



Genetic Polymorphisms in Cytokine Genes in Colombian Patients with Ocular Toxoplasmosis

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ABSTRACT Toxoplasmosis is caused by infection with the protozoan parasite *Toxoplasma gondii*, which has the capacity to infect all warm-blooded animals worldwide. Toxoplasmosis is a major cause of visual defects in the Colombian population; however, the association between genetic polymorphisms in cytokine genes and susceptibility to ocular toxoplasmosis has not been studied in this population. This work evaluates the associations between polymorphisms in genes coding for the cytokines tumor necrosis factor alpha (TNF- α) (rs1799964, rs1800629, rs1799724, rs1800630, and rs361525), interleukin 1 β (IL-1 β) (rs16944, rs1143634, and rs1143627), IL-1 α (rs1800587), gamma interferon (IFN- γ) (rs2430561), and IL-10 (rs1800896 and rs1800871) and the presence of ocular toxoplasmosis (OT) in a sample of a Colombian population (61 patients with OT and 116 healthy controls). Genotyping was performed with the “dideoxynucleotide (ddNTP) primer extension” technique. Functional-effect predictions of single nucleotide polymorphisms (SNPs) were done by using FuncPred. A polymorphism in the IL-10 gene promoter (-1082G/A) was significantly more prevalent in OT patients than in controls ($P = 1.93e-08$; odds ratio [OR] = 5.27e+03; 95% confidence interval [CI] = 3.18 to 8.739; Bonferroni correction [BONF] = 3.48e-07). In contrast, haplotype “AG” of the IL-10 gene promoter polymorphisms (rs1800896 and rs1800871) was present at a lower frequency in OT patients ($P = 7e-04$; OR = 0.10; 95% CI = 0.03 to 0.35). The +874A/T polymorphism of IFN- γ was associated with OT ($P = 3.37e-05$; OR = 4.2; 95% CI = 2.478 to 7.12; BONF = 6.07e-04). Haplotype “GAG” of the IL-1 β gene promoter polymorphisms (rs1143634, rs1143627, and rs16944) appeared to be significantly associated with OT ($P = 0.0494$). The IL-10, IFN- γ , and IL-1 β polymorphisms influence the development of OT in the Colombian population.

KEYWORDS ocular toxoplasmosis, single nucleotide polymorphisms, cytokines, susceptibility

Toxoplasmosis is caused by infection with the protozoan parasite *Toxoplasma gondii*, which has the capacity to infect all warm-blooded animals worldwide (1). It is estimated that 30 to 70% of the human population is infected with this parasite, and

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essentially the entire human population is at risk of infection (2). A limited number of people develop symptoms, suggesting that host susceptibility and strain disparity can play a role in the variability of clinical symptoms (3). For instance, in Colombia, where seroprevalence studies show that almost half of the population is infected (47% according a national study) (4), the incidence of symptomatic ocular toxoplasmosis (OT) is 3 new episodes per 100,000 inhabitants per year (5). It is estimated that around 10% of newly infected people develop the ocular form of toxoplasmosis (6). OT is characterized by intraocular inflammation and is the most common clinical manifestation of toxoplasmosis (7). Particularly, OT causes severe pathology in the eye in South America that is more severe than in other parts of the world, and it is characterized by deviated T helper 2 (Th2) immune responses (8, 9). Lesions can originate both from congenital infection and from infections acquired after birth (10, 11). The lesions can affect the macula and other layers of the retina and the choroid, resulting in retinochoroiditis, the most frequent cause of posterior uveitis in immunocompetent patients (12). Ocular manifestations can have an early or late onset, with primary or recurrent clinical manifestations, and present different degrees of ocular involvement that vary according to the immune status of the individual and by infection with different *T. gondii* strains (13–15). The relative contribution of the host inflammatory response elicited, versus parasite proliferation, to the development of retinal destruction has not yet been completely defined (16). Cytokines, chemokines, and their receptors play a key role in the regulation of the type and magnitude of the immune response. Whether the ocular manifestations resulting from infection by *T. gondii* are attributable to host or parasite genetic factors, differences in the exposure rate, or all these factors remains uncertain (17).

One important issue to study is the polymorphisms in genes coding for cytokines involved in the immune response to *Toxoplasma*. Infection in humans is characterized by the presence of high levels of cytokines, such as interleukin 12 (IL-12), tumor necrosis factor alpha (TNF- α), and gamma interferon (IFN- γ), all of which have been associated with ocular pathology (18). Besides the involvement of both CD8⁺ T lymphocytes and natural killer (NK) cells, the immune response to *T. gondii* infection induces a strong Th1 response orchestrated by CD4⁺ T cells and is dominated by the production of pro-inflammatory mediators. However, while the Th1 response prevents parasite replication, a strong Th1 response may also cause immune-mediated tissue damage, contributing to the severity of ocular toxoplasmosis. More recently, Th17 cells, characterized by the production of IL-17, a potent inducer of inflammation, were identified as key contributors to immunopathological responses in OT (19, 20). Taken together, a variety of gene polymorphisms might be involved in OT and may create an individual risk profile for a given patient (21).

