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ASTHMA

Involvement of IL-10 Gene Promoter Polymorphisms in the Susceptibility for Childhood Asthma

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Abstract Asthma and atopy have a complex background that may result from the interaction of genes and the environment. Interleukin (IL)-10 is known to play various roles in immune-regulating and anti-inflammatory responses. The aim of this study was to evaluate the possible effect of the IL-10 promoter polymorphisms on susceptibility to childhood asthma. We recruited 333 patients with atopic asthma, 55 with nonatopic asthma, and 248 normal controls. We performed a genetic association study of three genetic polymorphisms (IL-10 –1082A>G, IL-10 –819T>C, and IL-10 –592A>C) of the IL-10 promoter. There was no difference between atopic asthma, nonatopic asthma, and normal controls with respect to allele, genotype, or haplotype frequencies of these IL-10 polymorphisms. However, the −1082A>G polymorphism and ATA haplotype in the IL-10 promoter gene were associated with airway hyper responsiveness (AHR) and the –819T>C, –592A>C, and ATA and ACC haplotypes were also shown to be related to serum eosinophil cationic protein (ECP). Our results suggest that the polymorphisms within the IL-10 promoter may have a disease-modifying effect in the asthmatic airway.

Keywords Asthma · Child · Polymorphism · Genetic · Interleukin-10

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Introduction

Asthma is a chronic airway inflammatory disease characterized by variable airflow obstruction and airway hyper responsiveness (AHR) [1]. Asthma and atopy have a complex background that may result from the interaction of genes and the environment. Among various immunological factors, regulatory T (Treg) cells are known to play a key role in balancing immune responses to maintain and acquire tolerance against allergens [2]. Treg cells contribute to development and persistence of allergic disease through various proteins, including interleukin (IL)-10.

IL-10 is produced by Th2 lymphocytes, monocytes, and epithelial cells. It is known to play various roles in immune-regulating and anti-inflammatory responses. Several polymorphic sites within the promoter region of the IL-10 gene have been described, and three single nucleotide polymorphisms (SNPs) at positions −1082, −819, and −592 from the transcription initiation site have been identified [3]. The −1082 SNP is a G-to-A substitution and lies within a putative transcription factor binding site [4]. The −1082G allele is known to be associated with high in vitro IL-10 production [3]. The –819 SNP presents a dimorphic polymorphism, a C-to-T substitution, and may affect an estrogen-responsive element [5]. The –592 SNP is a C-to-A substitution and lies within a region with a negative regulatory function [4]. These polymorphisms exhibit strong linkage disequilibrium and appear in three potential haplotypes: GCC, ACC, and ATA. The haplotype GCC has been associated with high production of IL-10 [6]. Several studies examined the association of IL-10 polymorphisms with allergic diseases. However, the results have been inconsistent [7–13].

The aim of this study was to evaluate the possible effect of the IL-10 promoter polymorphisms on susceptibility to asthma in Korean children.

Materials and Methods

Study Subjects

We recruited 395 boys and 186 girls. Among them, 278 had atopic asthma (AA), 55 had nonatopic asthma (NAA), and 248 were normal controls (NC). We performed spirometry, methacholine challenge test, and skin test while measuring the total eosinophil count and the serum levels of total IgE and eosinophil cationic protein (ECP) in all subjects.

Asthma diagnosis was made in accordance with the American Thoracic Society (ATS). In short, current asthma was defined as recurrent wheezing or coughs in the absence of a cold in the preceding 12 months with a physician's diagnosis, and AHR upon methacholine challenge (PC_{20} $\leq$16 mg/ml) or at least 12% reversibility of forced expiratory volume in 1 s (FEV_1) after inhalation of β_2 agonist [14]. Atopy was defined as a positive skin test result to more than one extract of the common local aeroallergens, including *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cockroach, dog dander, cat dander, and fungus, and nonatopy was defined as a negative skin test result and serum IgE concentration less than 100 IU/ml. Patients treated with systemic corticosteroids because of asthma exacerbation in the preceding 1 month were excluded from this study.

Normal controls were age-matched healthy children who visited the hospital for general health workups and had no history of wheezing, recurrent or chronic diseases, infection during the preceding 2 weeks, or hypersensitivity to methacholine. Normal controls were also nonatopy and did not take any medications. Written consent was obtained from all participants before enrollment in the study, which had been previously approved by the Severance Hospital Institutional Review Board.

