

Single nucleotide polymorphisms of the *IL18* gene are associated with atopic eczema

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Background: Human IL-18 is an inflammatory cytokine that plays a role in atopic diseases, such as atopic eczema (AE), by enhancing IL-4 and IL-13 production and stimulating the synthesis of IgE.

Objective: To evaluate associations of polymorphisms in the *IL18* gene on chromosome 11q22 with AE, we performed genotyping for single nucleotide polymorphisms (SNPs) in the *IL18* gene in 225 patients with AE and 175 healthy control volunteers.

Methods: Genotyping was performed by means of restriction fragment length polymorphism analysis.

Results: Analyses revealed significant associations of SNPs +113[t/g] and +127[c/t] in exon 1, -137[g/c] in promoter region 1, and -133[c/g] in promoter region 2 with AE. These associations were not directly dependent on a specific subtype of AE or the concomitant manifestation of allergic rhinitis or asthma. On the functional level, the amount of IL-18 in the supernatants of PBMCs of patients with AE stimulated with *Staphylococcus aureus* enterotoxin B was significantly higher than that in healthy control subjects. In parallel, the amount of active IL-18 in the sera of patients with AE was enhanced at the exacerbation of their disease.

Conclusion: In conclusion, our data suggest that SNPs in the *IL18* gene might be involved in the development of AE by contributing to a functional dysregulation of the IL-18 production *in vivo*. (J Allergy Clin Immunol 2005;115:828-33.)

Key words: *IL-18, IgE, atopic eczema, allergy, polymorphism*

Atopic eczema (AE) is a chronic inflammatory skin disease that is strongly affected by its genetic background.¹ About 80% of adult patients with AE have sensitizations against common aeroallergens and food allergens with high levels of allergen-specific and total serum IgE.^{2,3} Learning more about the specific genetic

Abbreviations used

AE: Atopic eczema

SEB: *Staphylococcus aureus* enterotoxin B

SNP: Single nucleotide polymorphism

predisposition underlying AE and its pathophysiologic characteristics is necessary to elucidate predictive genetic factors and allow the identification of patients at risk at early stages of life.

IL-18 is produced by a wide range of immune cells, such as monocytes, macrophages, and dendritic cells, and has been recognized as an important regulator of the innate and the acquired immune system.⁴ To be effective, the inactive precursor of IL-18 has to be cleaved by caspase 1 (IL-1-converting enzyme) into its active variant. As a characteristic feature, IL-18 plays a paradigmatic role in the immunologic network: in synergy with IL-12, it induces the production of IFN- γ in T_H1 cells, B cells, and natural killer cells, thereby stimulating T_H1-mediated immune responses and inhibiting IgE synthesis (Fig 1).⁵⁻⁷ In previous studies, single nucleotide polymorphisms (SNPs) in the *IL18* gene⁸ have been associated with different chronic inflammatory diseases, such as Crohn's disease⁹ or diabetes mellitus.¹⁰ In contrast, animal studies have indicated that IL-18 might also initiate T_H2 responses and upregulate IgE production through stimulation of IL-4 and IL-13 synthesis in mast cells and basophils (Fig 1).⁴ In line with this, increased levels of IL-18 have been found in the sera of patients with atopic diseases.¹¹⁻¹⁴ Furthermore, it has been shown that binding of IL-18 to the IL-18 receptor induces the IL-1 receptor-associated kinase/TNF receptor-associated factor 6 signal transduction pathway, leading to nuclear factor κ B activation, which might cause upregulated expression of cytokines like IL-4 and IL-13.^{15,16} The gene for human IL-18 is located at chromosome 11q22.2-22.3, a region that has been linked to atopy-related traits.¹⁷ However, recently, no association could be found with juvenile idiopathic arthritis and bronchial asthma.¹⁸ The *IL18* gene is composed of 6 exons, and its expression is regulated by 2 distinct promoters upstream of exon 1 (promoter 1) and exon 2 (promoter 2),¹⁵ with the start codon located in exon 2. In the present study we investigated SNPs in promoter 1 (-137[g/c]), promoter 2 (-133[c/g] and -132[a/g]), and exon 1 (113[t/g] and 127[c/t]). Recently, the SNP +127(g/t) in exon 1 has been designated as a susceptibility locus

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for seasonal allergic rhinitis, whereas SNPs +113 [t/g] and +127 [g/t] in exon 1 and -137 [g/c] in promoter 1 have been shown to be associated with high IgE serum levels and specific sensitizations.¹⁹

In this study we conducted a case-control study to evaluate possible associations of SNPs in the *IL18* gene with susceptibility to AE and its specific subtypes.

