Early-Life Environmental Factors, IFN- γ Methylation Patterns, and Childhood Allergic Rhinitis

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Keywords

Children · Allergic rhinitis · Early-life environment · Methylation · IFN- γ

Abstract

Background: While early-life risk factors are known to influence the risk of allergies, the biological mechanisms underlying this observation are unclear. The aim of this study was to examine whether DNA methylation in childhood could underlie the association between early-life risk factors and allergic rhinitis (AR). **Methods:** In total, 234 patients, aged 6 years, were recruited, i.e., 114 were patients with AR (AR group) and 120 healthy children without AR (NAR group). The DNA methylation patterns of the IFN- γ promoter regions in CD4⁺ cells were analyzed using bisulfite sequencing. The percentage of Th1 was investigated by flow cytometry. The relationship among DNA methylation, early-life environment, and AR was examined. **Results:** After adjusting for several likely confounders, there was a higher likelihood of AR in children who had mothers with allergies than in children who had nonallergic mothers (OR = 5.19; 95% CI 1.18–29.41),

in children who were born in autumn or winter than in children who were born in the summer or spring (OR = 2.69; 95% CI 1.34–5.40), and in children who lived with indoor carpet or wallpaper than in children who lived without indoor carpet or wallpaper (OR = 4.14; 95% CI 2.05–8.30). Compared to the NAR group, the AR group had higher mean methylation levels of the promoter region in IFN- γ , and lower numbers of IFN- γ +CD4⁺ cells were associated with autumn-winter birthdates. The season of birth had an indirect effect on AR at 6 years, which was mediated by the mean IFN- γ promoter methylation level. **Conclusions:** This study suggests that early-life environments affect AR, and this is supported by the finding of IFN- γ methylation as a mediator of the effect of an individual's season of birth on AR. © 2019 S. Karger AG, Basel

Introduction

The prevalence of allergic disease including allergic rhinitis (AR) has increased over the past few decades in most countries worldwide [1, 2]. This change has oc-

curred very rapidly, suggesting that there might be additional factors affecting the prevalence of allergic disease beyond genetic changes. It is possible that the increase in the prevalence of allergic diseases, including AR, is associated with complex gene-environment interactions. Previous studies have shown that environmental factors contribute to the growing epidemic of allergies worldwide, which are accompanied by epigenetic changes in some cases [3].

The developmental origins of health and disease hypothesis suggests that a child's early-life environment influences the child's risk for chronic diseases such as AR [4]. Recent studies have focused on early-life risk factors and the development of allergic diseases later in life to understand the effect of exposure on the development of atopic disease. The association of prenatal and early-life exposures and the subsequent development of allergy-related outcomes with focuses on different breastfeeding characteristics [5], mode of delivery [6], the microbiome [7], indoor allergens [8], and air pollution exposure [9] during the first months and years of life have been previously reported. In several studies, the season of birth has also been associated with the risk of developing allergic disease including rhinitis [10, 11] and hay fever [12]; however, the mechanisms underlying such effects remain unknown.

Environmental pollutants play an important role in the occurrence and progression of allergic diseases, and their effects may be regulated through genetic and epigenetic mechanisms. Epigenetic changes in DNA methylation are one way in which transcriptional responses to changing environments can be established and maintained over long periods of time. DNA methylation in adulthood is associated with the season of birth, suggesting that this association arises postnatally and could mediate the effect of season of birth on allergies [11]. The TH1/TH2 paradigm assumes that a predominance of TH2 cell activation and an inadequate TH1 cell response are responsible for the development of allergies. We did not find any correlation between *IL-4* methylation and mRNA expression in the pediatric AR population in our previous study; however, we found that decreased *IFN-γ* mRNA expression was related to high levels of methylation of *IFN-γ* in CD4⁺ T cells [13]. *IFN-γ* is a Th1-associated cytokine gene that can be regulated by methylation and that plays an important role in modifying the development of innate and adaptive immune responses [14]. Prenatal environmental tobacco smoke exposure and subsequent house dust mite-induced asthmatic murine models have also demonstrated increased methylation in *IFN-γ* [15].