Polymerase Chain Reaction Amplification and Genotyping

Genomic DNA was extracted from whole blood of all study subjects using a FlexiGene DNA kit (Qiagen, Valencia, CA, USA). The PCR restriction fragment length polymorphism (RFLP) method was used to determine the genotypes of patients and controls for the target polymorphisms [15].

The IL-10 –592 genotype was determined by using an RsaI site introduced with a mismatch into the PCR primer next to the A/C transition. Amplification with the primers –592 S: 5′-cct agg tca cag tga cgt gg-3′ and −592 AS: 5′-ggt gag cac tac ctg act agc-3′ generated a 412-bp product. Digestion with RsaI (New England BioLabs, Ipswich, MA, USA) yields 176- and 236-bp fragments when A is at position –592.

The IL-10 −819 genotype was determined by using a MaeIII site introduced with a mismatch into the PCR primer next to the T/C transition. Amplification with the primer −819 S: 5′-tca ttc tat gtg ctg gag atg g-3′ and –819 AS: 5′-tgg ggg aag tgg gta aga gt-3′ generated a 209-bp product. Digestion with MaeIII (Roche Diagnostics, Indianapolis, IN, USA) yields 125- and 84-bp fragments when T is at position −819.

The IL-10 –1082 genotype was determined by using a MnlI (New England BioLabs) site introduced with a mismatch into the PCR primer next to the G/A transition. Amplification with the primer −1082 S: 5′-ctc gct gca acc caa ctg gc-3′ and –1082 AS: 5′-tct tac cta tcc cta ctt cc-3′ generated a 139-bp product. Digestion with MnlI yields 106- and 33-bp fragments when G is at position –1082.

Genomic DNA was amplified in a 25-µl PCR reaction under the following cycling conditions: denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min. This was followed by final extension at 72°C for 10 min in a thermal cycler (model 9700; PerkinElmer, Foster City, CA, USA). For amplification of the IL-10 −1082 polymorphism, an initial activation step of 95°C for 15 min preceded the cycling program, and annealing at 55°C in a cycling program. Amplified PCR products were digested with a restriction enzyme and analyzed on a 3% agarose gel stained with ethidium bromide.

Spirometry and Methacholine Challenge Test

Spirometry (VIASYS Healthcare Inc., Conshohocken, PA, USA) was performed and flow volume curves were obtained according to the ATS guidelines before and after bronchodilator inhalation [16].

A methacholine challenge test was performed according to a standard procedure [14]. Each subject inhaled increasing concentrations of methacholine (0.075, 0.15, 0.31, 0.62, 1.25, 2.5, 5, 10, 25, and 50 mg/ml) nebulized by a Mefar MB3 dosimeter (Brescia, Italy) until FEV_1 was reduced by 20% from a postnebulized saline value. Bronchial response to methacholine was expressed as a provocative concentration causing a 20% decrease in FEV_1 (PC_{20} in mg/ml) and was calculated by linear interpolation of the log dose–response curve.

Measurement of Blood Eosinophil Count, Serum Total IgE, and Eosinophil Cationic Protein

Peripheral venous blood was collected from patients and control subjects. An eosinophil count was performed on an aliquot of the whole venous blood using the NE-8000 system (Sysmex, Kobe, Japan.) The serum fraction was

then separated from whole blood and serum total IgE and ECP were measured using the CAP system (Pharmacia-Upjohn, Uppsala, Sweden).

Statistical Analysis

A χ^2 test was used to detect a significant departure in genotype frequency from the Hardy-Weinberg equilibrium at each single nucleotide polymorphism (SNP) and assess differences in genotype frequency between patients and controls. Logistic regression models were used for analyses of SNPs and haplotypes, controlling age and sex as covariates with three alternative models: codominant, dominant, and recessive. Haplotypes of the IL-10 promoter gene were analyzed using Haploview ver. 2.05 based on an EM algorithm [17]. Linkage disequilibrium between loci was measured using the absolute value of r^2 [18]. Differences in the mean value of the phenotypic characteristics were compared using an ANOVA test and Student's *t*-test. Statistical analyses were undertaken using SPSS ver. 18 (SPSS Inc., Chicago, IL, USA). P values < 0.05 were considered to be significant.

