Prenatal exposure to allergen, DNA methylation, and allergy in grandoffspring mice

M. Niedzwiecki[1], **H. Zhu**[2], **L. Corson**[2], **G. Grunig**[3], **P. H. Factor**[4], **S. Chu**[2], **H. Jiang**[2], and **R. L. Miller**[1,2,5]

[1]Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, NY

[2]Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY

[3]Department of Environmental Health Sciences, New York University, Tuxedo, NY

[4]Division of Pulmonary, Critical Care and Sleep Medicine, Department of Medicine, Beth Israel Medical Center and Albert Einstein College of Medicine, New York, NY

[5]Department of Pediatrics, Columbia University College of Physicians and Surgeons, New York, NY, USA

Abstract

Background—Prenatal allergen exposure has been linked to both induction and protection of allergic sensitization in offspring. We hypothesized that prenatal exposure of mice (F0) to *Aspergillus fumigatus* (*A. fumigatus*) would be associated with decreased immunoglobulin (Ig) E and airway eosinophilia and alterations in CpG methylation of T-helper genes in third-generation mice (F2).

Methods—Female BALB/c mice were sensitized to *A. fumigatus* (62.5, 125, 1250 µg, or saline) and re-exposed to the same dose on days 7 and 14 (early) or days 12 and 17 (late) gestation. Grandoffspring were treated with *A. fumigatus* (62.5 µg) at 9 weeks. IgE, IgG_1, and IgG_{2a} levels and cell counts from bronchoalveolar lavage fluid were determined. Lung DNA was pyrosequenced at multiple sites in the interferon (IFN)-γ and interleukin (IL)-4 promoters.

Results—Grandoffspring of mothers dosed with 1250 µg early during pregnancy developed increased airway eosinophilia ($P < 0.05$). Grandoffspring of mothers dosed late in pregnancy developed lower IgE ($P < 0.05$) and airway eosinophilia ($P < 0.05$). Grandoffspring of mothers dosed early had lower methylation at IL-4 CpG^{-408} and CpG^{-393} compared to late dosed mice ($P < 0.005$ across all doses). Few correlations were found between methylation levels and airway eosinophilia and IgE.

Conclusion—Prenatal exposure to *A. fumigatus* late during pregnancy, but not early, was associated with lower IgE and airway eosinophilia in grandoffspring. Prenatal exposure to *A. fumigatus* was associated with changes in CpG methylation in the IFN-γ and IL-4 promoters that did not correlate consistently with indicators of allergic sensitization.

Correspondence Rachel L. Miller, MD, PH8E-101, Columbia University College of Physicians and Surgeons, 630 W, 168th St, New York, NY 10032, USA. Tel.: 212-305-7759, Fax: 212-305-2277, rlm14@columbia.edu.

Keywords

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Asthma in parents is associated with increased risk of asthma in children, with maternal asthma more strongly associated with risk compared to paternal (1). This pattern suggests that maternal exposures, particularly during gestation, may alter developmental programming, leading to molecular, physiologic, and metabolic adaptations in the offspring (1, 2). For example, prenatal allergen exposure may 'prime' T-helper (Th) cells toward a more proallergic Th2 phenotype (3). Previous studies of prenatal exposures on the development of allergy have been mixed, with exposures linked to both increased (4) and decreased (5) allergy in the offspring, likely due to differing effects of dose, timing of exposure during gestation, and other methodological variations.

An emerging paradigm is that epigenetic regulation of genes important to Th gene transcription and cytokine production may be responsible for the development of allergy (6). Cell systems have shown that site-specific CpG methylation of Th gene promoters can steer differentiation toward the Th2 phenotype (7, 8). In particular, the CpG^{-53} of the interferon (IFN)-γ promoter was shown to undergo rapid methylation during Th2 polarization that blocked ATF2-c/Jun and CREB transcription factor binding (7). More limited *in vivo* studies have been conducted. In one study, our group showed that combined exposure to diesel exhaust particles and allergen in mice altered methylation patterns at CpG sites in the IFN-γ and IL-4 promoters that correlated with changes in immunoglobulin (Ig) E production (9).

