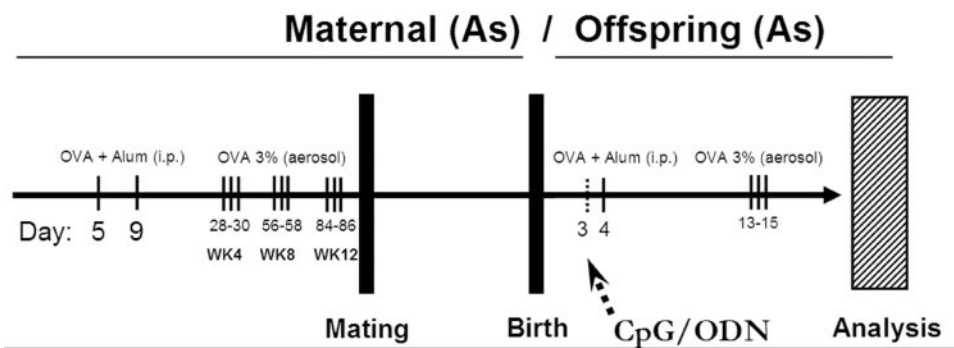
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³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; AI, airway inflammation; BAL, bronchoalveolar lavage; Cas, casein; DC, dendritic cell; ODN, oligodeoxynucleotide; Penh, enhanced pause.

A Basic Protocol



B Longterm Protocol



C Post-sensitization Protocol



FIGURE 1. A, Basic protocol. Newborn babies of OVA-sensitized and aerosol-challenged BALB/c mice received i.p. treatment with CpG ODNs or control ODN or PBS on day 3 and a single i.p. injection of OVA and alum (day 4), followed by exposure to aerosolized OVA on days 12–14 of life. B, Long-term effect of the CpG modification. Instead of analysis at 2 wk, the animals were analyzed at 4 or 6 wk of age; all animals received a 3-day OVA aerosol challenge at 2 wk and were rechallenged before analysis. C, Postsensitization administration of the CpG protocol modification. Instead of day 3, CpG (or ODN) was administered on day 12 (before aerosol challenge) or day 14 (middle of aerosol challenge period).

protocol derived from normal mothers (coding NoAs) and substitution of PBS for OVA in the final aerosol exposure of newborns derived from asthmatic mothers (coding AsNo). Similar protocols were used for studies with a second allergen, casein (Cas), with the modification that offspring of OVA-sensitized mice were given Cas injection i.p. and aerosol challenge with Cas (1% solution for 20 min.) In some experiments a conventional model with two injections of OVA (50 μ g/ml in alum i.p.) on days 1 and 5, followed by aerosol challenges (3% OVA in PBS) on days 9–11 in adult mice, was used.

CpG treatment was evaluated in the protocols summarized in Fig. 1. In the basic protocol, baby mice were treated on day 3 after birth with a single i.p. injection of either CpG 1826 or control nonstimulatory, non-CpG ODN 2138 (Coley Pharmaceuticals). After dose-response analysis with 5, 10, 20, and 30 μ g of CpG/mouse and 30 μ g of control ODN/mouse, subsequent experiments used a dosage of 20 μ g/mouse (Figs. 1A and 3). To investigate the duration of the CpG effect and perform lung mechanics studies, the animals were allowed to grow to 4 or 6 wk of age (long-term protocol; Fig. 1B). In this modification, all babies received CpG injection, OVA injection, and OVA challenge at 2 wk (as usual), but were kept for 4 or 6 wk, respectively, when analysis was performed after another 3-day OVA challenge. To test whether treatment with CpG of already sensitized babies would be effective, a postsensitization protocol was followed in which we injected CpG/ODN on day 12 or 14 of life (Fig. 1C). Finally, to test whether the injection of CpG to pregnant mothers would prevent the development of AHR and AI in the offspring, we injected 30 μ g/mouse of CpG or control ODN s.c. into pregnant mice on approximately day 14 of pregnancy (pregnant mother protocol).

Pulmonary function testing

The airway responsiveness of mice to increasing concentrations of aerosolized methacholine was measured using whole body plethysmography (Buxco). Briefly, each mouse was placed in a chamber, and continuous measurements of box pressure/time wave were calculated via a connected transducer and associated computer data acquisition system. The main indicator of airflow obstruction, enhanced pause (Penh), which shows a strong correlation with the airway resistance examined by standard evaluation methods, was calculated from the box waveform (22). After measurement of baseline Penh, aerosolized PBS or methacholine (MCh-acetyl- β -methylcholine chloride; Sigma-Aldrich) in increasing concentrations (6, 12, 25, 50, and 100 mg/ml) was nebulized through an inlet of the chamber for 1 min, and Penh measurements were made for 9 min after each dose. Penh values for the first 2 min and the last 2 min after each nebulization were discarded, and the values for 5 min in between were averaged and used to compare results.

Lung mechanics

To conduct additional evaluation of baseline parameters of lung mechanics via the flexiVent technique (SCIREQ), we extended the typical protocol to allow the baby mice to grow to a size compatible with the tracheal cannulation and other requirements of this procedure. The mice in this long-term protocol were 4 and 6 wk old (Fig. 1C). Mice were anesthetized and paralyzed with pentobarbital sodium (70 mg/kg i.p.) and doxacurium (0.5 mg/kg i.p.), tracheostomized with a 20-gauge cannula, and mechanically ventilated with a small animal ventilator (flexiVent; SCIREQ) at a tidal volume of 0.3 ml, a frequency of 2.5 Hz, and a fraction of inspired O_2 of