Results

Subject Characteristics

Clinical characteristics of the study subjects are summarized in Table 1. Blood eosinophils, serum ECP, and total IgE were higher in AA than in NAA or NC ($P \leq 0.01$). Baseline FEV_1 was lower in AA than in NC ($P < 0.01$).

Allele and Genotype Frequencies of IL-10 Promoter Gene

We performed a genetic association study of three genetic polymorphisms (IL-10 −1082A>G, IL-10 −819T>C, IL-10 −592A>C) of the IL-10 promoter. Table 2 shows the allele and genotype frequencies of three polymorphisms in the three groups of children. Overall, the results showed Hardy-Weinberg equilibrium in each subgroup. There is no difference among AA, NAA, and NC in allele or genotype frequencies of these IL-10 polymorphisms (Table 2).

Haplotype Frequencies of IL-10 Promoter Gene

The three polymorphisms in the IL-10 promoter gene were in linkage disequilibrium ($|D'|=1$), and three common haplotypes were constructed using the EM algorithm (Table 3). A haplotype with estimated frequencies below 0.05 were not included in either analysis. The frequency of the IL-10 promoter haplotypes ATA, ACC, and GCC was 63.9, 24.7, and 6.2%, respectively. There is no difference in the three haplotypes and their genotype distributions between AA, NAA, and NC (Tables 3 and 4).

Association of IL-10 Promoter Polymorphisms with Airway Hyper responsiveness

We examined the association of IL-10 promoter polymorphisms with AHR represented by methacholine PC_{20}. The homozygous AA genotype group of IL-10 –1082A>G showed significantly lower PC_{20} when compared with combined homozygous GG genotype and heterozygous AG genotype in asthmatic children ($P = 0.014$, Table 5). The ATA haplotype group showed lower PC_{20} compared with the GCC haplotype group in children with asthma ($P = 0.027$, Fig. 1a).

Association of IL-10 Promoter Polymorphisms with Serum ECP

We examined the association of IL-10 promoter polymorphisms with serum ECP in asthmatic children. The homozygous TT genotype group of IL-10 –819T>C showed lower serum ECP when compared with combined heterozygous CT genotype and homozygous CC genotype

Table 1 Clinical characteristics of the study subjects

	AA (n = 278)	NAA (n = 55)	NC (n = 248)	P value	
				AA vs. NAA	AA vs. NC
Age (years)	11.48 ± 4.06	10.07 ± 4.35	11.40 ± 4.33	0.07	0.63
Sex (M)	200 (71.9%)	37 (67.3%)	158 (63.7%)	0.56	0.10
IgE, log IU/ml	2.59 ± 0.53	1.73 ± 0.42	1.92 ± 0.65	<0.01	<0.01
Eosinophil count, log μl^{-1}	2.58 ± 0.34	2.36 ± 0.33	2.33 ± 0.39	<0.01	<0.01
ECP, log µg/l	1.20 ± 0.47	1.02 ± 0.41	1.01 ± 0.45	0.01	<0.01
Baseline FEV_1, % predicted	80.25 ± 13.63	83.98 ± 14.07	91.30 ± 10.42	0.11	<0.01

AA atopic asthma, *NAA* nonatopic asthma, *NC* normal controls, *n* number of patients, *ECP* eosinophil cationic protein, FEV_1 forced expiratory volume in 1 s

