

Letter to the Editor

Regulatory role of *IL10* genetic variations in determining allergen-induced T_H2 cytokine responses in children

To the Editor:

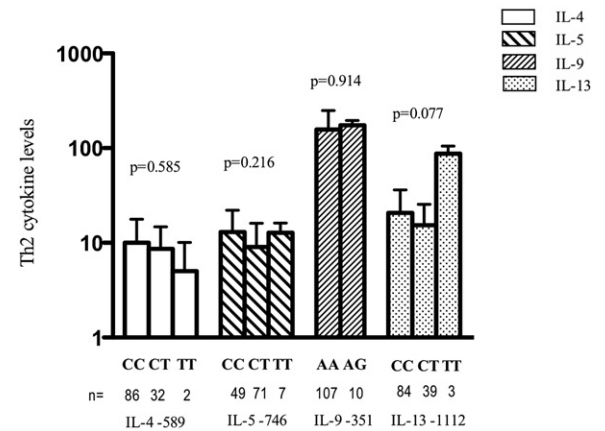
IL-10 is a key immunomodulatory cytokine the principal function of which is to limit the magnitude of immune responses. IL-10 is produced by a variety of cell types, including macrophages, dendritic cells, B cells, mast cells, eosinophils, and T cells ($CD4^+$ [T_H1 , T_H2 , and T_H9], $CD8^+$, and regulatory T cells). Production of suppressive IL-10 by T_H1 and T_H2 cells limits the effects of the proinflammatory cytokines they secrete, thereby controlling the immune response and preventing tissue damage.¹ Three single nucleotide polymorphisms (SNPs) in the promoter region (rs1800896, rs1800871, and rs1800872) of the *IL10* gene have been shown to alter IL-10 mRNA and protein levels² and are associated with both T_H2 -related³ and T_H1 -related diseases.⁴ T_H1 and T_H2 cytokine responses of PBMCs have generally been examined after stimulation with inhalant allergens, such as house dust mite (HDM), and studied in allergic rather than unselected subjects. Genetic factors might play a role in controlling PBMC cytokine release, and SNPs have been reported in several other T_H2 cytokine genes, including *IL4*, *IL5*, *IL9*, and *IL13*.⁵ Because current knowledge suggests a pivotal role for IL-10 in controlling immune responses, the aim of the current study was to examine the relative roles of promoter variants in *IL10* and T_H2 cytokine genes in determining cytokine production from HDM-stimulated PBMCs from the unselected Perth Infant Asthma Follow-up (PIAF) cohort. We hypothesized that *IL10* promoter variants that reduce IL-10 production would be associated with general upregulation of T_H2 cytokines.

The methods used are included in the [Methods](#) section of this article's Online Repository at www.jacionline.org. Briefly, DNA and PBMCs were collected from 11-year-old children who are participants in the ongoing PIAF longitudinal study. Promoter SNPs in cytokine genes were genotyped by using standard methods. *In vitro* studies on PBMC responses were done on cryobanked samples cultured with either 10 μ g/mL HDM or 1 μ g/mL PHA. IL-5, IL-10, and IL-13 protein levels in supernatants were measured by means of ELISA or time-resolved fluorescence, whereas *IL4*- and *IL9*-specific mRNA was quantitated by using Taqman PCR (Applied Biosystems, Foster City, Calif). Haplotypes of the 3 *IL10* promoter SNPs ($-1082G/A$, $-819C/T$, and $-592C/A$) were constructed with the Bayesian statistical-based program PHASE. All statistical analyses were conducted with SPSS for Windows Version 16.0 (SPSS, Inc, Chicago, Ill).