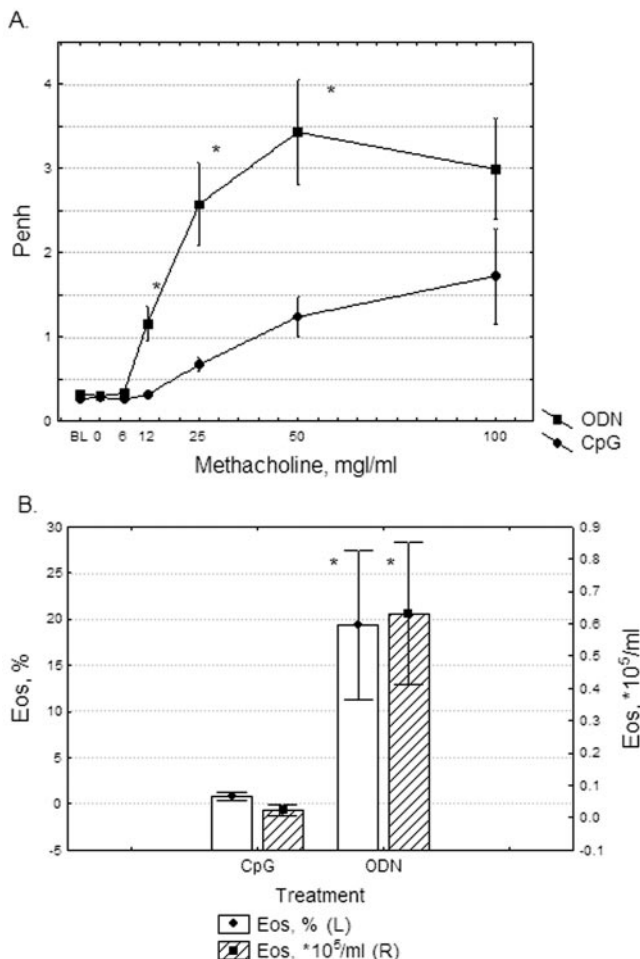


FIGURE 2. CpG effects on AHR (A) and BAL eosinophilia (B) in a conventional two-injection (days 1 and 8) and aerosol challenge (days 13–15) protocol. CpG treatment (30 μ g/mouse i.p. on day 0) prevented the AHR (A) and AI (B) otherwise seen after allergen challenge. $n = 12$. *, $p < 0.05$.

0.21 (room air). A positive end-expiratory pressure of 3 cm of H₂O was applied by submerging the expiratory line in water. Four sigh maneuvers to 3 $\times$ tidal volume were performed to ensure similar volume history and establish a stable baseline respiratory system resistance and elastance.

Lung mechanics were assessed using a modification of the optimal ventilator waveform method originally described by Lutchen et al. (23). Briefly, a forced oscillatory waveform consisting of 19 superimposed sinusoidal frequencies, ranging from 0.25 to 19.625 Hz, was delivered over 16 s as the input signal. The Fourier transform of airway opening pressure, the dependent output variable, divided by the Fourier transform of air flow, as determined by piston displacement, was used to calculate lung input impedance as a function of frequency. Data were fitted to the constant phase model, as described by Hantos et al. (24), to partition input impedance into airway resistance, inertance, compliance, tissue dampening (also known as tissue resistance, a value that represents resistance of lung tissue, as opposed to airway resistance which represents the resistance of bronchial tubing), and tissue elastance.

Pathologic analysis

After physiologic testing in airway-sensitized mice or postallergen-challenged mice, the animals were killed with sodium pentobarbital (Veterinary Laboratories). The chest wall was opened, and the animals were exsanguinated by cardiac puncture. The trachea was cannulated after blood collection. Bronchoalveolar lavage (BAL) was performed five times with 0.3 ml of sterile PBS instilled and harvested gently. Lavage fluid (recovery volume was ~90% of instilled) was collected and centrifuged at 1200 rpm (300 $\times$ g) for 10 min, and the cell pellet was resuspended in 0.5 ml of PBS. The total cell yield was quantified by hemocytometer. BAL differential cell counts were performed on cytocentrifuge slides prepared by centrifugation

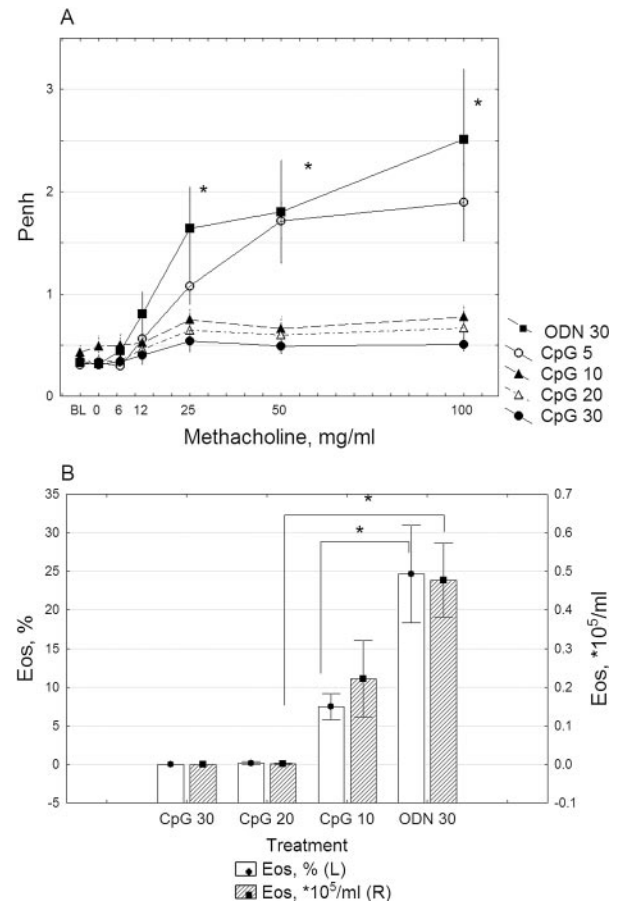


FIGURE 3. Dose-response CpG effect on Penh (A) and BAL eosinophilia (B). The Penh values in mice treated with 30 μ g/mouse control ODN were used to gauge the efficacy of CpG doses. CpG ODNs in a dose of 5 μ g/mouse had minimal effects, whereas 10-, 20-, and 30- μ g doses eliminated AHR equally (A). A dose of 10 μ g/mouse CpG did not effectively suppress airway eosinophilia (B), whereas 20- and 30- μ g doses were equally effective. *, $p < 0.05$.

