

Maternal allergy increases susceptibility to offspring allergy in association with T_H2-biased epigenetic alterations in a mouse model of peanut allergy

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Background: Although maternal atopy is a risk factor for the development of peanut allergy, this phenomenon has not been well characterized experimentally, and the mechanisms underlying offspring risk are unclear.

Objective: We sought to determine whether offspring of mothers with peanut allergy (O-PAM mice) are more susceptible to peanut allergy than offspring of naive mothers (O-NM mice) in a murine model and, if so, whether the susceptibility is linked to T_H2-biased epigenetic alterations.

Methods: Five-week-old O-PAM and O-NM mice were intragastrically sensitized to and challenged with peanut. Serum peanut-specific IgE levels, plasma histamine levels, anaphylactic reactions, and splenocyte and MLN cell cytokine production were measured. DNA methylation levels of the *Il4* gene promoter from splenocytes and MLN cells from sensitized offspring and splenocytes from unsensitized neonatal offspring were determined by means of pyrosequencing.

Results: O-PAM mice exhibited 3-fold higher peanut-specific IgE levels after peanut sensitization, as well as 5-fold higher histamine levels and significantly higher anaphylactic symptom scores after challenge than O-NM mice ($P < .05$ -.01). Cultured splenocytes and MLNs from O-PAM mice produced significantly more T_H2 cytokines than cells from O-NM mice ($P < .05$ -.01). Cells from O-PAM mice exhibited significantly

reduced DNA methylation at CpG sites of the *Il4* gene promoter than cells from O-NM mice. DNA methylation levels were inversely correlated with IL-4 and IgE production. O-PAM neonatal splenocyte hypomethylation of the *Il4* gene promoter was also present.

Conclusion: This study is the first to demonstrate that increased susceptibility to peanut allergy in O-PAM mice is associated with epigenetic alteration of the *Il4* gene promoter. This finding might provide insight into preventing the development of early-life allergy. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Peanut allergy, anaphylaxis mice, offspring, IgE, IL-4, DNA methylation

Food allergy, a growing public health concern in the United States, affects up to 8% of children and 4% of adults and is a major cause of anaphylaxis.¹⁻³ Among these food allergies, peanut allergy has attracted great public attention because of its prevalence, severity of reactions, and frequent lifelong persistence.⁴⁻⁷ In 1996, Hourihane et al⁸ reported that the prevalence of peanut and other allergies in the families of persons with peanut allergy was increased in successive generations in maternal but not paternal relatives. Additional clinical observational studies also show that maternal peanut allergy and other allergies increase the risk of a child having peanut allergy.⁹⁻¹¹ The reason for this is not known, and although allergy-associated genes have been identified, none are strongly associated with peanut or other food allergy.¹²

Peanut allergy, like other allergies, is a T_H2-mediated immune disorder characterized by increased expression of IL-4, IL-5, and IL-13 in human subjects¹³ and animal models.¹⁴⁻¹⁶ IL-4 is a key T_H2 cytokine required for B cells switching to IgE production, mast cell activation, and T_H2 cell differentiation.¹⁷ Higher IL-4/IFN- γ ratios were found in patients with persistent peanut allergy than in patients who naturally outgrew peanut allergy.¹⁸

Increasing evidence shows that epigenetic mechanisms are involved in regulating T-cell differentiation, cytokine expression, allergic sensitization, and allergic asthma development.¹⁹⁻²¹ CpG DNA methylation is a well-known epigenetic modification that affects chromatin remodeling and can drive cytokine expression in the absence of alterations in DNA sequences.²² DNA hypomethylation activates cytokine expression, whereas hypermethylation represses cytokine expression.²³⁻²⁵ DNA methylation status is dynamic and influenced by both external and internal factors. A study investigating the effect of inhalation of diesel exhaust particles and allergen on DNA demethylation status at several CpG sites of the *Il4* gene promoter showed that the level of DNA CpG⁻⁴⁰⁸ demethylation was inversely

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Abbreviations used

Con A: Concanavalin A
 CPE: Crude peanut extract
 CT: Cholera toxin
foxp3: Forkhead box P3
 MLN: Mesenteric lymph node
 NM: Naive mothers
 O-NM: Offspring of naive mothers
 O-PAM: Offspring of mothers with peanut allergy
 PAM: Mothers with peanut allergy
 PBL: Peripheral blood leukocyte

correlated with increased IgE production in a murine model of asthma.²⁶ Although it has been suggested that association of maternal atopy with increased offspring susceptibility to food allergy is linked to epigenetic changes,^{27,28} direct experimental evidence of such an association is lacking.

We hypothesized that maternal peanut allergy would increase propensity for peanut-specific IgE production, hypersensitivity reactions, T_H2 cytokine production, and T_H2 cytokine gene promoter hypomethylation in offspring. To test this possibility, we used a mouse model to compare IgE levels and anaphylactic reactions after suboptimal active peanut sensitization and challenge in offspring of mothers with peanut allergy (O-PAM) with those in offspring of naive mothers (O-NM). We also determined cytokine production and methylation status of the T_H2 signature cytokine *Il4* gene promoter at CpG sites in DNA from mesenteric lymph node (MLN) cells and splenocytes of offspring.

METHODS**Mice and reagents**

Six-week-old female and male C3H/HeJ mice purchased from the Jackson Laboratory (Bar Harbor, Me) were maintained on peanut-free chow under specific pathogen-free conditions according to standard guidelines for the care and use of animals.²⁹ Freshly ground whole roasted peanut and crude peanut extract (CPE) were prepared as described previously (see the [Methods](#) section in this article's Online Repository at www.jacionline.org).³⁰⁻³² Sources of reagents used in this study are provided in the [Methods](#) section in this article's Online Repository.

