Maternal allergy increases susceptibility to offspring allergy in association with Th2 biased epigenetic alterations in a mouse model of peanut allergy

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Abstract

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

Objective—To determine if offspring of peanut allergic mothers (O-PAM) are more susceptible to peanut allergy than offspring of naive mothers (O-NM) in a murine model; and if so whether the susceptibility is linked to Th2 biased epigenetic alterations.

Methods—Five-week-old O-PAM and O-NM were intragastrically (i.g.) sensitized to and challenged with peanut. Serum peanut-specific IgE, plasma histamine, anaphylactic reactions, and

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splenocyte (SPC) and mesenteric lymph node (MLN) cell cytokine production were measured. DNA methylation levels of the IL-4 gene promoter from sensitized offspring SPC and MLN cells and un-sensitized neonatal offspring SPCs were determined by pyrosequencing.

Results—O-PAM exhibited 3-fold higher peanut-specific IgE levels following peanut sensitization as well as 5-fold higher histamine levels and significantly higher anaphylactic symptom scores following challenge than O-NM ($p<0.05$-0.01). Cultured O-PAM SPCs and MLN produced significantly more Th2 cytokines than O-NM cells ($p<0.05$-0.01). O-PAM cells exhibited significantly reduced DNA methylation at CpG sites of the IL-4 gene promoter than O-NM cells. DNA methylation levels were inversely correlated with IL-4 and IgE production. O-PAM neonatal splenocyte hypomethylation of the IL-4 gene promoter was also present.

Conclusion—This study is the first to demonstrate that increased susceptibility to peanut allergy in offspring of peanut allergic mothers is associated with epigenetic alternation of the IL-4 gene promoter. This finding may provide an insight into preventing the development of early life allergy.

Clinical Implications—This study is the first to demonstrate that offspring of peanut allergic mothers are predisposed to develop peanut allergy, which is associated with epigenetic alternation of the IL-4 gene promoter. This suggests that an early life intervention that modifies the Th2 biased epigenetic status may reduce the incidence of allergy in high-risk offspring.

Keywords

Peanut allergy; anaphylaxis mice; offspring; IgE; IL-4; DNA methylation

Introduction

Food allergy, a growing public health concern in the U.S. 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 life-long persistence.[4-7] In 1996, Hourihane et al reported that prevalence of peanut and other allergies in the families of people with peanut allergy was increased in successive generations in maternal but not paternal relatives.[8] Additional clinical observational studies also show that maternal peanut allergy and other allergies increase the risk of a child to develop 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 Th2-mediated immune disorder characterized by increased expression of IL-4, IL-5, and IL-13 in humans [13] and animal models.[14-16] IL-4 is a key Th2 cytokine required for B cells switching to IgE production, mast cell activation and Th2 cell differentiation.[17] Higher IL-4/IFN-γ ratios were found in persistent peanut allergic patients 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 is 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 IL-4 promoter showed that the level of DNA CpG^{-408} demethylation was inversely 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 offspring propensity for peanut specific-IgE production, hypersensitivity reactions, Th2 cytokine production, and Th2 cytokine gene promoter hypomethylation. To test this possibility, we used a mouse model to compare IgE levels and anaphylactic reactions following suboptimal active peanut sensitization and challenge in offspring of peanut allergic mothers (O-PAM) to offspring of naïve mothers (O-NM). We also determined cytokine production and methylation status of the Th2 signature cytokine IL-4 gene promoter at CpG sites in DNA from offspring mesenteric lymph node cells (MLN), and splenocytes (SPCs).

Methods

Mice and reagents—Six-week (wk) 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) was prepared as described previously (Online Repository Method).[30-32] Sources of reagents used in this study are provided in the Online Repository Methods and Reagents.