of samples at 800 rpm for 5 min (Cytospin 2; Shandon). These slides were fixed in 95% ethanol and stained with Diff-Quick (VWR), a modified Wright-Giemsa stain, and a total of 200 cells were counted for each sample by microscopy. Macrophages, lymphocytes, neutrophils, and eosinophils were enumerated. After lavage, the lungs were instilled with 10% buffered formalin, removed, and fixed in the same solution. After paraffin embedding, sections for microscopy were stained with H&E. An index of pathologic changes in coded H&E-stained slides was derived by scoring the inflammatory cell infiltrates around airways and vessels for greatest severity (0, normal; 1, <3 cell diameter thick; 2, 4–10 cells thick; 3, >10 cells thick) and overall prevalence (0, normal; 1, <25% of sample; 2, 25–50%; 3, 51–75%; 4, >75%). The index was calculated by multiplying severity by prevalence, with a maximum possible score of 9.

IL-5 detection

Levels of IL-5 in BAL fluid were measured via multiplex Luminex xMAP assay using the Ab bead kits and equipment obtained from Luminex. The analysis was performed according to the manufacturer's instructions. Only the first 0.3-ml portion of the BAL fluid was used for cytokine assay.

Statistical analysis

Data are presented as the mean $\pm$ 0.95 SEM. Analysis was performed using the Statistica program (StatSoft). To estimate the significance of differences between means, the nonparametric Mann-Whitney *U* test, ANOVA with Tukey's honest significant differences for unequal *n* test, and Student-Newman-Keuls post-hoc test were used depending on the number of comparisons (25). Statistical significance was accepted at $p \leq 0.05$.

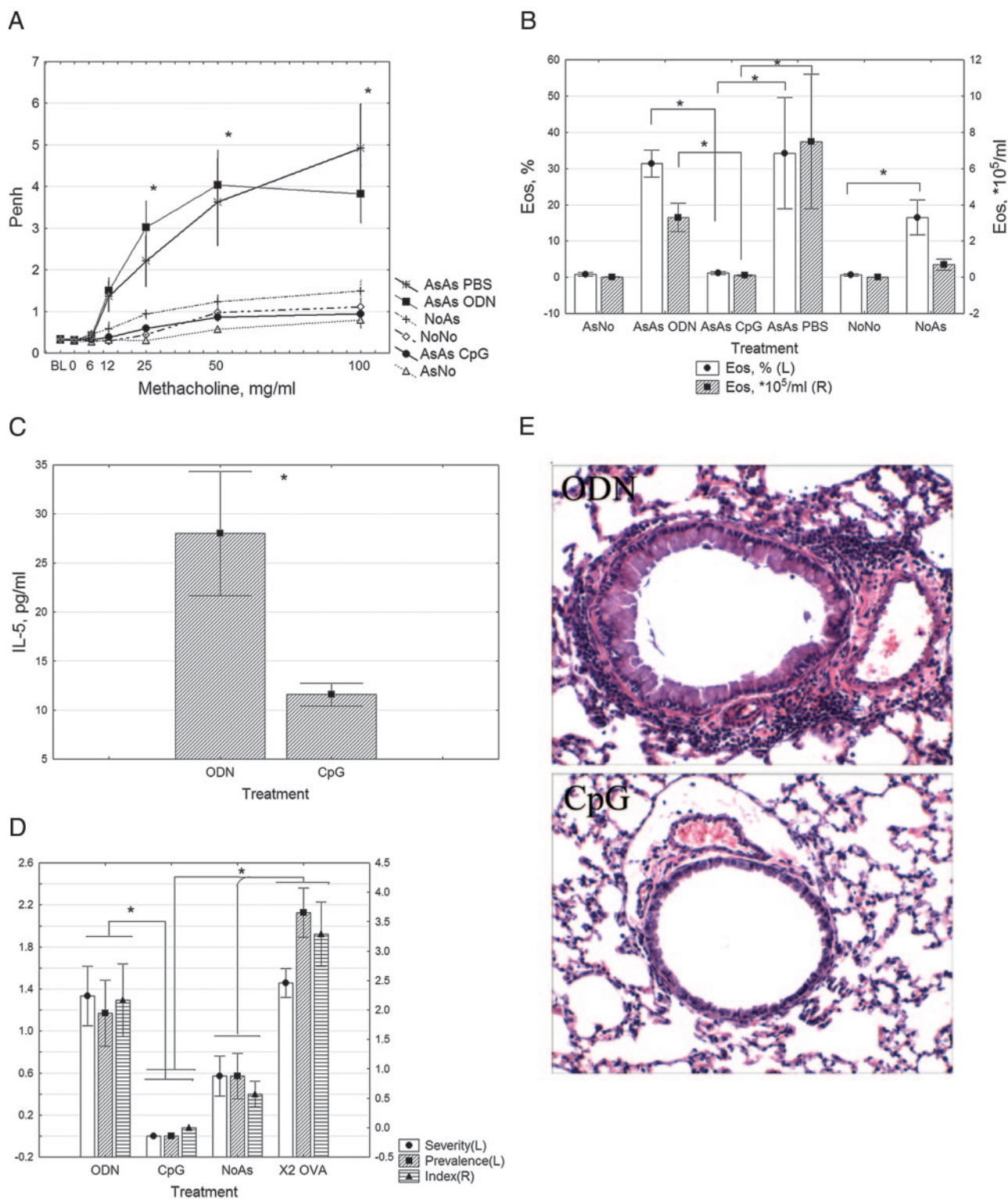


FIGURE 4. CpG effect in the basic protocol. **A**, Response to methacholine. Penh was increased in suboptimal offspring of asthmatic mothers (AsAs) treated with PBS or control ODN (positive controls), whereas Penh values in CpG-treated mice (AsAs CpG) were low and did not differ from those in nonsensitized babies of asthmatic mothers (AsNo), suboptimal babies of normal mothers (NoAs; maternal transmission controls), or completely untreated negative controls. For CpG, $n = 31$; for ODN, $n = 31$. *, $p < 0.05$. **B**, BAL analysis. Increased eosinophil counts in ODN- or PBS-treated positive controls (AsAs ODN and AsAs PBS) were absent in the CpG-treated suboptimal offspring of asthmatic mothers (AsAs CpG). The latter had values indistinguishable from negative controls. For CpG, $n = 31$; for ODN, $n = 31$. *, $p < 0.05$. **C**, CpG-treated offspring had significantly lower BAL IL-5 levels than ODN controls. For CpG, $n = 10$; for ODN, $n = 9$. *, $p < 0.05$. **D** and **E**, CpG treatment significantly reduced the accumulation of mononuclear cells and eosinophils around airways and vessels in the lung. For CpG, $n = 10$; for ODN, $n = 0$. *, $p < 0.05$. H&E stain; original magnification, $\times 200$.