Given the known effect of the early-life environment on the development of AR, together with the established role of *IFN-γ* methylation in allergic disease and our previous findings, we investigated whether methylation of the promoter of *IFN-γ* could be a molecular mechanism underlying the effect of the early-life environment on the risk of AR in children.

Methods

Study Subjects

One hundred fourteen patients with AR (AR group), who were born between January and December 2010 were recruited from the outpatient clinic of the Otolaryngology Department of Shanghai Children's Medical Center from January 2016 to August 2016. One hundred twenty healthy children undergoing a regular physical examination in our hospital were recruited as age- and gender-matched controls; these children constituted the no AR group (NAR group). All subjects were from the same region (Pudong area, Shanghai), with long-term residence in the locality since birth. None of the patients who were sampled were taking medications and had nasal allergy attacks. This study was approved by the ethics committee of Shanghai Children's Medical Center, and written informed consent was obtained from the parents or guardians of the children recruited into this study.

Clinical Evaluation

Patients in the AR group were diagnosed with AR in accordance with 2008 Allergic Rhinitis and Its Impact on Asthma (ARIA) guidelines [16]. All patients had experienced recurrent symptoms for at least 1 year, including 2 or more persistent symptoms of water-like tears, nasal itching, congestion, or sneezing lasting for more than 1 h every day. Additionally, the inclusion criteria required 1 or more positive serum IgE tests (SIgE ≥ 0.35 kU/mL) by Western blotting using the AllergyScreen™ human serum specific IgE allergen detection kit for specific inhalant allergens including house dust mites, cat/dog hair, molds, seasonal grass/tree and pollens, cockroaches, ragweed, *Artemisia argyi*, *Penicillium notatum*, and *Aspergillus fumigatus*. Members of the NAR group presented with no clinical features related to nasal diseases and did not have a cold or the flu at the time of recruitment. None of the subjects had a history of allergic disease or an atopic family history. Moreover, all of the controls were examined via serum SIgE tests, with no positive antigen-specific IgE reactions to any of the common allergens described above in the blood sample.

To control additional aspects of environmental exposure, we determined the following exclusion criteria: (1) recent major events such as a newly decorated house, relocation, transfer, separation from guardians, and hospitalization; (2) school outside of the same area as their residence; and (3) autoimmune diseases, congenital airway anomalies, heart diseases, or malignant tumors. Children who met any of these criteria were excluded.

Assessment Questionnaires

A sociodemographic questionnaire was sent to the parents of all of the study subjects. The questionnaire surveyed the factors that were reported to be associated with the risk of AR and includ-

Table 1. *IFN-γ* fragment primers for bisulfite sequencing

<i>IFN-γ</i> (NG_015840.1)	Forward primer sequence	Reverse primer sequence	Product size, bp
Sequencing F1 (CpG ⁻²⁹⁹ , CpG ⁻¹⁸⁹)	GTAAATGATTAATGTGTTTTGTGAATGAAG	TAAAATTTCCTTTAAACTCCTTAAATCC	194
Sequencing F2 (CpG ⁻⁵⁷ , CpG ⁺¹¹⁹ , CpG ⁺¹²⁵ , CpG ⁺¹⁶⁸)	GGATTTAAGGAGTTTAAAGGAAATTTTA	ACATATAAAATCCTAACAATAACAACCAAA	391

Site based on previous studies of *IFN-γ*.