Table E1 lists the study cohort characteristics, and Table E2 shows the SNP genotype and allele frequencies (both available in this article's Online Repository at www.jacionline.org). HDM-induced cytokine responses and nominated genotypes were determined for the study cohort ($n = 154$ -160). All SNPs were in Hardy-Weinberg equilibrium ($P > .05$), and allele frequencies were similar to those previously reported. Few subjects had the variant (G) *IL9* allele, and none were homozygous GG. Therefore analyses of the associations between the *IL9* promoter SNP and HDM-induced IL-9 cytokine levels were done on the AA and AG groups. No associations were found between the selected *IL4*, *IL5*, *IL9*, or *IL13* SNPs and respective PBMC HDM-induced

A

Th2 cytokine gene polymorphisms and corresponding Th2 cytokine levels in HDM stimulated PBMCs



B

IL-10 -592 C/A polymorphism and Th2 cytokine levels in HDM stimulated PBMCs

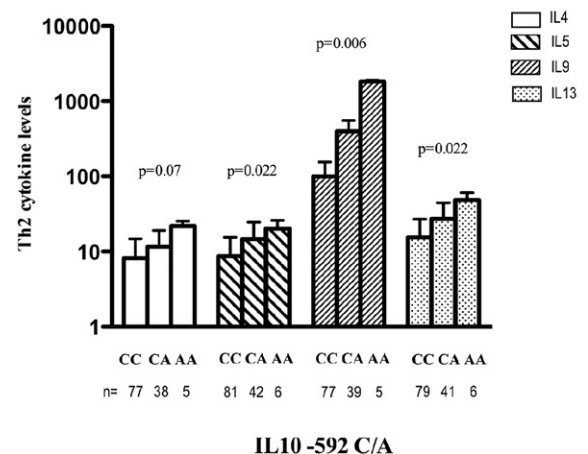


FIG 1. Relationship between T_H2 cytokine gene SNPs (A) and the *IL10* -592 C/A SNP (B) and mean levels of T_H2 cytokines produced in HDM-stimulated PBMCs. SNPs are listed on the x-axis, and log T_H2 cytokine levels (in picograms per milliliter for IL-5 and IL-13 and PCR units for IL-4 and IL-9) are listed on the y-axis.

cytokine levels (Fig 1, A). The *IL10* -592 and -819 SNPs were in complete linkage, as previously reported, and therefore only -592C/A was analyzed. The -592 C/A SNP and the ATA haplotype were associated with increased levels of IL-5, IL-9, and IL-13 and marginally significantly higher levels of IL-4 from HDM-stimulated PBMCs (Fig 1, B, and see Table E3 in this article's Online Repository at www.jacionline.org). There was a linear trend across the genotypes for IL-4 ($P = .017$), IL-5 ($P = .007$), IL-9 ($P = .001$), and IL-13 ($P = .006$) levels (Fig 1, B). There were no significant associations between IL-10 genotypes and haplotypes and HDM-induced IL-10 levels or between the

IL10 –1082 G/A SNP and the ACC or GCC haplotypes with HDM-induced T_H2 cytokine responses. In addition, there were no significant associations with PHA-induced cytokine responses (see Tables E4 and E5 in this article's Online Repository at www.jacionline.org). Results for atopic subjects were similar to those seen with the whole population (see Tables E6 and E7 in this article's Online Repository at www.jacionline.org).

In this study we found that T_H2 cytokine levels produced by HDM-stimulated PBMCs were associated with genetic variations in *IL10*. Surprisingly, genetic variations in the T_H2 cytokine genes themselves, despite their putative functional roles, were not associated with their respective cytokine levels. These data support a central and important role for IL-10 in regulating T_H2 cytokine responses in children because the low-producing *IL10* genotypes and haplotype were associated with higher levels of HDM-induced T_H2 cytokines. These results also suggest that the influence of IL-10 is more important than the SNPs in T_H2 coding genes in controlling T_H2 cytokine responses.⁵

A limited number of studies have reported using mitogens, LPS, or both as stimulants in the PBMC assays.^{6,7} The 589TT variant of *IL4* –589C/T was associated with higher levels of IL-4 cytokine production in PBMCs stimulated with PHA and phorbol 12-myristate 13-acetate (PMA). The –1112TT genotype of *IL13* –1112C/T was associated with significantly higher levels of IL-13 from PBMCs stimulated with the mitogens concanavalin A (ConA) and PMA. *IL10* –1082GG has been associated with increased levels of IL-10 from PBMCs stimulated with ConA, PMA/ionomycin, and LPS,² and the ATA haplotype was associated with lower levels of IL-10. In contrast, we found no associations between SNPs in genes encoding T_H2 cytokines and protein production and no associations between SNPs and haplotypes in the *IL10* promoter and IL-10 production in HDM-stimulated PBMCs. Another study found no difference related to the –1082 genotype in IL-10 levels from ConA-stimulated PBMCs.⁸ The reason for the lack of association between *IL10* SNPs/haplotype and IL-10 production in our study is unclear but might be due to a number of factors, including stimulus used, the unselected study cohort, or the mixed cell population used in the PBMC assays.⁹