Results

CpG validation and dose-response analysis

We first sought to validate the efficacy of our CpG reagent in a standard two-injection and aerosol challenge OVA model of asthma. After injection with control ODN, adult mice in this protocol showed substantial increases in Penh on methacholine challenge as well as AI, seen as eosinophilia in lung lavage. In contrast, adult CpG-treated mice showed significantly lower AHR (Fig. 2A) and airway eosinophilia (Fig. 2B), with levels comparable to those in untreated normal animals (not shown).

We next tested a range of dosages of CpG in asthma-susceptible babies in the basic (suboptimal) protocol (Fig. 1A). Treatment with control ODN at the maximal dosage had no effect on the expected development of AHR and AI in these mice (Fig. 3). As also shown in Fig. 3, CpG treatment at 5 $\mu\text{g}/\text{mouse}$ was ineffective. CpG treatment with 10 $\mu\text{g}/\text{mouse}$ significantly reduced AHR, but not AI, whereas doses of 20 or 30 μg of CpG/mouse significantly reduced both AHR and AI and were found to be almost equally effective (Fig. 3). These data show a dose-dependent action for CpG treatment and prompted the choice of a dose of 20 $\mu\text{g}/\text{mouse}$ for subsequent studies.

Effect of CpG treatment: basic protocol

The results obtained by treatment of baby mice from the maternal asthma transmission protocol are summarized in Fig. 4. CpG-treated offspring of asthmatic mothers (coded AsAs CpG) showed significantly lower AHR (Fig. 4A) and AI (Fig. 4B) compared with ODN-treated (AsAs ODN) or PBS-treated (AsAs PBS) controls. CpG treatment caused return of Penh and AI results to normal values, so that they were not statistically different from those in normal baby mice. Similarly, the eosinophil percentage in the CpG-treated group was 1.2%, which was not significantly different from those in normal animals (NoNo), nonsensitized offspring of asthmatic mothers (AsNo), and babies of normal mothers subjected to the basic suboptimal protocol (NoAs; see Fig. 1 for protocol). The latter had a tendency to slightly increased relative eosinophil counts; however, the values were significantly lower than in ODN controls. In contrast, BAL samples from the ODN- and PBS-treated mice contained $>30\%$ eosinophils.

Macrophage percentages were decreased proportionately to the increase in eosinophils in asthmatic animals. CpG treatment did not have a significant effect on either lymphocyte content or the small (0–2%) number of neutrophils present in lavage samples.

Histopathological evaluation showed that CpG-treated mice had significantly less allergic inflammation around airways and vessels than ODN-treated mice (Fig. 4, D and E). ODN-treated mice showed a degree of allergic lung inflammation similar to that in positive-control mice subjected to the two-injection protocol (as in Fig. 2). This qualitative impression is supported by semiquantitative scoring, as summarized in Fig. 4D.

To evaluate cytokine expression in the lungs of treated or control mice, we measured IL-5 levels. The data show that IL-5 levels in BAL fluid were significantly lower in CpG-treated babies than in ODN-treated controls (Fig. 4C). IL-5 levels correlated well with the percentage of eosinophil in the BAL ($r = 0.859$; $r^2 = 0.738$; $p < 0.0001$).

There was no effect of CpG in the absence of a developing allergic response (groups NoAs-CpG and NoNo-CpG both exhibited normal BAL yields and differentials and did not show evidence of AHR; data not shown).

To summarize, CpG treatment of baby mice with maternally transmitted susceptibility to asthma effectively prevented the development of increased Penh, BAL eosinophilia and IL-5 production, and pul-