Experimental protocols

Maternal protocol. To establish maternal peanut allergy, female C3H/HeJ mice on a peanut-free diet were orally sensitized with peanut, as previously described,^{15,16} with slight modification. Briefly, 6-week-old female mice were sensitized with peanut (10 mg) and cholera toxin (CT; 20 μ g) intragastrically weekly for 5 weeks and challenged at week 6 (200 mg of peanut per mouse). Our sensitization protocol differed from our previous standard protocol in that mice did not receive an additional boost (50 mg of peanut plus 10 mg of CT). Mice treated with the protocol used in our current study are termed mothers with peanut allergy (PAMs). As control animals, naive female mice were PBS sham sensitized and challenged; these mice are termed naive mothers (NMs). One week after peanut challenge, mice in both groups were bred with naive male subjects. All mice were fed peanut-free chow during gestation and lactation.

Determination of peanut protein levels in milk. During lactation, milk was collected from PAMs or NMs when their offspring were 10 to 15 days old with a mouse milking machine modified in our laboratory, as previously described.³³ An additional group of PAM lactating mice was administered 10 mg of peanut protein intragastrically to establish a positive control. Milk was collected 2 hours later. Levels of peanut protein in milk from these 3

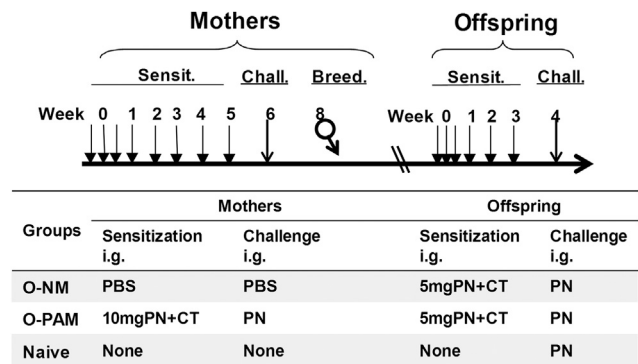


FIG 1. Experimental protocols. Peanut-sensitized female mice were orally challenged with peanut and then mated with naive male mice 1 week later. Five-week-old O-PAM and O-NM mice were sensitized weekly for 3 weeks and challenged at week 4. Offspring of PBS-challenged naive mothers served as normal control animals. *Breed.*, Breeding; *Chall.*, challenge; *i.g.*, intragastric; *PN*, peanut; *Sensit.*, sensitization.

TABLE I. Primers used for PCR amplification and pyrosequencing experiments

<i>Il4</i>		
PCR forward	GTTTTAAAGGGGTTTATAGTAGGAAGT	
PCR reverse	Biotin-AATTACCACTAAGTCTCCTCTACA	
Pyrosequencing	AGATTTTGTGATATTATTTGTTT	

groups were determined with a Veratox Peanut commercial kit (Neogen, Lansing, Mich), according to the manufacturer's instruction and as previously described.³⁴

Offspring protocol. O-PAM and O-NM, weaned at 4 weeks of age, were sensitized 1 week later with 3 weekly intragastric suboptimal doses of peanut (5 mg) and CT (10 μ g). A third group of age-matched peanut-naive offspring of peanut-free mothers (naive) served as normal control mice ([Fig 1](#)). All offspring were orally challenged at week 4 with 200 mg of ground peanut.

Measurement of peanut-specific antibodies. Sera were obtained from blood collected weekly during sensitization and 1 day before challenge by means of submandibular venipuncture to monitor antibody responses to peanut sensitization. Serum IgG antibodies were first depleted by using protein G-Sepharose (BioVisio, Milpitas, Calif) centrifugation to measure serum peanut-specific IgE levels.^{35,36} In brief, equal volumes of protein G-Sepharose and undiluted mouse serum were mixed and incubated at room temperature for 10 minutes and then centrifuged at 1200 rpm for 10 minutes at room temperature. Supernatants were collected, and the process was repeated twice. Peanut-specific IgE levels in protein G-depleted sera were measured by using previously described ELISA methods.^{37,38} Serum peanut-specific IgG₁ and IgG_{2a} levels were determined, as previously described.^{37,38}

Assessment of hypersensitivity reactions. Anaphylactic symptoms were evaluated 30 minutes after oral challenge by using the scoring system described previously (see the [Methods](#) section in this article's Online Repository at www.jacionline.org).¹⁵ In this scoring system 0 is no reaction, 1 is mild, 2 is moderate, 3 is severe, 4 is very severe, and 5 is death.³⁹ Core body temperatures were measured with a rectal probe (Harvard Apparatus, Holliston, Mass). To confirm that anaphylactic reactions were not IgG mediated, peanut-sensitized mice were pretreated with anti-Fc γ RIIB/Fc γ RIII mAb (mAb 2.4G2, 500 μ g per mouse) or isotype control antibody 24 hours before challenge, as previously described.^{40,41}

Histamine measurement. Histamine levels were measured with an enzyme immunoassay kit (ImmunoTECH, Marseille, France), as described by the manufacturer.

Cell culture and cytokine measurements. Offspring splenocytes and MLNs were prepared, as previously described,³³ and cultured in 24-well plates (4×10^6 /well/mL) in the presence or absence of CPE (200 μ g/mL) or concanavalin A (Con A; 2.5 μ g/mL). Seventy-two hours later,

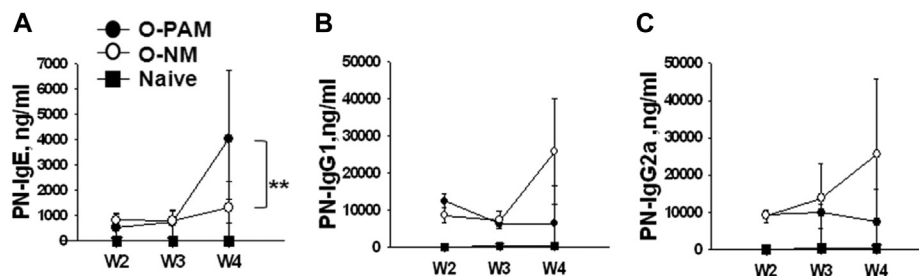


FIG 2. O-PAM mice have an increased IgE response to peanut sensitization. Blood was collected from offspring after peanut sensitization at indicated time points, and serum peanut-specific IgE (**A**), peanut-specific IgG₁ (**B**), and peanut-specific IgG_{2a} (**C**) levels were determined by using ELISA. ** $P < .01$ versus O-NM ($n = 8-11$ per group). PN, Peanut; W, week in sensitization protocol.