METHODS

Patients and diagnostic criteria

A total of 225 patients with AE²⁰ and 175 nonatopic healthy volunteers were recruited. The protocol was approved by the local ethics committee. AE was diagnosed on the basis of the diagnostic criteria of Hanifin and Rajka.²¹ The atopic status and the presence of concomitant allergic rhinoconjunctivitis and asthma and the disease severity were quantified by using the Diepgen score²² and the SCORAD system,²³ respectively. The same physician did the complete clinical scoring of all donors.

The patient collective used for genotyping consisted of 400 individuals. The genotype of the individuals was determined by using restriction fragment length polymorphism after PCR amplification.

Isolation of genomic DNA

Genomic DNA was isolated from peripheral blood, as reported elsewhere. Briefly, the whole blood was lysed, washed, and digested with proteinase K (Roche, Basel, Switzerland). After digestion, the solution was extracted 2 times with ethanol. DNA was ethanol precipitated and resuspended in TRIS buffer, as described previously.²⁴

Restriction fragment length polymorphism analysis

PCR for genotyping was performed with mutated primers, introducing restriction site for restriction.

Fragment polymorphism analysis of the respective genotype is shown in Table I. All enzymes were from NEB (Frankfurt, Germany). Restriction was performed in a volume of 10 μ L containing 5 μ L of PCR products and the buffer recommended by the supplier for 90 minutes at 37°C. The fragments were resolved on 12% polyacrylamide gel. The genotyping was performed blinded to the phenotypes. Restriction analysis was performed in duplicate, and SNPs were segregated correctly (Table I).

ELISA assay

Serum samples were collected in separator tubes and allowed to clot for 30 minutes before centrifugation for 10 minutes at approximately 1000g, and then they were stored at -20°C. ELISA assays were performed according to the manufacturers' instructions (R&D Systems, Wiesbaden, Germany). The sensitivity ranged from 25.6 to 1000 pg/mL for IL-18 and from 7.8 to 250 pg/mL for IL-12.

Analysis of total serum IgE and allergen-specific IgE levels

Serum levels of total and allergen-specific IgE against *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, birch pollen, timothy grass pollen, cat dander, hazelnut, peanut, milk, egg, *Aspergillus fumigatus*, *Candida albicans*, *Pityrosporum ovale*, and codfish in the sera of the patients were analyzed with the Immulite system (DPC Biermann, 61231 Bad Nauheim, Germany).

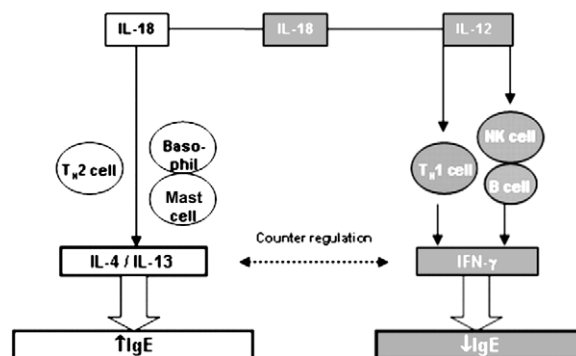


FIG 1. Summary of the role of IL-18 and IL-12 in the regulation of IgE synthesis and T_H1/T_H2 immune responses: schematic diagram summarizing the interaction of IL-18 and IL-12 in the immune system and the regulation of the IgE synthesis. NK, Natural killer cell.

