

Original article

A promoter polymorphism in the CD14 gene is associated with elevated levels of soluble CD14 but not with IgE or atopic diseases

Background: A polymorphism in the promoter region of the CD14 gene, C-159T, has been shown to be associated with increased levels of soluble CD14 (sCD14) and decreased serum immunoglobulin E (IgE) and the expression of a more severe atopic phenotype in previous studies.

Methods: To test if these associations are consistently found in different populations and different age groups, we genotyped 2048 children of different age groups as well as 888 adults from different regions of Germany for the CD14 C-159T polymorphism.

Results: While an association between this promoter polymorphism and levels of sCD14 could be confirmed in our study population (CC: 1017 ng/ml vs TT: 1370 ng/ml, $P = 0.03$), no association between CD14 C-159T genotypes and IgE levels or the prevalence of atopic diseases was seen.

Conclusions: The lack of association between CD14 genotypes and IgE as well as atopic outcomes in this large German study population seems to indicate that CD14 genotypes may not directly be involved in the development of allergies during childhood.

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The variation in the prevalence of atopic diseases such as asthma, allergic rhinoconjunctivitis, and atopic eczema is striking between different populations throughout the world (1). Differences in the genetic makeup of populations and environmental factors are thought to be responsible for these effects. Elevated levels of total serum immunoglobulin E (IgE) have consistently been shown to be associated with atopic diseases in population studies and are therefore considered a valuable tool to determine allergic disposition (2). The increased production of IgE is a hallmark of an immune system that is dominated by TH2-associated cytokines, such as interleukin (IL) 3–5, IL 13, and granulocyte-macrophage colony-stimulating factor. The genes for these cytokines have been mapped to a cluster on chromosome 5q which showed an association with total serum IgE levels in linkage studies (3, 4). It has further been suggested that CD14, a gene located within 3.0 kb of the cytokine gene

cluster on chromosome 5q31, plays a significant role in modulating total serum IgE levels (5). The CD14, an important receptor involved in the recognition of lipopolysaccharide (LPS) and other bacterial wall components (6, 7), is predominantly expressed on the surface of monocytes, macrophages and neutrophils but also detectable in soluble form in the serum (8). The complex formed between LPS, CD14 and Toll-like receptor 4 leads to a strong response by antigen presenting cells and the induction of TH1 cells (9), most likely mediated by IL 12 (10). Therefore, CD14 might be an important player in the balance between infectious and allergic disease and the early polarization towards a TH1 response.

Baldini et al. identified a polymorphism in the proximal promoter region of the CD14 gene (C-159T), which altered the levels of soluble CD14 (sCD14) and furthermore decreased the levels of total serum IgE in skin prick test (SPT)-positive children (11). However, in

SPT-negative and therefore nonatopic subjects, the effect of the CD14 polymorphism on total serum IgE levels was not observed. These findings suggested that the polymorphism in CD14 has some protective effect in children whose immune system is already oriented towards a TH2 response. A functional change in the CD14 promoter has been shown (12) and recently it has been proposed by Vercelli (13) that differences in the environmental exposure levels to endotoxin may modulate the effect of this polymorphism.

To test the potential role of the CD14 promoter polymorphism C-159T in different areas and at different times during life we performed a very large association study investigating the effects of CD14 polymorphisms on sCD14 and serum IgE levels (total $N = 2936$).

Methods

Subjects

In 1995 and 1996 a cross-sectional study was conducted in Munich, western Germany and in Dresden, eastern Germany. In Munich, 3679 children at age 6 and 3830 children at ages 9–11 were enrolled in the survey while in Dresden 3668 children participated (International Study of Asthma and Allergies in Childhood, ISAAC). In both study centres, questionnaires including the ISAAC core questions were sent through the schools to the families for self-completion. Children of all ages underwent SP testing and blood was obtained for serum IgE measurements and DNA extraction. Pulmonary function testing and bronchial challenge with hyperosmolar saline (4.5%) was performed in the older age group. Total serum IgE was measured in a 50% random sample of all children that had given blood in Dresden and all children with blood available in Munich. In this study, only children of German origin who were genotyped successfully and had IgE data available were included in the analysis (Munich age 6 years $n = 256$, Munich age 10 years $n = 1087$, Dresden age 10 years $n = 705$). The study methods for all phenotyping procedures have been described in detail elsewhere (14). Therefore, only methods pertaining to this analysis are given below. In addition, 888 adult, healthy and voluntary blood donors were selected randomly by the Institute of Clinical Immunology and Transfusion Medicine in Giessen, middle Germany, to participate in this study. The median age of adults recruited for this study was 33.9 years (range 18–67 years). In a randomly selected subgroup of 160 adults a structured interview was performed and a screening test for specific IgE was performed. In adults and children a subsample of 545 individuals was selected for measurements of sCD14 in the serum (Munich $n = 186$, Dresden $n = 170$ and Giessen $n = 189$). Selection for sCD14 measurements was random in adults. In children, an equal number of SPT-positive and -negative children from Munich and Dresden were selected.