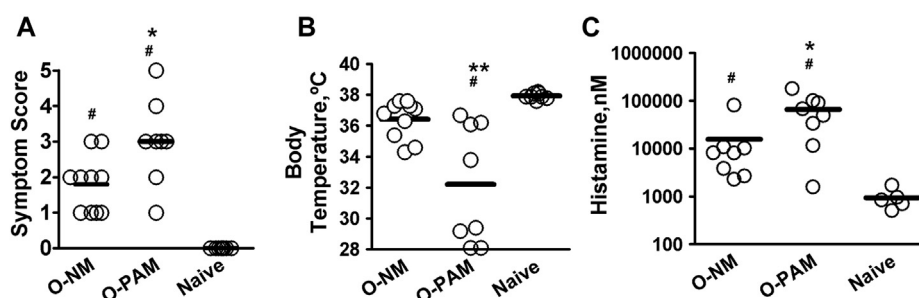


FIG 3. O-PAM mice have anaphylaxis after peanut oral challenge. Thirty minutes after challenge, anaphylactic symptoms in both O-NM and O-PAM mice were scored (**A**), core body temperatures were measured (**B**), and plasma histamine levels were determined (**C**). Histamine levels are presented in log scale. * $P < .05$ and ** $P < .01$ versus O-NM and # $P < .05$ versus normal control mice ($n = 8-11$ per group). Each dot represents an individual mouse. The horizontal bar indicates the mean.

supernatants were collected, and cytokine levels were determined by using ELISA. Splenocytes and MLN cells were then collected and preserved in Buffer RTL (Qiagen, Germantown, Md) for DNA methylation assays.

DNA pyrosequencing methylation analysis. Genomic DNA from MLN cells and splenocytes of offspring was isolated with an AllPrep mini DNA kit (Qiagen). DNA from the spleens of approximately 1-week-old offspring mice (unsensitized) and DNA from maternal peripheral blood leukocytes (PBLs) after challenge were extracted with the DNeasy Tissue/Blood kit (Qiagen), as previously described.⁴² Isolated DNA was then bisulfite converted with an Epitect plus DNA Bisulfite kit (Qiagen), per the manufacturer's instructions. Bisulfite-converted DNA was used to amplify promoter regions of the *Il4* gene by using PCR with the primers shown in Table I. PCR products were subjected to pyrosequencing by using the Pyromark Q24 Pyrosequencing system (Qiagen) with the sequencing primers for CpG⁻⁴⁰⁸ and CpG⁻³⁹³, as shown in Table I. Bisulfite-converted DNA was also used to amplify promoter regions of the *Ifng* and forkhead box P3 (*foxp3*) genes by using PCR, and PCR products were then subjected to pyrosequencing with sequencing primers. Primers, as listed in Table E1 in this article's Online Repository at www.jacionline.org, were chosen based on previous studies.^{26,42} Methylation levels of PCR products at each CpG site were determined by using PyroMark 24 run software (Qiagen).

Statistical analysis

Data were analyzed with the SigmaStat statistical software package (Systat, Chicago, Ill). Differences between 2 groups were analyzed by using *t* tests if the data were approximately normal or by using the Mann-Whitney rank sum test if the data were skewed. ANOVA followed by the Bonferroni *t* test was performed for all pairwise comparisons if the data were approximately normal. Differences among more than 2 groups were analyzed by using ANOVA on ranks, followed by all pairwise comparisons (Dunn method) if the data were not normally distributed. GraphPad Prism 4 software (GraphPad Software, La Jolla, Calif) was used for nonparametric (Spearman) correlation analysis. *P* values of less than .05 based on 2-tailed tests were considered statistically significant.

RESULTS

O-PAM produce increased peanut-specific IgE levels after oral peanut sensitization

PAMs had increased peanut-specific IgE levels after intragastric sensitization and exhibited hypersensitivity reactions after intragastric challenge but before breeding (see Fig E1 in this article's Online Repository at www.jacionline.org).

O-PAM and O-NM were sensitized by using a suboptimal protocol consisting of 3 weekly administration of 5 mg of peanut plus 10 μ g of CT (half the dose and less than half the duration of our standard sensitization protocol).¹⁵ Based on our preliminary results, suboptimal offspring sensitization more clearly distinguishes between O-PAM and O-NM sensitivity to allergy. Four weeks after the initial sensitization, O-PAM sera contained approximately 300% more peanut-specific IgE than O-NM sera (4061.2 ± 2739.6 vs 1280.9 ± 594.3 ng/mL; $P < .01$, $n = 8-11$ per group; Fig 2, A). Sera from normal control mice contained no detectable peanut-specific IgE. Peanut-specific IgG₁ and IgG_{2a} levels in sensitized O-PAM and O-NM mice were not significantly different, although they tended to be higher in O-NM than O-PAM mice ($n = 8-11$ per group; Fig 2, B and C). Thus O-PAM mice produce more IgE than O-NM mice in response to the same sensitization protocol.

O-PAM mice experience more severe anaphylaxis after oral peanut challenge

Only 2 (18%) of 11 peanut-challenged O-NM mice had severe reactions (score 3), and none exhibited very severe or fatal reactions. In contrast, 6 (75%) of 8 O-PAM mice exhibited severe

