

Demethylation of the *FCER1G* promoter leads to FcεRI overexpression on monocytes of patients with atopic dermatitis

Y. Liang, P. Wang, M. Zhao, G. Liang, H. Yin, G. Zhang, H. Wen & Q. Lu

Hunan Key Laboratory of Medical Epigenomics, Department of Dermatology, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China

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Keywords

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Correspondence

Qianjin Lu, MD, PhD, Hunan Key Laboratory of Medical Epigenomics, Department of Dermatology, The Second Xiangya Hospital, Central South University, #139 Renmin Middle Rd, Changsha, Hunan 410011, China.

Tel.: 86 13787097676

Fax: 86 731 85533525

E-mail: dermatology20091@yahoo.cn

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Abstract

Background: Overexpression of the high-affinity receptor for immunoglobulin E on atopic monocytes and dendritic cells is known to contribute to the pathogenesis of atopic dermatitis (AD). However, it remains unclear what is the underlying mechanism of FcεRI deregulation. It has been speculated that epigenetic deregulation may play a role.

Methods: Global DNA methylation levels of monocytes from 10 AD patients and 10 healthy controls were measured using a global DNA methylation kit. Bisulfite sequencing was performed to determine the methylation status of the *FCER1G* promoter region. FcεRIγ mRNA and FcεRI protein levels were detected by real-time RT-PCR, Western blotting, and flow cytometry, respectively. Patch methylation and the demethylating agent, 5-azacytidine, were used to determine the functional significance of methylation changes on FcεRI expression.

Results: Monocytes from AD patients show a global hypomethylation, as well as a locus-specific hypomethylation at *FCER1G* promoter, as compared to healthy controls. Furthermore, this hypomethylation of *FCER1G* is inversely correlated with its expression. Patch methylation in combination with luciferase reporter assay confirmed the direct relationship between methylation and expression. Moreover, treating healthy monocytes with 5-azacytidine caused a reduction in methylation levels and an induction in FcεRIγ transcription and surface expression of FcεRI.

Conclusion: Demethylation of specific regulatory elements within the *FCER1G* locus contributes to FcεRI overexpression on monocytes from patients with AD.

The high-affinity receptor for IgE (FcεRI) is strongly up-regulated on antigen-presenting cells (APCs) of patients with atopic dermatitis (AD). It is well accepted that this up-regulation plays an important role in the pathophysiological changes leading to hyperallergic responses in patients with AD. FcεRI at the surface of myeloid-derived APCs (monocytes and dendritic cells) presents IgE-bound antigens to T cells, thus leading to hypersensitivity reactions. The FcεRI form present on human APCs is a trimeric protein made up of one FcεRIα and two FcεRIγ subunits. The gamma subunits are postulated to be the key determinant of maturation and surface expression levels of FcεRI on APCs. Because of the critical role of gamma subunit in up-regulating FcεRI on

atopic APC, there has been considerable interest in revealing factors that regulate the FcεRI gamma subunit gene (*FCER1G*) (1, 2).

Allergies are on the rise worldwide, and environmental factors are thought to be crucial to this epidemiological phenomenon (3). Environmental factors can contribute to the onset as well as the progression of allergic disorders, such as asthma, by disrupting the epigenetic regulation of immunological gene activities (4). DNA methylation at CpG dinucleotides in the 5'-region of genes plays a critical role in regulating gene expression. The absence of methylation at CpG islands within gene regulatory elements is indicative of transcriptional activity, whereas methylation of cytosines

within these islands results in gene silencing (5). Two cis-elements (−445/−366 bp and −365/−264 bp) had been identified to be involved in the regulation of *FCER1G* gene transcription, both of which are part of an Alu repeat (6). Alu repeats are rich in CpG dinucleotides and are the primary targets for DNA methylation, which keep gene silencing and thereby prevent genomic instability (7). In this study, we investigated the putative role of DNA methylation in the regulation of FcεRIγ expression in monocytes of patients with AD. For our experiments, we chose to analyze monocytes instead of dendritic cells, because native dendritic cell numbers were too low to perform DNA methylation and correlation analyses.