Table 2 Allele and genotype frequencies of the IL-10 promoter polymorphisms

Loci	Genotype	AA (*n* = 278)	NAA (*n* = 55)	NC (*n* = 248)	*P* value*		
					AA vs. NAA	AA vs. NC	NAA vs. NC
–1082A>G	AA	244 (87.8%)	47 (85.5%)	215 (86.7%)	0.57	0.50	0.92
	AG	33 (11.9%)	7 (12.7%)	30 (12.1%)	0.29	0.24	0.87
	GG	1 (0.4%)	1 (1.8%)	3 (1.2%)	0.74	0.68	0.96
	Allele frequency (*q*)	0.06	0.08	0.07	0.57	0.49	0.92
–819T>C	TT	132 (47.5%)	23 (41.8%)	113 (45.6%)	0.16	0.68	0.29
	TC	116 (41.7%)	22 (40.0%)	108 (43.5%)	0.12	0.90	0.22
	CC	30 (10.8%)	10 (18.2%)	27 (10.9%)	0.38	0.64	0.52
	Allele frequency (*q*)	0.32	0.38	0.33	0.15	0.68	0.28
–592A>C	AA	120 (43.2%)	26 (47.3%)	111 (44.8%)	0.80	0.79	0.78
	AC	124 (44.6%)	19 (34.5%)	106 (42.7%)	0.23	0.98	0.38
	CC	34 (12.2%)	10 (18.2%)	31 (12.5%)	0.63	0.70	0.82
	Allele frequency (*q*)	0.35	0.36	0.34	0.80	0.78	0.77

AA atopic asthma, *NAA* nonatopic asthma, *NC* normal controls, *n* number of patients, *q* minor allele frequency

* Each *P* value was calculated with codominant, dominant, and recessive models. Logistic regression analysis was applied to control age and sex as covariables

Table 3 Haplotype frequencies of the IL-10 promoter gene

Haplotype	AA	NAA	NC	*P* value*		
				AA vs. NAA	AA vs. NC	NAA vs. NC
Ht1 (ATA)	0.68	0.63	0.68	0.57	0.65	0.57
Ht2 (ACC)	0.26	0.29	0.26	0.36	0.96	0.36
Ht3 (GCC)	0.06	0.09	0.07	0.34	0.57	0.34

AA atopic asthma, *NAA* nonatopic asthma, *NC* normal controls

* Each *P* value was calculated with codominant, dominant, and recessive models. Logistic regression analysis was used to control age and sex as covariables

Table 4 Genotype distributions of haplotypes of the IL-10 promoter gene

Haplotype	Genotype	AA (*n* = 278)	NAA (*n* = 55)	NC (*n* = 248)	*p* value*		
					AA vs. NAA	AA vs. NC	NAA vs. NC
Ht1 (ATA)	Ht1/Ht1	115 (47.1%)	23 (41.8%)	106 (42.7%)	0.64	0.80	0.64
	Ht1/–	125 (45.0%)	22 (40.0%)	108 (43.5%)	0.35	0.98	0.54
	–/–	38 (13.7%)	10 (18.2%)	34 (13.7%)	1.00	0.74	0.82
Ht2 (ACC)	Ht2/Ht2	18 (6.5%)	5 (9.1%)	15 (6.0%)	0.46	0.93	0.49
	Ht2/–	102 (36.7%)	20 (36.4%)	92 (37.1%)	0.58	1.00	0.63
	–/–	158 (56.8%)	30 (54.5%)	141 (56.9%)	0.46	0.83	0.44
Ht3 (GCC)	Ht3/Ht3	1 (0.4%)	1 (0.4%)	3 (1.2%)	0.32	0.36	0.69
	Ht3/–	27 (9.7%)	7 (12.7%)	26 (10.5%)	0.43	0.50	0.69
	–/–	250 (89.9%)	47 (85.5%)	219 (88.3%)	0.29	0.24	0.87

AA atopic asthma, *NAA* nonatopic asthma, *NC* normal controls

* Each *P* value was calculated with codominant, dominant, and recessive models. Logistic regression analysis was applied to control age, sex as covariables

Table 5 Clinical characteristics according to the IL-10 promoter polymorphisms within asthmatics

Loci	Genotype	FEV_1 (%, predicted)	PC_{20} (log mg/ml)	IgE (log IU/ml)	Eosinophil count (log/μl)	ECP (log μg/l)
–1082A>G	AA	80.94 ± 13.19	0.19 ± 0.62	2.45 ± 0.60	2.55 ± 0.35	1.18 ± 0.47
	AG or GG	82.32 ± 12.74	0.44 ± 0.53	2.40 ± 0.62	2.56 ± 0.40	1.10 ± 0.47
	P value	0.550	0.014	0.633	0.817	0.305
–819T>C	TT	81.03 ± 12.75	0.18 ± 0.62	2.47 ± 0.59	2.56 ± 0.36	1.11 ± 0.49
	TC or CC	81.19 ± 13.46	0.26 ± 0.61	2.43 ± 0.61	2.54 ± 0.35	1.22 ± 0.45
	P value	0.916	0.232	0.525	0.571	0.030
–592A>C	AA	80.41 ± 13.24	0.18 ± 0.63	2.43 ± 0.62	2.55 ± 0.35	1.10 ± 0.49
	AC or C	81.66 ± 13.03	0.26 ± 0.60	2.46 ± 0.59	2.55 ± 0.35	1.22 ± 0.45
	P value	0.426	0.254	0.627	0.933	0.021