Fig. 1. Locations and patterns of CpG sites investigated in the *IFN-γ* promoter region. **a** CpG sites under investigation relative to the translation start site (TSS). **b** Differential patterns of methylation of the *IFN-γ* promoter at CpG sites between the AR and NAR groups. Values are presented as means ± SD. **p* < 0.05 compared with the NAR groups.

ed the following variables: gender, gestational age (preterm [<37 weeks], term [37–41 weeks], or postterm [≥ 42 weeks]), mode of delivery (vaginal or cesarean section), age at weaning (<6 , 6–12, or >12 months), history of parental allergic disease (yes or no, parents with AR, allergic conjunctivitis, asthma, or eczema were defined as having an allergic diseases), and parental educational level (high school and below, college, or graduate and above). The environmental assessment included the home address, season of birth (spring-summer [March to August] or autumn-winter [September to February]), secondhand smoke exposure (yes or no), and indoor carpet or wallpaper (yes or no) in the child's home during their first 2 years of life.

Sample Collection and CD4⁺ T Cell Isolation

Peripheral blood mononuclear cells (PBMC) were collected and isolated by centrifugation on a Ficoll gradient and resuspended in RPMI medium supplemented with 10% heat-inactivated fetal calf serum and 2% penicillin-streptomycin (Sigma, St. Louis, MO, USA). Flow cytometry sorting of CD4⁺ T lymphocytes was performed. Cell purity was greater than 98%, with a count greater than 1×10^6 after sorting.

DNA Extraction and Bisulfate Conversion

A centrifugal column blood genomic DNA extraction kit (QIAamp DNA Micro Kit, Qiagen) was used for genomic DNA extraction from the purified CD4⁺ T-cell population. A NanoDrop ND-1,000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to assess the concentration of the extracted DNA.

Methylation treatment was performed with an EZ DNA Methylation-Gold Kit (Zymo Research) to convert unmethylated cytosines to uracil and to leave the methylated cytosines unchanged. Polymerase chain reaction (PCR) amplification was performed with the DNA samples with 3 fragment primers of the target genes. The total reaction volume was 15 μ L, including 1.5 μ L of 1 $\times$ Hot-StarTaq buffer, 1 μ L of Mg^{2+} (25 mM), 0.5 μ L of dNTP (10 mM), 0.5 μ L of each forward and reverse primer (10 μ M), 0.15 μ L of Hot-StarTaq polymerase (5 U/ μ L), 1 μ L of template DNA (100 ng), and 2 μ L of ddH₂O. The reaction conditions were as follows: predenaturation at 95 °C for 15 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, for a total of 40 cycles, followed by a final extension at 72 °C for 5 min. PCR products were purified with a Qiagen QIA Quick Purification Kit (catalog No. 28104) and stored at -20 °C (Table 1).

Fig. 2. Flow cytometric evaluation of Th1 and Th2 in PBMC of the AR and NAR groups. **a** CD4+(CD3+CD8-) T cells were gated in PBMC. Th1 cells (IFN- γ + CD4+ T cells) were gated in CD4+ T cells in the AR and NAR groups. Values in the dot plots indicate the percentages of different cell subsets among the total CD4+ T cells. The y axis in the image of AR group shares with the NAR group. **b** The lower and upper lines of the box indicate the 25th

and 75th percentiles, and the center line in the box indicates the median. The whiskers extend the range to the 10th and 90th percentiles, and the circles denote the 5th and 95th percentiles (Mann-Whitney U test). **c** Correlations of the mean methylation statuses in the promoter CpG islands of *IFN- γ* with their flow cytometric levels (Spearman's rank correlation).

Target Amplification and Purification

The target areas for *IFN- γ* (NG_015840.1) were chosen based on previous studies of epigenetic regulation following inhaled environmental exposures [17, 18]. Data were downloaded from the University of California at Santa Cruz (UCSC) website (<http://genome.ucsc.edu/>; GRCh37/hg19), including the area 2 kb upstream of the 5' end and the full-length gene sequence. CpG islands in the target sequence were predicted with the appropriate software from <http://emboss.bioinformatics.nl/cgi-bin/emboss/cpgplot>. Premier 3 software was used for the analysis (Fig. 1a).