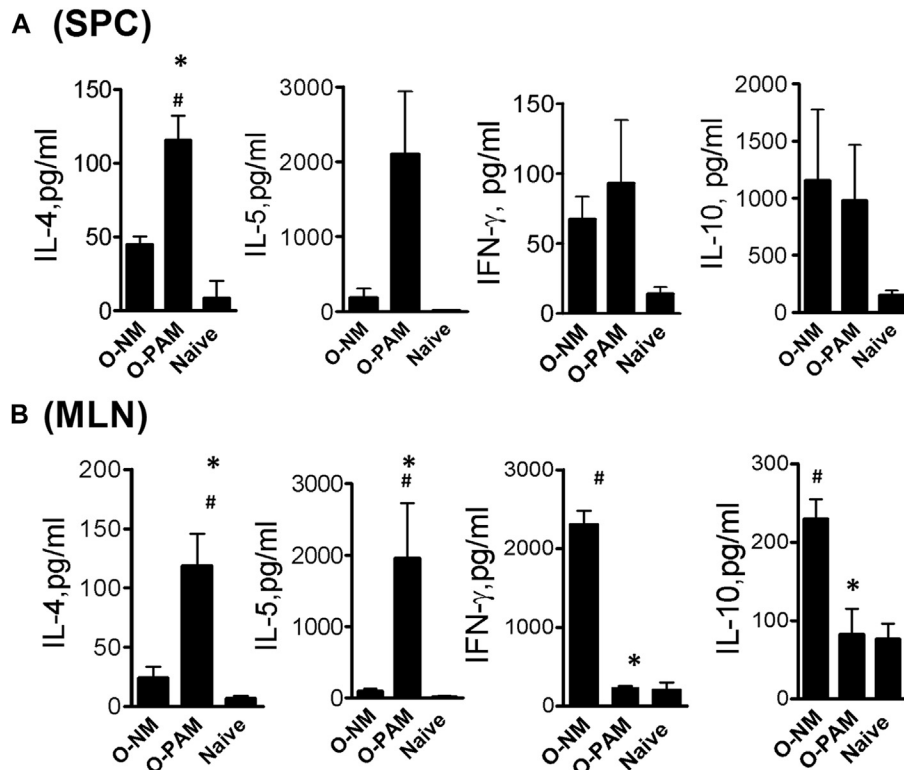


FIG 4. Cytokine secretion by offspring splenocyte (SPC; **A**) and MLN (**B**) cultures. Splenocytes and MLN cells were prepared and cultured for 72 hours in the presence or absence of CPE. Culture supernatant IL-4, IFN-γ, and IL-10 levels were determined. Data are expressed as means $\pm$ SDs of each group. * $P < .05$ versus O-NM and # $P < .05$ versus normal control mice ($n = 8-11$ per group).

reactions, including 1 fatal and 1 near-fatal reaction. Mean anaphylactic symptom scores of O-PAM mice were significantly higher than those of O-NM mice (3.0 ± 1.2 vs 1.8 ± 0.9 , respectively; $P < .05$; $n = 8-11$ per group; Fig 3, A). Naive control mice showed no sign of anaphylaxis after challenge. Core body temperatures in O-PAM mice were significantly less than those of O-NM mice (mean, $32.2^\circ\text{C} \pm 3.9^\circ\text{C}$ vs $36.4^\circ\text{C} \pm 1.3^\circ\text{C}$; $P < .01$; $n = 8-11$ per group; Fig 3, B) and normal control mice ($37.9^\circ\text{C} \pm 0.2^\circ\text{C}$, $P < .01$, $n = 8-11$ per group). Consistent with the physiologic changes, plasma histamine levels in O-PAM mice averaged 420% higher than those of O-NM mice ($66,175.2 \pm 20,072.6$ vs $15,724.8 \pm 9,271.1$ nmol/L; $P < .05$; $n = 8-11$ per group; Fig 3, C). In addition, suppression of IgG-mediated anaphylaxis by administration of anti-FcγRIIB/FcγRIII mAb (2.4G2) 24 hours before challenge in O-PAM mice had no effect on challenge-induced anaphylactic reactions or decreased core body temperatures (see Fig E2 in this article's Online Repository at www.jacionline.org).

Splenocytes and MLN cells from O-PAM mice secrete more IL-4

We compared production of the signature T_H2 cytokine IL-4 by splenocytes and MLN cells isolated from peanut-sensitized and challenged O-PAM, O-NM, and naive control mice after *in vitro* peanut protein stimulation. Peanut-stimulated splenocytes from O-PAM mice produced significantly more IL-4 than splenocytes from O-NM mice ($P < .05$; $n = 8-11$ per group; Fig 4, A). IL-10 and IFN-γ production did not differ. IL-4 and

IL-5 levels were also significantly higher in MLN cell cultures from O-PAM mice than in cultures from O-NM mice after peanut stimulation ($P < .05$; $n = 8-11$ per group; Fig 4, B). MLN cells from O-PAM mice also produced significantly less IL-10 and IFN-γ than those from O-NM mice ($P < .05$; $n = 8-11$ per group; Fig 4, B). Cytokine production by peanut-stimulated splenocytes and MLNs from normal control offspring was negligible, and cytokine levels were not detected in cultures with medium alone (data not shown).

Il4 gene promoter DNA methylation was reduced at CpG sites in splenocytes and MLN cells in O-PAM mice

To determine the differences in DNA methylation, we analyzed both global methylation using a global DNA methylation ELISA kit and site-specific methylation using pyrosequencing. In the global DNA methylation ELISA kit the methylated fraction of DNA is recognized by anti-5-methylcytosine antibody and quantified through an ELISA-like reaction. We found no significant differences in global DNA methylation of MLN cells between O-PAM, O-NM, and normal control mice after challenge (see Fig E3, A, in this article's Online Repository at www.jacionline.org). We next determined the DNA methylation status of the *Il4*, *foxp3*, and *Ifng* gene promoters in DNA from splenocytes and MLNs of O-PAM, O-NM, and normal control mice. The percentage of *Il4* gene promoter methylation at CpG⁻⁴⁰⁸ and CpG⁻³⁹³ in MLN cells and splenocytes from O-PAM mice was significantly less than in DNA from O-NM

