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Allergen-Independent Maternal Transmission of Asthma Susceptibility

Kaoru Hamada,‡ Yasue Suzuki,‡ Alejandra Goldman,§ Yao Yu Ning,§ Carroll Goldsmith,‡ Aiyappa Palecanda,‡ Brent Coull,‡ Cedric Hubeau,‡ and Lester Kobzik 3‡§

Maternal asthma is a risk factor for development of asthma in children, but mechanisms remain unclear. Offspring of asthmatic mother mice (sensitized and repeatedly exposed to OVA Ag) showed airway hyperresponsiveness and allergic pulmonary inflammation after an intentionally suboptimal OVA sensitization and exposure protocol that had little effect on normal offspring. Similar results were obtained when offspring of OVA-allergic mothers were exposed to an unrelated allergen, casein, indicating that the maternal effect is allergen independent and not transferred by OVA-specific Abs. Premating treatment with neutralizing anti-IL-4 Ab or reduction of maternal allergen exposure abrogated the maternal effect, showing a critical mechanistic role for IL-4 and suggesting an additional benefit of allergen avoidance. The Journal of Immunology, 2003, 170: 1683–1689.


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4 Abbreviations used in this paper: AHR, airway hyperresponsiveness; AI, allergic inflammation; BAL, bronchoalveolar lavage; Cs, casein; Penh, enhanced pause; inflammation (AI), and serum OVA-specific IgE, which are features that recall human asthma in this commonly used model (17, 18). After mating, we compared offspring of normal or asthmatic mother mice for susceptibility to development of the asthma phenotype (AHR and AI) upon exposure to allergen (OVA) in early life. Our strategy was to use an intentionally suboptimal protocol in which only a single i.p. injection was used for sensitization, rather than the two injections that consistently give robust AHR and AI when animals are subsequently challenged with aerosolized allergen (19, 20). After observing increased susceptibility in this basic model, we investigated whether or not the maternal effect is allergen specific by challenge of baby mice with a second, unrelated allergen, casein (Cs). We also identified a critical role for active allergic inflammation in the asthmatic mother mouse by manipulation of premating allergen exposures and by premating treatment of mothers with neutralizing anti-IL-4 Ab.

Materials and Methods

Animals

Newborn BALB/c mice were obtained commercially from Harlan Sprague Dawley (Indianapolis, IN) as litters with their mother mouse at day 2 of age or by in-house breeding, as described below. Each mother and litter was housed separately, fed a 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/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 (St. Louis, MO).

Allergen sensitization and exposure

Maternal sensitization was achieved by initial i.p. injections of 0.1 ml PBS containing OVA (5 μg) and alum (1 mg) 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, Braintree, MA) using a Pari LT2 nebulizer (Sun Medical Supply, Kansas City, KS) connected to an air compressor (PulmoAID; DeVilbiss, Somerset, PA) (21). Immediately after the last aerosol exposure, the female mice were placed in cages with male mice to allow mating. At ≈ day 18 of pregnancy, some mice were further exposed to an aerosol challenge of OVA for each of 3 consecutive days, as above. After birth, baby mice were treated with a single i.p. injection of OVA and alum on day 3. On the next day, the baby mice were exposed to aerosolized OVA, as above. Physiologic and pathologic analysis was performed the next day (age day
The experimental protocol is summarized in Fig. 1. In some experiments, female mice were injected with rat anti-mouse IL-4 Ab (1 mg i.p., clone 11B.11; National Cancer Institute, Frederick, MD). Controls for the four major variables of the protocol included omission of treatment for the first three (maternal sensitization, OVA aerosol exposure of pregnant mice, newborn i.p. sensitization) and substitution of PBS for OVA aerosol exposure of newborns. Treatment groups are coded by their exposures in these four stages, using O for OVA, P for PBS, and Cs for no treatment, as summarized in Table I. Similar protocols were used for studies with a second allergen, Cs, with the modification that aerosol challenge with Cs was performed using a 1% solution for 20 min.