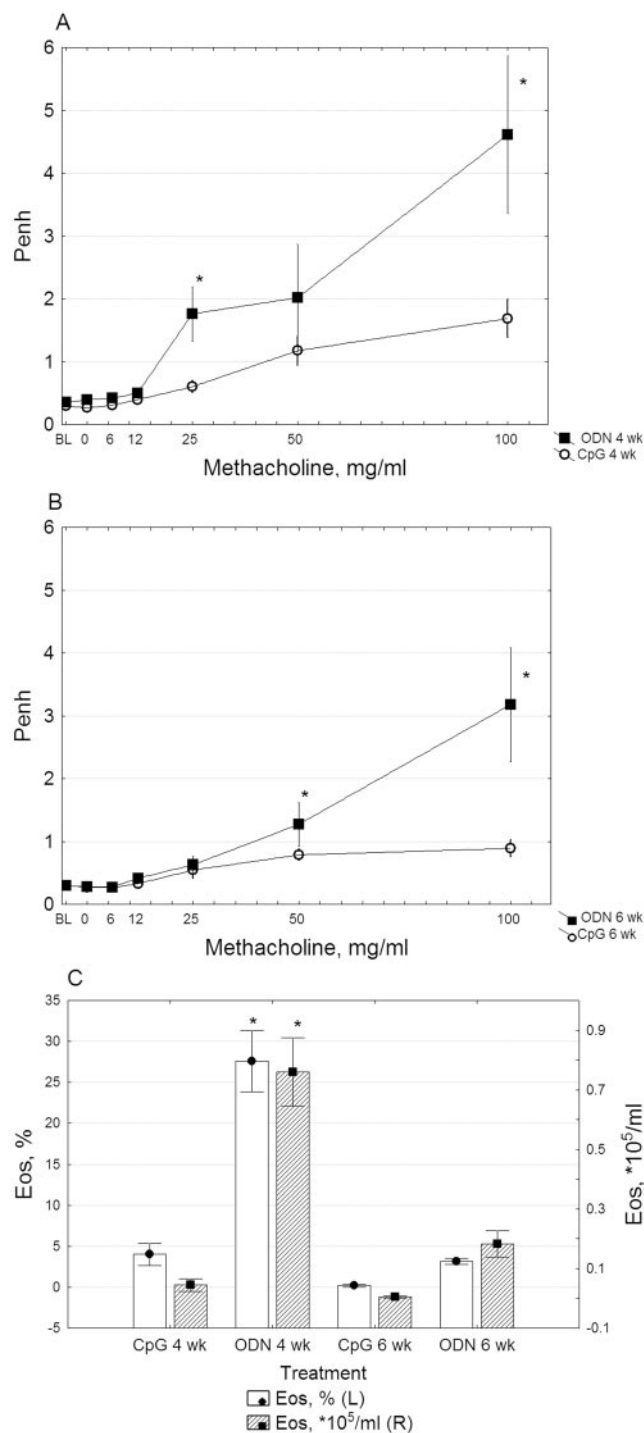


FIGURE 5. Long-term CpG effect: Penh at 4 wk (A) and 6 wk (B), and BAL eosinophilia (C). The CpG-treated mice showed significantly lower AHR (A) and AI (B) at the 4 wk point. At 6 wk, a statistically significant effect of CpG on AHR was seen, but BAL eosinophilia was substantially reduced in positive controls, precluding analysis of the CpG effect. For each group, $n = 6$. *, $p < 0.05$.

monary allergic inflammation compared with ODN- or PBS-treated controls.

Duration of effect analysis

To determine the duration of the CpG effect after a single i.p. injection on day 3 of life, we analyzed 4- and 6-wk-old animals in the long-term protocol (see Fig. 1B). We reasoned that the use of

larger (older) animals might also allow us to compare results of whole-body plethysmography with more invasive analysis of lung mechanics (flexiVent), an approach that is not anatomically feasible at 2 wk of age (the time of our usual analysis). We found that *Penh* was significantly lower in CpG-treated offspring of asthmatic mice compared with ODN controls at both 4 wk (Fig. 5A) and 6 wk (Fig. 5B) of age. In the 4-wk-old mice, we observed a clear difference, with marked eosinophilia in control ODN-treated mice and absence of eosinophilic inflammation in the CpG-treated group (Fig. 5C). However, by 6 wk, BAL eosinophilia was substantially reduced and close to normal values in the positive control ODN-treated group, precluding evaluation of the effect, if any, of CpG treatment at this time point in this protocol. The data suggest that the effect of CpG may last longer than the maternally transmitted susceptibility (see *Discussion*).

Results of lung mechanics evaluation are summarized in Fig. 6. Basal airway compliance was significantly higher (improved, closer to normal) in CpG-treated babies than in ODN controls. There was significantly reduced tissue damping in CpG-treated mice (closer to normal). There also was a trend to decreased airway resistance in CpG-treated mice; however, this was not statistically significant. Hence, the data obtained with flexiVent analysis of basal pulmonary function were consistent with the plethysmography results and showed that CpG treatment improved pulmonary function (see *Discussion*). Based on measurements of pulmonary function and allergic inflammation, the protective effect of the single CpG injection lasts up to 6 wk in this model.

Effect of CpG after sensitization

This experiment was designed to test the hypothesis that CpG not only can prevent sensitization of susceptible offspring, but can also prevent allergy in already sensitized offspring of asthmatic mother mice. CpG effectively reversed susceptibility to allergic effects of OVA aerosols when administered on day 12 (before the first day of aerosolized OVA challenge; see Fig. 1C for protocol). Both AHR and AI were substantially blocked in CpG-treated offspring compared with ODN-treated offspring (Fig. 7, A and C). In contrast, treatment on day 14 (in the middle of OVA aerosol challenges) had no effect on AHR (Fig. 7B), but still caused a decrease in airway eosinophilia (Fig. 7C).

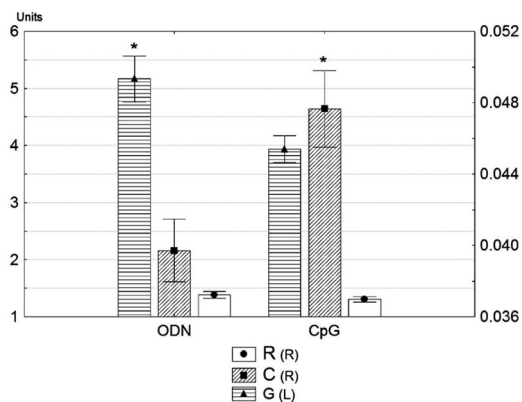


FIGURE 6. Lung mechanics: baseline parameters. In the CpG group, lung resistance (R) was slightly lower, whereas lung compliance (C) was significantly increased, and tissue dampening (G) was significantly decreased compared with the ODN control group, which indicates an improvement in the baseline parameters in the CpG-treated group. Total $n = 13$. *, $p < 0.05$.