Functional genetic polymorphisms in cytokine genes may interfere with or enhance the expression of cytokines and play a role in the genetic regulation of inflammatory responses and resistance or susceptibility to infectious diseases. Single nucleotide polymorphisms (SNPs) are useful to identify polymorphisms associated with susceptibility to a particular disease. Natural selection has favored the introduction of biallelic SNPs into cytokine genes, which results in variation in the production and level of protein rather than variation in its quality (22). Previous studies indicated that polymorphisms in the IL-1, IL-10, IFN- γ , and TNF- α genes or promoters are associated with a higher frequency of OT in people from Brazil (21, 23–25). Not unexpectedly, host cytokine gene polymorphisms have been a focus of interest in toxoplasmic chorioretinitis. Cytokines, in particular IFN- γ and TNF- α , play an essential role in resistance to *T. gondii* infections (26, 27). These cytokines activate innate immunity and macrophages, a major first line of defense.

Polymorphisms in genes encoding various cytokines have been shown to be connected with susceptibility to parasitic diseases. Indeed, individuals in Brazil who were homozygous for the A allele (+874T/A) of the IFN- γ gene had a high risk of OT if they possessed the A/A genotype, compared to a negative-control group (23). In addition, experimental data demonstrated a relevant role for the anti-inflammatory

cytokine IL-10 in modulating acute OT; thus, the IL-10 gene polymorphism (IL-10 –1082A allele; AA and AG genotypes) was associated with the occurrence of OT (24). More recently, a study conducted by Cordeiro et al. (28) similarly associated an IL-6 polymorphism (–174G/C) with the occurrence, but not recurrence, of OT in Brazilian patients. Those authors also showed that the recurrence of toxoplasmic retinochoroiditis was associated with an IL-1 α (–889C/T) polymorphism, related to an increase in IL-1 α expression (24). Another study in Poland demonstrated that the major C allele at the IL-1 β +3954C>T SNP was significantly more frequently detected among fetuses and neonates with congenital *T. gondii* infection than among uninfected persons. The outcomes reported in that study suggested that the presence of a mutated T allele in or marking the gene with this IL-1 β SNP has a protective function against the development of congenital toxoplasmosis (infection acquired *in utero* during gestation). However, the mechanism of the role of the IL-1 β +3954C>T SNP has yet to be investigated in a detailed molecular study (29). A study of a TNF- α gene polymorphism (–308G/A) in Brazil also demonstrated that the occurrence or recurrence of toxoplasmic retinochoroiditis was not associated with this polymorphism (21). Together, data from those studies suggest that the genetic control of the immune response is relevant for the pathogenesis of toxoplasmic chorioretinitis.

However, given the complexity of both parasite biology and the host immune system, it is unlikely that genetic variation at a single locus, as shown by SNP analyses, would provide an adequate explanation for the interindividual differences in host immune responses that result in diverse clinical manifestations. To this end, the identification of gene-gene interactions could enhance the power and accuracy of predicting disease outcomes of a complex disorder (30). For a better description of the genetic architecture of disease susceptibility and unambiguous identification of factors responsible for both causality and predisposition to a disease, functional appraisal of disease-associated polymorphisms is essential. There is widespread recognition that differences in gene expression may be an important source of phenotypic diversity in complex diseases (31, 32) and that noncoding polymorphisms contribute to the variance and etiology of a trait by regulating the expression of nearby genes.

To explore the plausible regulatory mechanisms exerted by cytokine SNPs, we have characterized allele-specific events by studying their transcriptional differences in terms of reporter gene activities and allelic expression imbalance (AEI). Basu et al. provided detailed insights into the molecular effects of *cis*-regulatory variants in controlling cytokine gene expression in *Plasmodium falciparum*-mediated malaria. However, that study underscored the possibility that this complex trait involves even more complex regulatory intricacies than previously anticipated (33). To date, few studies have reported an association between IL-10, TNF- α , IL-1, and IFN- γ gene polymorphisms and the development of OT, and none of those studies was conducted with a Colombian population. In this paper, we conducted a case-control study to investigate the association between TNF- α (rs1799964, rs1800629, rs1799724, rs1800630, and rs361525), IL-1 β (rs16944, rs1143634, and rs1143627), IL-1 α (rs1800587), IFN- γ (rs2430561), and IL-10 (rs1800896 and rs1800871) gene polymorphisms and the risk of OT in a Colombian population.

RESULTS

Genetic ancestries of cases and controls are similar. The apportionment of genetic ancestral contributions in cases and controls was estimated as the mean of each ancestry across individuals, using Admixture v1.3 software. Considering the historical formation of the Colombian population, we assumed an essentially trihybrid contribution from Native Americans, Europeans, and Africans (i.e., $K = 3$). Ancestry analyses were conducted by using the Human Genome Diversity Project and Centre d'Etude du Polymorphisme Humain (HGDP-CEPH) populations as a reference (for Africa $n = 43$ cases [$P = 0.07$] and $n = 10$ controls [$P = 0.05$]; for Europe, $n = 43$ cases [$P = 0.68$] and $n = 10$ controls [$P = 0.70$]; and for America, $n = 43$ cases [$P = 0.25$] and $n = 10$ controls [$P = 0.25$]). No differences in the genetic ancestries in cases and

controls were detected ($P = 0.91$). The ancestry information for cases and controls was similar to that previously reported by Ossa et al. (34) for the Central West Andean region of Colombia (where the present study was carried out), with the European contribution to the genetic background being higher for this population.