Materials and subjects

Patients with AD ($n = 10$, 13–38 years, mean age: 20.9 years) were recruited from the outpatient dermatology clinic and in-patient ward at the Second Xiangya Hospital, Central South University. Atopic dermatitis diagnoses were determined using the Hanifin and Rajka's diagnostic criteria (8), and disease activity was assessed using the SCORing Atopic Dermatitis (SCORAD) index (9). Healthy control subjects ($n = 10$, 20–34 years, mean age: 23.9 years) were recruited from the medical staff at the Second Xiangya Hospital. Patients and controls were age and sex matched in all experiments, and monocyte samples from each group were paired and studied in parallel. This study was approved by the human ethics committee of the Central South University Xiangya Medical College, and written informed consent was obtained from all subjects. The information of AD patients and control subjects is provided in Table 1.

Table 1 Demographics and medications for patients and control subjects

Sample no.	Age/gender	SCORAD	System medications
P1	28/F	37.5	Antihistamine
P2	23/F	61.4	Medical herbs
P3	16/M	40.1	None
P4	13/F	49.9	None
P5	15/M	39.8	Antihistamine
P6	16/F	35.9	None
P7	38/M	44.6	None
P8	19/F	57.2	Antihistamine
P9	25/F	43.2	None
P10	16/F	53.6	None
C1	20/F	–	–
C2	20/M	–	–
C3	28/F	–	–
C4	24/M	–	–
C5	28/F	–	–
C6	21/F	–	–
C7	20/F	–	–
C8	24/F	–	–
C9	34/M	–	–
C10	20/F	–	–

Monocytes isolation

A total of 60 ml of venous peripheral blood was drawn from each patient and control subject and preserved in heparin. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Hypaque density gradient centrifugation (Hengxin Chemical Reagent Co., Shanghai, China). CD14⁺ monocytes were isolated by positive selection using magnetic beads, according to the protocol provided by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany).

5-azaC treatment

Healthy donor monocytes were cultured in RPMI-1640 containing 0.3 g/l l-glutamine (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) fetal calf serum (FCS, heat inactivated; Sigma Aldrich), 100 U/ml penicillin (Sigma Aldrich), 0.1 mg/ml streptomycin and 1 mg/ml LPS (Sigma Aldrich). Twelve hours after plating, cells were treated with 3 μM of 5-azacytidine (Sigma Aldrich) or vehicle control in growth medium for 3 days.

RNA Isolation and real-time qPCR

Total RNA was extracted from monocytes using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer. Real-time PCR was performed using a Rotor-Gene 3000 Real-time PCR instrument (Corbett Research, Australia). FcεRIγ and β-actin mRNA were amplified by SYBR real-time PCR using the One Step PrimeScript RT-PCR Kit (TaKaRa Biotech, Dalian, China). β-actin mRNA was used as the internal control. The PCR primers were 5'-AGCTGGCCCTACCCCTA-TAA-3' and 5'-TTAGCTGGAGTTGGGAATGG-3' for human FcεRIγ gene and 5'-GCACCACA CTTCTACAAT-GAGC-3' and 5'-GGATAGCACAGCCTGGATAGCAAC-3' for human β-actin gene. Relative FcεRIγ mRNA expression normalized to β-actin mRNA was calculated using the equation: $2^{-\Delta C_t}$, where $\Delta C_t = (C_{T_{Fc\epsilon RI\gamma}} - C_{T_{\beta\text{-actin}}})$ (10). The fold difference in FcεRIγ mRNA expression between 5-azaC-treated and 5-azaC-untreated monocytes normalized to β-actin mRNA was calculated using the following equation: $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_{T_{Fc\epsilon RI\gamma}} - C_{T_{\beta\text{-actin}}})_{\text{treated}} - (C_{T_{Fc\epsilon RI\gamma}} - C_{T_{\beta\text{-actin}}})_{\text{untreated}}$ (11).

