Maternal and cord blood miR-223 expression associates with prenatal tobacco smoke exposure and low regulatory T-cell numbers

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Background: There is evidence that microRNAs (miRNAs) are sensitive to environmental stressors, including tobacco smoke. On the other hand, miRNAs are involved in immune regulation, such as regulatory T (Treg) cell differentiation. The aim of the present study was to investigate the association between prenatal tobacco smoke exposure, miRNAs, and Treg cell numbers.
Methods: Within a prospective mother-child study (Lifestyle and Environmental Factors and Their Influence on Newborns Allergy Risk), we analyzed the expression of miR-155 and miR-223 together with Treg cell numbers in maternal blood during pregnancy, as well as in cord blood (n = 441). Tobacco smoke exposure was assessed based on questionnaire answers and maternal urine cotinine levels. Additionally, the concentration of smoking-related volatile organic compounds was measured in dwellings of study participants.
Results: Both maternal and cord blood miR-223 expressions were positively correlated with maternal urine cotinine levels. An association was also found between maternal miR-223 expression and indoor concentrations of benzene and toluene. High miR-223 expression was associated with lower Treg cell numbers in maternal and cord blood. Furthermore, children with lower Treg cell numbers at birth had a higher risk of atopic dermatitis during the first 3 years of life. The concentration of the toluene metabolite S-benzylmercapturic acid in maternal urine was associated with decreased cord blood, but not maternal blood, miR-155 expression. A relationship between miR-155 expression and Treg cell numbers was not found.
Conclusions: For the first time, we show that maternal tobacco smoke exposure during pregnancy correlates with the level of miRNA-223 expression in blood, with an effect on children's cord blood Treg cell numbers and subsequent allergy risk. (J Allergy Clin Immunol 2014;133:543-50.)

Key words: *miR-223, miR-155, regulatory T cells, cord blood, pregnancy, tobacco smoke*

Programming of the immune system, particularly with regard to tolerance development, starts before birth. In addition to genetic predisposition and intrinsic factors, such as the *in utero* microenvironment and maternal immune status,[1] environmental exposure during pregnancy, such as microbial[2-4] or chemical[5-7] exposure, is known to have an influence on children's immune system development. Within the Lifestyle and Environmental Factors and Their Influence on Newborns Allergy Risk (LINA) study, we found out that maternal smoking/exposure to tobacco smoke during pregnancy leads to lower regulatory T (Treg) cell numbers at birth, with the consequence of an increased risk of atopic dermatitis and allergic sensitization during the first year of life.[8] It is more and more evident that environmental exposures, particularly tobacco smoke, induce epigenetic changes, such as DNA methylation,[9,10] histone modification,[11] or altered microRNA (miRNA) expression,[12] and that these modulate epithelial cell differentiation, immune cell differentiation, or both in a long-lasting manner.[13]

Recently, it has been shown that prenatal tobacco smoke exposure affects global and gene-specific DNA methylation.[14] Izzotti et al[15] demonstrated robust changes in miRNA expression in the lungs of rats subchronically exposed to environmental tobacco smoke (ETS). The authors analyzed 484 miRNAs, 126 of which were significantly downregulated (eg, miR-155 and miR-223) and 7 of which were upregulated. In a human study Schembri et al[16] found 28 miRNAs to be differentially expressed in airway epithelium from current smokers compared with subjects who have never smoked. Furthermore, it has been found that exposure to metal-rich particulate matter modified the expression of candidate miRNA in peripheral blood leukocytes.[17] Thus it is apparent that miRNA expression is modulated by environmental agents. On the other hand, miRNAs have been reported to modulate many aspects of the immune response, such as differentiation, proliferation, cell fate determination, and intracellular signaling pathways.[18,19] As has already been demonstrated for DNA methylation, miRNAs might also represent a link between the environmental exposures and resulting immune responses. However, thus far, a link between

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Abbreviations used
ETS: Environmental tobacco smoke
FOXP3: Forkhead box protein 3
gDNA: Genomic DNA
LINA: Lifestyle and Environmental Factors and Their Influence on Newborns Allergy Risk
miRNA: MicroRNA
MR: Mean ratio
RT: Reverse transcription
SBMA: S-benzylmercapturic acid
SPMA: S-phenylmercapturic acid
Teff: Effector T
Treg: Regulatory T
TSDR: Treg cell–specific demethylated region
VOC: Volatile organic compound

environmental exposure, miRNA expression, and Treg cell numbers has not been shown.

Using microarrays, Cobb[20] reported that 35 miRNAs were preferentially expressed in Treg cells compared with other immune cell subtypes, at least in the murine system. Of these, miR-155 and miR-223 have been described as playing pivotal roles in Treg cell formation or function.[20-22] In the present study we investigate the potential link between maternal tobacco smoke exposure, miRNA expression, and Treg cell development. Within the prospective birth cohort study LINA, we analyzed the expression of miR-155 and miR-223 together with Treg cell numbers in maternal blood samples during pregnancy, as well as in cord blood, considering maternal tobacco smoke exposure as an environmental effect.