The purified PCR products were cloned into pMD18-T simple vectors with a TAKARA T Carrier Link Kit (D101A) and plated onto KAN-X-GAL plates for blue-white screening. Positive colonies were inoculated into LB-amp+ tubes (volume ratio 1,000:1), cultured at 37°C, and shaken at 180 rpm for more than 3 h. PCR identification was performed using specific PCR primers. The PCR products were identified by 1.5% agarose gel electrophoresis.

Sanger Sequencing of Bacterial Clones

To address the heterogeneity of DNA methylation patterns, 10 or more clones/alleles were sequenced per patient for each of the targeted loci. Bacterial suspensions of 10 positive clones identified by PCR were randomly selected from each sample and sequenced. The methylation ratio of the CG loci in the 10 clones was assessed by template sequence alignment using Sequencher software.

Flow Cytometric Analysis

T-cell subsets were phenotyped by flow cytometry as previously described [13]. Flow cytometry sorting of the CD4+ T lymphocytes was performed. The cell purity was greater than 98%, with a count above 1×10^6 after sorting. An evaluation of intracellular IFN- γ was performed in PBMC stimulated with an agent (Cell Stimulation, eBioscience, San Diego, CA, USA) for 5 h. At the end of the stimulation period, the PBMC were mixed with the appropriate volume of fluorescence dye-conjugated monoclonal antibodies ($5 \mu\text{L}$: 1×10^6 cells/mL) and fixed with fixation and per-

Table 2. Distribution and relationship between potential risk factors and AR in 6-year-old children

Variable	AR group (n = 114)	NAR group (n = 120)	p
<i>Demographics</i>			
Male/female ratio	71/43	78/42	0.685
Weight, kg	21.28±2.81	19.63±4.47	0.913
Height, mm	115.31±4.76	116.88±7.06	0.258
<i>Potential risk factors</i>			
Age at weaning (months)			0.152
<6	43 (37.7)	37 (30.8)	
6–12	59 (51.8)	60 (50.0)	
>12	12 (10.5)	23 (19.2)	
Gestational age (weeks)			0.245
Term	96 (84.2)	100 (83.3)	
Preterm	7 (6.1)	3 (2.5)	
Post-term	11 (9.6)	17 (14.2)	
Mode of delivery			0.284
Vaginal	48 (42.1)	42 (35.0)	
Cesarean	66 (57.9)	78 (65.0)	
Maternal allergic history			
No	66 (57.9)	114 (95.0)	<0.001
Yes	48 (42.1)	6 (5.0)	
Maternal education			
High school and below	44 (38.6)	72 (60.0)	0.005
College	52 (45.6)	39 (32.5)	
Graduate and above	18 (15.8)	9 (7.5)	
Season of birth			<0.001
Spring-summer	45 (39.47)	72 (60.0)	
Autumn-winter	69 (60.53)	48 (40.0)	
<i>Exposure during the first 2 years of life</i>			
Secondhand smoke exposure			0.001
No	54 (47.4)	27 (22.5)	
Yes	60 (52.6)	93 (77.5)	
Indoor carpet or wallpaper			
No	38 (33.3)	69 (57.5)	0.019
Yes	76 (66.7)	51 (42.5)	

Values are presented as means ± SD or numbers (%) unless otherwise stated. The χ^2 or Student *t* test was performed. Significant results are in bold.

meabilization reagent (Intraprep; Beckman Coulter, Danvers, MA, USA). Next, the cells were stained with anti-human CD3-PerCP (PerCP; eBioscience), anti-human CD8-fluorescein isothiocyanate (FITC; eBioscience) and anti-human IFN- γ -phycoerythrin (PE, 0.5 μ g/10⁶ cells; eBioscience). IgG isotype controls were utilized for each antibody and patient. The cell pellets were resuspended in 400 μ L of PBS containing 0.2% w/v paraformaldehyde (Sigma) for flow cytometric analysis after washing and centrifugation.