Cell preparation

PBMCs were isolated from 20 mL of heparinized blood of patients with AE and healthy control donors by using Lymphoprep (Progen, Heidelberg, Germany). PBMCs were resuspended at a density of 10⁶/mL in RPMI-1640 medium (Biochrom, Berlin, Germany) supplemented with heat-inactivated FCS, L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μ g/mL). PBMCs (10⁶/mL) were cultured in the presence or absence of 100 ng/mL *Staphylococcus aureus* enterotoxin B (SEB; Sigma Chemical Co, St Louis, Mo) for 24 hours in a final volume of 1 mL in a round-bottom tube (Falcon 2059; Becton Dickinson Labware, Lincoln Park, NJ) in a humidified atmosphere containing 5% CO₂.

Statistical analysis

Statistical analysis with the Wilcoxon test was performed with SPSS 12.0 for Windows (SPSS, Chicago, Ill). Calculated values shown were percentages of positive cells or means $\pm$ SEM. Additionally, the frequencies of the genotypes between the different groups were compared by using the Cochran-Armitage trends test.²⁵ Odds ratios of the wild-type allele and the heterozygote mutant allele against the homozygote mutant allele are given.

RESULTS

A total of 225 patients with AE were included in this study. One hundred ninety-one of these patients were classified as having an extrinsic subtype of AE with concomitant allergic rhinitis (n = 186), allergic asthma (n = 44), or both. The average age of these patients was 30.8 $\pm$ 13 years. The total serum IgE level in the extrinsic subgroup was 1399 $\pm$ 1796.3 kU/L, and the SCORAD score was 47.9 $\pm$ 22.11. The patients of the intrinsic nonatopic subgroup (n = 34) were at an average age of 26.3 $\pm$ 12.1 years, the total serum IgE level was 58.49 $\pm$ 121.59 kU/L, and the SCORAD score was 34.9 $\pm$ 18.5. None of the patients of the intrinsic nonatopic subgroup had any sensitizations against aeroallergens or food allergens, concomitant allergic rhinitis, or allergic asthma. A total of 175 healthy donors without any phenotype of AE (average age, 41 $\pm$ 18.4 years) has been used as control group.

TABLE I. Overview of primers used for restriction fragment polymorphism analysis

Polymorphism	Primer	Annealing temperature	Enzyme
Exon 1, 113	5'-ccaaggagtgccgacagcagctct-3'; 5'-ggggcagccgcttagcagctag-3'	50°C	<i>NheI</i>
Exon 1, 127	5'-atgcttctaatggactaagga-3'; 5'-agactgcagcaggtgtgtgcc-3'	56°C	<i>MscI</i>
Promoter 1, -137	5'-atgcttctaatggactaagga-3'; 5'-gtaatactactatttcatgaatt-3'	50°C	<i>EcoRI</i>
Promoter 2, -133	5'-gtattcataagctgaaactcccg-3'; 5'-tggtctatggcattagccttac-3'	53°C	<i>SmaI</i>
Promoter 2, -132	5'-gtattcataagctgaaactcccg-3'; 5'-tggtctatggcattagccttac-3'	53°C	<i>AseI</i>

TABLE II. Allelic frequency of common polymorphisms in the human *IL18* gene in subtypes of AE

Frequency (%)	Group 1	Group 2	Group 3	Group 4
Exon 1 -113G*	28.74	30.21	31.08	19.76
Exon 1 127T*	28.61	28.57	29.49	19.23
Promoter 1 -137C*	29.09	29.59	32.89	18.59
Promoter 2 -132G	24.55	21.88	20.51	25.0
Promoter 2 -133G*	33.43	34.69	30.77	25.42

Percentages given represent the allelic frequency of the polymorphic alleles differing from the published wild type within each group.

Group 1, Patients with extrinsic AE and sensitizations against house dust mite; group 2, patients with extrinsic AE without sensitizations against house dust mite; group 3, patients with nonatopic AE without any sensitizations; and group 4, healthy, nonatopic control subjects.

Polymorphisms in the *IL18* gene are more frequent in patients with AE

To evaluate the allelic frequencies of the polymorphic alleles in the regulatory region of the *IL18* gene, we performed restriction fragment length polymorphism PCR experiments. Table II shows the genotype and allele distributions of all 5 polymorphisms investigated.