Epigenetic regulation also may explain partially the heritability of asthma (2). A gestational diet high in folic acid, a methyl donor for methylation reactions, induced allergic airway inflammation in offspring mice (F1) in association with hypermethylation of RUNX3, a T-cell regulator (10). Differences in levels of global DNA methylation among dendritic cells, and in their antigen-presenting activity, derived from offspring mice also were associated with the presence or absence of an asthma-like phenotype in the mother (11). Epidemiologic studies additionally have demonstrated associations between prenatal exposures, including tobacco smoke and polycyclic aromatic hydrocarbons (PAH), with differential methylation patterns in several asthma candidate genes and symptoms in children (12, 13). Also, prenatal smoking, determined using retrospective questionnaire, was associated with greater asthma risk in the grandchildren (14). Others have suggested that the timing of exposure during gestation may be important, with folic acid supplementation either during the first trimester or during late pregnancy being associated with asthma-related symptoms in young children in separate studies (15, 16).

We hypothesized that allergen exposure during gestation may alter the allergic phenotype through multiple generations of mice and that such changes would be associated with DNA methylation of Th genes. We also hypothesized that these associations may vary depending on the timing of prenatal exposure. Given our previous report that prenatal *Aspergillus fumigatus* (*A. fumigatus*) administration combined with diesel exhaust particles was associated with reduced IgE in sensitized offspring (F1) (17), here we exposed female mice (F0) to multiple doses of *A. fumigatus* during the early or late period of gestation and determined the effects on IgE, airway eosinophilia, and Th gene IFN-γ and IL-4 promoter methylation in the lungs of their grandoffspring (F2).

Methods

Aspergillus fumigatus sensitization

BALB/c female mice were exposed to *A. fumigatus* (62.5 µg, 125 µg, or 1.25 mg) intranasally in 50 µl of saline, or saline alone, five times 4 days apart, beginning 20 days prior to mating. Pregnant mice were retreated with same dose of *A. fumigatus* or saline on days 7 and 14 (early gestation) or days 12 and 17 (late gestation) after mating (Fig. 1). At 10– 12 weeks of age, offspring (F1) were mated with littermates. Their offspring (F2, grandoffspring) at 9 weeks of age were treated with six doses of *A. fumigatus* (62.5 µg in 50 µl), each dose 4 days apart, and IgE, IgG_1, and IgG_2 levels were measured.

Bronchoalveolar lavage (BAL), airway cellular analysis, and airway hyper-reactivity

Mice were killed at median age of 12.5 weeks, and BAL was performed. One hundred cells were counted for each sample from 10 randomly chosen viewing fields, and eosinophils, lymphocytes, macrophages, and neutrophils were quantified by a blinded reader. At median age of 15.5 weeks, mice were anesthetized and intubated with a 20-g catheter inserted directly into the trachea, placed on a flexiVent ventilator, and airway resistance was determined.

DNA methylation

DNA was extracted from lung tissue using the Wizard SV Genomic DNA Purification System (Promega, Madison, WI, USA). Purified DNA (400 ng) underwent bisulfite modification using the EZ DNA Methylation-Gold kit (Zymo, Irvine, CA, USA). Bisulfite-converted DNA (20 ng) was used in PCR with primers that spanned two regions of the IFN-γ promoter (CpG^{-205} and CpG^{-190}; CpG^{-53} and CpG^{-45}, relative to transcription start site) and one region of the IL-4 promoter (CpG^{-408}, CpG^{-393}) (9) (Table 1). PCR products were pyrosequenced, and methylation levels at each CpG site were determined using PyroMark 24 run software (Qiagen) as described (9).

Nonparametric statistical analyses were performed. These details, as well as additional methods, are provided in the online supplement.

Results

IgE in adult female mice and grandoffspring

Adult female mice treated with *A. fumigatus* developed dosedependent increases in total IgE levels compared to mice treated with saline ($P < 0.0001$ Kruskal–Wallis, Fig. S1). Grandoffspring (F2) of mothers (F0) who received *A. fumigatus* 1250 µg developed lower levels of IgE following sensitization with *A. fumigatus* 62.5 µg compared to grandoffspring of mothers who were treated with saline or *A. fumigatus* 125 µg during pregnancy ($P < 0.05$, Dunn–Nemenyi). When the grandoffspring were stratified according to the timing of prenatal dose (early *vs* late pregnancy), grandoffspring of mice who were treated with *A. fumigatus* 62.5 or 1250 µg later in pregnancy, but not early, developed reduced IgE levels following sensitization to *A. fumigatus* compared to grandoffspring of mice that were treated with saline or 125 µg (Fig. 2A,B, $P < 0.05$, Dunn–Nemenyi). Significant reductions in IgE levels of grandoffspring also were found when comparing the levels after the fifth dose of *A. fumigatus* in the grandoffspring when compared to saline (data not shown, Kruskal–Wallis, $P < 0.0001$).