The stained and fixed samples were injected into a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), and the data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA) according to the manufacturer's instructions. In total, 10,000 CD3+CD8[−] lymphocyte events were typically acquired for analysis of the frequency of intracellular cytokine staining. Cytokine production was confirmed based on staining of the isotype control. The results are expressed as the percentage of cy-

tokine-positive lymphocytes in the stimulated culture. The percentage of cytokine-producing T-helper cells (IFN- γ +CD4⁺) was calculated within the total CD3+CD8[−] subpopulation (Fig. 2a). There was no difference in mean fluorescence intensity for each sample or among the different groups.

Statistical Analysis

All subjects who met the inclusion criteria were included in the analysis. All normally distributed numeric data are expressed as means (±SD). With regard to the basic characteristics and potential risk factors, the differences between groups were measured using the χ^2 test for categorical variables or *t* tests for continuous variables. *p* < 0.05 was considered statistically significant. In the logistic regression analyses, model 1 was adjusted for gender and model 2 was adjusted for several likely confounders such as gender, age at weaning, mode of delivery, maternal education level, and second-

Table 3. Early-life environmental risk factors for AR in children according to multivariable logistic regression models

Variable	Model 1 adjusted ^a OR	Model 2 adjusted ^b OR
Maternal allergic history		
No	1	1
Yes	6.06 (2.35–29.83)	5.9 (1.18–29.41)
Maternal education		
High school and below	1	1
College	2.18 (1.24–3.82)	2.96 (0.41–6.21)
Graduate and above	3.24 (1.34–7.86)	2.60 (0.84–8.02)
Season of birth		
Spring-summer	1	1
Autumn-winter	3.43 (1.28–5.25)	2.69 (1.34–5.40)
Indoor carpet or wallpaper exposure		
No	1	1
Yes	2.70 (1.58–4.60)	4.14 (2.05–8.30)

Values in parentheses are 95% CI. Significant results are in bold at $p < 0.05$. ^a For gender. ^b For gender, age at weaning, mode of delivery, and secondhand smoke exposure.

hand smoke exposure. Between-group and within-group comparisons of the mean methylation levels of the *IFN- γ* promoter region were performed using parametric tests. Medians and 25/75 and 10/90 percentiles were used when continuous variables were not normally distributed as a percentage of the *IFN- γ* + CD4+ T cells. The Mann-Whitney U test was further performed when comparing 2 groups. Multiple regression analyses were conducted to assess each component of the proposed mediation model. The mediation analyses were tested using the bootstrapping method with bias-corrected confidence estimates in PROCESS version 3.1 for SPSS after controlling for the covariates of gender and any other significant risk factors we identified in the multivariable logistic regression (<http://www.processmacro.org/download.html>) [19]. The 95% CI of the indirect effects was obtained with 5,000 bootstrap resamples [20, 21]. The Z score and p value were determined by the Sobel test for the indirect effect [22]. Spearman's rank correlation was used to analyze the associations between the level of methylation and the percentage of *IFN- γ* + CD4+ T cells. $p < 0.05$ was considered statistically significant. All statistical analyses were conducted using SPSS 17.0 (Statistical Package for the Social Sciences Inc., Chicago, IL, USA), and the data were plotted using GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, CA, USA).

The statistical power for this study was calculated using G*Power 2 software (<http://www.psych.uni-duesseldorf.de/aap/projects/gpower/>). The statistical power for the comparison of AR versus NAR was 95% with the current sample size; the α was 0.05 and the β was 0.2.

Results

Distribution of Characteristics among the AR and NAR Groups

A total of 114 patients with AR and 120 controls without AR were enrolled into the study. Eighty-two (71.9%)

of the allergic children exhibited markedly high levels of house dust mite-specific IgE; 20 (17.5%) of the allergic children showed high ragweed-specific IgE, and 12 (10.5%) had high seasonal grass/tree and pollen-specific IgE levels. As shown in Table 2, maternal allergic history ($p < 0.001$), maternal education level ($p = 0.005$), season of birth ($p < 0.001$), and indoor carpet or wallpaper exposure ($p = 0.019$) were significantly associated with AR. However, no significant differences were found between the 2 groups in terms of the age at weaning, gestational age, mode of delivery, or secondhand smoke exposure.