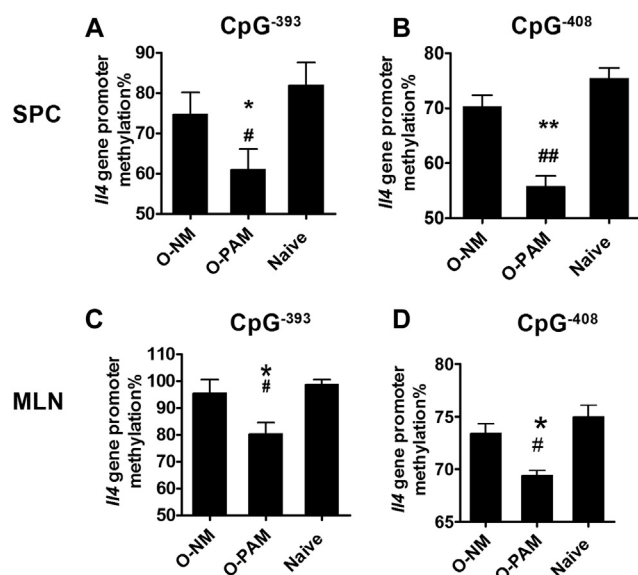


FIG 5. Methylation at the CpG⁻⁴⁰⁸ and CpG⁻³⁹³ sites of the *IL4* gene promoter. Purified DNA from sensitized and challenged offspring splenocytes (SPC) and MLN cells underwent bisulfite treatment, PCR amplification, and pyrosequencing. Percentages of DNA methylation at the CpG⁻⁴⁰⁸ and CpG⁻³⁹³ sites of the *IL4* gene promoters in offspring splenocytes (A and B) and MLN cells (C and D) are shown. Data are expressed as means $\pm$ SDs of each group. * $P < .05$ and ** $P < .01$ versus O-NM and # $P < .05$ and ### $P < .01$ versus normal control mice ($n = 5-8$ per group).

and normal control mice (Fig 5, A and B, for splenocytes and Fig 5, C and D, for MLNs; all $P < .05$ or $P < .01$; $n = 5-8$ per group). Thus splenocyte and MLN DNA from O-PAM mice have a hypomethylated *IL4* gene promoter profile. In addition, PBLs from PAMs also showed significantly reduced methylation at CpG⁻⁴⁰⁸ and CpG⁻³⁹³ sites of the *IL4* gene promoter compared with PBLs from native mothers (see Fig E4 in this article's Online Repository at www.jacionline.org). However, we did not observe a significant difference in methylation levels at the *Ifng* and *foxp3* gene promoters in MLN cells from O-PAM and O-NM mice (see Fig E3, B and C), indicating that additional factors, such as histone modifications,⁴³ might contribute to expression of these proteins.

Offspring spleen and MLN cell DNA methylation levels at CpG⁻⁴⁰⁸ of the *IL4* gene promoter inversely correlate with IL-4 and IgE production

Correlation analysis revealed that the percentage of methylation at CpG⁻⁴⁰⁸ but not CpG⁻³⁹³ of the *IL4* gene promoter in DNA in splenocytes and MLN cells was inversely correlated with *in vitro* IL-4 production and serum peanut-specific IgE levels ($P < .05$; $n = 10-12$; Fig 6).

Splenocytes from unsensitized neonatal O-PAM mice produce more Con A-stimulated IL-4 and are hypomethylated at CpG sites of the *IL4* gene promoter

We quantitated IL-4 secretion from neonatal splenocytes from O-PAM and O-NM mice that had not been immunized with peanut and CT. Splenocytes from both groups did not respond to

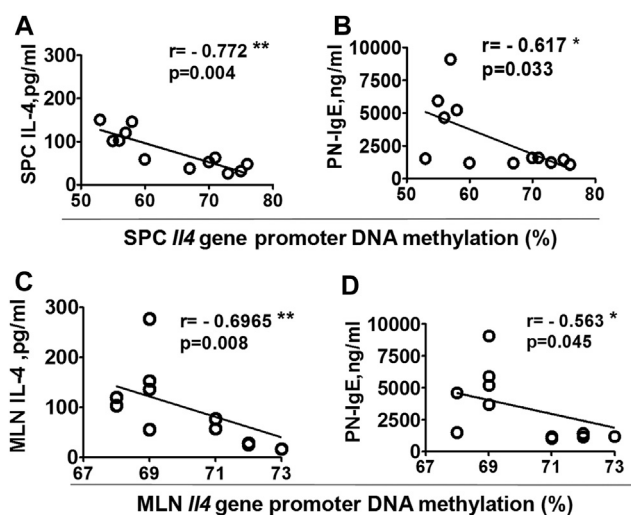


FIG 6. Correlation between DNA methylation at *IL4* gene promoter and IL-4 production in SPC or MLN cells cultures from peanut sensitized offspring (A and C). Correlation between DNA methylation at *IL4* gene promoter and PN specific IgE in serum from sensitized offspring (B and D). Nonparametric (Spearman) correlation was used. * $P < .05$ and ** $P < .01$ ($n = 10-12$). Each dot represents an individual mouse. PN, Peanut; SPC, splenocyte.

CPE stimulation (data not shown). However, Con A-stimulated IL-4 secretion was higher in O-PAM mice than in O-NM mice ($P < .001$; $n = 5-8$ per group; Fig 7, A). In addition, splenocyte DNA from unsensitized neonatal O-PAM mice was less methylated at the CpG⁻⁴⁰⁸ and CpG⁻³⁹³ sites of the *IL4* gene promoter than splenocyte DNA from unsensitized neonatal O-NM mice ($P < .05$; $n = 5-8$ per group; Fig 7, B and C).

DISCUSSION

Although epidemiologic studies show that maternal atopy is a significant risk factor for development of peanut allergy,^{8,11} the present study provides the first experimental evidence that maternal peanut allergy increases offspring susceptibility to IgE-mediated peanut hypersensitivity. We demonstrated that a suboptimal oral sensitization regimen induces only a low level of IgE production and mild anaphylactic reactions in O-NM mice but robust IgE production and severe reactions, including fatal/near-fatal reactions, in O-PAM mice. The increased severity of reactions was associated with markedly increased postchallenge plasma histamine levels. It is well known that IgG and IgE induce anaphylactic reactions in mice.⁴⁴ In the present study we demonstrated that the increased peanut hypersensitivity was not IgG mediated because peanut-specific IgG₁ and IgG_{2a} levels in O-PAM and O-NM mice did not differ and because Fc γ RIII blockade did not attenuate anaphylactic reactions in O-PAM mice. This is consistent with previous observations that anaphylactic responses to ingested antigens in the mouse are nearly always IgE mediated.⁴⁵

Our observations with enteric sensitization of offspring are consistent with the results of a previous study, in which the O-PAM and O-NM mice were sensitized epicutaneously with egg white for 4 weeks.⁴⁶ It was demonstrated that egg white-specific IgE levels were significantly higher in O-PAM mice than in O-NM mice, suggesting that maternal atopy is an important allergen-independent risk factor for offspring IgE hypersensitization.