Eosinophilic inflammation in the grandoffspring

Grandoffspring of mice who were challenged with the highest *A. fumigatus* dose (1250 µg) during early gestation developed greater levels of airway eosinophilia following sensitization with *A. fumigatus* compared to grandoffspring of mice who were challenged with *A. fumigatus* 62.5 µg (Dunn–Nemenyi $P < 0.05$, Fig. 3A). Grandoffspring of mice who received *A. fumigatus* 1250 µg dosed late during pregnancy developed lower levels of airway eosinophilia following sensitization with *A. fumigatus* compared to grandoffspring of mice who were treated during pregnancy with saline or *A. fumigatus* 62.5 µg (Dunn–Nemenyi $P < 0.05$, Fig. 3B).

Lung DNA methylation at the IFN-γ and IL-4 promoters

Levels of methylation at CpG^{-408} and CpG^{-393} in the IL-4 promoter were lower among grandoffspring of mice dosed early in all experimental groups compared to those dosed later (Kruskal–Wallis, $P < 0.0005$, Fig. 4A,B). Following stratification by timing of exposure, significant differences in methylation levels at CpG^{-393} and CpG^{-408} across *A. fumigatus* doses were detected only among mice prenatally dosed early (Kruskal–Wallis, $P = 0.03$ and $P = 0.029$, Fig. 4A,B). Levels of methylation at CpG^{-205} and CpG^{-190} in the IFN-γ promoter also were lower among grandoffspring of mice dosed early in all experimental groups compared to those dosed later (Kruskal–Wallis, $P < 0.0005$, Fig. 4C,D). DNA methylation in IFN-γ CpG^{-53} or CpG^{-45} did not differ by timing (Kruskal–Wallis, $P = 0.81$ and $P = 0.76$, Fig. S3) or across treatment groups. Following stratification by timing of prenatal exposure, significant differences across experimental doses were not detected in the early dosed group.

Additional results are provided in the Supporting Information.

Discussion

Maternal exposure during pregnancy (F0) to *A. fumigatus* was associated with reductions in IgE and airway eosinophilia in grandoffspring (F2). Upon stratification by timing of prenatal allergen exposure, the reductions in IgE and airway eosinophilia were only observed in the late dosed offspring. Conversely, early gestational exposure to allergen produced an increase in airway eosinophilia. Grandoffspring of mice following early prenatal exposure to allergen had lower lung DNA methylation levels at multiple CpG sites at both the IL-4 and IFN-γ promoters compared to offspring of late dosed mice. Grandoffspring of mice dosed early in pregnancy showed evidence of allergen dose-dependent differences in CpG^{-393} and CpG^{-408} DNA methylation at the IL-4 promoter. Only a few borderline and significant correlations were found in IL-4 and IFN-γ methylation with indicators of allergic sensitization (i.e., IgE, airway eosinophilia) in the F2 offspring. Collectively, these results indicate that maternal prenatal exposure to *A. fumigatus* dosed later in pregnancy may affect the allergic airway responses across multiple generations. Epigenetic changes in IL-4 and IFNγ occur in grandoffspring mice (F2) following prenatal exposure to allergen, but those measured here do not appear responsible for the changes in IgE and airway eosinophilia.

Previously, we found that sensitization to *A. fumigatus* in combination with prenatal exposure to diesel exhaust particles led to a reduction in IgE and airway eosinophilia in the F1 offspring (17). While the current study did not test F1 mice, the two studies together suggest that the effects of prenatal administration of *A. fumigatus* on IgE may extend through the third generation under certain conditions.