In the multivariate logistic regression for model 1 (Table 3), after adjusting for gender, AR was related to the risk factors of maternal allergic history, autumn-winter birthdate, and indoor carpet or wallpaper exposure. In model 2 (Table 3), the relationships between AR and maternal allergic history, autumn-winter birthdates, and indoor carpet or wallpaper exposure remained statistically significant after adjusting for all confounders. In addition, the interactions between the covariates in the multivariate models showed no significant differences.

Comparison of IFN- γ Promoter Methylation Patterns in the AR and NAR Groups

The DNA methylation levels around the *IFN- γ* promoter site in CD4+ T cells were measured. The mean methylation statuses of 6 CG loci (–299, –189, –57, +119, +125, and +168) around the promoter region of *IFN- γ* in the AR group were significantly higher than those in the NAR group ($p < 0.001$; Fig. 1a). Among all of the loci, the differences in methylation levels at loci –299, –189, –57,

Table 4. Influence of season of birth on mean *IFN-γ* promoter methylation levels in the AR and NAR groups

Variables	AR group	Mean <i>IFN-γ</i> promoter methylation levels	NAR group	Mean <i>IFN-γ</i> promoter methylation levels	<i>p</i> value ^a
Total	114	86.35±0.73	120	77.68±0.88	<0.001
Season of birth					
Spring-summer	45	80.97±1.40	72	78.99±1.02	0.284
Autumn-winter	69	88.96±0.67	48	75.76±1.56	0.001
<i>p</i> value ^b		<0.001		0.078	

Values are presented as means ± SD or numbers. ^a Comparison between AR and NAR groups by Student's *t* test. ^b Comparison between the categories of each variable for AR and NAR group by Student's *t* test. Significant results are in bold.

Fig. 3. Coefficients (β) and the indirect effects of season of birth on AR at 6 years through the mean DNA methylation level in the promoter of *IFN-γ* (indirect effect $Z = 2.85$; 95% CI 0.196–0.753, $p = 0.004$). A-path: season of birth variable predicts AR at 6 years. B-path: mean *IFN-γ* promoter methylation levels predict AR at 6 years after controlling for season of birth. C'-path (direct effect): season of birth predicts AR at 6 years after controlling for mean *IFN-γ* promoter methylation levels. C-path (total effect): season of birth predicts AR at 6 years. ** $p < 0.01$. *** $p < 0.001$.

and +168 between the AR and NAR groups were significantly different ($p = 0.006$, $p = 0.029$, $p = 0.023$, and $p = 0.032$; Fig. 1b).

Percentage of *IFN-γ*+ CD4+ T Cells and Correlation with the Individual Methylation Level

Flow cytometry revealed that, compared to patients in the NAR group (median: 10.64%, IQR 4.87–6.63), patients with AR had a significantly lower percentage of peripheral Th lymphocytes producing *IFN-γ* (*IFN-γ*+CD4+) in CD4+ cells (median: 5.5%, IQR 1.54–2.69) ($p = 0.001$) (Fig. 2b). There was a significant negative correlation between the individual methylation level and the percentage of *IFN-γ*+CD4+ T cells ($r = -0.392$, $p < 0.001$) (Fig. 2c).

Mediator of the Mean *IFN-γ* Promoter Methylation Level in an Indirect Effect of Season of Birth on AR

After controlling for gender and other risk factors, first, it was found that season of birth was positively associated with AR at 6 years (as opposed to no AR) (C-path; total effect coefficient [β] = 0.669, $t[232] = 4.528$,