**Pulmonary function testing**

Airway responsiveness of mice to increasing concentrations of aerosolized methacholine was measured using whole body plethysmography (Buxco, Sharon, CT). Briefly, each mouse was placed in a chamber, and continuous measurements of airway resistance were calculated via a connected transducer and associated computer data acquisition system. The main indicator of airflow obstruction, enhanced pause (Penh), which shows 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 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 taken for 9 min after each dose. Penh values for the first 5 min after nebulization were averaged and used to compare results across treatment groups and individual mice.

**Pathologic analysis**

After physiologic testing in airway-sensitized mice or postallergen-challenged mice, the animals were euthanized with sodium pentobarbital (Vetar, California, CA). Briefly, each mouse was placed in a chamber, and continuous measurements of airway resistance were calculated via a connected transducer and associated computer data acquisition system. The main indicator of airflow obstruction, enhanced pause (Penh), which shows 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 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 taken for 9 min after each dose. Penh values for the first 5 min after nebulization were averaged and used to compare results across treatment groups and individual mice.

**Statistical analysis**

Data are presented as mean ± SE. ANOVA analysis of differences among group means was performed using Fisher’s protected least significant difference test and the Statview software program (Abacus Concepts, Berkeley, CA). Statistical significance was accepted when p ≤ 0.05.

**FIGURE 1.** Schematic of main protocol. BALB/c female mice received i.p. injections of 0.5 ml PBS containing OVA (5 μg) and alum (1 mg) at days 3, 7 and 11 of age and were exposed to aerosols of allergen (3% v/v) for 10 min on 3 consecutive days at 4, 8, and 12 wk of age, followed by mating. At day 18 of pregnancy, some mice were further exposed to aerosolized OVA. After birth, newborns received a single i.p. injection of OVA and alum (day 3), followed by exposure to aerosolized OVA on days 12–14 of life. Physiologic (plethysmography) and pathologic analysis was performed the next day (19, 22). Treatment groups are coded by exposures in these four stages, using O for OVA, P for PBS, and Cs for no treatment (see Table I).

**FIGURE 2.** Evaluation of asthmatic status of female, future mother mice. The efficacy of the sensitization and aerosol challenge protocol used to generate asthmatic mothers was evaluated in subsets of mice after the aerosol exposures at 4, 8, and 12 wk, and after analysis of offspring mice. As shown here, the i.p. sensitized female mice exhibited marked AHR to OVA aerosol (indicated by increased Penh, A) and AI (BAL eosinophils × 107, B) after exposure to OVA aerosol at 4 wk of age, but not after exposure to aerosols of the vehicle PBS (C). Similar results were seen at the other time points analyzed (8, 12, and 2 wk postnatal; data not shown).
Results
Susceptibility to OVA-specific allergic airways disease

The basic protocol for these studies (summarized in Fig. 1) was performed on mice derived from either normal or OVA-sensitized/exposed mothers. The efficacy of the protocol in creating allergic airway disease in the female (future mother) mice was evaluated after each of the aerosolized allergen challenges at 4, 8, and 12 wk of age. The results showed AHR and AI after allergen exposure (Fig. 2). We subjected the offspring obtained by mating of asthmatic or normal mice to an intentionally suboptimal sensitization with OVA (a single i.p. injection) before aerosolized allergen challenge on days 12–14 of life. The experimental design results in four major variables that define the status of the young mice at the end of the protocol. These variables and the coding system used to designate them are summarized in Table I.