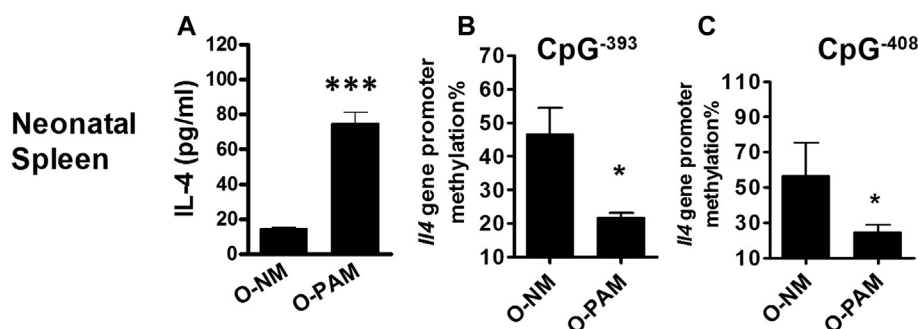


FIG 7. Neonatal offspring splenocyte IL-4 cytokine levels and CpG methylation at the CpG⁻⁴⁰⁸ and CpG⁻³⁹³ sites of the *Il4* gene promoter. Splenocytes from unimmunized neonatal offspring were prepared and cultured for 72 hours in the presence of Con A. IL-4 levels in culture supernatants were determined by using ELISA. DNA methylation of the *Il4* gene promoter from unimmunized neonatal splenocytes was determined as described in Fig 5. **P* < .05 and ****P* < .001 (*n* = 5–8 per group). Data are expressed as means ± SDs of each group.

Our data also indicate that maternal peanut allergy did not increase offspring susceptibility to peanut allergy by maternal milk or amniotic fluid transmission of peanut allergen because our mice were on a peanut-free diet and because there was no detectable peanut in milk (see Fig E5 in this article's Online Repository at www.jacionline.org) or amniotic fluid (data not shown) from PAMs, whereas peanut allergens were detectable in the milk of postpartum mice fed peanuts (see Fig E5). The results of our published peanut–egg white experiments, which established that IgE hypersensitivity in O-PAM mice is not allergen specific, further excludes a role for neonatal passive sensitization through maternal milk. Thus alternative mechanisms must be responsible for the ability of maternal atopy to increase the susceptibility of offspring to antigen sensitization.

T_H2 cytokines play a central role in the pathogenesis of food allergy.⁴⁷ IL-4, a signature cytokine of T_H2-biased immunity, promotes IgE production and the differentiation of naive CD4⁺ T cells into T_H2 cells.⁴⁸ In this study peanut-restimulated MLN cells and splenocytes from O-PAM mice produced significantly more IL-4 than cells from O-NM mice, demonstrating the presence of T_H2-biased immunity in O-PAM mice. In addition, IL-10 and IFN- γ production by MLN cells, but not splenocytes, from O-PAM mice was impaired. This might be because the gut mucosal immune response was more affected than the peripheral immune response in these mice with food allergy. However, a causative role for IL-4 in impaired IL-10 and IFN- γ production in this model requires further investigation. In addition, we found that increased IL-4 levels from splenocytes in response to Con A stimulation were present in O-PAM mice that had not themselves been immunized with peanut plus CT, indicating that some inherited epigenetic changes might promote *Il4* gene transcription and atopy development regardless of the allergen involved.

Epigenetic mechanisms have been suggested to explain why maternal allergy increases the risk of allergy development by offspring.²⁸ However, our data provide the initial experimental evidence that maternal peanut allergy induces T_H2-promoting epigenetic alterations in offspring. Altered DNA methylation status at CpG sites in the gene regulatory region is an important marker of epigenetic regulation of cytokine expression. Reduced DNA methylation at the *Il4* gene promoter increases IL-4 expression by increasing the accessibility of transcription factors to the regulatory region.^{24,25} In an asthma model Liu et al²⁶ investigated

CpG sites (CpG⁻⁴⁰⁸, CpG⁻³⁹³, CpG⁻³³⁹, and CpG⁻³¹⁴ in the *Il4* proximal promoter region and CpG⁺¹⁰¹, CpG⁺¹¹³, CpG⁺¹⁸⁵, CpG⁺²²⁵, and CpG⁺²²⁹ in the first intron) relative to the first start codon of the *Il4* gene. In that study antigen plus diesel exposure induced hypomethylation at CpG⁻⁴⁰⁸ of the *Il4* gene promoter, and the degree of methylation at CpG⁻⁴⁰⁸ correlated inversely with IgE levels. In the present study maternal peanut allergy was associated with hypomethylation at CpG⁻⁴⁰⁸ and CpG⁻³⁹³ of the *Il4* gene promoter in splenocytes and MLNs in O-PAM mice after antigen sensitization and challenge compared with that seen in O-NM and normal control mice. The same pattern of hypomethylation of the *Il4* gene promoter was also present in 1-week-old neonatal mice in the O-PAM group before sensitization. Methylation at CpG⁻⁴⁰⁸, but not CpG⁻³⁹³, correlated inversely with IL-4 and IgE production. These data are the first evidence that maternal peanut allergy induces *Il4* gene epigenetic changes and that this might play a key role in offspring predisposition to peanut allergy. The data also support previous evidence that CpG⁻⁴⁰⁸ is a functionally important CpG residue at the *Il4* gene promoter,²⁶ due at least in part to its location in the proximal promoter region of the *Il4* gene.^{26,42}

In conclusion, we demonstrate that O-PAM mice are more susceptible to development of peanut allergy than offspring of mothers without peanut allergy. Maternal peanut allergy induces epigenetic alteration of the *Il4* gene promoter in their offspring, which correlates with a T_H2-biased response (IL-4 and IgE production). These findings might provide insights into developing a preventive intervention by modifying early-life epigenetic status.