$p < 0.001$). It was also found that season of birth was positively related to the DNA methylation level in the promoter of *IFN-γ* (A-path; $\beta = 0.332$, $t[232] = 5.328$, $p = 0.009$). Second, the results indicated that the mediator, the mean DNA methylation level in the promoter of *IFN-γ*, was positively associated with AR at 6 years (B-path; $\beta = 0.327$, $t[232] = 5.115$, $p < 0.001$) after controlling for season of birth. Further mediation analysis confirmed the mediating role of the mean *IFN-γ* promoter methylation level in the association between season of birth and AR at 6 years (indirect effect $Z = 2.85$; 95% CI = 0.196–0.753, $p = 0.004$). In addition, the results indicated that the direct effect of season of birth on AR at 6 years became nonsignificant (C'-path; direct effect $\beta = 0.167$; 95% CI = –0.183 to 1.346) when the mean *IFN-γ* promoter methylation level was controlled for, thus suggesting full mediation by the *IFN-γ* promoter methylation level (Fig. 3) (online suppl. Table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000495304). No other risk factors in our study were found to be significant in the mediation model (data not shown).

We compared the mean *IFN-γ* promoter methylation levels between the AR and NAR groups stratified by season of birth (Table 4). The mean methylation levels in the AR group were significantly higher than those in the NAR group ($p < 0.001$). Children born in autumn-winter had significantly higher methylation levels in the AR group than in the NAR group ($p = 0.001$). Autumn-winter birth had a significant impact on methylation levels in AR group ($p < 0.001$).

Discussion

In this study, we describe the effects of early-life environment on AR at 6 years of age and report that early-life environment-associated DNA methylation levels around the *IFN-γ* promoter site in CD4⁺ T cells present at that time as a mediator, constituting a potential mechanism of the sustained effect of season of birth on the risk of AR. The results also demonstrated that the percentage of IFN- γ (IFN- γ +CD4⁺) in CD4⁺ cells was significantly lower in the AR group than in the NAR group.

In our study, maternal allergic history was a strong risk factor of AR at age 6 years among children in this geographic area. This finding is supported by previous research that suggests that having parents with allergies, specifically maternally allergies, is associated with a high likelihood of childhood allergy development [23, 24]. Due to the susceptibility of children to allergic diseases in early life, regulatory T cells that influence the early steps of allergy initiation could be involved [25]. In addition, maternal allergic status could influence the cytokine milieu experienced by the fetus, including lower IFN- γ responses [26] or increased percentages of IL-4+CD4⁺ T cells and a reduced Treg/Th2 ratio in the cord blood [27]. This topic is one that warrants further exploration in subsequent studies. However, we did not find that a maternal allergic history impacted the childhood AR development mediated by *IFN-γ* promoter methylation. The finding is consistent with a recent report that maternal allergy correlated with impaired Tregs in neonates, which could enhance the susceptibility of offspring to allergic diseases in early childhood due to an imbalance of Th1 and Th2 cells [25].

Although we found that the effect of fine particulate matter 2.5 may be mediated through epigenetic modification of the *IFN-γ* promoter region in a previous study [13], we further explored other environmental aspects as AR risk factors mediated by *IFN-γ* methylation such as season of birth. We found that season of birth was a risk

factor for AR; autumn-winter birthdates increased the risk of AR 2.7 times in 6-year-old children in this geographic locale. The methylation level of the *IFN-γ* promoter was also higher in children born during the autumn and winter compared to those born during the spring and summer in the AR group. Several possible explanations have been suggested to elucidate the link between season of birth and risk of AR. Possible mechanisms associated with the season of birth could produce long-lasting differences in disease risk, including seasonal fluctuations in levels of allergens such as pollen [28], a reduced vitamin synthesis due to lower UV-B levels during autumn and winter [29], the seasonality of early-life respiratory viral infections [30], and possible seasonal variations in maternal nutrition [31]. Recently, one epigenome-wide association study was based on methylation measured in whole-blood DNA collected from 367 participants aged 18 years from the UK. The author reported that 92 CpG sites were associated with specific seasons of birth and 4 of these associations were replicated in an independent series of 207 children aged 8 years from The Netherlands [11]. The indirect effect of season of birth on AR at 6 years was found to be mediated by the level of methylation of the *IFN-γ* promoter methylation in our study. Emerging evidence suggests that environmental allergy triggers can alter DNA methylation in the *IFN-γ* promoter, thereby modifying the allergic disease risk in murine studies [17, 32]. *IFN-γ* methylation has also been shown to vary by asthma diagnosis among monozygotic twins, which is consistent with mediation by environmental exposure [33]. Although some studies have shown that the environment affects DNA methylation, with some exposures leading to increased DNA methylation and long-term gene silencing [34], further research is needed to determine whether exposed subjects develop epigenetic alterations over time and which alterations increase the risk of disease.