Case-control association analysis indicates that the IL-10 –1082A/G and the IFN- γ +874A/T polymorphisms are associated with ocular toxoplasmosis. We investigated the distribution of 12 SNPs in 61 Colombian OT patients (cases) and 116 healthy controls. The genotype distribution of all polymorphisms did not deviate significantly from the Hardy-Weinberg equilibrium (HWE). The frequencies of the genotypes of polymorphisms in proinflammatory and anti-inflammatory cytokine genes in cases and controls are presented in Table 1. Among the cytokine genes, significantly higher frequencies of IL-1, IFN- γ , and IL-10 polymorphisms were observed for the OT group. We found that the IFN- γ polymorphism at this position (+874) was most frequently found in the OT group. Notably, the GG genotype with the IL-10 –1082A/G polymorphism was highly associated with the OT phenotype ($P = 1.47e-05$). Among variants of IL-10, the strongest association was found for the SNP –1082A/G (rs1800896), where the GG genotype was highly prevalent in patients in comparison to healthy subjects. The IL-10 –819G/A polymorphism was not associated with OT ($P = 0.756$).

In Table 2, we present the frequencies of the TNF- α –238G/A, TNF- α –308G/A, TNF- α –1031T/C, TNF- α –857C/T, and TNF- α –863C/A polymorphisms. No differences between the observed and expected distributions of genotypes were found between cases and controls. Our study is the first to investigate the association between TNF- α gene polymorphisms and the occurrence of OT in Colombian populations. Our finding was unexpected, as the role of TNF- α in OT seems to be relevant. In addition, for the IL-1 SNPs, we did not find significant differences in the frequencies of the G/A genotype.

The allele distributions (minor allele frequency [MAF]) of the polymorphisms in cytokines genes for each group are summarized in Table 2. In order to determine whether the less-represented alleles of SNPs in candidate genes (IL-1, IFN- γ , TNF- α , and IL-10 genes) were an independent risk factor for toxoplasmosis, we performed an association test (Table 3).

Most of the SNPs analyzed had no significant effect, while the differences proved to be significant for the rs1800896 and rs2430561 SNPs after the application of the Bonferroni correction (BONF). In our study, the –1082G allele ($P = 1.93e-08$; odds ratio [OR] = 5.27; 95% confidence interval [CI] = 3.18 to 8.73; BONF = $3.48e-07$) and the +874A allele ($P = 3.37e-05$; OR = 4.2; 95% CI = 2.478 to 7.12; BONF = $6.07e-04$) were present at high frequencies and were significantly represented in OT patients compared with controls. In the case of the rs1143627 polymorphism, it initially showed a significant effect ($P = 0.05969$; OR = 1.53; 95% CI = 0.98 to 2.37) that disappeared when the Bonferroni correction was applied (BONF = 1.00).

So far, several studies have shown some involvement of the proinflammatory cytokine IL-1 β in the immune response after *T. gondii* infection. No associations between the observed and expected distributions of the IL-1 α –889T/C, IL-1 β +3954G/A, IL-1 β –511C/T, TNF- α –308G/A, TNF- α –238C/T, TNF- α –863C/A, TNF- α –857G/A, and TNF- α –1031T/C alleles were found. There were no significant differences in the allelic frequencies of each of these polymorphisms in patients with OT compared to control subjects ($P = 0.4916$, $P = 0.7099$, $P = 0.169$, $P = 0.8345$, $P = 0.9696$, $P = 0.5503$, $P = 0.6428$, and $P = 0.843$, respectively).

Haplotypes of the IL-1 β and IL-10 gene promoter polymorphisms are associated with susceptibility to ocular toxoplasmosis. In order to analyze the haplotype effect of cytokine genes, we considered those genes with enough SNPs analyzed (at least two in each gene). The IFN- γ gene was not analyzed by the haplotype approach. We also estimated the TNF- α haplotype frequencies (rs1799964, rs1800629, rs1799724, rs1800630, and rs361525) and evaluated the association of these variants with OT. We observed five haplotype combinations (TGGCC, TGACC, CGGAC, TAGCC, and CGGCT).