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 PN) and cholera toxin (20 μg CT) intragastrically (i.g.) weekly for 5 weeks (wks) and challenged at wk 6 (200 mg PN/mouse). Our sensitization protocol differed from our previous standard protocol in that mice did not receive an additional boost (50 mg PN+10 mg CT). Mice treated with the protocol used in our current study are termed peanut allergic mothers (PAM). As controls, naïve females were PBS (Phosphate Buffered Saline) sham sensitized and challenged; these mice are termed naïve mothers (NM). One-week after peanut challenge, mice in both groups were bred with naive males. All mice were fed peanut-free chow during gestation and lactation.

Determination of peanut protein in milk: During lactation, milk was collected from PAM or NM when their offspring were 10-15 days old with a mouse milking machine modified in our laboratory as described.[33] To establish a positive control, an additional group of PAM

lactating mice was administered 10mg of peanut protein intragastrically. Milk was collected 2 hours later. The levels of peanut protein in milk from these three groups were detected using a Veratox® Peanut commercial kit (Neogen Corp, Lansing, Mich) following the manufacturer's instruction and as previously described.[34]

Offspring protocol—Offspring of peanut allergic mothers (O-PAM) and offspring of naive mothers (O-NM), weaned at 4 wks of age, were sensitized one week later by 3 weekly i.g. suboptimal doses of peanut (5mg) and CT (10μg). A third group of age-matched peanut-naive offspring of peanut-free mothers (Naive) served as normal controls (Figure 1). All offspring were orally challenged at week 4 with 200 mg of ground peanut.

Measurement of peanut-specific antibodies—To monitor antibody responses to peanut sensitization, sera were obtained from blood collected weekly during sensitization and one day prior to challenge by submandibular venipuncture. To measure serum peanut-specific IgE, serum IgG antibodies were first depleted by protein G–Sepharose (BioVisio, Milpitas, CA) centrifugation.[35;36] In brief, equal volumes of protein G–Sepharose and undiluted mouse serum were mixed and incubated at room temperature for 10 minutes, 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 using previously described ELISA methods.[37;38] Serum peanut-specific IgG1 and IgG2a were determined as previously described.[37;38]

Assessment of hypersensitivity reactions—Anaphylactic symptoms were evaluated 30 minutes following oral challenge utilizing the scoring system described previously (Online Repository Methods).[15] In this scoring system, 0 is no reaction, 1 is mild, 2 is moderate, 3 is severe, 4 very severe, 5 is death.[39] Core body temperatures were measured using a rectal probe (Harvard Apparatus, NJ). To confirm that anaphylactic reactions were not IgG mediated, peanut sensitized mice were pretreated with anti-FcγRIIB/FcγRIII mAb (mAb 2.4G2, 500μg/mouse) or isotype control antibody 24 hours prior to challenge as previously described.[40;41]

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

Cell culture and cytokine measurements—Offspring SPCs 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 μg/ml) or Con A (2.5 μg/mi). 72 hrs later, supernatants were collected and cytokine levels were determined by ELISA. SPCs and MLN cells were then collected and preserved in Buffer RTL (Qiagen, MD) for DNA methylation assays.

DNA pyrosequencing methylation analysis—Genomic DNA from offspring MLN cells and SPCs was isolated using an AllPrep mini DNA kit (Qiagen, MD). DNA from the spleens of approximately 1 week old offspring mice (unsensitized) and DNA from maternal peripheral blood leucocytes (PBL) post challenge were extracted using DNeasy Tissue/ Blood kit (Qiagen, MD) as previously described.[42] Isolated DNA was then bisulfite converted using an Epitect plus DNA Bisulfite kit (Qiagen, MD) as per manufacturer's

instructions. Bisulfite converted DNA was used to amplify promoter regions of the IL-4 gene by PCR, using the primers in Table I. PCR products were subjected to pyrosequencing using the Pyromark Q24 Pyrosequencing system (Qiagen, MD) using the sequencing primers for CpG^{-408} and CpG^{-393} in Table I. Bisulfite converted DNA was also used to amplify promoter regions of the IFN-γ and Foxp3 genes by PCR and PCR products were then subjected to pyrosequencing using sequencing primers. Primers, which are listed in Table EI, were chosen based on previous studies.[26;42] Methylation levels of PCR products at each CpG site were determined using PyroMark 24 run software (Qiagen,MD)