Clinical implications: This study is the first to demonstrate that O-PAM mice are predisposed to peanut allergy, which is associated with epigenetic alteration of the *Il4* gene promoter. This suggests that an early-life intervention that modifies the T_H2-biased epigenetic status might reduce the incidence of allergy in high-risk offspring.

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METHODS

Reagents

CT was purchased from List Biological Laboratories (Campbell, Calif). Con A, collagenase, and avidin-peroxidase were purchased from Sigma-Aldrich (St Louis, Mo). DNase I was purchased from Roche (Branford, Conn). Antibodies for ELISA were purchased from BD Pharmingen (San Diego, Calif) or R&D Systems (Minneapolis, Minn). DNA methylation reagents were purchased from Qiagen.

CPE preparation

Freshly ground whole roasted peanut and CPE were prepared, as previously described.^{E1,E2} Endotoxin levels in ground peanut were tested with the Pyrogen Plus assay kit (Lonza, Basel, Switzerland), as previously described,^{E3} and were less than the detectable level. Briefly, peanuts were ground with a coffee grinder (Krups, Peoria, Ill) followed by a mortar and pestle to make a smooth paste. The paste was defatted by washing with at least 20 volumes (wt/vol) of cold acetone and air-dried overnight at 4°C. Protein was extracted from dried powder by agitating in PBS supplemented with a protease inhibitor cocktail without EDTA (Roche Diagnostics, Indianapolis, Ind) overnight at 4°C. After centrifugation at 2,500g for 20 minutes at 4°C, the supernatant was collected, filtered, and centrifuged at 12,000g for 3 minutes. Protein concentrations were determined with a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Vernon Hills, Ill). The extract was stored at -80°C.

Evaluation of anaphylaxis

Anaphylactic symptoms were evaluated 30 minutes after oral challenge by using the scoring system described previously^{E4}: 0, no symptoms; 1, scratching and rubbing around the snout and head; 2, puffiness around the eyes and snout, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, cyanosis around the mouth and the tail; 4, no activity after prodding or tremor and convulsion; and 5, death. Cage identities were concealed during visual assessment of anaphylactic symptoms.

Determination of global DNA methylation

Genomic DNA from MLN cells was purified as described in "DNA pyrosequencing methylation analysis" section in the print text. Global DNA methylation was determined by using the MethylFlash Methylated DNA Quantification Kit (Colorimetric; Epigentek Group, New York, NY).^{E5} This kit measures the methylcytosine content as a percentage of total cytosine content. Using a DNA concentration of 20 µg/mL, the purified DNA was added to wells in an ELISA plate. The methylated fraction of DNA was quantified by using 5-methylcytosine-specific antibodies. The amount of methylated DNA was proportional to the OD intensity in an ELISA plate reader at 450 nm. DNA methylation was calculated by using the following formula:

$$[(OD_{\text{sample}} - OD_{M3})/S] / [((OD_{M4} - OD_{M3}) \times 2)/P] \times 100\%,$$

where *OD* is optical density; *M3* is the negative control, an unmethylated polynucleotide containing 50% of cytosine; *S* is the amount of input sample DNA in nanograms; *M4* is the positive control, a methylated polynucleotide containing 50% of 5-methylcytosine; and *P* is the amount of input positive control in nanograms. The amount of methylated DNA was expressed as a percentage of total DNA.

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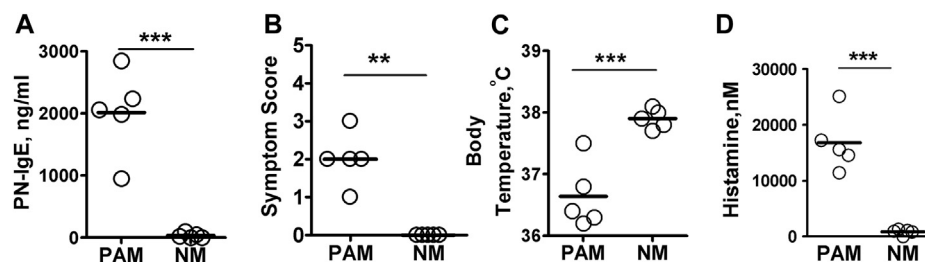


FIG E1. Mothers with peanut allergy exhibited hypersensitivity reactions after oral peanut challenge and before mating. Blood was collected from PAMs and NMs 1 day before challenge. **A**, Serum peanut-specific IgE levels were measured by using ELISA. **B-D**, Anaphylaxis scores (Fig E1, *B*) and core body temperatures (Fig E1, *C*) were measured and plasma histamine levels (Fig E1, *D*) were determined 30 minutes after challenge. ** $P < .01$ and *** $P < .001$ versus NMs ($n = 5$ per group). Each dot represents an individual mouse. Horizontal bars indicate means. Data shown are representative of 2 individual experiments.

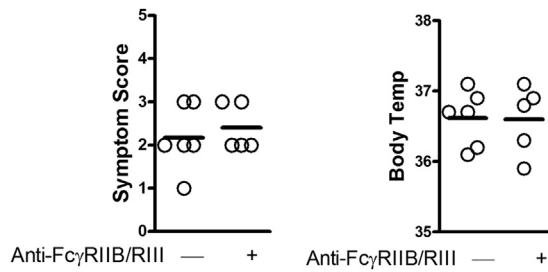


FIG E2. Anaphylaxis scores and core body temperatures with or without anti-FcγRIIB/RIII mAb administration in peanut-sensitized O-PAM mice are shown after oral challenge (n = 5-6). *Horizontal lines* represent the means of each group.