Informed written consent was obtained from all adults and the parents of children included in the study. All study methods were approved by the ethics committees of the University of Münster, the University of Giessen and the Bavarian Medical Council.

Questionnaire and structured interview

Self-administered questionnaires including the ISAAC core questions were used in children to assess symptoms of asthma, allergic

rinitis and atopic eczema (15). Children whose parents reported a doctor's diagnosis of either asthma, recurrent spastic or recurrent asthmatic bronchitis were classified as having asthma. The definition of 'wheeze ever' was based on the ISAAC question: 'Has your child ever had wheezing?'. Current wheeze was defined as wheezing in the last 12 months. The definition of atopic dermatitis and hay fever was based on a parental report of a doctor's diagnosis of atopic eczema and hay fever, respectively.

In adults, structured interviews were performed in a randomly selected subgroup of 160 adults from Giessen assessing a personal medical history of hay fever or house dust allergy, asthma, and atopic dermatitis. Furthermore, information on the family history of atopic diseases (first-degree relatives) was obtained using a questionnaire.

Skin prick test

In children, the sensitivity to six common aeroallergens (*Dermaphagoides pteronyssinus*, *D. farinae*, *Alternaria tenuis*, cat dander, grass, and tree mixture) was assessed by a SPT using lancets and extracts from ALK (ALK, Horsholm, Denmark) according to IS-AAC phase II protocols. All allergen extracts were standardized. A child was considered sensitized if a wheal reaction of more than 3 mm was present to at least one specific allergen after subtraction of the negative control.

Total and specific serum IgE measurements

In children, total serum IgE levels were measured using the Insulite system (DPC Biermann, Bad Nauheim, Germany). In adults, serum IgE was measured by using a N Latex IgE mono Test (Behring Nephelometer Analyzer II, Dade Behring, Deerfield, IL). The concentration of specific IgE against a panel of aeroallergens (mixed grass pollen, birch pollen, mugwort pollen, *D. pteronyssinus*, cat dander, dog dander, *Cladosporium herbarum*) was tested by Fluorescence Enzyme Immuno Assay (SX1 CAP; Pharmacia, Lund, Sweden) in all children and a subgroup of 160 adults in which the structured interview was performed.

Soluble CD14 measurements

Serum sCD14 levels were measured by the Institute of Clinical Immunology and Transfusion Medicine in Giessen using the commercially available enzyme-linked immunosorbent assay kit Quantikine® sCD14 ELISA (supplied by R&D Systems, Minneapolis, MN).

Molecular genetic methods

In children, genomic DNA was extracted by a standard salting out method from whole blood (16). To amplify the promoter region of the CD14 gene primers, 5'-GTGCCAACAGATGAGGTTAC-3' and 5'-CCTCTGTGAACCCTGATCAC-3' were used according to standard polymerase chain reaction (PCR) protocols (11). The PCR products were digested with 1 unit of the restriction endonuclease *AV*AI (New England Biolabs GmbH, Frankfurt, Germany) electrophoresed on 2% agarose gels and visualized with ethidium bromide staining and ultraviolet illumination.

For the adult population samples from Giessen, genotyping of CD14 C-159T polymorphism was performed by a PCR sequence-specific priming (PCR-SSP) technology. The PCR-SSP genotyping primers were: CD14-159T allele-specific primer 5'-AGA ATC CTT

CCT GTT ACG GT-3', CD14-159C allele-specific primer 5'-GAA TTC TTC CTG TTA CGG C-3', and CD14 common primer 5'-GCT GCC TCT GAC AGT TTA TG-3'. The PCR products were visualized after agarose gel electrophoresis and ethidium bromide staining. The C-reactive protein gene was additionally used as an internal amplification control. For each subsequent series of PCR runs, a positive and a negative control from individuals, tested by DNA sequencing was included.