Flow cytometric analysis

CD14⁺ monocytes suspensions (1×10^5 cells) were incubated with anti-human FcεRIα antibodies (Upstate, Lake Placid, NY, USA) for 30 min at room temperature, then washed four times in 2 ml PBS/BSA and centrifuged at 400 *g* for 5 min. Cells were then resuspended and incubated in solution containing PE-labeled secondary antibodies (Abcam, Cambridge, UK) for 30 min at room temperature before being washed another four times. Fluorescence intensities were determined using a FACScalibur system (Becton Dickinson, Franklin Lakes, NJ, USA) and CellQuest software (Becton Dickinson). FcεRI expression levels were evaluated by

calculating the mean fluorescence intensity (MFI) and the proportion of positive cells.

Western blotting

Monocytes were lysed, and proteins were extracted and separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked in TBST buffer containing 5% nonfat dry milk, blotted with the indicated antibodies, and developed with Lumi-nol/Enhancer Solution (Pierce, Rockford, IL, USA). FcεRIγ and β-actin antibodies were purchased from Upstate (1 : 200) and Santa Cruz Biotechnology (1 : 2000; CA, USA). Band intensities of FcεRIγ and β-actin protein were quantified using Quantity one software (Bio-Rad, Hercules, CA, USA), and the normalized intensity of FcεRIγ is the ratio of background-corrected raw intensities of protein of FcεRIγ/β-actin.

Genomic DNA extraction and measurement of global DNA methylation

Genomic DNA was isolated from monocytes using the TIANamp Genomic DNA kit (Tiagen Biotech, Beijing, China). Global DNA methylation was measured using the MethyLamp™ Global DNA Methylation Quantification Kit and instructions provided by the manufacturer (Epigentek Group, Farmingdale, NY, USA). This kit yields accurate measures of methylcytosine content as a percentage of total cytosine content. Briefly, DNA was immobilized on a strip well specifically treated to have a high affinity for DNA. DNA methylation was then quantified by an enzyme-linked immunosorbent assay-like reaction using a 5-methylcytosine antibody. The amount of methylated DNA is proportional to the optical density (OD) intensity, and the degree of DNA methylation can be calculated using the following formula:

$$\% \text{Methylation} = (\text{OD (sample-blank)} / 2) / \text{OD (positive control-blank)} \times 100\%$$

where 'blank' is buffer without DNA and 'positive control' is methylated control DNA.

Bisulfite sequencing

Genomic DNA bisulfite conversion was performed using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA, USA). The 158-bp (−465 to −308 bp) *FCER1G* promoter fragment was amplified by nested PCR and cloned into pGEM-T vector (Promega, Madison, WI, USA). Ten independent clones were sequenced for each of the amplified fragments. The first-round PCR primers were 5'-GTTTAAGATTAGTTTGATT AATATGGAGAA-3' and 5'-ACCCACAAAATAAA AAA AATTTTAC-3', and the second-round PCR primers were 5'-GGAGAAATTTTATT TTTATTAAAAAT-3' and 5'-AT TACCCAACTAAAATACAATAAC-3' for *FCER1G* promoter fragment.

Patch methylation and luciferase activity assay

A 239-bp fragment containing the human *FCER1G* promoter (−486 to −248 bp) was cloned into *pGL3-Basic* (Promega, Madison, WI, USA). The regions of interest were then excised using the appropriate restriction endonucleases (*KpnI* and *BglII* both from NEB, Ipswich, MA, USA), gel purified, and methylated with *SssI* and S-adenosylmethionine (NEB) using instructions provided by the manufacturer. Methylated fragments were then ligated back into the expression constructs upstream of the luciferase ORF, and methylation was confirmed by digesting with the methylation-sensitive restriction endonucleases *AciI* (NEB). Controls included mock-methylated constructs similarly generated but omitting the *SssI*. The methylated or mock-methylated *pGL3-FCER1G* constructs were then transfected into THP-1 cells by electroporation, and β-galactosidase luciferase expression constructs (*pRL-CMV*; Promega) were used as transfection controls. Twenty-four hours later, the cells were washed twice with PBS, suspended in 400 μl of reporter lysis buffer (Promega), and lysed by freezing and thawing. Insoluble material was removed by centrifugation, and luciferase or β-galactosidase levels were measured. We provide a more detailed description of this procedure in a previous publication (12).