TABLE 1 Genotypic frequencies for each SNP evaluated

Official gene symbol	Alternative gene names	Gene ID	Polymorphism		Genotype, no. (%) of individuals						P value	
			rs ID ^a	Position in gene	Chromosome location	Cases	Healthy controls					
TNF	TNF- α , DIF, TNFA, TNFSF2, TNLG1F	7124	rs1799964	-1031	6:31574531	TT, 39 (67.2)	TC, 18 (31.0)	CC, 1 (1.7)	TT, 76 (65.5)	TC, 38 (32.8)	CC, 2 (1.7)	
TNF	TNF- α , DIF, TNFA, TNFSF2, TNLG1F	7124	rs1800630	-863	6:31574699	CC, 39 (76.5)	CA, 10 (19.6)	AA, 2 (3.9)	CC, 88 (77.2)	CA, 26 (22.8)	AA, 0 (0.0)	
TNF	TNF- α , DIF, TNFA, TNFSF2, TNLG1F	7124	rs1799724	-857	6:31574705	GG, 35 (61.4)	GA, 21 (36.8)	AA, 1 (1.8)	GG, 78 (67.2)	GA, 34 (29.3)	AA, 4 (3.5)	
TNF	TNF- α , DIF, TNFA, TNFSF2, TNLG1F	7124	rs361525	-238	6:31575324	CC, 53 (89.8)	CT, 5 (8.5)	TT, 1 (1.7)	CC, 103 (88.8)	CT, 12 (10.3)	TT, 1 (0.9)	
TNF	TNF- α , DIF, TNFA, TNFSF2, TNLG1F	7124	rs1800629	-308	6:31575254	GG, 51 (85.0)	GA, 9 (15.0)	AA, 0 (0.0)	GG, 100 (86.2)	GA, 16 (13.8)	AA, 0 (0.0)	
IL1A	IL-1, IL-1A, IL1F1, IL-1-ALPHA	3552	rs1800587	-889	2:112785383	GG, 33 (55.0)	GA, 23 (38.3)	AA, 4 (6.7)	GG, 59 (50.9)	GA, 46 (39.7)	AA, 11 (9.5)	
IL1B	IL-1, IL1F2, IL-1-BETA	3553	rs16944	-511	2:112837290	GG, 15 (24.6)	GA, 29 (47.5)	AA, 17 (27.9)	GG, 37 (31.9)	GA, 56 (48.3)	AA, 23 (19.8)	0.391
IL1B	IL-1, IL1F2, IL-1-BETA	3553	rs1143634	3954	2:112832813	GG, 41 (68.3)	GA, 17 (28.3)	AA, 2 (3.3)	GG, 83 (71.5)	GA, 29 (25.0)	AA, 4 (3.5)	
IL1B	IL-1, IL1F2, IL-1-BETA	3553	rs1143627	-31	2:112836810	GG, 14 (22.9)	GA, 27 (44.3)	AA, 20 (32.8)	GG, 37 (31.9)	GA, 55 (47.4)	AA, 24 (20.7)	0.169
IFNG	IFG, IFI	3458	rs2430561	874	12:68158742	TT, 2 (4.3)	TA, 21 (44.7)	AA, 24 (51.1)	TT, 47 (40.5)	TA, 46 (39.7)	AA, 23 (19.8)	
IL-10	CSIF, TGIF, GVHDS, IL-10, IL10A	3586	rs1800871	-819	1:206773289	GG, 34 (57.6)	GA, 19 (32.2)	AA, 6 (10.2)	GG, 61 (52.6)	GA, 44 (37.9)	AA, 11 (9.5)	0.756
IL-10	CSIF, TGIF, GVHDS, IL-10, IL10A	3586	rs1800896	-1082	1:206773552	AA, 8 (15.1)	AG, 14 (26.4)	GG, 31 (58.5)	AA, 57 (50.0)	AG, 40 (35.1)	GG, 17 (14.9)	1.47e-05

^ars ID, reference SNP ID number.

TABLE 2 Allelic distribution of cytokine gene polymorphisms in Colombian patients with ocular toxoplasmosis and healthy controls^a

Chr	dbSNP ID	A1	A2	MAF		GMAF
				Cases	Controls	
1	rs1800871	G	A	0.2627	0.2845	0.43
1	rs1800896	A	G	0.283	0.3246	0.27
2	rs1800587	G	A	0.2583	0.2931	0.27
2	rs1143634	G	A	0.175	0.1595	0.13
2	rs1143627	G	A	0.4508	0.444	0.47
2	rs16944	G	A	0.4836	0.4397	0.49
6	rs1799964	T	C	0.1724	0.181	0.22
6	rs1800630	C	A	0.1373	0.114	0.15
6	rs1799724	G	A	0.2018	0.181	0.10
6	rs1800629	G	A	0.075	0.06897	0.09
6	rs361525	C	T	0.05932	0.6034	0.06
12	rs2430561	T	A	0.266	0.3966	0.28

^aChr, chromosome; A1, allele 1; A2, allele 2; MAF, minor allele frequency; GMAF, global minor allele frequency.

However, a significant association of the distributions of the haplotype frequencies between cases and healthy controls was not found.

Haplotype "GAA" of the IL-1 β gene promoter polymorphisms (rs16944, rs1143634, and rs1143627) appeared to be significantly associated with susceptibility to ocular toxoplasmosis ($P = 0.038$). Haplotypes "GG" and "GA" of the IL-10 gene promoter polymorphism (rs1800896 and rs1800871) are also significantly associated with OT ($P = 4.0132e-07$ and $P = 1.1072e-06$, respectively). Frequencies are shown in Table 4.