FEV_1 forced expiratory volume in 1 s, *ECP* eosinophil cationic protein

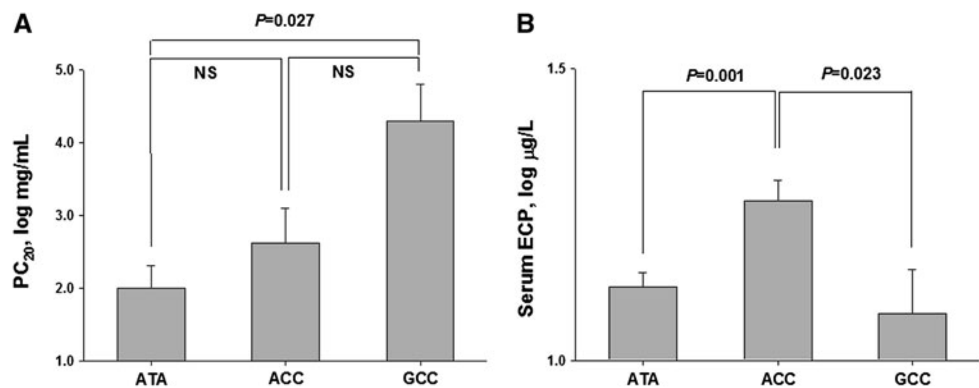

Fig. 1 Airway hyper responsiveness and serum eosinophil cationic protein in asthma, with children stratified by interleukin (IL)-10 promoter haplotype

($P = 0.03$). The homozygous AA genotype group of IL-10 –592A>C also showed lower serum ECP when compared with combined heterozygous AC genotype and homozygous CC genotype ($P = 0.021$, Table 5).

The ACC haplotype group showed higher serum ECP compared with the ATA haplotype ($P = 0.001$) and the GCC haplotype group ($P = 0.023$, Fig. 1b). In the genotypic distributions of haplotype 1 (ATA/ATA, ATA/–, –/–), serum ECP was lower in the ATA/ATA group than in the –/– group ($P = 0.01$, Fig. 2a). In the genotypic distributions of haplotype 2 (ACC/ACC, ACC/–, –/–), serum ECP was higher in the ACC/ACC group than in the ACC/– group ($P = 0.004$) and the –/– group ($P = 0.001$, Fig. 2b).

Discussion

In this study, three single nucleotide polymorphisms (IL-10 –1082A>G, IL-10 −819T>C, IL-10 −592A>C) in the promoter region of the IL-10 gene were investigated to establish the role of IL-10 in susceptibility to asthma and atopy in Korean children. There was no difference between

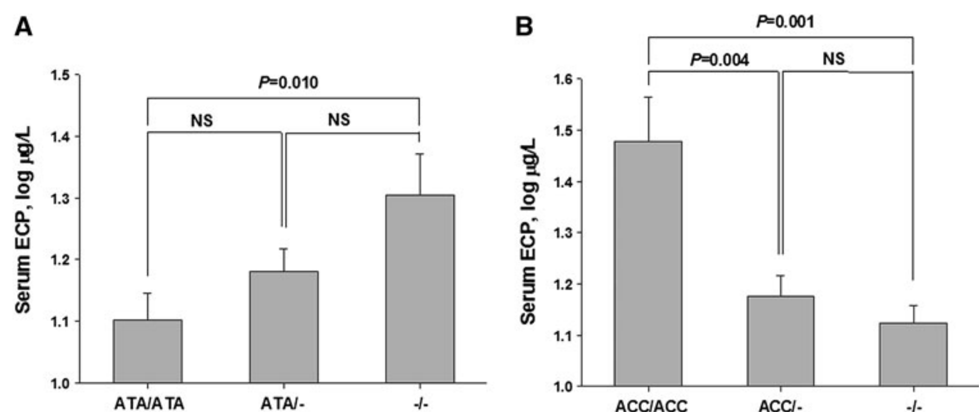

Fig. 2 Serum eosinophil cationic protein in asthma, with children stratified by genotype distributions of haplotypes of the interleukin (IL)-10 promoter gene