Statistical analysis

To test the differences in methylation levels and FcεRIγ expression, we compared both patients with AD and healthy controls by using independent-samples *T*-test. Quantitative values were compared with both 5-azacytine-treated group and 5-azacytine-untreated control group by using paired samples *T*-test, all *T*-test results are given as mean ± SD, respectively. Correlation analysis between the methylation level of *FCER1G* promoter and FcεRIγ expression was studied by using Pearson's correlation coefficient. All tests were two-tailed, and *P* < 0.05 was considered statistically significant. Statistical analysis was performed with SPSS 16.0 software (SPSS, Chicago, IL, USA).

Results

Global DNA methylation and methylation of the *FCER1G* promoter region in monocytes of patients with AD

To assess global DNA methylation levels in patients with AD, CD14⁺ monocytes were isolated from 10 AD patients and 10 age- and sex-matched healthy control subjects. We observed a significant decrease in global methylation level in CD14⁺ monocytes from AD patients as compared to the healthy controls (*P* = 0.019, Fig. 1A).

Because of the critical role of FcεRIγ chain in determining the surface expression on monocytes and DC, we next detected the methylation levels of the *FCER1G* promoter regulatory elements. A 158-bp (−465 bp to −308 bp) sequence encompassing two above-mentioned regulatory cis-elements has been analyzed by using bisulfite sequencing, and the mean methylation level of this region (including all of eight

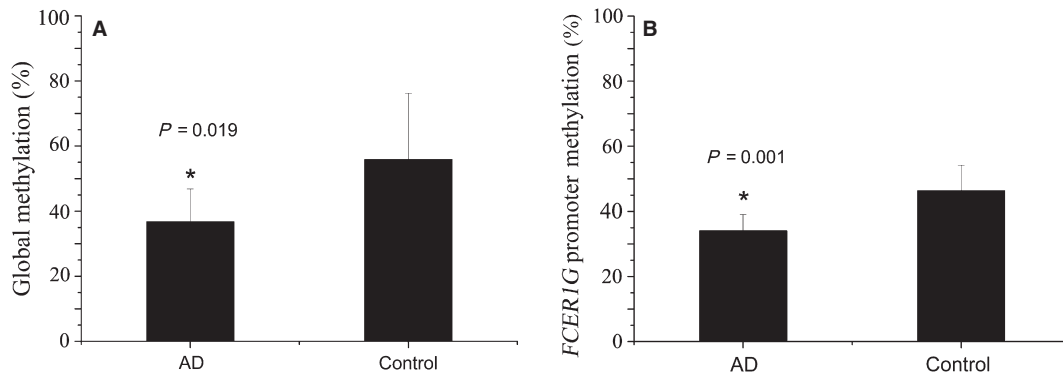


Figure 1 Reduced global DNA methylation and *FCER1G* methylation in monocytes of patients with atopic dermatitis (AD). (A) Mean ($\pm$ SD) global methylation (%) in CD14⁺ monocytes from AD patients and control subjects. Global DNA methylation was measured using the Methylamp™ Global DNA Methylation Quantification Kit. (B)

CpG pair sites at position -428, -426, -392, -375, -358, -349, -341, -333 bp) was statistically significantly reduced in monocytes of patients with AD relative to healthy control monocytes ($P = 0.001$, Fig. 1B).