![FIGURE 3. Maternal allergy promotes development of asthma phenotype in baby mice. A. Airway responsiveness (Penh) was significantly increased in offspring of OVA-sensitized and exposed mothers, with or without allergen exposure during pregnancy (○, O/O/O/O; ■, O/-/O/O), over that seen in offspring of normal mothers (△, O/-/-/O/O) as well as other controls (□, O/O/-/-/O/O; □, O/O/-/-/P; ○, -/-/-/-/O/O). *, **, p < 0.01 vs all other groups at 12–100 mg/ml methacholine, n ≥ 16, each group; B, offspring of OVA-sensitized and exposed mother mice subjected to OVA sensitization and aerosol exposure showed increased eosinophils (× 105) in BAL samples (O/O/O/O, O/-/-/O/O; *, †, p < 0.01 vs controls, n ≥ 16). Histopathology showed accumulation of lymphocytes and eosinophils around airways and vessels and goblet cell hyperplasia (C, O/O/O/O; D, O/-/-/-/O/O; ×200). Offspring of normal mother mice subjected to the same OVA sensitization and aerosol exposure (E, -/-/-/-/O/O) manifest a lesser, albeit detectable, degree of pathologic change compared with normal lung seen in normal controls exposed only to (PBS) aerosols (F, -/-/-/-/P). See also scoring of changes in Table II.](http://www.jimmunol.org/)

### Table II. Quantitative analysis of histopathologic changes

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Inflammation Index</th>
<th>Goblet Cell Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>O/O/O/O</td>
<td>42</td>
<td>2.4 ± 0.3*</td>
<td>1.4 ± 0.1*</td>
</tr>
<tr>
<td>O/-/-/-/O/O</td>
<td>29</td>
<td>2.4 ± 0.4*</td>
<td>1.7 ± 0.2*</td>
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<tr>
<td>-/-/-/-/-/O/O</td>
<td>44</td>
<td>0.8 ± 0.2</td>
<td>0.9 ± 0.1</td>
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<tr>
<td>O/O/-/-/-/P</td>
<td>12</td>
<td>0 ± 0</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>-/-/-/-/-/-/P</td>
<td>8</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>O/O/Cs/Cs</td>
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<td>2.0 ± 0.6*</td>
<td>1.7 ± 0.3*</td>
</tr>
<tr>
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<td>2.0 ± 0.6*</td>
<td>2.5 ± 0.9*</td>
</tr>
<tr>
<td>-/-/-/-/-/-/Cs/Cs</td>
<td>14</td>
<td>1.1 ± 0.2</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

*An index of pathologic changes in coded H&E slides was derived by scoring the inflammatory cell infiltrates around airways and vessels for greatest severity (0, normal; 1, ≤3/b cell diameter thick; 2, 4–10 cells thick; 3, ≥10 cells thick) and overall extent (0, normal; 1, ≤25% of sample; 2, 25–50%; 3, 51–75%; 4, ≥75%). The index was calculated by multiplying severity by extent, with a maximum possible score of 9. The extent of goblet cell hyperplasia in airway epithelium was scored on a similar 0–3 scale.

*, p < 0.01 vs -/-/-/-/O/O, O/O/-/-/P, -/-/-/-/-/P groups.

†, p < 0.01 vs -/-/-/-/Cs/Cs, -/-/-/-/-/P groups, ANOVA; similar results obtained using nonparametric analysis (data not shown).
Babies from asthmatic, but not normal, mother mice showed: 1) airway hyperresponsiveness to methacholine (increased Penh; Fig. 3A); 2) increased eosinophils on BAL (Fig. 3B); and 3) robust pathologic changes of AI (eosinophil and mononuclear cell infiltration around airway and vessels and goblet cell hyperplasia) (Fig. 3, C and D). Results of semiquantitative scoring of histology support the qualitative changes illustrated in Fig. 3, C–F, and are presented in Table II. It is noteworthy that the maternal effect was seen whether or not OVA-allergic mothers were subjected to an additional OVA aerosol challenge during pregnancy (i.e., O/O/O/O vs O/O/O/O; Fig. 3). Treatment groups in which sequential components of the sensitization protocol were omitted or replaced with control PBS were tested and mostly showed minimal airway responsiveness and AI.