Coexpression analysis identifies connectivity of the IL-1 β and IL-1 α genes mediated by Ribonuclease k6 (RNASE6). We simultaneously analyzed the 12 SNPs for the most significant gene-gene interactions associated with OT. Most of the polymorphisms involved in multifactor dimensionality reduction (MDR) combinations were not associated with disease in the single-site analysis. We further evaluated a possible epistatic effect among the less-represented alleles of SNPs in candidate genes. To this end, we applied a statistical approach to perform an analysis of the combined epistatic effect of SNPs. We did not find a significant risk for ocular toxoplasmosis in the presence of any MAF alleles from the rs1800896 and rs2430561 SNPs (Table 5). Coexpression analyses identified a connectivity of the IL-1 β and IL-1 α genes, mediated by RNASE6. No coexpression with IL-10, IFN- γ , and TNF- α (Fig. 1) was observed. Additional work is needed to assess the still unknown gene-gene interactions. Raj et al. performed an expression quantitative trait locus (eQTL) study on purified CD4⁺ T cells and monocytes from 461 healthy individuals and found that susceptibility alleles for certain diseases had an overrepresentation of either T cell-specific or monocyte-specific eQTLs (35). We

TABLE 3 Association analysis of MAF alleles in cytokine polymorphisms in patients with ocular toxoplasmosis and healthy controls^a

Chr	Gene	Position	CHISQ	P	OR	L95	U95	BONF
1	IL-10 -819G/A	206773288	0.1851	0.6671	0.8962	0.5439	1.477	1
1	IL-10 -1082A/G	206773551	45.04	1.93e-08	5.27	3.18	8.739	3.48e-07
2	IL-1 α -889G/A	112785382	0.473	0.4916	0.8401	0.5111	1.381	1
2	IL-1 β +3954G/A	112832812	0.1384	0.7099	1.118	0.6212	2.012	1
2	IL-1β -31G/A	112836809	3.546	0.05969	1.526	0.9819	2.371	1
2	IL-1 β -511G/A	112837289	1.892	0.169	1.361	0.8768	2.112	1
6	TNF- α -1031T/C	31574530	0.03925	0.843	0.9425	0.5244	1.694	1
6	TNF- α -863C/A	31574698	0.3567	0.5503	1.236	0.6161	2.48	1
6	TNF- α -857G/A	31574704	0.2151	0.6428	1.143	0.6489	2.015	1
6	TNF- α -308G/A	31575253	0.04365	0.8345	1.095	0.4687	2.556	1
6	TNF- α -238C/T	31575323	0.001451	0.9696	0.982	0.3853	2.503	1
9	IFN-γ +874T/A	68158741	30.48	3.37e-05	4.2	2.478	7.12	6.07e-04

^aChr, chromosome; CHISQ, chi-square value; OR, odds ratio; L95 and U95, lower and upper bounds of the 95% confidence intervals for the odds ratio, respectively; BONF, Bonferroni correction. Boldface indicates that statistical differences are significant.

TABLE 4 Haplotype frequencies of IL-10, TNF- α , and IL-1 β gene polymorphisms in patients with ocular toxoplasmosis^b

SNP	Haplotype	Frequency	P value ^a
IL-10 gene region (rs1800896, rs1800871)	GG	0.405	4.01e-03
	GA	0.319	1.11e-02
	AA	0.231	0.0321
	AG	0.045	0.001
TNF- α gene region (rs1799964, rs11800629, rs1799724, rs1800630, rs361525)	TGGCC	0.569	NA
	TGACC	0.162	0.6
	CGGAC	0.111	0.72
	TAGCC	0.07	0.6
	CGGCT	0.04	0.73
IL-1 β gene region (rs16944, rs1143634, rs1143627)	AGG	0.458	NA
	GGA	0.357	0.61
	GAA	0.159	0.038
	GGG	0.017	0.71

^aNA, not applicable.

^bBoldface indicates that statistical differences are significant.

used the SCAN database to check for coexpression between the genes identified in this paper and the genes identified by Raj et al., whose SNPs had P values of <10⁻⁸ (36). We found that there was coexpression between these two groups of genes (Table 6).

DISCUSSION

Ocular toxoplasmosis can cause visual alterations, retinochoroiditis, and, in some cases, loss of sight. *T. gondii* infection induces a response by T helper 1 cells, which produce IFN- γ , a cytokine that is involved in resistance to toxoplasmosis (37). Chorioretinitis during active OT involves all chorioretinal layers. This inflammatory process is characterized by the appearance of exudative, white-appearing lesions with soft borders; during the active phase, these lesions evolve with necrosis and the deposition of pigment, resulting in chronic inactive and irreversible atrophic and hyperpigmented retinal scars, which are sequelae of the acute process. The effector cells that attack and destroy infected retinal neurons include CD4⁺ and CD8⁺ T cells, B cells, and macrophages. Macrophages have also been occasionally detected in the choroid underlying retinal lesions (38). In patients with active retinochoroidal lesions due to congenital infection, an expansion of monocytes and NK cells in blood was found (39). The expansion, migration, and activation of these cells might be associated with chemokine/cytokine cross talk (40). Information concerning intraocular cytokine levels in OT has been obtained from aqueous humor (AH) samples, but further studies are needed in order to determine the precise source of these mediators and their contributions to pathogenesis.