METHODS

For more detailed information, please see the Methods section in this article's Online Repository at www.jacionline.org.

Study design

Six-hundred twenty-nine mother-child pairs (622 mothers and 7 twin pairs) were recruited within the prospective birth cohort study LINA from March 2006 until December 2008 in Leipzig, Germany. Blood samples were obtained during pregnancy (34th week of gestation), at birth (cord blood), and every year thereafter. Data on confounding variables, prenatal exposure, lifestyle factors, and children's disease outcomes were obtained from questionnaires filled in by the parents 4 weeks before birth and every year on the child's birthday. Atopic dermatitis was assessed by parental report of doctor-diagnosed atopic dermatitis: "Has a doctor diagnosed atopic dermatitis in your child during the last 12 months?" The present investigation comprises study participants with miRNA and Treg cell analyses, as well as volatile organic compound (VOC)/metabolite measurements (see Fig E1 in this article's Online Repository at www.jacionline.org). Participation was voluntary, and informed consent was given by the parents. This study was approved by the Ethics Committee of the University of Leipzig (file reference 046-2006, 160-2008).

VOC measurement

Indoor measurement of VOCs (benzene, toluene, m+p-xylene, and o-xylene) was performed in the dwellings of study participants between the 34th and 36th weeks of gestation by using passive samplers. For details, see the Methods section in this article's Online Repository.

Metabolite measurement

The toluene and benzene metabolites S-benzylmercapturic acid (SBMA) and S-phenylmercapturic acid (SPMA), respectively, and the nicotine metabolite cotinine were analyzed in the urine of pregnant mothers (34th week). For details, see the Methods section in this article's Online Repository.

Real-time PCR for miRNAs

Total RNA was isolated from cell cultures by using peqGOLD Trifast and from blood cells and serum by using peqGOLD RNAPure (PEQLAB Biotechnologie GmbH, Erlangen, Germany). The reverse transcription (RT) of miRNA into cDNA was performed by using the miRCURY LNA Universal RT kit, according to the manufacturer's instructions (Exiqon A/S, Vedbaek, Denmark). The amount of total RNA for RT was 100 and 200 ng for serum and cell culture/blood cell samples, respectively.

The expression of miRNA species was detected by means of PCR with the miRCURY LNA Universal RT miRNA PCR LNA PCR primer sets hsa-miR-155, hsa-miR-223 and the reference gene PCR primer set SNORD44 (hsa, Exiqon A/S). The expression of miRNA was calculated semiquantitatively by using the $\Delta\Delta$ cycle threshold method for cell culture and blood cell samples by normalizing toward a reference gene. Serum samples were normalized to the used serum volume of 100 μL and to the lowest measured value.

Quantification of Treg cells

Measurement of Treg cells numbers was performed by means of forkhead box protein 3 *(FOXP3)* methylation–specific real-time PCR. For details, see the Methods section in this article's Online Repository.

In vitro separation and activation of immune cell populations

Different immune cell populations (granulocytes, effector T [Teff] cells, Treg cells, and PBMCs) were isolated and analyzed for miR-155 and miR-223 expression. For details, see the Methods section in this article's Online Repository.

Statistical analysis

Statistical tests were performed with Statistica for Windows (Version 10.0; StatSoft [Europe], Hamburg, Germany). Because measured Treg cell numbers, VOCs, and metabolite concentrations were not normally distributed, a logarithmic transformation was performed. Medians of miRNA expression were compared by using the Wilcoxon test. Linear regression models were used to analyze the association between VOCs, metabolites, and miRNA expression in blood samples; to analyze the relation between smoking during pregnancy and VOC concentrations at home and metabolite concentrations in urine; and to analyze the relationship between miRNA expression (maternal blood, 34th week of gestation, and cord blood) and Treg cell numbers in maternal and cord blood. Linear regression models were adjusted for possible confounding factors given in detail in the figure legends. Data are presented as mean ratios (MRs), which are the back-transformed effects from the regression model of the logarithmically transformed outcome. For the relation between Treg cell numbers at birth (for this purpose, Treg cell numbers were categorized by median) and atopic dermatitis during the first 3 years of life, odds ratios were adjusted for month of birth, sex, maternal atopic dermatitis, maternal smoking/ETS exposure at home, siblings, parental education, cat and/or dog ownership, and breast-feeding until 6 months were calculated. We used maternal atopic dermatitis instead of parental atopy because maternal atopic dermatitis was found to be the stronger predictor for children's atopic dermatitis.[1] The χ^2 test was used to test the relationship between the analyzed subcohort and the entire LINA cohort. All P values of less than .05 were considered significant.

RESULTS

Characteristics of the study population

Characteristics of the study population are listed in Table I. There were no differences in the distribution of considered