**Allergen-specific IgE**

To investigate the contribution of allergen-specific IgE, we measured serum anti-OVA IgE. The characterization of a purified anti-OVA IgE mAb (2C6) developed and used as a reference reagent for the standard curves in these ELISA is presented in Fig. 4, A and B. Serum OVA-specific IgE was increased in offspring of asthmatic mothers (Fig. 4C), including unsensitized offspring that did not show AHR or AI (e.g., the O/O/O/P group; see Figs. 3 and 4). Similarly, elevated levels of OVA-specific IgG were detected in asthmatic mothers, their offspring, and breast milk from asthmatic mothers (data not shown). These data and the similar absence of AHR and AI in unsensitized offspring exposed to OVA allergen aerosols (O/O/O/O) indicate that allergen-specific Ab is transferred from mother to offspring in this model, but is not sufficient to confer sensitization and allow development of AHR and AI upon challenge with aerosolized allergen. This indicated that the maternal effect might be allergen independent and represents a more generalized increase in allergic susceptibility, as suggested by the increased immediate contact hypersensitivity to a second allergen observed in offspring of OVA-allergic female mice (25, 26).

**Susceptibility to respiratory allergy to a different allergen**

To more directly test the role of allergen and/or Ab in maternal transfer of susceptibility, we replaced the OVA allergen used for sensitization and challenge of baby mice with Cs. This was based on development of a mouse model of asthma using this second,
distinct protein Ag (bovine Cs). Analysis showed marked AHR and AI in baby mice that were sensitized with two i.p. injections of Cs with alum adjuvant before challenge with aerosolized Cs allergen, with minimal response to aerosolized PBS (Fig. 5, A and D). Similar results were found in adult mice (results not shown), confirming that Cs could be used like OVA to create mouse models of allergic airway disease. We modified the previous protocol (as outlined in Fig. 1) to test the response of babies born to OVA-allergic and exposed mothers to sensitization with a single i.p. injection of Cs, followed by challenge with Cs aerosols (days 12–14) and evaluation (day 15). Babies from OVA-asthmatic, but not normal, mother mice showed marked susceptibility to sensitization by the single i.p. treatment with Cs. This was manifest as: 1) AHR normal, mother mice showed marked susceptibility to sensitization created by the last premating aerosol challenge is required for the maternal effect. In a second set of experiments, we administered neutralizing anti-IL-4 (24) or control rat IgG Ab to female mice just after completion of their last premating OVA aerosol (week 12; see Fig. 1). Offspring of anti-IL-4-treated mother mice showed markedly decreased AHR and AI compared with their IgG-treated counterparts, in sensitization and challenge protocols using either the same Ag (OVA; Fig. 7A) or a different Ag (Cs; Fig. 7, B and C).

These data implicate maternal IL-4 induced by active allergic inflammation in the period just before pregnancy as necessary for maternal transmission of susceptibility. However, we also considered the possibility that persistence and transfer of rat anti-IL-4 Abs does not mediate the maternal inactivation of offspring produced in the (future) mother mice just before mating might persist and act on the developing immune system of their offspring during pregnancy. We focused these initial studies on IL-4, a cytokine well characterized for its role in allergic airway inflammation (27, 28).

To test these postulates, we performed two related experiments. In the first, we omitted the final premating OVA aerosol challenge of sensitized female mice (as well as omitting any allergen exposure during pregnancy). Offspring of mice treated this way no longer showed susceptibility to sensitization to either OVA or Cs, with no AHR (Fig. 6, A and B) or AI (results with Cs, Fig. 6C; similar results with OVA, data not shown) detected after aerosol challenge. These findings indicate that active allergic inflammation created by the last premating aerosol challenge is required for the maternal effect. In a second set of experiments, we administered neutralizing anti-IL-4 (24) or control rat IgG Ab to female mice just after completion of their last premating OVA aerosol (week 12; see Fig. 1). Offspring of anti-IL-4-treated mother mice showed markedly decreased AHR and AI compared with their IgG-treated counterparts, in sensitization and challenge protocols using either the same Ag (OVA; Fig. 7A) or a different Ag (Cs; Fig. 7, B and C).