Statistical analysis—Data were analyzed using the SigmaStat statistical software package (Systat, Chicago, IL). Differences between two groups were analyzed by t test if the data were approximately normal or by Mann-Whitney Rank Sum Test if the data were skewed. An ANOVA followed by a Bonferroni t test was performed for all pair-wise comparisons if the data were approximately normal. Differences among more than two groups were analyzed by ANOVA on ranks followed by all pair-wise comparisons (Dunn's method) if the data were not normally distributed. Graphpad Prism 4 was used for non-parametric (Spearman) correlation analysis. P values less than 0.05, based on 2-tailed tests, were considered statistically significant.

Results

Offspring of peanut allergic mothers produce increased peanut-specific IgE following oral peanut sensitization

PAM mothers had increased peanut specific IgE levels following i.g. sensitization and exhibited hypersensitivity reactions following i.g. challenge before breeding (Figure E1 in the Online Repository).

O-PAM and O-NM were sensitized using a suboptimal protocol consisting of 3 weekly 5 mg PN+10 µg CT administrations (half the dose and less than half the duration of our standard sensitization protocol).[15] Based on our preliminary results, sub-optimal 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<0.01, N=8-11/group, Fig 2A). Sera from normal control mice contained no detectable peanut-specific IgE. Peanut-specific IgG1 and IgG2a 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 (N=8-11/group, Fig 2B&C). Thus, O-PAM produce more IgE than O-NM in response to the same sensitization protocol.

Offspring of peanut allergic mothers develop more severe anaphylaxis following oral peanut challenge

Only 2 of 11 (18%) peanut challenged O-NM mice developed severe reactions (score 3) and none exhibited very severe or fatal reactions. In contrast, 6 of 8 (75%) O-PAM exhibited severe reactions, including one fatal and one near-fatal reaction. Mean anaphylactic symptom scores of O-PAM were significantly higher than O-NM (O-PAM 3.0±1.2 vs. O-

NM 1.8±0.9, p<0.05, N=8-11/group, Fig 3A). Naive control mice showed no sign of anaphylaxis following challenge. O-PAM core body temperatures were significantly below those of O-NM (mean: 32.2 ±3.9° C vs. 36.4 ±1.3° C; p< 0.01; N=8-11/group, Fig 3B) and normal control mice (37.9±0.2 °C, p<0.01, N=8-11/group). Consistent with the physiological changes, O-PAM plasma histamine levels averaged 420% higher than those of O-NM (66175.2±20072.6 vs. 15724.8±9271.1 nM, *p*< 0.05, N=8-11/group, Fig 3C). In addition, suppression of IgG-mediated anaphylaxis by administration of anti-FcgRIIB/ FcgRIII monoclonal antibody (2.4G2) 24 hours prior to challenge in O-PAM had no effect on challenge-induced anaphylactic reactions or decreased core body temperatures (Figure E2 in the Online Repository).

Splenocytes and mesenteric lymph node cells from offspring of peanut allergic mothers secrete more IL-4

We compared production of the signature Th2 cytokine, IL-4, by SPCs and MLN cells isolated from peanut-sensitized and challenged O-PAM, O-NM and naïve control mice following in vitro peanut protein stimulation. Peanut stimulated SPCs from O-PAM produced significantly more IL-4 than O-NM SPCs (p<0.05, N=8-11/group, Fig 4A). IL-10 and IFN-γ production did not differ. IL-4 and IL-5 levels were also significantly higher in O-PAM MLN cell cultures than in O-NM cultures following peanut stimulation (p<0.05, N=8-11/group, Fig 4B). O-PAM MLN cells also produced significantly less IL-10 and IFN-γ than O-NM MLN cells (*p*<0.05, N=8-11/group, Fig 4B). Cytokine production by peanut-stimulated SPCs and MLNs from normal control offspring was negligible, and cytokines were not detected in medium alone cultures (data not shown).