Statistical analysis

Chi-square statistics as well as Cochran–Armitage trend tests were used to compare qualitative traits between groups. Total serum IgE and serum sCD14 levels were summarized descriptively with geometric mean values and respective 95% confidence intervals. As sCD14 levels were not normally distributed but skewed towards lower levels, values were log-transformed for statistical analysis and retransformed for their graphical depiction. Analysis of variance and appropriate contrasts on log-transformed values (IgE and sCD14) were used to compare the CC and TT genotypes. A nominal level of significance ($\alpha = 0.05$) was used for all statistical tests. Calculations were performed with the SAS software package (version 8.2; SAS Institute GmbH, Heidelberg, Germany) and the SPSS software package (version 10.0).

Results

Demographic characteristics of the study population are shown in Table 1. In terms of these characteristics, children participating in genotyping did not differ significantly from subjects not genotyped and adults selected for a structured interview and specific IgE measurements were not statistically different from the total adult study population (data not shown). While the prevalence of atopy was lower in children from Munich at age 6 (11.3%) no significant difference in atopy was found between children aged 9 and 11 from Munich (24.6%) and Dresden (26.2%) and adults from Giessen (24.8%).

Using a restriction enzyme-based test and PCR-SSP we determined the frequency of a C to T transition at position bp-159 from the transcription start site in the promoter region of the CD14 gene, 2971 samples were genotyped successfully (99.7% of all individuals with DNA available). The allele frequencies for CC, CT and

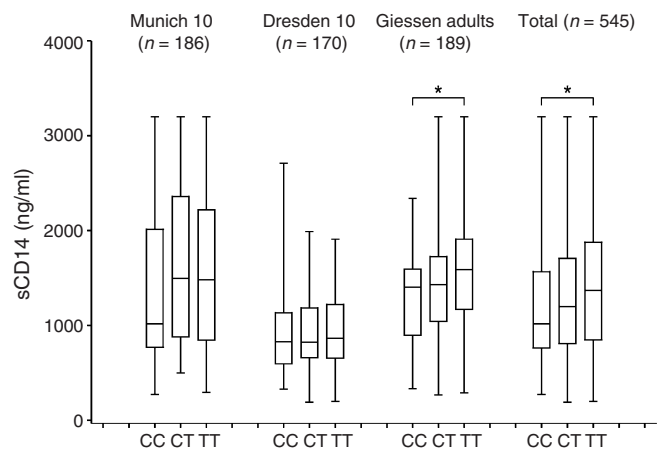


Figure 1. Box plots of serum levels of soluble CD14 (sCD14) in ng/ml by CD14 C-159T genotypes in children and adults. Box plots illustrate median values and interquartile distance.

TT were 0.28, 0.50, and 0.23 for children in Munich ($n = 1343$), 0.28, 0.50, and 0.21 for those in Dresden ($n = 705$), and 0.26, 0.53, and 0.21 in Giessen ($n = 888$), respectively. These results are in agreement with the predicted Hardy–Weinberg equilibrium.

The association between CD14 C-159T and serum levels of sCD14 was investigated in three subsamples of children and adults (Munich $n = 186$, Dresden $n = 170$ and Giessen $n = 189$; total $N = 545$). In all subsamples a trend for elevated sCD14 levels in carriers of TT genotypes was found (Fig. 1). This trend reached statistical significance in the Giessen population only (CC: 1404 ng/ml vs TT: 1588 ng/ml, $P < 0.04$). When all three populations were analysed together, an overall statistical significant increase of sCD14 levels was observed (CC: 1017 ng/ml vs TT: 1370 ng/ml, $P < 0.03$).

Furthermore, geometric mean values of log-transformed serum IgE levels were tested for associations with CD14 C-159T genotypes (Fig. 2A). No significant differences in serum IgE were detected between CD14 genotypes in the samples from Munich, Dresden, and Giessen. When the population was stratified for SPT reactivity, no significant association was detected between CD14 C-159 genotypes and any atopic phenotype including serum IgE levels (Fig. 2B).