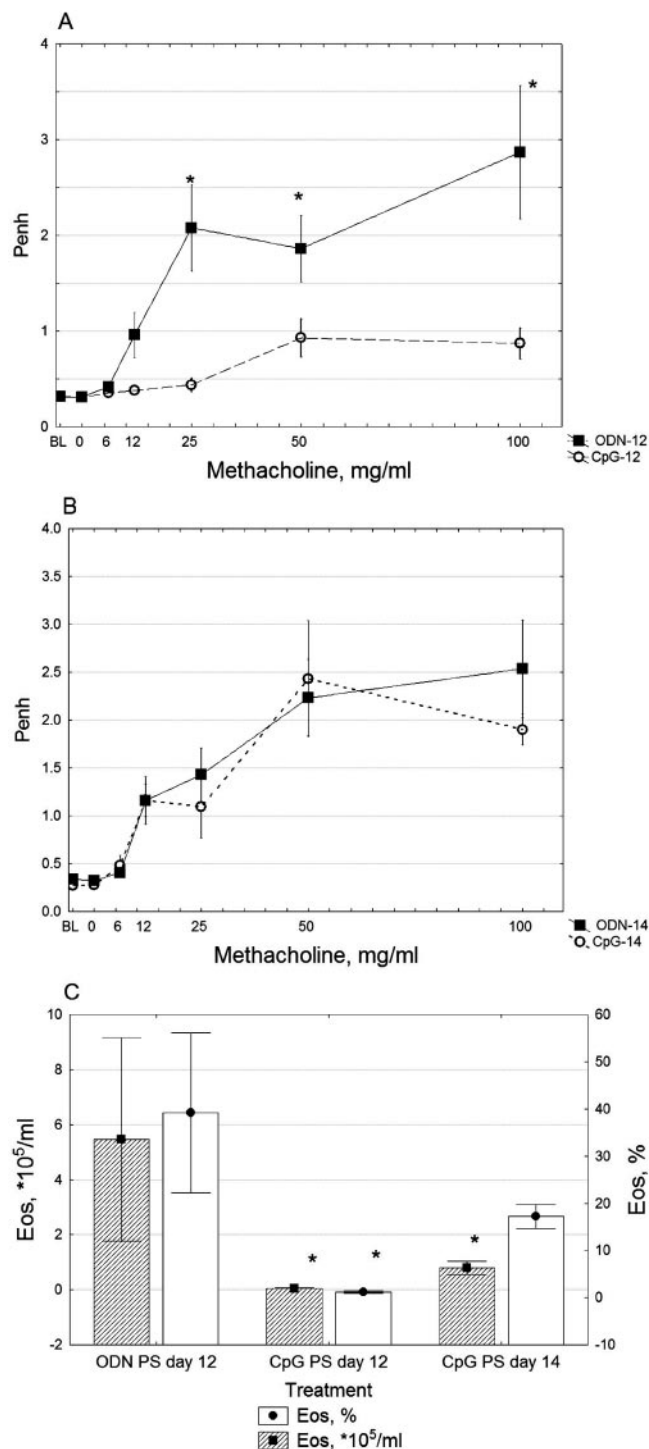


FIGURE 7. Postsensitization administration of CpG on days 12 and 14. On day 12, CpG effectively abrogated both AHR (A) and AI (C), whereas in the day 14 group (B), AHR was no different from that in ODN controls. For CpG (ODN) day 12, $n = 12$ (10); for CpG (ODN) day 14, $n = 12$ (6). *, $p < 0.05$.

Effect of unrelated allergen

It was previously shown in this model that the effect of maternal asthma transmission is not allergen specific. We hypothesized that CpG ODNs would be effective to counteract susceptibility to unrelated allergens as well. Hence, the offspring of OVA-allergic mothers were sensitized and challenged with a second, unrelated allergen, Cas, instead of OVA as in the original protocol.

The control group, treated with non-CpG ODN, showed increased susceptibility to the development of AHR and AI after sensitization and challenge with Cas. In contrast, CpG treatment of baby mice in this protocol using a second allergen (Cas) showed significantly lower AHR (Fig. 8A), AI (Fig. 8B), and pulmonary inflammation (Fig. 8, C and D) compared with ODN-treated controls. This suggests that the mechanism of CpG action is not allergen specific and is effective to reverse the broadly increased susceptibility to allergy seen in neonates in our model.

CpG treatment of pregnant mothers

To test the potential efficacy of CpG administration during pregnancy, we administered CpGs or control ODNs (30 μ g/mouse) as s.c. injections to pregnant, OVA-sensitized and challenged mice on approximately day 14 of gestation. The offspring of these mothers were subjected to the suboptimal sensitization protocol as summarized in Fig. 1A. In some cases we observed decreased litter size (three babies instead of the usual six to eight), premature delivery, and decreased growth rates of offspring. Such litters were not included in analysis (see *Discussion*). After OVA aerosol challenges, we evaluated the Penh and airway eosinophilia. Interestingly, babies of CpG-treated mothers showed significantly reduced AHR

(Fig. 9A), but not reduced eosinophilia (Fig. 9B), compared with ODN controls.

Discussion

The major finding reported is that in vivo treatment with CpG ODNs reverses the increased susceptibility to allergic asthma seen in a mouse model of maternal transmission of asthma risk. Specifically, CpG treatment on day 3 after birth prevented the development of AHR and eosinophilic AI in offspring of asthmatic mother mice otherwise seen in untreated or control ODN-treated offspring. Indeed, the CpG-treated offspring were indistinguishable from offspring of normal mothers in terms of pulmonary function, eosinophil recruitment and IL-5 levels in BAL, and lung parenchymal inflammation. Additional findings were that 1) the protective effect from a single CpG injection lasts for (at least) 4 wk, and for 6 wk in case of AHR, in our model; 2) CpG was effective not only when administered before allergen sensitization, but also when used to revert pre-existing sensitization; and 3) CpG also reversed the susceptibility to unrelated, non-OVA allergens seen in this model of maternal transmission of asthma risk.