IL-4 gene promoter DNA methylation was reduced at CpG sites in spleen and mesenteric lymph node cells in offspring of peanut allergic mothers

To determine the differences in DNA methylation, we analyzed both global methylation using a global DNA methylation ELISA kit and site-specific methylation by 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 controls following challenge (Figure E3A in the Online Repository). We next determined the DNA methylation status of the IL-4, Foxp3 and IFN-γ genes promoters in DNA from SPCs and MLNs of O-PAM, O-NM and normal controls. The percent of IL-4 promoter methylation at CpG^{-408} and CpG^{-393} in MLN cells and SPCs from O-PAM was significantly less than in DNA from O-NM and normal controls (Fig 5A and B for SPCs, C and D for MLN, all *p* < 0.05 or *p*<0.01, N=5-8/group). Thus, O-PAM SPC and MLN DNA have a hypomethylated IL-4 promoter profile. In addition, peripheral blood leukocytes (PBL) from PNA mothers also showed significantly reduced methylation at CpG^{-408} and CpG^{-393} sites of the IL-4 promoter as compared to PBL from native mothers (Figure E4 in the Online Repository). However, we did not observe a significant difference in methylation levels at the IFN- γ and Foxp3 gene promoters in MLN cells from O-PAM and O-NM (Figure E3B&C in the Online Repository), indicating that additional factors, such as histone modifications,[43] may contribute to expression of these proteins.

Offspring spleen and mesenteric lymph node cell DNA methylation levels at CpG^{-408} of the IL-4 gene promoter inversely correlate with IL-4 and IgE production

Correlation analysis revealed that the percent of methylation at CpG $^{-408}$ but not CpG $^{-393}$ of the IL-4 promoter in DNA in SPCs and MLN cells was inversely correlated with in vitro IL-4 production and serum peanut specific IgE levels (p<0.05, N=10-12, Fig 6 A-D).

Splenocytes from un-sensitized neonatal offspring of peanut allergic mothers produce more ConA stimulated IL-4 and are hypomethylated at CpG sites of the IL-4 gene promoter

We quantitated IL-4 secretion by from neonatal splenocytes from O-PAM and O-NM that had not been immunized with PN and CT. SPCs from both groups did not respond to CPE stimulation (data not shown). However, Con A stimulated IL-4 secretion was higher in O-PAM than in O-NM (Fig 7A, *p*<0.001, N=5-8/group). In addition, splenocyte DNA from unsensitized neonatal O-PAM was less methylated at the CpG^{-408} and CpG^{-393} sites of the IL-4 promoter than splenocyte DNA from unsensitized neonatal O-NM (Fig 7 B&C, *p*<0.05, N=5-8/group).

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, but robust IgE production and severe reactions, including fatal/near fatal reactions in O-PAM. The increased severity of reactions was associated with markedly elevated post-challenge plasma histamine levels. It is well known that IgG as well as IgE induces 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 IgG2a levels in O-PAM and O-NM did not differ, and because FcγRIII blockade did not attenuate O-PAM anaphylactic reactions. 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 offspring of peanut allergic mothers and naïve mothers 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 than O-NM, suggesting that maternal atopy is an important, allergen-independent risk factor for offspring IgE hyper-sensitization.

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 (Figure E5 in the Online Repository) or amniotic fluid (data not shown) from PAM, while peanut allergens were detectable in the milk of post-partum mice that were fed peanuts (Figure E5 in the Online Repository). The results of our published peanut-egg white

experiments, which established that IgE hypersensitivity in O-PAM is not allergen-specific, further excludes a role for neonatal passive sensitization though maternal milk. Thus, alternative mechanisms must be responsible for the ability of maternal atopy to increase susceptibility of offspring to antigen sensitization.