These data implicate maternal IL-4 induced by active allergic inflammation in the period just before pregnancy as necessary for maternal transmission of susceptibility. However, we also considered the possibility that persistence and transfer of rat anti-IL-4 Ab to the circulation of newborns. This could block development of AHR and AI in the offspring (similar to direct treatment with anti-IL-4 (29), but would preclude any interpretation of the role of IL-4 in earlier events in the mother.

**FIGURE 5.** Role of active maternal allergic inflammation. Omission at 12 wk of the premating 3 days of aerosol exposure to OVA in female mice resulted in absence of development of airway hyperresponsiveness in babies upon sensitization to A, OVA (○, O/−/−/O/O vs ■, O/−/O/O, △, −/−/−/O/O) and B, Cs (○, O/−/−/Cs/Cs, vs ■, O/−/−/Cs/Cs, △, −/−/−/Cs/Cs) *p < 0.01 vs two other groups at 25–100 mg/ml methacholine; n ≥ 16. C, BAL results from a representative experiment show that omission of premating OVA exposure (O/−/−/−/Cs/Cs) results in diminished eosinophilia (× 105) upon sensitization and challenge of offspring with Cs. *, p < 0.01 vs O/−/−/Cs/Cs.

**FIGURE 6.** Role of active maternal allergic inflammation. Omission at 12 wk of the premating 3 days of aerosol exposure to OVA in female mice resulted in absence of development of airway hyperresponsiveness in babies upon sensitization to A, OVA (○, O/−/−/−/O/O vs ■, O/−/−/O/O, △, −/−/−/O/O) and B, Cs (○, O/−/−/Cs/Cs, vs ■, O/−/−/Cs/Cs, △, −/−/−/Cs/Cs) *p < 0.01 vs two other groups at 25–100 mg/ml methacholine; n ≥ 16. C, BAL results from a representative experiment show that omission of premating OVA exposure (O/−/−/−/Cs/Cs) results in diminished eosinophilia (× 105) upon sensitization and challenge of offspring with Cs. *, p < 0.01 vs O/−/−/Cs/Cs.

**FIGURE 7.** Effect of IL-4 blockade. Treatment of OVA-sensitized and exposed female mice with neutralizing anti-IL-4 Ab (clone 11B.11 (24), but not control rat IgG (1 mg i.p.) after completion of the last premating OVA aerosol exposure at 12 wk markedly diminished development of airway hyperresponsiveness in offspring sensitized and exposed either to A, OVA (■, O/−/−/−/O/O + control IgG; ○, O/−/−/−/O/O + anti-IL-4; □, −/−/−/O/O) or B, Cs (■, O/−/−/−/Cs/Cs + control IgG; ○, O/−/−/−/Cs/Cs + anti-IL-4; □, −/−/−/Cs/Cs) *p < 0.01 vs two other groups at 25–100, 50–100 mg/ml methacholine, respectively. C, BAL results from a representative experiment show that treatment with anti-IL-4 (O/−/−/−/Cs/Cs + anti-IL-4) results in diminished eosinophilia (× 105) upon sensitization and challenge of offspring with Cs. *, p < 0.01 vs O/−/−/Cs/Cs.
mouse. To address this possibility, we first measured the concentration of rat IgG in serum samples taken from babies of treated mothers at day 3 after birth. As shown in Fig. 8A, we did detect circulating anti–IL-4 in these newborns (mean ± SE, 49 ± 8 ng/ml; n = 14). To test the functional effect, if any, of this persistent anti–IL-4, we measured the serum levels of baby mice on day 3 of life after injection with different amounts of rat anti–IL-4 on day 2. As shown in Fig. 8A, injection of 10 μg i.p. resulted in levels markedly higher (630 ± 67 ng/ml; n = 12) than that seen in serum of babies of mothers treated with 1 mg i.p. before mating. The functional effect of this amount of circulating anti–IL-4 was tested by i.p. injection on day 3 of 10 μg anti–IL-4 into a cohort of offspring of asthmatic mothers before i.p. injection with allergen on day 4, aerosol allergen challenge days 12–14, and analysis on day 15, as per the usual protocol. This treatment had no effect on the development of AHR in response to Cs (Fig. 8B). Analysis of anti–IL-4 persistence. The first direct demonstration that transfer of mediator(s) from mother to offspring of asthmatic mothers before i.p. injection with allergen. There were three major findings. First, the data did indeed show development of the asthma phenotype in offspring of asthmatic (but not normal) mother mice. The data provide the first direct demonstration that transfer of asthma susceptibility can occur by biologic mechanisms in a model wherein genetic and environmental influences are excluded. Second, this model demonstrated that the maternal effect is allergen independent. This finding argues against a critical role for transfer of Ag or specific Abs per se, although the data do not exclude the possibility that Ag-specific immunity can also be transferred under some conditions. Third, the studies of this model also demonstrated a requirement for active maternal allergic inflammation in the period just before or during early gestation, and a critical function for IL-4.