AA, NAA, and NC in allele, genotype, or haplotype frequencies of these IL-10 polymorphisms. However, the −1082A>G polymorphism and ATA haplotype in the IL-10 promoter gene were associated with AHR, and the –819T>C, –592A>C, and ATA and ACC haplotypes were also shown to be related to serum ECP.

IL-10 expression might be important for the inflammatory cascade and regulation of the airway tone after allergic sensitization and challenge in asthma [19, 20]. Therefore, low levels of IL-10 might be associated with the pathogenesis of asthma, especially severe atopic asthma [19]. On the other hand, it has been shown that asthma has a complex immune mechanism, and there are numerous interactions among inflammatory mediators. For example, the serum human leukocyte antigen (HLA)-G level was high and the serum IL-10 level was low in patients with atopic asthma [21]. Although these polymorphisms have been reported to influence IL-10 expression, there are still conflicting findings regarding their direct associations [22, 23]. A potential reason for these inconsistent results is that there are gene-to-gene and gene-to-environment interactions.

Nevertheless, it has been reported that the IL-10 promoter gene SNP may affect the pathogenesis of allergic diseases. The IL-10 –1082A>G, IL-10 –819T>C and IL-10 –592A>C polymorphisms were associated with asthma, and their interactions with genes involved in the vitamin D pathway affected an asthma risk, but the effects were not uniform across populations [24]. The IL-10 –1082A>G polymorphism might affect asthma inception and also can modify the allergenecity of dust mite exposure, but this association was not replicated in another study [25]. Another recent study about SNPs in genes involved in the development and function of Treg cells showed that the IL-10 –819T>C and –592A>C polymorphisms were associated with asthma in patients aged 6-8 years, but the analyses of gene-to-gene interactions did not show any association with asthma and atopy [26]. In our previous study, we showed that the –819T>C and –592A>C polymorphisms in the IL-10 promoter gene were associated with susceptibility to atopic dermatitis (AD) and blood eosinophil count [11]. The ATA haplotype is also associated with susceptibility to atopic eczema [11]. However, we did not show that there was any association between IL-10 promoter SNPs and asthma susceptibility in this study. The main reasons for such discrepancies may be the phenotype heterogeneity, ethnic differences, or various environmental exposures.

Recent reports on patients with AD suggested that IL-10 genotype or haplotype was associated with eosinophil counts and total serum IgE [8, 10, 11]. However, IL-10 genotype or haplotype had no significant effect on serum IgE or eosinophil counts in our study on asthmatic children. Although IL-10 promoter polymorphisms had no direct effect on eosinophil counts, the IL-10 –819T>C and –592 polymorphisms and ACC haplotype and its genotypic distributions had a significant effect on serum ECP.

The AA genotype showed more AHR than AG or GG genotype of –1082A>G. The –1082A polymorphism exhibited significantly less promoter activity than –1082G [12]. The ATA haplotype group represented more AHR than other groups in our study. The ATA haplotype has also been associated with lower transcriptional activity than the other haplotypes, with low IL-10 production [27, 28], and low expression of the IL-10 gene is thought to favor asthma susceptibility. So our results are partly consistent with those of previous reports, although IL-10 promoter polymorphisms did not show any association with asthma susceptibility. We showed the association between asthma and IL-10 polymorphisms in Korean children for the first time, while we analyzed three candidate polymorphisms without full gene screening of IL-10 in relatively small subjects.

In conclusion, there was no significant difference in allele, genotype, and haplotype frequencies among IL-10 –1082 G/A, –819 T/C, and –592 A/C polymorphisms in children with asthma. However, AHR and serum ECP are associated with genotype or haplotype frequencies and the genotype distributions of the IL-10 gene promoter region. The polymorphisms within the IL-10 promoter may have a disease-modifying effect in the asthmatic airway.

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