Similar to results in the mouse model of ocular toxoplasmosis, resistance to OT is associated with the ability to produce IL-12 and IFN- γ in response to parasite antigens (41). Infection by *Toxoplasma* induces high levels of IFN- γ in humans, as can be deduced from reports of both asymptomatic and symptomatic *T. gondii*-seropositive individuals, compared to negative controls (42). However, by comparing the levels of this cytokine in individuals with disease to those in individuals who are infected but without symptoms, only a slight increase in the level of this cytokine was reported for

TABLE 5 Gene-gene interactions between the IL-10 -1082A/G and IFN- γ +874T/A gene polymorphisms

rs1800896 haplotype	No. of individuals with rs2430561 haplotype (frequency)		
	A/A	A/T	T/T
G/G	20 (0.112)	19 (0.107)	5 (0.028)
G/A	9 (0.050)	22 (0.124)	20 (0.112)
A/A	17 (0.096)	23 (0.129)	24 (0.135)

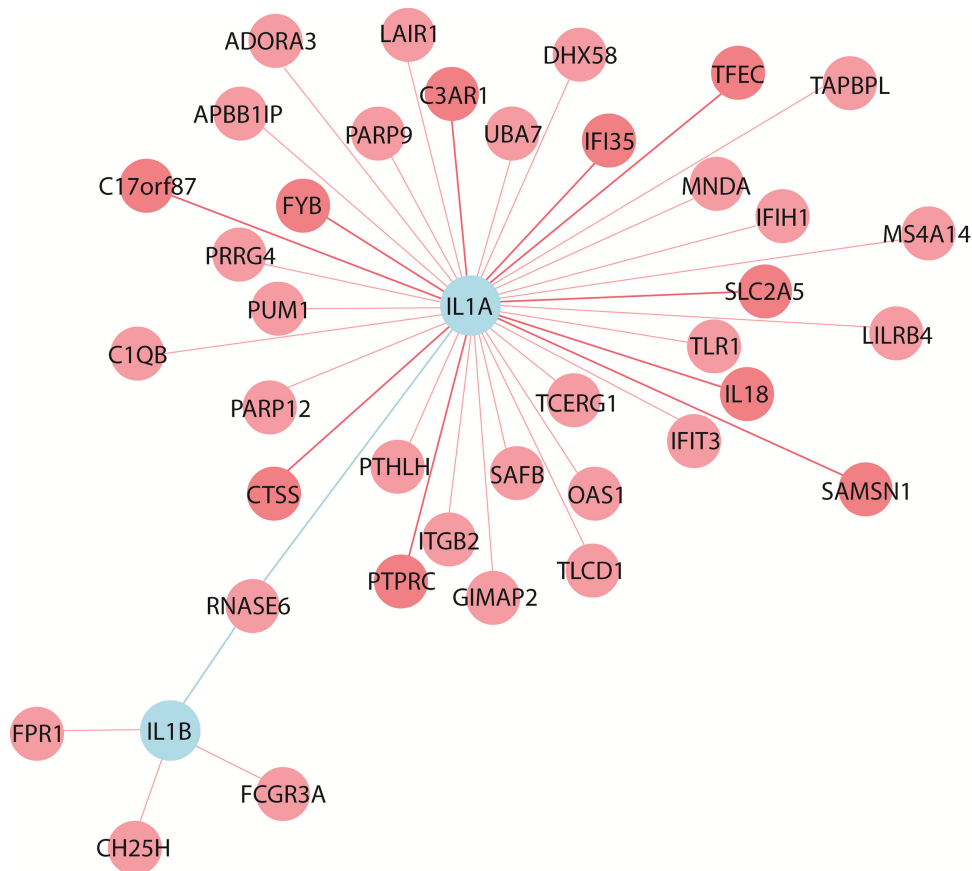


FIG 1 Coexpression analysis of IL-1 α , IL-1 β , TNF- α , IFN- γ , and IL-10, using NP *de novo* coexpression analysis (<http://www.wzgenomics.cn/NPdenovo/index.php>). The reference data set was HBT (The Human Brain Transcriptome), the minimum Pearson correlation coefficient was 0.8, and the maximum number of nodes of the network was 80.

T. gondii-seropositive asymptomatic individuals compared to individuals with OT (43). During our studies concerning intraocular cytokine levels in OT, we analyzed only a very few patients in order to establish differences in the levels of cytokines between those with vertically transmitted OT and those with OT acquired after birth. However, an analysis of the IFN- γ /IL-10 ratio in *ex vivo*-stimulated peripheral mononuclear cells suggests that patients who acquired infection *in utero* have higher levels of IL-10 production (9).

To explain why infected people can become sick although they produce IFN- γ , an imbalance in the cytokine network should be explored. Polymorphisms in the cytokine-encoding genes, including proinflammatory cytokines such as TNF- α , IL-6, IL-12, and IFN- γ and anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGF- β), were previously shown to be associated with several diseases (44, 45).