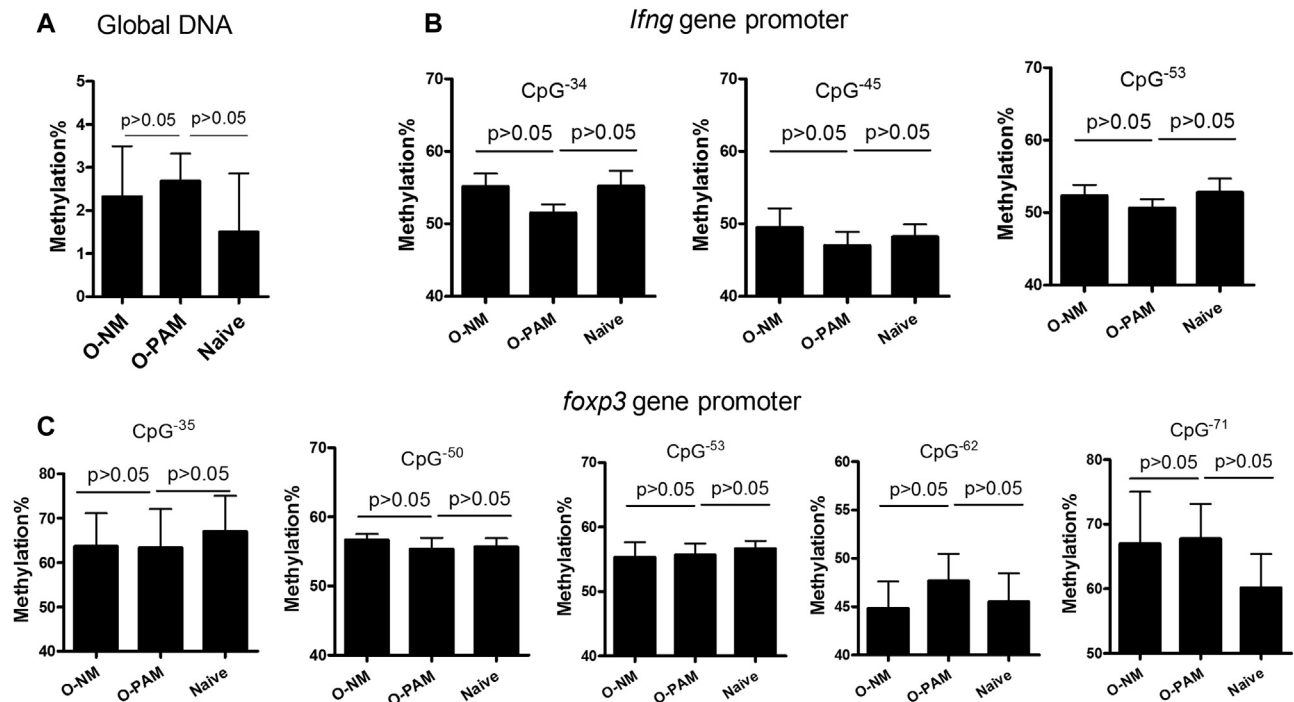


FIG E3. **A**, Global methylation of MLN cells in O-PAM, O-NM, and normal control mice after challenge was measured by using the global DNA methylation ELISA kit (Epigentek). In this ELISA kit the methylated fraction of DNA is recognized by 5-methylcytosine antibody and quantified through an ELISA-like reaction ($n = 6-7$ per group). Both $P > .05$ for O-PAM versus O-NM and O-PAM versus naive mice. **B**, Purified DNA from sensitized and challenged offspring MLN cells underwent bisulfite treatment, PCR amplification, and pyrosequencing. **B** and **C**, Percentage of DNA methylation of the *Ifng* and *foxp3* gene promoters in offspring MLN cells. Data are expressed as means $\pm$ SDs of each group ($n = 5-6$ per group).

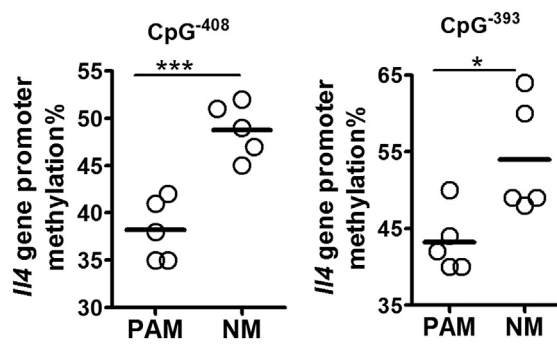


FIG E4. DNA methylation at CpG⁻⁴⁰⁸ and CpG⁻³⁹³ sites of the *IL4* gene promoter from PBLs of PAMs and NMs before breeding. Purified DNA from mothers' PBLs before breeding underwent bisulfite treatment, PCR amplification, and pyrosequencing. * $P < .05$ and *** $P < .001$ versus NMs ($n = 5$).

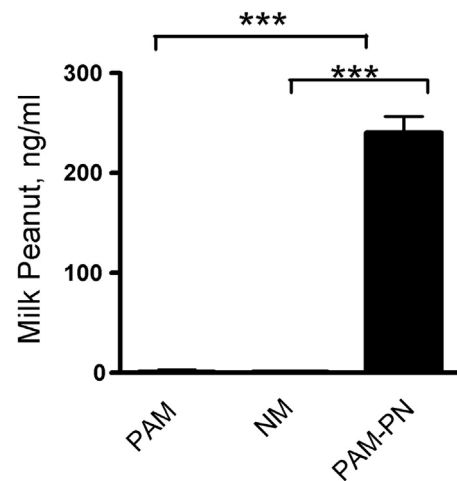


FIG E5. Breast milk was collected from lactating PAMs and NMs when their offspring were 10 to 15 days old by using a mouse milking machine modified in our laboratory. An additional group of lactating PAM mice was inoculated intragastrically with 10 mg of peanut protein (*PAM-PN*). Milk was collected 2 hours after peanut protein feeding. Milk was diluted 1:2 in PBS, and peanut protein levels were detected with a commercial kit (Neogen Corp; $n = 3-4$). *** $P < .001$.

TABLE E1. Primers used for PCR amplification and pyrosequencing experiments

Mouse <i>foxp3</i>	
PCR forward	TATATTTT TAGATGATT TGTAAGGGTAAA
PCR reverse	Biotin-TCACCTTAATAAAATAAACTACTA
Pyrosequencing	AAAAAATTGGATTATTAGAA
Mouse <i>Ifng</i>	
PCR forward	TGGTGTGAAGTAAAAGTGTTTTTAGA
PCR reverse	Biotin-TACACCTCTCTAACTTCCAATT
Pyrosequencing 1	AAAAAAAAATTTGTGAAAATA
Pyrosequencing 2	GAATGGTATAGGTGGGTA