The mechanism by which genetic variants of *IL10* affect T_H2 cytokine output in response to HDM stimulation in our study is unclear. Antigens such as HDM generally elicit a strong T_H2 immune response, with increased levels of IL-4, IL-5, and IL-13, as well as induction of the T_H2 transcription factor GATA-3, and levels of *IL10* mRNA and protein were decreased in children with asthma, a disease characterized by increased T_H2 cytokine levels.³ Numerous studies of the IL-10 signaling pathway have used LPS exposure and subsequent activation of the Toll-like receptor 4 pathway through MyD88 to gain insight into the mechanisms by which IL-10 mediates its anti-inflammatory response.¹⁰ Some limitations of our study include the low numbers in some genotype groups and the potential influence of other genes not examined.

In conclusion, this study examined associations between promoter SNPs in *IL10* and several T_H2 cytokine genes with cytokine production in HDM-stimulated PBMCs from an

unselected cohort of 11-year-old children. Our results support an important role for IL-10 in modulating T_H2 cytokine responses to HDM, specifically that the low-IL-10-producing genotypes and haplotype and not genetic variants in T_H2 cytokine genes were associated with increased levels of HDM-induced T_H2 cytokines providing further evidence of a pivotal role for IL-10 in controlling proinflammatory cytokine responses.

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METHODS

The PIAF cohort

The longitudinal community-based PIAF cohort consists of 253 unselected white children recruited antenatally from expectant mothers attending a local metropolitan hospital (Osborne Park Hospital, Perth, Australia) between June 1987 and November 1990. Exclusion criteria included premature birth, congenital abnormalities, and respiratory symptoms in the first month after birth. Details of the population and the recruitment process have been published elsewhere.^{E1} The cohort was assessed in infancy; at 1, 6, and 12 months; and during childhood at 6 and 11 years, with the 18-year follow-up currently being completed. At each time point lung function, airway responsiveness and skin prick test responses to common allergens were assessed, and a comprehensive questionnaire on respiratory health was completed. At the 6- and 11-year follow-up visits, blood was collected for IgE measurement and extraction of DNA, and PBMCs were collected at the 11-year follow-up visit. Written parental consent was obtained for each follow-up, and verbal agreement was obtained from the children at the 11-year assessment. Data from the 11-year follow-up were used in this study. The study was approved by the Ethics Committee of Princess Margaret Hospital for Children.

Genotyping

DNA was isolated from PBMCs, as previously described.^{E2} SNPs in the promoter regions of *IL10* (−1082G/A rs1800896, −819C/T rs1800871, and −592C/A rs1800872), *IL4* (−489C/T rs2243250), *IL5* (−746C/T rs2069812), *IL9* (−351A/G rs1799962), and *IL13* (−1112C/T rs1800925) were genotyped by using standard methods (PCR-RFLP or Sequenom Mass-ARRAY [Sequenom, Inc, San Diego, Calif]).

Cellular studies

Cryobanked PBMCs were cultured for 48 hours in AIM-V (Life Technologies, Carlsbad, Calif) medium with 4×10^{-5} mol/L 2-mercaptoethanol alone or with 10 mg/mL HDM extract (prepared in house) or 1 mg/mL PHA (Murex Diagnostics Ltd, Dartford, United Kingdom), as previously described.^{E3,E4} Two hundred microliters of cells was cultured in triplicate in round-bottom 96-well plates (Nunc, Roskilde, Denmark) at a concentration of 1.2×10^6 cells/mL. After stimulation, cell pellets were resuspended in RNeasy Lysis Buffer (Qiagen, Crawley, Australia) and stored at −20°C. Cultures were performed in batches by the same team using carefully standardized reagents throughout, with progressive cross-validation of growth media and allergen batches throughout the period over which experiments were performed to avoid reagent-associated variations in *in vitro* reactivity.