It is well recognized that differences in gene expression may be an important source of phenotypic diversity in complex diseases and that noncoding polymorphisms contribute to the variance and etiology of a trait by regulating the expression of nearby genes (31, 46). The present study is the first to demonstrate the association between polymorphisms in the IL-10 gene promoter (-819G/A and -1082A/G) and OT in a Colombian population. The link between OT and the GG genotype at the IL-10 promoter SNPs provides evidence that abnormalities in the genetic control of cytokine levels may be relevant in influencing the human immune response in OT. IL-10 is an anti-inflammatory and immune-regulatory cytokine, which induces T cell anergy by downregulating the expression of the genes coding for the costimulatory molecule B7-1/B7-2, major histocompatibility complex (MHC) class II, proinflammatory cytokines

TABLE 6 Coexpression between genes identified in this paper and those identified by Raj et al.,^a as determined by using the SCAN database^b

SNP	Gene	Coexpressed gene	P value
rs5016378	ABCC4	TNF	0.00003
rs6496603	ANPEP	TNF	0.00003
rs3857405	ARSB	TNF	0.00003
rs10882987	AVPI1	IL1A	0.00003
rs11571700	BRCA2	IL1A	0.00001
rs7335538	CARS2	IL-10	0.0001
rs10823760	CDH23	IL1A	0.00003
rs2185415	CDH23	IL1A	0.00003
rs12782689	CDH23	IL1A	0.00006
rs12763836	CDH23	IL1A	0.0001
rs12772205	CDH23	IL1A	0.0001
rs10873263	COQ6	TNF	0.00006
rs10503215	CSMD1	IL-10	0.00009
rs3849831	CSMD1	TNF	0.0001
rs260709	EDAR	TNF	0.000002
rs12090415	EPHB2	TNF	0.00003
rs16947233	FBXW8	TNF	0.00007
rs7133609	FBXW8	TNF	0.0001
rs6549191	FRMD4B	IL-10	0.0001
rs488532	GCNT2	IL-10	0.00003
rs4853066	HK2	IL1A	0.0001
rs2069727	IFNG	C1orf112	0.00009
rs2069727	IFNG	CUL7	0.0001
rs2069722	IFNG	DPYSL2	0.00008
rs2069722	IFNG	FOXJ2	0.00004
rs2069727	IFNG	LRRCC1	0.00004
rs2069727	IFNG	MSH5	0.0001
rs2069727	IFNG	RAB23	0.00006
rs2069727	IFNG	SLC4A7	0.0001
rs2069718	IFNG	SPIRE1	0.0001
rs2069727	IFNG	TIFA	0.0001
rs3024491	IL-10	CD52	0.0001
rs1304037	IL1A	IER3	0.0001
rs1304037	IL1A	MRPS7	0.000001
rs1800587	IL1A	MRPS7	0.00004
rs9407340	KANK1	IL-10	0.0001
rs6853658	KCNIP4	TNF	0.00002
rs655487	KCNMA1	TNF	0.0001
rs2219172	L3MBTL4	IL-10	0.0001
rs11710266	LIMD1	TNF	0.0001
rs2667975	LYN	IL-10	0.00003
rs373696	LYRM4	IL-10	0.00007
rs195063	MAN1A1	IL1A	0.0001
rs3791328	MGAT5	TNF	0.00006
rs7174277	MYO1E	TNF	0.00001
rs6692267	NEGR1	IL1A	0.00005
rs11908460	PLCB1	TNF	0.0001
rs13325518	PPM1L	TNF	0.000002
rs16831830	PPM1L	TNF	0.0001
rs10771415	PZP	TNF	0.0001
rs4714758	TMEM63B	IL-10	0.0001
rs228587	TPK1	IL1A	0.0001
rs10840100	TRIM66	TNF	0.00003
rs1286422	TTC7B	IL-10	0.00007
rs1286439	TTC7B	IL-10	0.00007
rs1290434	TTC7B	IL-10	0.00007
rs2343	TTC7B	TNF	0.00005
rs1396049	TXK	IL1A	0.00007
rs16954363	VPS53	TNF	0.00009
rs12450330	VPS53	TNF	0.0001
rs16954271	VPS53	TNF	0.0001
rs7207469	VPS53	TNF	0.0001
rs7220509	VPS53	TNF	0.0001
rs292553	WDR91	IL1A	0.0001

(Continued on next page)

TABLE 6 (Continued)

SNP	Gene	Coexpressed gene	P value
rs292557	WDR91	IL1A	0.0001
rs4504197	ZMAT3	TNF	0.00006
rs6769215	ZMAT3	TNF	0.00006

^aSee reference 35.

^bP values for coexpression according to the SCAN database are included.

(IFN- γ , TNF- α , and IL-12), and chemokines secreted by activated macrophages (47, 48). One of the most important SNPs in the promoter region of the IL-10 gene is the -1082A/G (rs1800896) polymorphism, which may have an effect on the transcriptional binding site; thus, this SNP may alter the rate of gene expression. It has been reported that 50 to 75% of the variation in IL-10 production is genetically controlled (49).

The human IL-10 gene is located on chromosome 1 and has been mapped to the junction between 1q31 and 1q32 (50). Several polymorphic sites in the IL-10 gene have been identified. Three polymorphic sites (-1082A/G [rs1800896] -819C/T [rs1800871], and -592C/A [rs1800872]) are located in the promoter region of the gene. Individuals homozygous for the -1082G allele have higher circulating IL-10 levels, higher expression levels of IL-10 mRNA, and higher levels of production of IL-10 following *in vitro* stimulation (51, 52). Indeed, the presence of the G allele at position -1082 correlates with a higher level of IL-10 protein production *in vitro* and in the pleural fluid of patients with active tuberculosis (50, 53, 54). Data from those studies suggest that carriers of the -1082G allele are likely to have a high risk for the progression of OT because the -1082G allele may suppress the immune response by increasing the expression level of IL-10.