Increased FcεRIγ expression inverse correlation with *FCER1G* promoter methylation in monocytes of patients with AD

We found that FcεRIγ mRNA and protein levels were both increased in monocytes of patients with AD as compared to healthy control monocytes (Fig. 2A,B). Furthermore, we found that the average methylation level of all eight CG pairs in the 158-bp *FCER1G* promoter fragment was inversely related to FcεRIγ expression in monocytes from patients with AD ($n = 10$, $R = -0.711$, $P = 0.021$, Fig. 2C) and healthy controls ($n = 10$, $R = -0.697$, $P = 0.025$, Fig. 2D).

Effect of methylation on *FCER1G* activation

Having demonstrated the inverse correlation between DNA methylation and expression in *FCER1G*, we next wanted to test whether methylation plays a direct role in silencing *FCER1G*. 'Patch' methylation in combination with luciferase assay was utilized. A 239-bp fragment from the *FCER1G* promoter was cloned into *pGL3-Basic* vector, and then the regions were excised and methylated *in vitro* with *SssI* and S-adenosylmethionine, prior to ligating back into the expression construct. Controls included β -galactosidase controls as well as mock-methylated constructs. Low luciferase activities were observed when the *FCER1G* promoter was methylated, indicating that methylation suppresses promoter activity (Fig. 3).

Effect of inhibiting DNA methylation in monocytes on FcεRI expression

To test the relationship between *FCER1G* promoter demethylation and gene expression induction, as well as the subsequent increase in surface FcεRI expression, we treated

monocytes from healthy control donors with the DNA methylation inhibitor 5-azacytidine and measured FcεRIγ and surface FcεRI expression 72 h later. We observed a reduction in *FCER1G* promoter methylation levels (Fig. 4A), an increase in FcεRIγ expression, and a corresponding increase in surface FcεRI expression (Fig. 4B–E).

Discussion

In this study, we found that CD14⁺ monocytes from patients with AD were globally hypomethylated relative to healthy controls. Furthermore, methylation within the *FCER1G* promoter was significantly reduced in monocytes from patients with AD, and this change correlated with an up-regulation in FcεRIγ mRNA and protein levels. We confirmed the direct relationship between *FCER1G* promoter methylation and its expression using patch methylation in combination with luciferase assay. Furthermore, we found that when methylation was inhibited in healthy monocytes with 5-azacytidine, the FcεRI expression increased. Together, these results suggest that the demethylation of specific regulatory elements contributes to FcεRI overexpression on monocytes from patients with AD, which in turn leads to hyperallergic responses.

Atopic monocytes with high levels of FcεRI are thought to play an important role in the pathogenesis of AD. This is because FcεRI-bearing monocytes might differentiate into inflammatory dendritic epidermal cells (IDEC), which amplify allergic inflammatory reactions in skin by stimulating T cells, and may also be involved in switching from Th2-pre-dominant early phase AD to chronic AD (1, 13). To our knowledge, our study shows for the first time that changes in the epigenetic regulation of the *FCER1G* gene may provide an explanation for the pathological up-regulation of FcεRI on monocytes of patients with AD, but it is difficult further to analyze the DNA methylation status of IDEC from patients with AD because of limited amount of IDEC. This finding is particularly interesting in light of the other research showing that epigenetic changes are key to the establishment and maintenance of the Th2 bias in AD (14).