Th2 cytokines play a central role in the pathogenesis of food allergy.(47) IL-4, a signature cytokine of Th2 biased immunity, promotes IgE production and the differentiation of naïve CD4$^+$ T cells into Th2 cells.(48) In this study, PN-restimulated O-PAM MLN cells and SPCs produced significantly more IL-4 than O-NM cells, demonstrating the presence of Th2 biased immunity in O-PAM. In addition, IL-10 and IFN-γ production by MLN cells, but not SPCs from O-PAM was impaired. This might be because the gut mucosal immune response was more affected than the peripheral immune response in these food allergic mice. 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 SPCs in response to ConA stimulation were present in O-PAM that had not themselves been immunized with PN + CT, indicating that some inherited epigenetic changes may promote IL-4 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 Th2-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 IL-4 promoter increases IL-4 expression by increasing the accessibility of transcription factors to the regulatory region.(24;25) In an asthma model, Liu et al(26) investigated CpG sites (CpG-408, CpG-393, CpG-339, and CpG-314 in the IL-4 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 IL-4 gene. In that study, antigen plus diesel exposure induced hypomethylation at CpG-408 of the IL-4 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 IL-4 promoter in SPCs and MLNs in O-PAM following antigen sensitization and challenge, as compared to O-NM and normal controls. The same pattern of hypomethylation of the IL-4 promoter was also present in one-week-old neonatal mice in the O-PAM group prior to sensitization. Methylation at CpG residue (-408), but not CpG-393, correlated inversely with IL-4 and IgE production. These data are the first evidence that maternal peanut allergy induces IL-4 gene epigenetic changes, and that this may play a key role in offspring predisposition to develop peanut allergy. The data also support previous evidence that CpG^{-408} is a functionally important CpG residue at the IL-4 promoter,(26) due at least partially to its location in the proximal promoter region of the IL-4 gene.(26;42)

In conclusion, we demonstrate that offspring of peanut allergic mothers are more susceptible to development of peanut allergy than offspring of non-peanut allergic mothers. Maternal peanut allergy induces epigenetic alternation of the IL-4 promoter in their offspring, which correlates with a Th2 biased responses (IL-4 and IgE production). These findings may

provide insights into developing a preventive intervention by modifying early life epigenetic status.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Con A	concanavalin A
CT	cholera toxin
FBS	fetal bovine serum
i.g	intragastric gavage, or intragastrically
MLNs	mesenteric lymph nodes
SPCs	splenocytes
PAM	peanut allergic mothers
NM	naive mothers
O-PAM	offspring of peanut allergic mothers
O-NM	offspring of naive mother

PA	peanut allergy
PBS	Phosphate buffered saline
Th	T helper cells
wk (s)	week (s)

	Mothers		Offspring	
Groups	Sensitization i.g.	Challenge i.g.	Sensitization i.g.	Challenge i.g.
O-NM	PBS	PBS	5mgPN+CT	PN
O-PAM	10mgPN+CT	PN	5mgPN+CT	PN
Naïve	None	None	None	PN
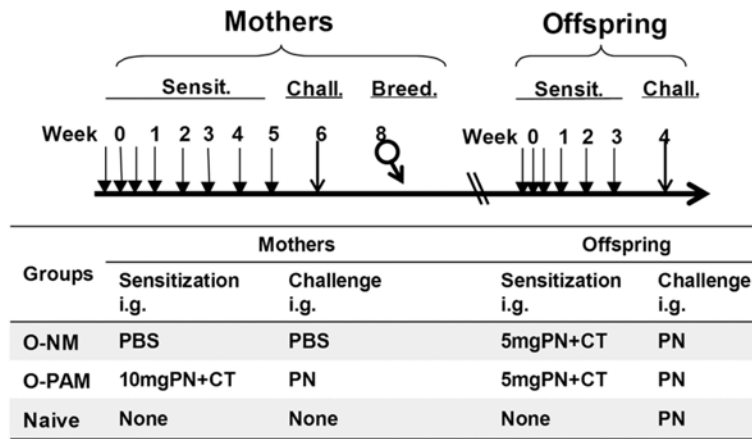
Figure 1. Experimental protocols