Frequencies of polymorphic alleles 113[t/g] and 127[c/t] in exon 1, -137[g/c] in promoter 1, and -133[c/g] in promoter 2 of the *IL18* gene significantly differed between patients with the extrinsic subtype of AE with sensitizations against house dust mite (group 1), patients with extrinsic AE without sensitizations against house dust mite (group 2), and patients with the nonatopic intrinsic variant of AE (group 3) in comparison with healthy control volunteers (group 4; Tables II and III). Patients with AE with genetic variations in the *IL18* gene regions studied did not differ in regard to sex, SCORAD score, or serum IgE level (Table IV).

PBMCs of patients with AE stimulated with SEB produce high amounts of IL-18

To assess the capacity of PBMCs of patients with AE and healthy control volunteers to produce active IL-18 protein, functional experiments with PBMCs of patients with AE without genetic variations at the *IL18* gene sites studied here; patients with AE with allelic variations at regions +113[t/g], +127[c/t], -137[g/c], and -133[c/g] of the *IL18* gene; and healthy control donors have been performed. Analyses of the cell culture supernatants of these PBMCs after 24 hours of stimulation with SEB revealed a significantly enhanced amount of IL-18 pro-

TABLE III. Results of association analyses of subtypes of AE with *IL18* SNPs

Groups 1, 2, and 3 vs group 4	Trend test	Odds ratio	Confidence interval of the odds ratios
Exon 1, 113G*	0.0011	2.7627	0.9071-8.4140
Exon 1, 127T*	0.0011	2.7731	0.9107-8.4441
Promoter 1, -137C*	0.0001	4.2821	1.2413-14.7716
Promoter 2, -132G	0.5585	0.386	0.1114-1.3400
Promoter 2, -133G*	0.0111	1.7426	0.8116-3.7416

Calculations for the phenotype-specific sensitizations for 225 patients with AE, including the extrinsic form of AE, with (group 1) and without (group 2) sensitizations against house dust mite and patients with the nonatopic form of AE (group 3) in comparison with 175 healthy nonatopic volunteers (group 4) are shown. A *P* value of less than .05 was considered significant.

*Significant associations were seen for -137C in promoter 1, 113G in exon 1, 127T in exon 1, and -133G in promoter 2.

duced by PBMCs of patients with AE with genetic variations of the *IL18* gene (Fig 2, A). In parallel, no upregulation of the IL-12 production after 24 hours of stimulation with SEB was detectable in the subgroup of patients with AE with genetic variations in the respective *IL18* gene regions (Fig 2, B).

Patients with AE display higher IL-18 and lower IL-12 serum levels than healthy control subjects

To evaluate whether a dysregulation on the IL-18 and IL-12 protein level might be a characteristic feature of AE, we performed ELISA assays of sera of patients with AE and healthy control subjects. Patients with AE at the exacerbation of the disease (ie, SCORAD >40) displayed significantly higher serum levels of the active variant of IL-18 protein (Fig 3, A). In contrast, the level of IL-12 detectable in the sera of the same patients with AE was significantly lower than in healthy, nonatopic control volunteers (Fig 3, B).

DISCUSSION

IL-18 has previously been designated as IFN- γ -inducing factor and requires cleavage to be transformed into its active form. Similar to IL-1 β , IL-18 is synthesized as a 24-kd precursor protein, which is then enzymatically cleaved to an 18-kd mature IL-18 protein.⁴ In the absence of IL-12, IL-18 induces the production of IgE and promotes immune mechanisms of the T_H2 type, whereas