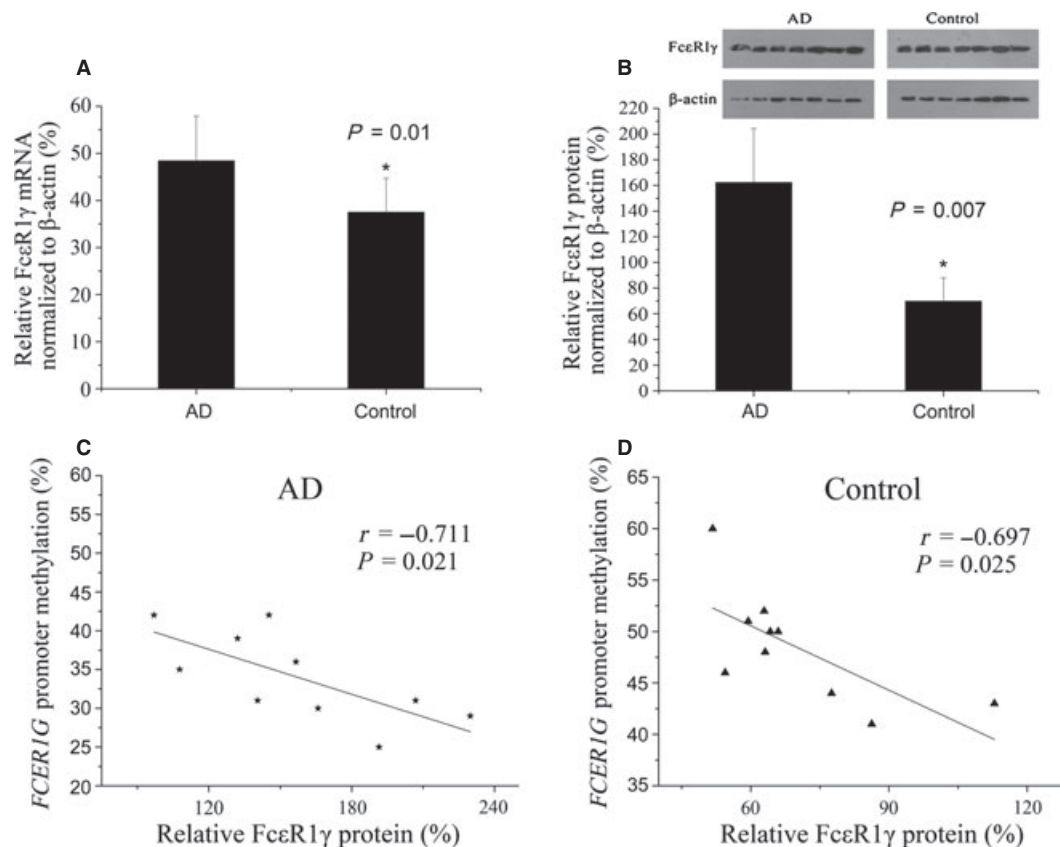


Figure 2 Increased FcεRIγ expression is inversely correlated with FCER1G promoter methylation in monocytes of patients with atopic dermatitis (AD). (A) FcεRIγ mRNA levels in CD14⁺ monocytes from the patients with AD ($n = 10$) and healthy controls ($n = 10$) were assessed by quantitative real-time PCR and calculated using the equation: $2^{-\Delta\Delta C_T}$, where $\Delta C_T = (C_{T_{Fc\epsilon RI\gamma}} - C_{T_{\beta\text{-actin}}})$. Relative FcεRIγ mRNA expression was normalized to the percentage (%) of β-actin mRNA input copy number. Values represent mean $\pm$ SD. (B) Quantification of band intensities in FcεRIγ Western blots of protein lysates from CD14⁺ monocytes of patients with AD ($n = 10$) and healthy

controls ($n = 10$). Band intensities of FcεRIγ were normalized to the percentage (%) of β-actin band intensity. Values represent mean $\pm$ SD. (C) Correlation between the mean methylation level (%) of the FCER1G promoter (Y-axis) and relative FcεRIγ protein expression (X-axis) in CD14⁺ monocytes from the patients with AD ($n = 10$). (D) Correlation between the mean methylation level (%) of the FCER1G promoter (Y-axis) and relative FcεRIγ protein expression (X-axis) in CD14⁺ monocytes from healthy controls ($n = 10$). Atopic dermatitis and healthy controls were compared using independent-samples *T*-test; correlation was analyzed using Pearson's correlation coefficient.