Peanut-sensitized female mice were orally challenged with peanut, and then mated with naïve males one week later. Five-wk-old offspring of peanut allergic mothers (O-PAM) and naïve mothers (O-NM) were sensitized weekly for 3 wks and challenged at week 4. Offspring of PBS challenged naïve mothers served as normal controls. Sensit: Sensitization; Chall: Challenge; Breed: Breeding.
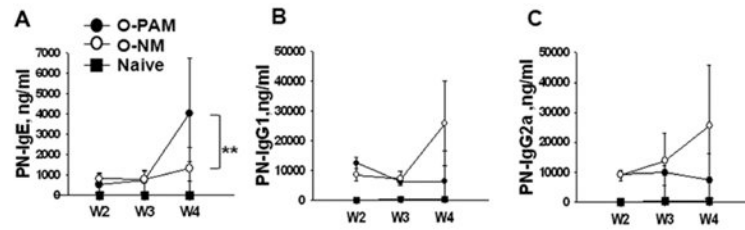
Figure 2. Offspring of peanut allergic mothers have an increased IgE response to peanut sensitization

Blood was collected from offspring following peanut sensitization at indicated time-points, and serum peanut-specific IgE levels (A), peanut-specific IgG1 levels (B) and peanut-specific IgG2a levels (C) were determined by ELISA. **p<0.01, vs. O-NM (N=8-11/group). W: week in sensitization protocol.
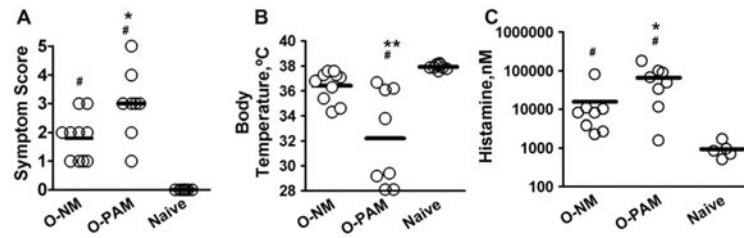
Figure 3. Offspring of peanut allergic mothers develop anaphylaxis following peanut oral challenge