In addition, the number of positive SPT tests against common allergens was not associated with CD14 genotypes (data not shown). Elevated sCD14 levels did not correlate with decreased total serum IgE levels (data not shown).

In Table 2, the prevalence of asthma, bronchial reactivity (BHR), atopic dermatitis, hay fever and SPT reactivity is presented by CD14 genotypes at different ages. The prevalence of these atopic phenotypes was not associated with CD14 genotypes in any of the tested populations.

Table 1. Descriptive characteristics of study populations

	Munich 6 ($n = 256$)	Munich 10 ($n = 1087$)	Dresden 10 ($n = 705$)	Giessen adults ($n = 891$)
Male gender (%)	50.8	48.4	51.5	62.0
Mean age in years (SD)	5.9 (0.4)	9.5 (0.5)	9.7 (0.6)	34.1 (11.2)
Atopy (SPT-positive, %)	11.3	24.6	26.2	24.8*
Geometric mean serum IgE (log-transformed IU/ml)	39.4	64.8	57.6	46.2
Frequency T allele	0.49	0.47	0.47	0.47

SPT, skin prick test; IgE, immunoglobulin E.

* Determined in a subgroup of 160 individuals by structured interview.

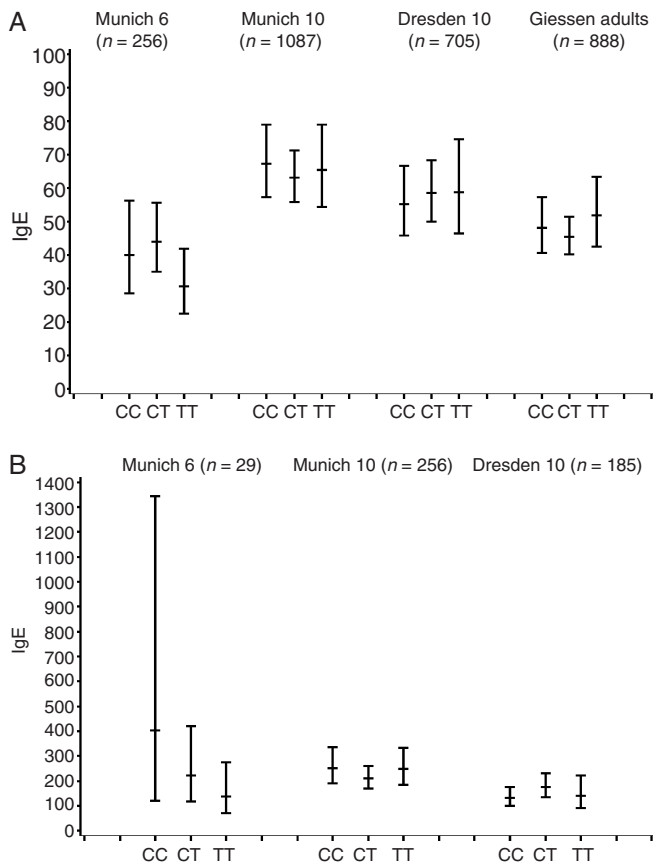


Figure 2. (A) Geometric mean values of serum immunoglobulin E (IgE) levels (IU/ml) by CD14 C-159T genotypes in four population samples. (B) Geometric mean values of serum IgE levels (IU/ml) by CD14 C-159T genotypes in atopic children (defined as skin prick test-positive).

Discussion

Using a very large, carefully phenotyped German study population of different age groups we genotyped 2048 children and 888 adults for the promoter polymorphism C-159T in the CD14 gene. A statistically significant association between the CD14 C-159T polymorphism and sCD14 levels was observed. However, we could not find any association between the CD14 promoter polymorphism and total serum IgE levels or the number of positive SPTs. Furthermore, no correlation between the

CD14 promoter polymorphism and atopic phenotypes was detected.

The results of association studies in human populations remain controversial. After the initial report of a promoter polymorphism in the CD14 gene and its association with serum IgE level (11), a number of studies have investigated this association in different populations. While some authors could replicate the results from the initial Tucson study (17–19), others failed to do so (20, 21). Some of these studies may have lacked the adequate size and power (22) but also differences in ethnicity and age may have contributed to the observed discrepancies. Our study population is the largest to date genotyped for CD14 C-159T with data on atopy status, serum IgE and sCD14 available. Thus, the power to detect an association if present was rather high. As the study population consisted of individuals of German origin only, it seems unlikely that population stratification has influenced our results. Effect modification by age is furthermore unlikely as the effect was consistent among the three different age groups investigated.