Cytokine analyses

Levels of IL-5, IL-10, and IL-13 protein in supernatants were measured by means of ELISA or time-resolved fluorescence. Coating and biotin-labeled detection antibodies for these measurements were purchased from BD PharMingen (San Jose, Calif). mRNA levels for IL-4 and IL-9 were measured from the 48-hour cell culture pellets and quantitated with Taqman.^{E5}

Statistical analyses

Because levels of cytokines (IL-4, IL-5, IL-9, and IL-13) stimulated with HDM and PHA were in approximate log-normal distributions, natural log transformations were conducted before the parametric statistical analyses. Haplotypes of the 3 SNPs in the *IL10* gene promoter (−1082G/A, −819C/T, and −592C/A) were constructed by using the Bayesian statistical-based program PHASE, version 2.0.2.^{E6} The ATA haplotype consisted of the −1082A, −819T, and −592A alleles. The differences in cytokine levels between genotypes and haplotypes were investigated by using the independent-samples *t* test and ANOVA. Linear regression was also used to clarify the associations of genotypes and haplotypes of *IL10* with TH2 cytokines. All statistical analyses were conducted with SPSS software (SPSS for Windows Version 16.0), and a *P* value of less than .05 was considered significant.

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TABLE E1. Details of the 130 children with PBMC responses to HDM included in the study

Male sex, no. (%)	75 (57)
Age, mean (SD [range])	11.1 (0.90 [9.28-13.47])
Atopy (positive SPT response), no. (%)	71 (54.6)
Total IgE (kU/L), mean (SD [range])	207.29 (628.12 [1.0-6642.0])
Airway responsive to histamine (PD ₂₀ <7.8 mg/mL), no. (%)	45 (34.6)

SPT, Skin prick test.

TABLE E2. Genotype and allele frequencies for the SNPs analyzed in this study

<i>IL10</i> -1082 rs1800896 (n = 156)	AA (no. [%]): 53 (34.0)	AG (no. [%]): 65 (41.7)	GG (no. [%]): 38 (24.4)	A allele (%): 54.8	GCC haplotype (%): 45
<i>IL10</i> -819 rs1800871 (n = 157)	CC (no. [%]): 89 (56.7)	CT (no. [%]): 59 (37.6)	TT (no. [%]): 9 (5.7)	C allele (%): 75.5	ACC haplotype (%): 30
<i>IL10</i> -592 rs1800872 (n = 157)	CC (no. [%]): 89 (56.7)	AC (no. [%]): 59 (37.6)	AA (no. [%]): 9 (5.7)	C allele (%): 75.5	ATA haplotype (%): 25
<i>IL4</i> -589C/T rs2243250 (n = 160)	CC (no. [%]): 114 (71.2)	CT (no. [%]): 44 (27.5)	TT (no. [%]): 2 (1.2)	C allele (%): 85.0	
<i>IL5</i> -746C/T rs2069812 (n = 154)	CC (no. [%]): 65 (42.2)	CT (no. [%]): 79 (51.3)	TT (no. [%]): 10 (6.5)	C allele (%): 67.9	
<i>IL9</i> -351A/G rs1799962 (n = 156)	AA (no. [%]): 143 (91.7)	AG (no. [%]): 13 (8.3)	GG (no. [%]): 0	A allele (%): 95.8	
<i>IL13</i> -1112 C/T rs1800925 (n = 155)	CC (no. [%]): 102 (65.8)	CT (no. [%]): 50 (32.3)	TT (no. [%]): 3 (1.9)	C allele (%): 82.0	

TABLE E3. Associations between the *IL10* –592C/A SNP and *IL10* ATA haplotype (0, 1, or 2 copies of the haplotype) with T_H2 cytokine production in HDM-stimulated PBMCs