Some limitations to this study merit discussion. We have used Penh measurement via whole-body plethysmography to evaluate

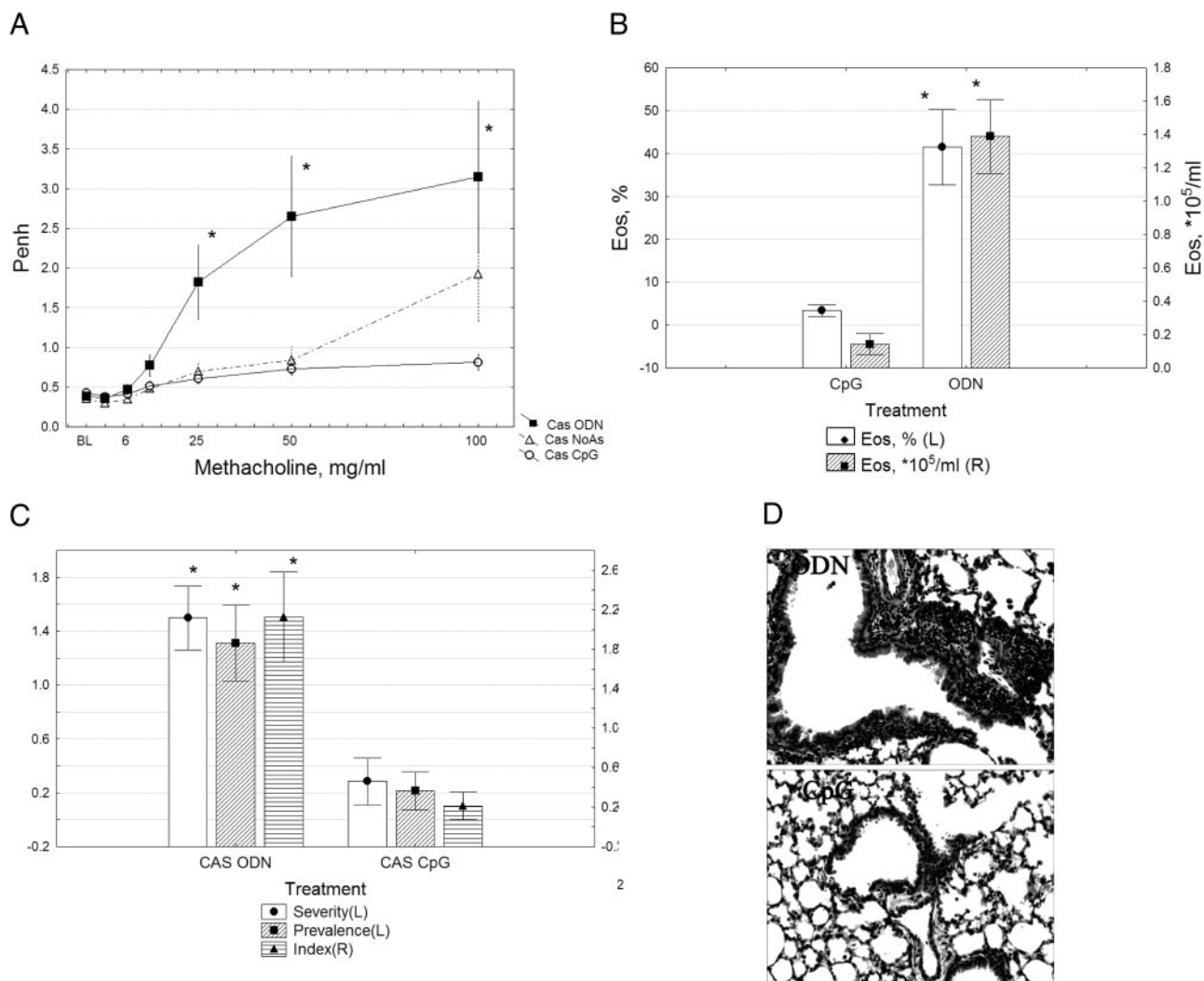


FIGURE 8. CpG effect on unrelated allergen: Penh (A), BAL (B), and lung histology (C) scoring and micrograph (D). Offspring of OVA-sensitized mice sensitized and challenged with casein were treated with control ODN and showed increased Penh, BAL eosinophilia, and pulmonary inflammation. The CpG treatment significantly reduced AHR (A), AI (B), and infiltration in the lung (C and D). For CpG, $n = 9$; for ODN, $n = 8$. *, $p < 0.05$. Lung sections were stained with H&E; original magnification, $\times 200$.

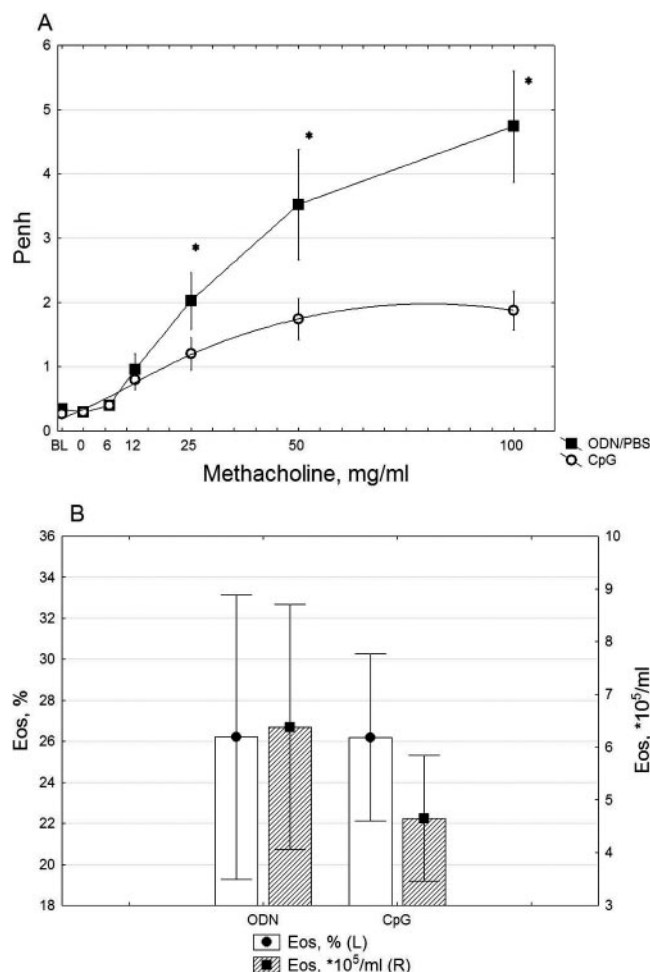


FIGURE 9. Effect of pregnancy CpG treatment on offspring Penh (A) and BAL eosinophilia (B). Susceptible mothers were treated with either control ODN or CpG during pregnancy. Babies of CpG-treated mothers revealed significantly reduced Penh, but not reduced eosinophilia, compared with babies of ODN-treated mothers. For CpG, $n = 18$; for ODN, $n = 9$. *, $p < 0.05$.