Our findings differ in part from those previously published by Baldini et al. who also reported an increase of sCD14 and a concomitant decrease of total serum IgE levels in atopic (SPT-positive) children from Tucson with a TT genotype (11). While this association was present in Tucson children of Caucasian origin, no such association was found in Hispanics. Koppelman and coworkers also observed increased serum IgE levels in atopic subjects with a CC genotype in a population of Dutch adults. In this study, also the number of positive SPTs was increased in carriers of the CC genotype (17). We did not observe a statistically significant increase in the number of positive SPTs in our population. However, the number of allergens tested in our population was limited to six allergens while in the Dutch study 12 allergens were tested. Thus, differences in numbers of positive SPTs would have been harder to detect in our population.

Functional *in vitro* studies on the CD14 promoter revealed that the polymorphism C-159T leads to a decreased affinity of DNA/protein interactions at a GC box that contains a binding site for SP1, SP2, and SP3 transcription factors (12). In luciferase reporter studies a 20% increase in gene expression after endotoxin stimulation was observed in constructs carrying the T at position bp-159 of the promoter compared with constructs containing the C allele. This increased promoter

Table 2. Prevalence of atopic diseases (in %) by CD14 C-159T genotypes and city/age groups

	Munich 6 (n = 256)			Munich 10 (n = 1087)			Dresden 10 (n = 705)			Giessen adults (n = 160)		
	CC	CT	TT	CC	CT	TT	CC	CT	TT	CC	CT	TT
Asthma	7.7	12.2	8.2	11.2	12.3	11.1	7.0	10.7	8.0	5.4	5.4	7.1
Hay fever	1.6	4.8	1.7	9.1	8.3	7.0	8.7	9.7	8.7	19.4	23.9	29.8
Atopic dermatitis	15.6	11.9	13.6	16.6	20.5	18.3	20.2	16.2	19.6	4.2	10.0	7.3

activity of the T allele was confirmed by our results as carriers of the T allele show higher levels of sCD14. However, although the effect of the CD14 promoter polymorphism C-159T on the expression of CD14 has been consistent across several populations and in functional analyses, the evidence for an effect of elevated sCD14 levels on serum IgE levels is less convincing. *In vitro* studies have shown that stimulation with endotoxin enhances IL-4-dependent IgE synthesis through a monocyte-dependent mechanism (23). The role of CD14 and altered CD14 expression by the CD14 C-159T promoter polymorphism in this process is, however, not yet clear. The lack of association between CD14 genotypes and IgE as well as atopic outcomes in our study and others (20) favours the notion that CD14 genotypes may not be directly involved in the development of atopic diseases.

Differences in environmental exposure have been suggested to contribute to the discrepant results reported above (24). An 'endotoxin switch' has been proposed by Vercelli, where the CD14 promoter polymorphism changes the threshold at which environmental endotoxin stimulation leads to a TH2 immune response. According to this hypothesis, the CD14 promoter polymorphism would not influence the development of atopy at very low or very high concentrations of environmental endotoxin. An alternative explanation for these diverse results may be proposed. The CD14 is only one part of the LPS signalling pathway and many cofactors such as LPS-binding protein, Toll-like receptor 4 and others are

involved in the signal transduction of endotoxin stimulation. The translation of endotoxin stimulation into an IgE-related response may furthermore be complex and may involve regulation through timing of stimulation, molecular interaction with other immune pathways and external signals apart from endotoxin. Thus, the relevance of the CD14 promoter polymorphisms may be dependent on the presence or absence of other, so far unknown exposures or genetic cofactors in different populations. Further studies in diverse environments with well-characterized environmental exposures will be needed to understand these mechanisms.

In conclusion, our results confirm that CD14 genotypes are related to the expression of sCD14. This effect was strongest in our adult study population. However, no association was found between CD14 genotypes and serum IgE levels or atopic diseases in this large German population sample comprising different age groups from different geographical areas but the same ethnic origin.

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