Thirty minutes following challenge, O-NM and O-PAM anaphylactic symptoms were scored (A), core body temperatures were measured (B), and plasma histamine levels were determined (C). Histamine levels are presented in log scale. *$p<0.05$,**$p<0.01$, vs. O-NM; # $p< 0.05$, vs. normal control (N=8-11/group). Each dot represents an individual mouse. Horizontal bar indicates the mean.
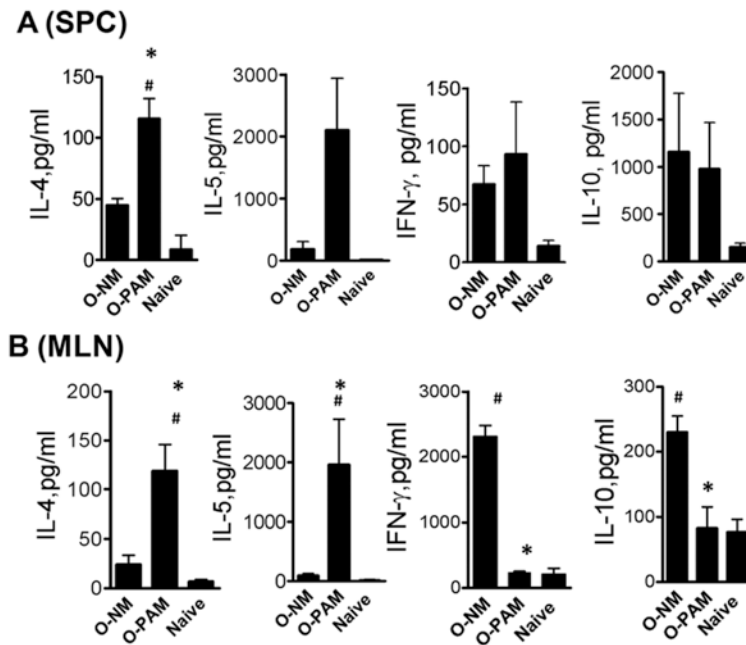
Figure 4. Cytokine secretion by offspring SPC and MLN cultures
SPC and MLN cells were prepared and cultured for 72 hours in the presence or absence of crude peanut extract. SPC (A), and MLN (B). Culture supernatant IL-4, IFN-g and IL-10 levels were determined. Data are expressed as means ± SDs of each group. *, $p < 0.05$ vs. O-NM; #, $p < 0.05$ vs. normal control (N=8-11/group).
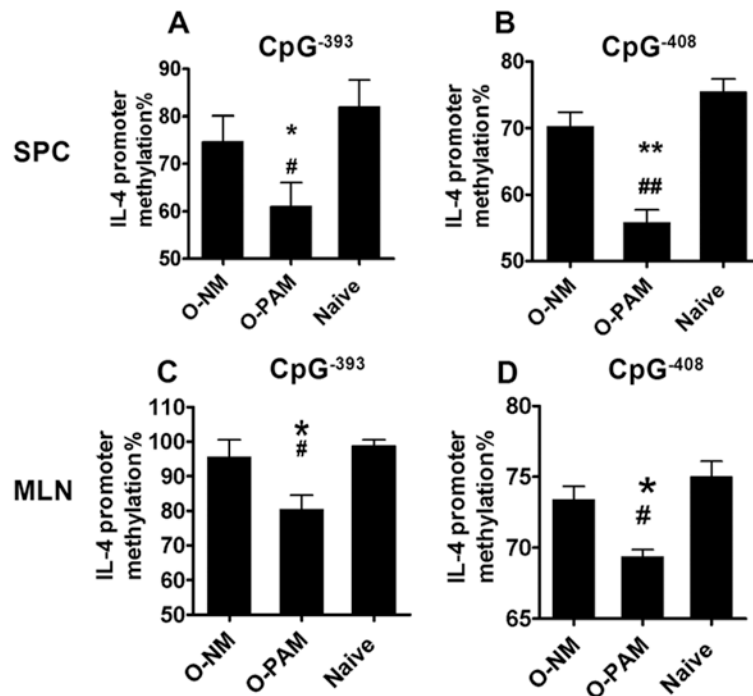
Figure 5. Methylation at the CpG^{-408} and CpG^{-393} sites of the IL-4 promoter
Purified DNA from sensitized and challenged offspring SPCs and MLN cells underwent bisulfite treatment, PCR amplification, and pyrosequencing. Percent of DNA methylation at CpG^{-408} and CpG^{-393} sites of the IL-4 promoters in offspring SPCs (A&B) and MLN cells (C&D). Data are expressed as means ± SDs of each group.*p < 0.05; **p < 0.01 vs.O-NM; [#] p < 0.05; [##] p < 0.01 vs. normal control (N=5-8/group).
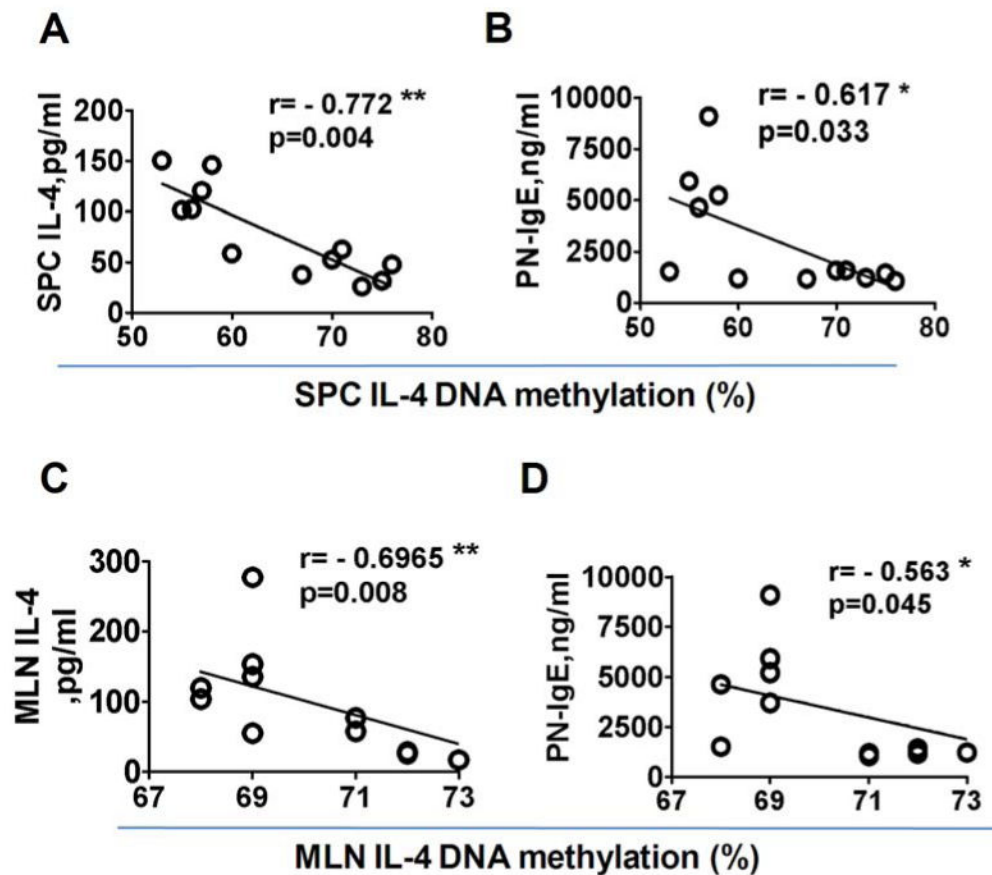
Figure 6. Correlation between percent DNA methylation of the IL-4 promoter, IL-4 production and peanut-sensitized offspring serum IgE levels (O-PAM+O-NM)