Allergy-relevant changes in global or gene-specific methylation patterns might be induced by environmental factors. For example, children with prenatal tobacco exposure display a global hypomethylation at the AluYb8 repeat element in buccal cells (15). Benzopyrene, a polycyclic aromatic hydrocarbon (PAH), can decrease global DNA methylation, inhibit DNA methyltransferase (DNMT) expression *in vitro*, and interfere with recruitment of the methylation machinery (16–18). Another study has suggested that diesel exhaust causes hypermethylation of CpG sites within the IFN-γ promoter and hypomethylation of the IL-4 promoter and correlates with increased induction of IgE in response to intranasal challenge with the allergen *Aspergillus fumigatus* (19).

It will be of interest to investigate whether the changes in global and FCER1G promoter methylation that we observed

in our atopic monocyte group correlate with exposure to specific environmental factors. Interestingly, in a mouse model of allergic asthma, exposure to dust mite antigens activates TLR4 and increases the expression of a unique set of miRNAs that includes miRNA-16, miRNA-21, and miRNA-126. Selective blockade of miRNA-126 leads to amelioration of asthma symptoms and a diminished Th2 response, inflammation, and airway hyper-responsiveness through an miR-126-mediated suppression of GATA-3 expression (20). Furthermore, in a previous study, we found that miR-126 regulates DNA methylation and induces DNA hypomethylation by targeting DNMT1 in CD4 T cells of patients with lupus (21). Together these results illustrate the possibility that allergen-induced changes in TLR4-miR126 pathway activity lead to monocyte DNA hypomethylation and subsequent hyperallergic responses.

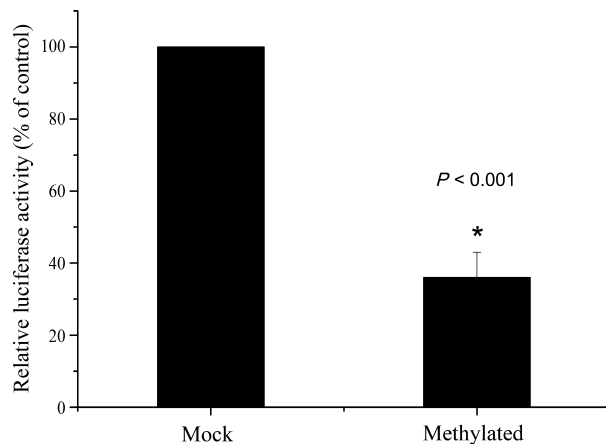


Figure 3 Effect of promoter methylation on *FCER1G* gene activity. Comparison of luciferase activity in THP-1 cells cotransfected with a pRL-CMV vector containing β -galactosidase and a pGL3 vector containing a luciferase gene driven by a methylated or mock-methylated *FCER1G* promoter fragment (–486 to –248 bp). After 24 h, luciferase activity in cell lysates was determined, related to β -galactosidase activity, and relative luciferase activity was normalized to the activity of a mock-methylated control pGL3 vector. Results are presented as the ratio (%) of luciferase activity to mock-methylated control. Values represent mean $\pm$ SD (three independent experiments per group).

Highly publicized studies have shown a link between the use of folic acid supplements during early pregnancy and increased risk of asthma, wheezing, and respiratory disease in children (22–24). The above view was challenged by a subsequent study, which showed that higher serum folate levels are associated with a lower risk of high total serum IgE concentrations, atopy, and wheezing (25). Shedding light on this controversy are findings suggesting that polymorphisms in the folate metabolism gene methylenetetrahydrofolate reductase (*MTHFR*) are in association with atopy, asthma, or both (26). The seemingly conflicted findings highlight the need for more further research on effects of folic acid supplementation on the pathogenesis of allergic diseases. Interestingly, higher serum folate levels might increase DNMT1 expression and activity and thereby prevent DNA hypomethylation (27, 28). Thus, the timing of methyl donor supplementation might play a pivotal role in determining its effect on the development of allergic disease.