IFN-γ gene expression is tightly regulated in early life, and strong negative control of IFN- γ production in CD4⁺ T cells has been associated with the risk of subsequent development of atopy. Recent studies have demonstrated the potency of *IFN-γ* promoter methylation as a mechanism for control of human *IFN-γ* gene expression [35] and cytokine expression [18], which may be an important contributing factor in the development of atopy in childhood. Our finding of the negative correlation between *IFN-γ* methylation level and flow cytometric level was consistent with previous reports. Although altered DNA methylation patterns in CD4⁺ T cells indicate the importance of epigenetic mechanisms in allergic diseases, the

observed difference in *IFN-γ* DNA methylation between patients and controls was small (AR: 86.35% vs. NAR: 77.68%) in our study. Our findings agree with a recent quantitative study of genome-wide methylation in CD4+ T cells from monozygotic twins, only 1 of whom had an autoimmune disease, i.e., psoriasis; in that study, the affected and unaffected siblings were observed to have a substantial number of small differences, with a significant correlation with gene expression [36]. Moreover, a study of DNA methylation in CD4+ T cells from patients with the autoimmune disease systemic lupus erythematosus also reported widespread small changes in DNA methylation [37].

In addition, indoor carpet or wallpaper in the child's home during their first 2 years of life became insignificant after adjusting for multiple confounders, such as age at weaning, mode of delivery, maternal education level, and secondhand smoke exposure. This finding was consistent with previous reports that showed that perinatal indoor aeroallergen exposure does not seem to affect the development of childhood AR [8]. However, interior decoration materials such as chemical fiber carpets and wallpapers contain formaldehyde and some toxic elements, which may increase children's allergic symptoms [38].

Our study had numerous limitations. First, given our small sample size, it is possible that we overestimated the significance of the association of certain risk factors for AR with the DNA methylation level of *IFN-γ*. Furthermore, there are several factors that could confound the association between season of birth and the DNA methylation levels of *IFN-γ* in children with AR, including ambient air pollution and proportions of different leukocyte subsets; however, we tried to control for these variables by recruiting subjects for this study who lived in the same region and measuring the average DNA methylation levels in *IFN-γ* of homogeneous populations of leukocytes. Therefore, the conclusions of this study may be limited to those within the specific geographic locale studied, and

future studies should examine a sample of patients from a different geographic area to determine the generalizability of these findings.

Conclusions

In this study, we examined how early-life environmental risk factors, such as maternal allergic history, season of birth, and indoor carpet or wallpaper exposure, might be related to the development of AR in 6-year-old individuals. The methylation profile of the *IFN-γ* promoter gene could be the mediating mechanism through which the transcriptional responses to season of birth are established and maintained in childhood.

Acknowledgement

We acknowledge all of the participants for their help with data collection and all of the children and their parents for participating in this study.

Disclosure Statement

The authors declare that they have no competing interests.

Funding Sources

All phases of this study were supported by the Chinese National Natural Science Foundation (81502815) and Shanghai Jiao Tong University (YG2016MS29).

Author Contributions

Y.L., X.R., B.M., F.J., and J.C. conceived and designed the experiments. Y.L., X.R., and B.M. performed the experiments. Y.L., F.J., and J.C. analyzed the data. Y.L., X.R., B.M., F.J., and J.C. contributed reagents/materials/analysis tools. Y.L., X.R., F.J., and J.C. wrote this paper.

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