Percent of offspring SPC and MLN DNA methylation at the CpG^{-408} site of the IL-4 promoter was plotted versus SPC and MLN IL-4 and serum IgE (A-D). Non-parametric (Spearman) correlation was used. *p< 0.05; **, p<0.01(N=10-12). Each dot represents an individual mouse.

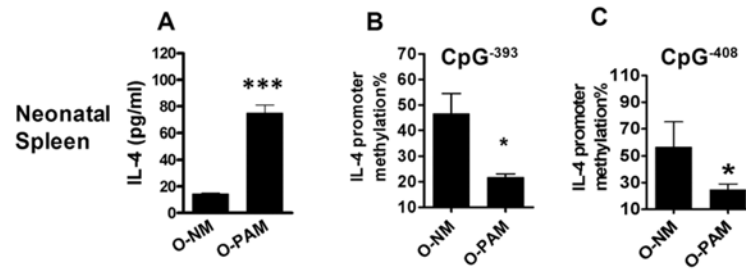**Figure 7. Neonatal offspring SPC IL-4 cytokine levels and CpG methylation at CpG^{-408} and CpG^{-393} sites of the IL-4 promoter**

SPCs from unimmunized neonatal offspring were prepared and cultured for 72 hours in the presence of ConA. IL-4 levels in culture supernatants were determined by ELISA. DNA methylation of the IL-4 promoter from unimmunized neonatal SPCs was determined as described in Figure 5. *, $p<0.05$; ***, $p<0.001$(N=5-8/group). Data are expressed as means ± SDs of each group.

Table I

Primers used for PCR amplification and pyrosequencing experiments

IL-4	
PCR forward	GTTTTTAAGGGGTTTTTATAGTAGGAAGT
PCR reverse	Biotin-AATTACCACTAACTCTCCTCCTACA
Pyrosequencing	AGATTTTTTTGATATTATTTTGTTT