In summary, our results reveal that demethylation of specific regulatory elements within the *FCER1G* locus contributes to FcεRI overexpression on monocytes from patients with AD. These findings should help elucidate the role of epigenetic changes in the pathogenesis of AD. Further research is necessary to determine the causes leading to the deregulation of epigenetic gene regulatory mechanisms in patients

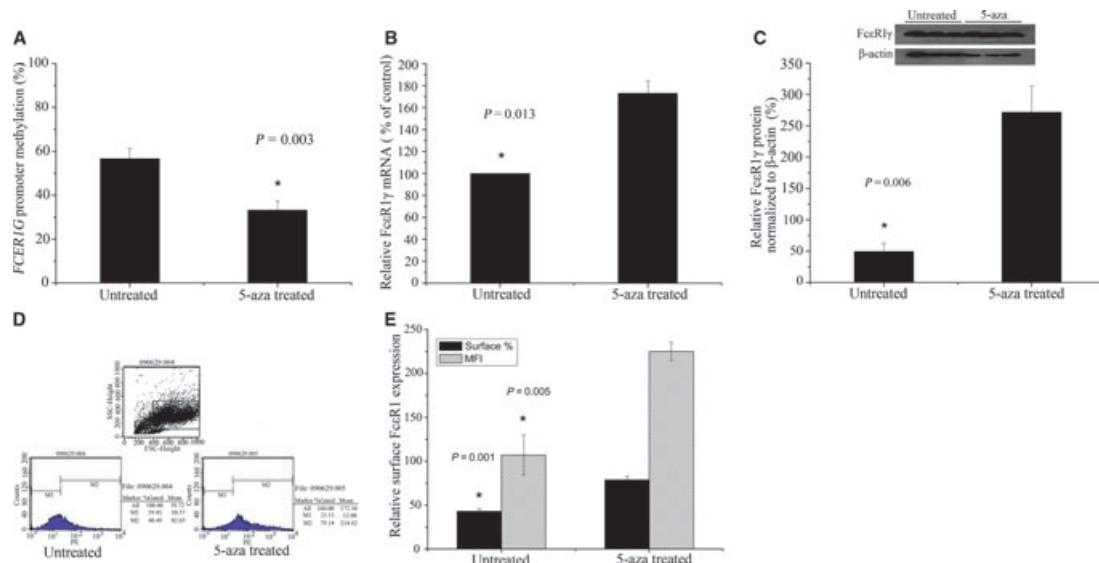


Figure 4 Effect of inhibiting DNA methylation in monocytes on FcεRI expression. CD14⁺ monocytes from three individual healthy donors were treated with 5-azacytidine (5-azaC treated) or vehicle control (untreated) for 72 h. (A) The mean methylation percentage (%) within the *FCER1G* promoter regulatory element in 5-azaC-treated and in 5-azaC-untreated CD14⁺ monocytes was determined using bisulfite sequencing. (B) FcεRI γ mRNA expression levels in 5-azaC treated and in untreated CD14⁺ monocytes were assessed by quantitative real-time PCR. The percentage (%) of FcεRI γ mRNA was calculated by $2^{-\Delta\Delta C_t}$ -method normalized to β -actin, relative to

FcεRI γ mRNA levels of untreated control. Values represent mean $\pm$ SD. (C) Quantification of band intensities in FcεRI γ western blots of protein lysates from 5-azaC-treated and 5-azaC-untreated CD14⁺ monocytes. Band intensities of FcεRI γ were normalized to the percentage (%) of β -actin band intensity. Values represent mean $\pm$ SD. (D)–(E) FACS analysis of the percentage of cells expressing FcεRI (surface %) and the mean fluorescent intensity (MFI) in 5-azaC-treated and in 5-azaC-untreated CD14⁺ monocytes. All comparison analyses between 5-azaC-treated and 5-azaC-untreated were performed by paired samples *T*-test.

with AD, as this may lead to the development of novel therapeutic interventions for AD.

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Conflict of interest

The authors have no financial conflicts of interest.

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