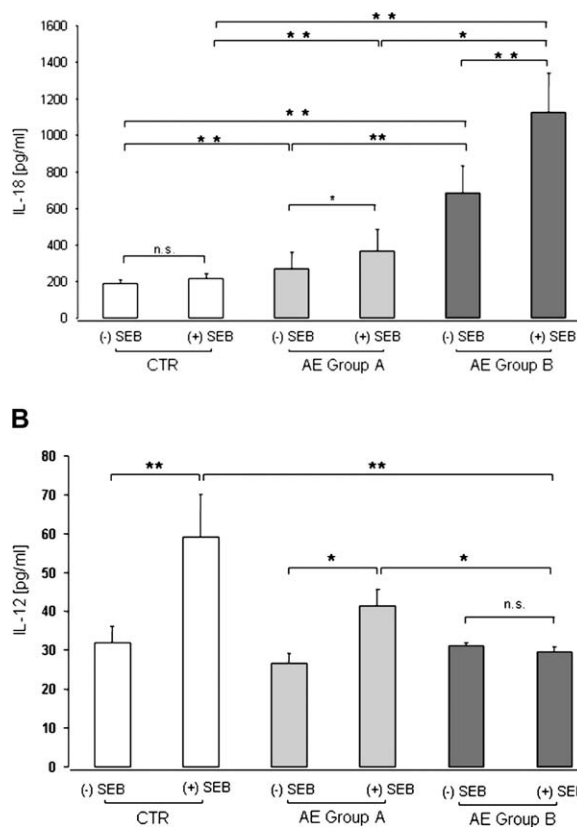


FIG 2. PBMCs of patients with AE with allelic variations in the *IL18* gene produce higher levels of IL-18 and lower levels of IL-12 after stimulation with SEB. **A**, IL-18 levels in the supernatant of PBMCs of patients with AE without allelic variations in the *IL18* gene (AE group A, $n = 11$), patients with AE with allelic variations in the *IL18* gene (AE group B, $n = 15$), and healthy volunteers ($n = 20$) stimulated with SEB for 24 hours (+ SEB) or left unstimulated (– SEB) are shown on the y-axis. CTR, Healthy control subjects; ns, not significant. * $P < .05$, ** $P < .01$. **B**, IL-12 levels in the supernatant of PBMCs of patients with AE without allelic variations in the *IL18* gene (AE group A, $n = 11$), patients with AE with allelic variations in the *IL18* gene (AE group B, $n = 15$), and healthy volunteers ($n = 18$) stimulated with SEB for 24 hours (+ SEB) or left unstimulated (– SEB) are shown on the y-axis. CTR, Healthy control subjects; ns, not significant. * $P < .05$, ** $P < .01$.

TABLE IV. Clinical parameters of patients with AE with wild-type alleles at the respective *IL18* gene regions (AE group A) and patients with AE with genetic variations (ie, SNPs +113[t/g], +127[c/t], –137[g/c], and –133[c/g] of the *IL18* gene [AE group B])

Parameter	AE group A	AE group B
Sex	61% female, 39% male	48% female, 52% male
SCORAD	45.72 ± 21.95	46.14 ± 21.83
IgE level (kU/L)	1311.84 ± 1758.60	1056.14 ± 1529.10

in contrast, in the presence of IL-12, IL-18 synergizes with IL-12 to promote immune responses of the T_H1 type.²⁶ Together, these counterregulations have been shown to be of critical immunologic importance for the activation of the innate immune system and defense against microbial infections through the induction of IFN- γ . The function of IL-18 in the human immune system is very heterogeneous: most importantly, IL-18 plays a crucial role in the control

of the balance between T_H1 - and T_H2 -dominated immune responses and the regulation of the IgE synthesis. A characteristic feature of the pathophysiology of AE is a dysbalance on the level of T_H2 and T_H1 immune responses and the control of these mechanisms. In this context, it might be of critical immunologic importance that several cell types that are involved in the IgE-mediated network of AE, such as keratinocytes²⁷ or distinct dendritic cell subtypes, constitutively express variable amounts of IL-18.^{28,29} These properties predispose IL-18 to be an important modulator of AE. The gene for human IL-18 is located on chromosome 11q22.2-22.3 (locus ID 3606, National Center for Biotechnology Information, National Institutes of Health) and has been designated as a candidate region for atopy previously.¹⁷ In this study we provide evidence for an association of polymorphisms in the *IL18* gene with a functional dysregulation of the IL-18 production in a subgroup of patients with AE *in vitro*, which might be of clinical relevance for the pathophysiology of AE *in vivo*.²⁸ Most importantly, this association occurs independently of the subtype of