–592C/A	ATA	No.	GM	95% CI		P value
				Lower	Upper	
IL-4 cytokine responses						
CC	0	77	8.18	6.59	10.15	.07
CA	1	38	11.60	7.45	18.05	
AA	2	5	21.93	3.47	138.50	
IL-5 cytokine responses						
CC	0	81	8.67	6.85	10.97	.022
CA	1	42	14.60	9.92	21.47	
AA	2	6	20.22	5.79	70.60	
IL-9 cytokine responses						
CC	0	77	99.85	55.52	179.61	.006
CA	1	39	399.14	154.50	1,031.12	
AA	2	5	1,810.76	91.39	35,882.33	
IL-13 cytokine responses						
CC	0	79	15.40	11.56	20.52	.022
CA	1	41	27.10	17.46	42.10	
AA	2	6	48.20	12.41	187.20	

GM, Geometric mean.

TABLE E4. Associations between the *IL10* –592C/A SNP and cytokine production in PHA-stimulated PBMCs

–592C/A	No.	GM	95% CI		P value
			Lower	Upper	
IL-5 cytokine responses					
CC	81	251.59	209.81	301.72	.733
CT	42	245.82	183.10	330.07	
TT	6	330.83	124.72	877.43	
IL-10 cytokine responses					
CC	80	132.94	111.01	159.22	.366
CT	42	105.54	79.36	140.36	
TT	6	125.32	46.75	335.89	
IL-13 cytokine responses					
CC	81	1203.63	978.87	1480.15	.439
CT	42	1449.54	1144.13	1836.28	
TT	6	1606.16	988.91	2608.68	

GM, Geometric mean.

TABLE E5. Associations between T_H2 cytokine SNPs and cytokine production in PHA-stimulated PBMCs

	No.	GM	95% CI		<i>P</i> value
			Lower	Upper	
Associations between <i>IL5</i> –746C/T and IL-5 cytokine responses					
CC	49	230.79	180.08	295.74	.497
CT	72	270.72	220.61	332.22	
TT	7	204.71	100.22	418.17	
Associations between <i>IL13</i> –1112C/T and IL-13 cytokine responses					
CC	84	1,246.88	1,024.34	1,517.77	.816
CT	39	1,369.50	1,047.12	1,791.13	
TT	3	1,517.17	226.85	10,145.65	

GM, Geometric mean.

TABLE E6. Associations between SNPs in T_H2 cytokine genes and T_H2 cytokine production in HDM-stimulated PBMCs from subjects with positive skin prick test responses

	No.	GM	95% CI		P value
			Lower	Upper	
Associations between <i>IL4</i> –589C/T and IL-4 cytokine responses					
CC	28	12.30	7.12	21.25	.29
CT/TT	11	7.63	4.68	12.45	
Associations between <i>IL5</i> –746C/T and IL-5 cytokine responses					
CC	20	19.71	10.45	37.18	.62
CT/TT	18	15.82	8.18	30.64	
Associations between <i>IL9</i> –351A/G and IL-9 cytokine responses					
AA	33	265.68	87.53	806.42	.96
AG	3	242.21	0.045	1,318,097.54	
Associations between <i>IL13</i> –1112C/T and IL-13 cytokine responses					
CC	24	25.27	13.56	47.09	.82
CT/TT	14	22.72	10.87	47.46	

GM, Geometric mean.

TABLE E7. Associations between the *IL10* –592C/A SNP and *IL10* ATA haplotype (0, 1, or 2 copies) with T_H2 cytokine production in HDM-stimulated PBMCs from subjects with positive skin prick test responses

stimulated T cells from subjects with positive skin prick test responses						
–592C/A	ATA	No.	GM	95% CI		P value
				Lower	Upper	
IL-4 cytokine responses						
CC	0	21	7.80	4.76	12.80	.087
CA/AA	1/2	18	15.64	7.87	31.10	
IL-5 cytokine responses						
CC	0	21	11.06	6.72	18.21	.008
CA/AA	1/2	18	32.94	16.80	64.591	
IL-9 cytokine responses						
CC	0	21	144.45	40.57	514.25	.076
CA/AA	1/2	18	834.22	168.63	4127.40	
IL-13 cytokine responses						
CC	0	21	15.70	9.10	27.10	.029
CA/AA	1/2	17	41.68	19.80	87.76	

GM, Geometric mean.