pulmonary function in our model. This approach reflects in large part the technical difficulties of using invasive methods on 2-wk-old small mouse pups. We are aware of the ongoing discussion in the literature about whether Penh measurement via whole-body plethysmography truly represents AHR and whether it is a valid technique for different strains of laboratory animals (26). However, it is worth noting that analysis of responses to aerosolized OVA in sensitized, BALB/c strain mice (i.e., as in our model) is the experimental setting where Penh values correlate best and to an (arguably) acceptable degree with more invasive measures (22–28). In the subset of experiments we performed to study the duration of the CpG effect, we were able to find similar trends in Penh and basal pulmonary function tests using the invasive flexiVent approach in older, larger mice. Although we also attempted to measure responses to aerosolized methacholine during the flexiVent analysis, technical problems precluded satisfactory testing, e.g., possibly related to condensation of aerosol on the smaller diameter tubing used for tracheal cannulation in the 4- and 6-wk-old mice.

Although we made a point to use non-CpG oligonucleotides as a control for CpG effects, we are aware of recent findings that some non-CpG ODN can also show CpG-independent, immune stimulatory effects (29, 30). However, such effects are qualitatively dif-

ferent from those of CpG motif-containing ODNs, and most importantly have not been found using the specific control ODN sequence 2138 we used (31). Finally, we also found that there was no significant difference between treatment with non-CpG ODN and injection of PBS vehicle alone (Fig. 4).

Starting CpG treatment after allergen challenge resulted in a diminished CpG effect (Fig. 7). Additional experimentation is required to determine whether this reflects initiation of irreversible effects (by allergen before CpG) or insufficient time for optimal CpG effect.

It was reported that inhibition of Th2 responses achieved by CpG is reversible, and in the long-term setting, CpG treatment must be repeated or its effect is lost (18). We found in our experiments that the effect of a single CpG injection on day 3 of life remains present up to 4 wk, and for 6 wk in case of AHR. It is unlikely that this duration is explained by the persistence of CpG ODNs in the treated mice, because the half-life of CpGs is much shorter (days (Coley Pharmaceuticals manufacturer's information)). The data also suggest a waning or loss of increased susceptibility, but the resolution of this question awaits the results of ongoing studies. Despite this limitation, we can conclude that the single therapy on day 3 was effective at reversing the maternal effect in both very young mice (Fig. 4) and already-weaned, 4- to 6-wk-old mice (Fig. 5).

Although CpG mechanisms might include effects on Ab levels, we have not investigated this possibility in this model because of previous findings that the maternal effect and allergy development in the neonates in our model are Ab independent (as detailed previously (19)). These data include the presence of anti-OVA IgE and IgG in the offspring of asthmatic mother mice, even if the baby mice are not sensitized or exposed. We interpret this as reflecting transfer of maternal Ab to baby mice, some of which is likely to occur across the placenta, and some of which occurs via breast milk. The latter route is supported by our unpublished observations of robust serum levels of anti-OVA IgG and IgE in normal baby mice (tested at 2 wk of age, without any sensitization or exposure to OVA) adoptively nursed by OVA-sensitized/challenged asthmatic mother mice. The observation that increased susceptibility occurs to an allergen different from the maternal allergen (i.e., Cas vs OVA) also argues against a causal role for Ab in this model.

Although we observed an intriguing effect of CpG treatment of pregnant mice, this approach has the limitations that experimental manipulations might either nonspecifically (e.g., via stress) or mechanistically alter the normal course of pregnancy, disturbing normal, pregnancy-favorable Th1/Th2 balance. Indeed, we observed decreased litter size in ~50% of treated mothers, and there were several cases of premature deliveries or stillbirths. Because some vaccinations are compatible and safe for pregnant women, immune modulation during this period is theoretically possible. The basis of the discordance in effect on offspring susceptibility to AHR and AI is unknown. It can be hypothesized that CpG could act through effects on maternal cytokine levels (e.g., IL-4) as reported by Agrawal et al. (17), or on fetal lung development, points not addressed in these studies. This observation of discordant changes in AHR and AI is similar to findings in experiments that alter regulatory T cell populations in susceptible neonates (32) and requires additional study.

The mechanisms by which CpGs modulate immune responses are the subject of substantial current interest. For example, although it was shown that interaction of CpG with TLR9 is necessary for the CpG effect (33–35), there are data suggesting that internalization of the CpG molecules may occur via another, as yet unidentified receptor that is expressed on dendritic cells (DCs) (36). One interesting postulate for the effects of CpG ODNs on

atopic airway inflammation is that administration of CpG alone is effective at promoting Th1-type responses, whereas maximal protection against AI (and reduction of Th2-type responses) requires the coadministration of allergen and CpG ODNs (37). In this scenario the formation of immune tolerance is achieved: allergen in the lungs in the presence of CpG-ODN promotes a Th1 milieu and also, perhaps by inducing the release of IL-10 and TGF- β , promotes Ag-specific regulatory T cells that can down-regulate both Th1 and Th2 responses and tolerize local DCs. Based on these and other studies, DCs are an important (if not primary) target for the CpG effect.

The primary cell targets for the CpG are DCs, macrophages, and B cells. The functional state of these cells in neonates differs in complex ways from that in adults (reviewed in Ref. 38). We speculate that in utero influences on neonatal DC function/phenotype could play a central role in the mechanisms of maternal asthma transmission. Studies are underway to investigate this postulate and to test the effect of CpGs on DC function in the neonates with asthma susceptibility in our model.

Our results support the importance of environmental exposures very early in life. Exposure to Ag in early life can cause asthma, whereas exposure to CpG at the same time point can protect against asthma in a vulnerable host.

Finally, there are encouraging results in early human trials using CpGs in adult volunteers (39). Our finding of beneficial effects of CpG in offspring of asthmatic mothers with increased risk of asthma suggests that this approach may ultimately have therapeutic value and merits additional experimental study.

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Disclosures

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