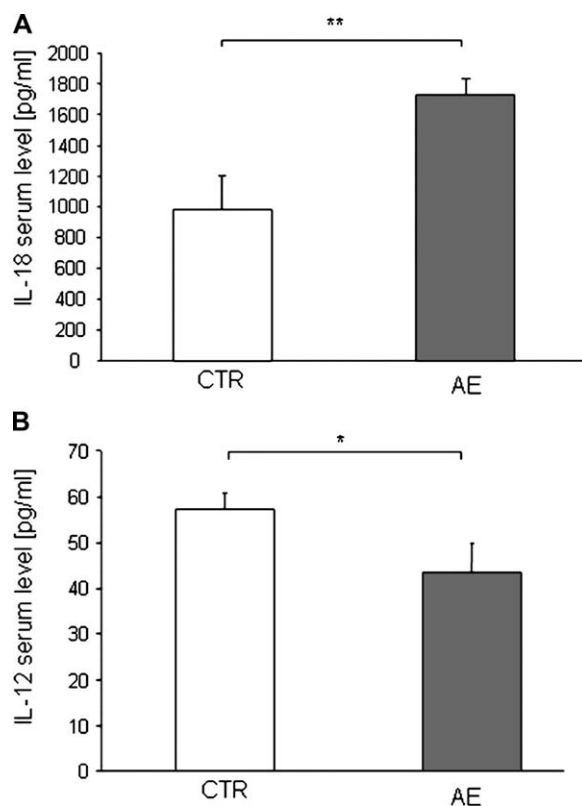


FIG 3. Patients with AE display enhanced IL-18 serum levels. IL-18 (A) and IL-12 (B) serum levels of patients at the exacerbation state of AE (n = 13) and healthy control volunteers (n = 13) are depicted in picograms per milliliter. CTR, Healthy control subjects; ns, not significant. * $P < .05$; ** $P < .01$.

AE, such as the allergic, extrinsic, or nonatopic intrinsic subtypes. In addition, the association of these SNPs was independent from the concomitant occurrence of allergic rhinitis or asthma bronchiale (data not shown) or specific sensitizations to house dust mite allergen in patients with AE. Because microbial stimuli, such as *Staphylococcus aureus* enterotoxins, play a major role in the pathophysiology of AE, a dysbalanced production of IL-18 on the basis of genetic defects might contribute to the enhanced IL-18 production on one hand and a relatively reduced production of IL-12 of PBMCs of patients with AE on the other hand observed here. Together, this might lead to a paradoxical initiation and amplification of T_H2 responses in AE after microbial stimulation. In our study the highest association with AE was seen for SNP -137[g/c], located in the GATA3 binding site of the *IL18* gene, which has been shown to be strongly involved in T_H2 differentiation.¹⁶ Most interestingly, the highest frequency of this polymorphic allele has been detected in patients with the intrinsic and nonatopic subtype of AE, indicating a role of this SNP for the development of a GATA3-dependent T_H2 predominance, especially in patients with low serum IgE levels without any sensitizations. However, one has to bear in mind that substantial linkage disequilibrium between all variants of the *IL18*

gene has been found¹⁹ reflected, as in this study, by the overall strong association of all SNPs studied with AE. In addition, the existence of 2 promoters and different protein forms of IL-18 indicate an extremely complex regulation of its expression.¹⁹ Given the biologic function of IL-18, gene-gene or protein-protein interactions could also be relevant, which have been seen for other cytokines and their receptors, such as influences of different polymorphisms in the *IL4*, *IL13*, and *IL4RA* genes.^{24,30}

Taken together, this is the first study showing a genetic and functional effect of SNPs in the *IL18* gene on the clinical phenotype of AE. To bias the dysregulation on the IL-18 level might open up new strategies for therapeutic approaches aimed at counteracting the genetic and functional abnormalities in atopic diseases.

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