After fertilization and before implantation, methylation marks are re-established *de novo* via the actions of DNA methyltransferases (18). Early gestational exposure to *A. fumigatus* potentially could have downregulated these methyltransferases, lowering methylation levels

among the early dosed groups. However, if this mechanism was biologically important here, one may have predicted that associations between methylation levels and IgE in the grandoffspring would have been observed if the exposure occurred early during pregnancy instead of later. Instead, these observations may be more consistent with evidence that methylation changes may occur throughout gestation (19). Alternately, because the reductions in IgE were most pronounced among mice prenatally exposed later in gestation, one could speculate that prenatal allergen exposure induced Th cytokine shifts among F0 mice that were transferred to F1 mice across the placenta or through breast milk (20), and possibly induced their own epigenetic marks that subsequently were inherited by the F2 mice.

Only a few studies to date have examined the influence of timing of environmental exposures within gestation on allergic phenotypes in offspring. In one study, relatively high-dose ovalbumin (OVA) administration in BALB/c mice early in gestation was associated with lower OVA-IgE levels compared to OVA exposure late in gestation (21). In a human cohort, increased exposure to PAHs and fine particulate matter ($PM_{2.5}$) in the second month of gestation was associated with lower cord IgE levels and relative increases in $CD3^+$ and $CD4^+$ lymphocytes. Increased exposure to PAH and $PM_{2.5}$ in mid- to late pregnancy was associated with opposite findings (22, 23), suggesting that the timing of exposure during gestation may have a distinct effect on B-cell and T-cell-related outcomes relevant to allergic sensitization.

Another novel aspect of this study is the focus on lung DNA methylation of Th genes in the grandoffspring that varied by both timing of prenatal allergen exposure and dose. The strongest results suggest that methylation levels of both cytokine genes were lower among mice whose prenatal exposures were earlier in gestation. The absence of correlations between gene methylation and IgE indicates that methylation of these CpG sites does not appear to regulate directly the IgE response in these mice. Instead, the observed differences in methylation following prenatal exposure to *A. fumigatus* suggest that epigenetic regulation occurred in association with environmental exposures two generations ago. The biological significance of these epigenetic changes is uncertain. One could speculate that the effect of prenatal allergen exposure on allergic immune responses measured here in F2 mice was a result of significant epigenetic changes in unmeasured genes like Foxp3, a gene susceptible to both DNA methylation and histone modification and implicated in the regulation of allergy (24).

The relative importance of methylation of individual CpG sites in the IFN-γ and IL-4 promoters, in relation to gene expression, is not established (25). For example, although significant changes in methylation levels at CpG^{-205} in IFN-γ were observed with allergen treatment, it is uncertain whether these led to sustained effects on IFN-γ gene expression, particularly when methylation at CpG^{-53} and CpG^{-45} was unchanged. Moreover, the balance of Th1 and Th2 cytokines is thought to contribute to the outcome of the immune response (26). Increases in the methylation of both the IFN-γ and IL-4 promoters may fail to induce relative shifts in cytokine production. Also, the absolute changes in methylation level required to affect the cytokine gene transcription are unknown. The levels of methylation of the IFN-γ promoter measured in this study were much higher than those reported in previous studies of peripheral $CD4^+$ cells in mice (9). These observations may reflect differences in gene methylation in lung *vs* $CD4^+$ cells (27).

Methylation levels at IFN-γ CpG^{-205} and CpG^{-190} were associated negatively with airway eosinophilia. Because increased IFN-γ expression is generally associated with Th1 responses and reduced eosinophilia (28), this finding appears to be counterintuitive.

However, IFN-γ production can stimulate eosinophil activation and survival in some models (29, 30).

These results also add to a limited number of studies that suggest the timing of prenatal exposures may modify epigenetic outcomes. For example, the study of the Dutch Hunger Winter in 1944–1945 found that famine was associated with changes in methylation in several genes implicated in metabolic disease and growth that were modified by timing of famine during pregnancy (31). A study of baboons demonstrated that the extent of global methylation in offspring kidneys varied by timing of prenatal dietary restriction (32).