Some advantages and limitations of the experimental design merit discussion. Use of a mouse model allowed us to experimentally exclude two other potentially important mechanisms for maternal influence on asthma risk in humans: transmission of susceptibility genes and effects of maternal behavior (e.g., smoking). Consequently, the demonstration of maternal transmission of asthma risk in our model does not address the likely contribution of these other pathways to final outcomes in people. Mouse (and other animal) models of asthma are imperfect replicates of the human disorder. Nevertheless, analysis of the asthma phenotype in mice (airway hyperresponsiveness, allergic pulmonary inflammation, and allergen-specific Abs) has provided many useful insights into pathogenesis (27, 30–32).

Intervention with neutralizing Ab and manipulation of the model provided data that IL-4 induced by the last premating OVA challenge is a necessary mediator for maternal effects on offspring. We observed a remarkable persistence of the rat anti-mouse IL-4 in the serum of offspring of treated mother mice. These findings are similar to the long t1/2 in mouse serum reported for rat anti–IL-5 (33). However, direct testing showed that 10-fold higher serum concentrations of anti–IL-4 were ineffective at blocking the susceptibility of newborn mice of allergic mothers to allergic sensitization, supporting an important mechanistic role for IL-4 at some point in the maternal/fetal interaction. Whether this represents persistence of IL-4 induced by the premating exacerbation of our protocol and subsequent transplacental transfer or an indirect effect of IL-4 on other immune cells and mediators that in turn

FIGURE 8. Analysis of anti–IL-4 persistence. The concentration of rat IgG in serum samples taken from 3-day-old babies of mothers treated with anti–IL-4 before mating is shown in A and can be compared with concentrations measured 24 h after injection of 10 μg i.p. into normal 2-day-old baby mice. B and C. A cohort of offspring of asthmatic mothers received treatment on day 3 with 10 μg anti–IL-4 before i.p. injection with allergen on day 4, aerosol allergen challenge days 12–14, and analysis on day 15, as per the usual protocol. This treatment had no effect on the development of AHR in response to Cs (Fig. 8B). Analysis of anti–IL-4 persistence. The first direct demonstration that transfer of mediator(s) from mother to offspring of asthmatic mothers before i.p. injection with allergen. There were three major findings. First, the data did indeed show development of the asthma phenotype in offspring of asthmatic (but not normal) mother mice. The data provide the first direct demonstration that transfer of asthma susceptibility can occur by biologic mechanisms in a model wherein genetic and environmental influences are excluded. Second, this model demonstrated that the maternal effect is allergen independent. This finding argues against a critical role for transfer of Ag or specific Abs per se, although the data do not exclude the possibility that Ag-specific immunity can also be transferred under some conditions. Third, the studies of this model also demonstrated a requirement for active maternal allergic inflammation in the period just before or during early gestation, and a critical function for IL-4.

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affect the developing individual remains to be determined. In addition, this model will be useful to further analyze the potential of other pharmacologic (e.g., anti-inflammatory) or public health (e.g., allergen avoidance) interventions on the maternal transmission of asthma risk.

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References