Limitations of this study warrant discussion. The observations may be specific to BALB/c mice. The use of whole lung tissue for DNA extraction, which included a mixture of cell types, may have impeded our ability to detect significant methylation changes among the treatment groups. The effect of inbreeding the F1 generation, rather than mating F1 females with 'fresh' male mice, is also unknown. We did not measure IFN-γ and IL-4 cytokine levels and thus were unable to correlate the promoter methylation with cytokine production. The lack of F0 and F1 measures prevented the analysis of *A. fumigatus* exposure on allergy and CpG methylation in these generations. Additionally, because we did not continue the experiment through the F3 generation, we cannot conclude that these findings reflect transgenerational inheritance (18). Some experiments, including the airway hyper-reactivity experiments and measures of eosinophilia, could have been underpowered. Finally, the observed batch effect for IgE may stem from assay variability. However, IgE levels were compared primarily across all *A. fumigatus* doses within each timing group, not between timing groups, diminishing to some extent the impact of assay variability.

In summary, these findings suggest that *A. fumigatus* exposure during the prenatal period affects allergic immune responses in grandoffspring. Timing of prenatal allergen exposure determined the allergic response in grandoffspring, with exposures late in pregnancy diminishing the systemic and airway allergic response. Exposures early in pregnancy induced greater airway eosinophilia. While changes in DNA methylation were observed in grandoffspring in association with prenatal allergen exposure, they do not appear responsible for the observed changes in IgE or airway eosinophilia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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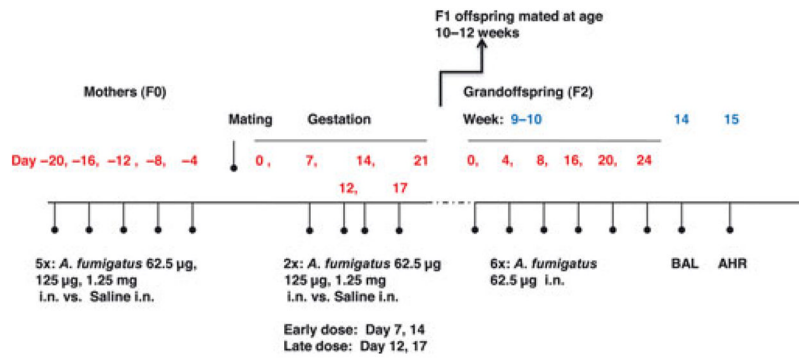
Figure 1.
Experimental protocol. AHR, Airway hyper-reactivity; BAL, Bronchoalveolar lavage; i.n, intranasal; 5×: five doses of *Aspergillus fumigatus*; 6×: six doses of *A. fumigatus.*
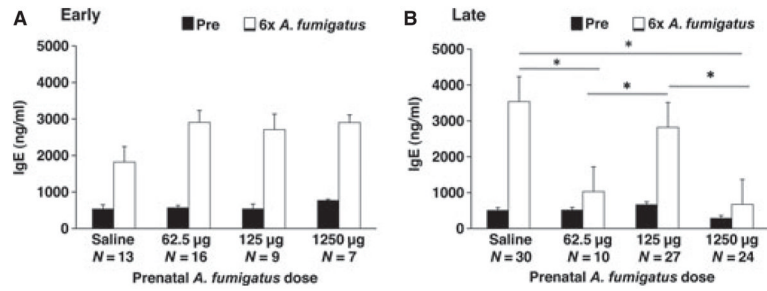
Figure 2.
Mean IgE levels in grandoffspring (F2) following sensitization of female mice (F0) to *Aspergillus fumigatus.* IgE data are presented as mean ± standard error. (A) Early dosed: No changes in IgE were detected across treatment groups. (B) Late dosed: IgE was reduced in grandoffspring (F2) of mothers treated with 62.5 and 1250 μg of *A. fumigatus*, compared to saline and 125 μg. *$P < 0.05$.

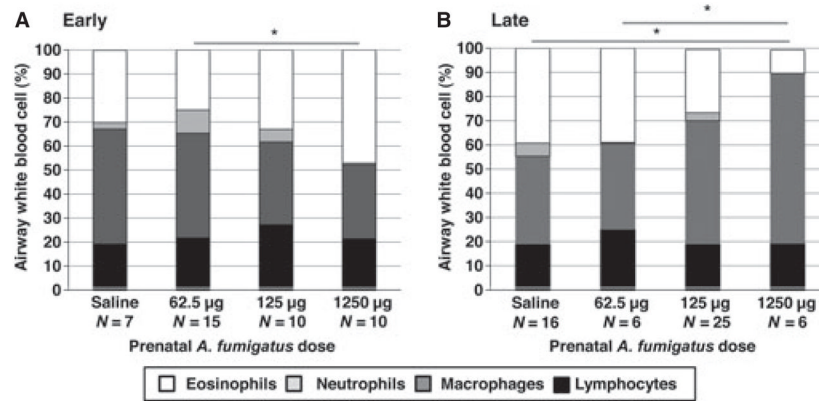**Figure 3.**
BAL counts in grandoffspring (F2) following sensitization of female mice (F0) to
Aspergillus fumigatus. One hundred cells in total were counted for each sample from 10
randomly chosen viewing fields, and eosinophils, lymphocytes, macrophages, and
neutrophils were quantified by a blinded reader. BAL data are presented as percents of total
cell counts. (A) Early dosed: Percent eosinophils in bronchoalveolar lavage fluid was
increased in grandoffspring (F2) of mice treated with 1250 µg of *A. fumigatus* (47.1 ±
3.9%), compared to 62.5 µg (25.0 ± 6.7%). (B) Late dosed: Percent eosinophils in
bronchoalveolar lavage fluid was decreased in grandoffspring (F2) of mice treated with
1250 µg of *A. fumigatus* (9.7 ± 4.8%), compared to saline (39.3 ± 3.7%) and 62.5 µg (39.0
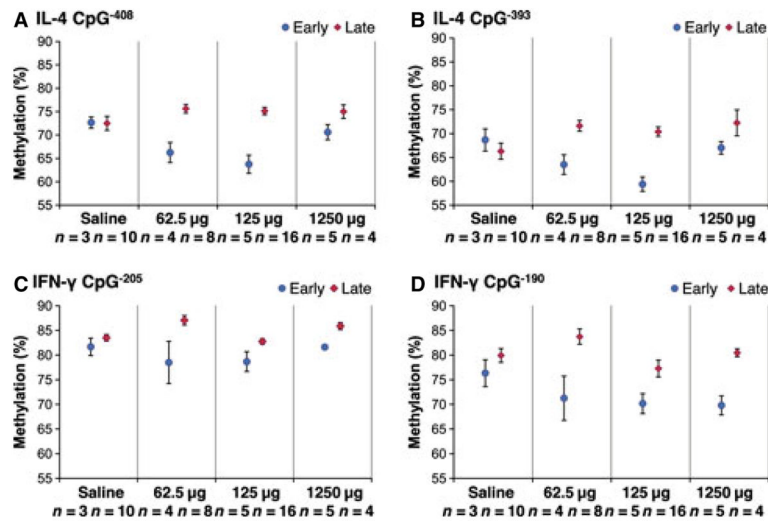± 4.6%). *$P < 0.05$.

Figure 4.
CpG methylation at the IL-4 and IFN-γ promoters in grandoffspring (F2) following
sensitization of female mice (F0) to *Aspergillus fumigatus*. Methylation data are presented
as mean ± standard error. (A) IL-4 CpG^{-408}: Methylation in grandoffspring (F2) of mice
dosed early with *A. fumigatus* was lower than in grandoffspring of mice dosed late ($P<$
0.0005). (B) IL-4 CpG^{-393}: Methylation in grandoffspring (F2) of mice dosed early with *A.
fumigatus* was lower than in grandoffspring of mice dosed late ($P<$ 0.0005). (C) IFN-γ
CpG^{-205}: Methylation in grandoffspring (F2) of mice dosed early with *A. fumigatus* was
lower than in grandoffspring of mice dosed late ($P<$ 0.0005). (D) IFN-γ CpG^{-190}:
Methylation in grandoffspring (F2) of mice dosed early with *A. fumigatus* was lower than in
grandoffspring of mice dosed late ($P<$ 0.0005).

Table 1

Primers used for PCR amplification and pyrosequencing experiments

IFN-γ	
PCR forward	TGGTGTGAAGTAAAAGTGTTTTTAGA
PCR reverse	biotin-TACACCTCTCTAACTTCCAATTTT
Pyrosequencing 1	GAATGGTATAGGTGGGTA
Pyrosequencing 2	AAAAAAAATTTGTGAAAATA
IL-4	
PCR forward	GTTTTTAAGGGGTTTTTATAGTAGGAAGT
PCR reverse	biotin-AATTACCACTAACTCTCCTCCTACA
Pyrosequencing	AGATTTTTTTGATATTATTTTGTTT