IFN- γ is an important Th1 cytokine that is secreted by NK cells and T cells, and its production plays a critical role in macrophage activation in order to control *Toxoplasma* infection. The IFN- γ -encoding gene is located on chromosome 12q24 and consists of four exons with three intervening regions (55). A polymorphism in the first intron of the IFN- γ gene at position 874 (rs2430561) directly influences IFN- γ production levels (56). The IFN- γ +874T/A SNP is located within a putative nuclear factor κ B (NF- κ B) binding site, and NF- κ B specifically binds to the DNA sequence containing the T allele (57). Thus, the T allele might be responsible for the induction of IFN- γ production at a higher level. Indeed, it has been shown that the T and A alleles most likely correlate with high and low expression levels of IFN- γ , respectively (58, 59). The +874T allele is absolutely linked to the 12-CA-repeat microsatellite, while the +874A allele is adjacent to a non-12-CA repeat (56). Our results indicate that the A allele was significantly associated with the development of OT. However, for revealing the biological significance of these SNPs in susceptibility to ocular toxoplasmosis, further studies are needed in different populations.

IL-1 β , a proinflammatory pleiotropic cytokine, is a member of the IL-1 family that possesses the ability to stimulate the expression of genes associated with inflammation and the immune response, including cyclooxygenase type 2, phospholipase A₂, and inducible nitric oxide synthase (60). Additionally, another important proinflammatory property of IL-1 β is its capacity to increase the expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) on endothelial and other cell surfaces. IL-1 α and IL-1 β bind to IL-1 receptor type I, eliciting signal transduction and the corresponding biological effects. At least three SNPs in the IL-1 β gene have been reported, all of which represent a C/T base transition at positions -511 and -31 in the promoter and at position +3954 in the exon (61-64). Despite the importance of IL-1 β during the immune response to *T. gondii*, no associations of the allelic frequencies of these polymorphisms between patients and controls were found.

Data presented in this work refer to specific SNPs in some of the cytokine genes involved in the immune response to *T. gondii*; however, additional studies are required to explain how these SNPs can affect the immune response in OT. SNPs may influence protein conformation (topology), promoter activity, pre-mRNA and RNA splicing, or

transcriptional regulation affecting cytokine production (65). Some SNPs in the IL-4 and TNF- α genes create a new binding site for the OCT-1 transcription factor affecting gene expression (66, 67). In addition, the variability in IL-10 production associated with the presence of the IL-10 -819G/A polymorphism is related to the activation of poly(ADP-ribose) polymerase 1 (PPAR-1) and the suppression of IL-10 transcription (68). Further studies to examine the relationship between SNPs in cytokine genes and the presence of recurrences, a major concern in OT, would be useful. We have not yet analyzed the relationship of SNPs with recurrences in this cohort.

Our data suggest that genetic susceptibility to ocular involvement during *Toxoplasma* infection exists, which provides some possible clues to understanding why some *Toxoplasma*-infected people have ocular involvement. Although the eye has immune privilege, the ocular immune response is connected with the peripheral response. We previously demonstrated that patients with ocular toxoplasmosis have different peripheral immune responses compared to those of patients without ocular involvement (9). Both retina and brain are damaged by *Toxoplasma* infection in humans. The presence of particular receptors in neural cells (including the retina) and the absence of mechanisms in the eye to compensate for the effects of IL-10 and IFN- γ gene polymorphisms are possible explanations for this tissue tropism.

Functional studies are needed to understand how our SNPs affect the cytokine balance in OT. The clinical presentation of OT is heterogenous in terms of the size and number of lesions and the presence and location of recurrences (macula, optic nerve, and peripheral retina). Different cytokine profiles might contribute to this clinical heterogeneity.

A key objective in biological research is to identify all molecules within a living cell and how they interact. However, the functions of many genes are still not understood. Gene coexpression networks can be used for various purposes, including candidate disease gene prioritization, functional gene annotation, and the identification of regulatory genes (69). Coexpression analysis identified the connectivity of the IL-1 β and IL-1 α genes mediated by RNASE6.

Our study provides evidence that common genetic variants in Th1 (IL-1, IFN- γ , and TNF- α) and Th2 (IL-10) genes are associated with the risk of developing OT in patients from Colombia. These results are similar to those of previous studies of patients with OT in Brazil (70) and congenital toxoplasmosis in Poland (29) and are consonant in their broad conclusions and generalizations about the proinflammatory and downmodulatory cytokine genes that are associated with our findings in a cohort of patients with congenital toxoplasmosis in the United States. These genes include P2X7R (70, 71), NALP1 (72–74), ALOX12 (75), HLA classes I and II (76–79), and ERAP1 (80), all of which influence the immune response. Our findings are in agreement with current knowledge about immunity to *T. gondii*.

In future studies, it may be useful for devising diagnostic and therapeutic approaches in OT to determine whether these polymorphisms alter cytokine production in patients with ocular compromise and if they influence the clinical presentation of disease. To this end, the identification of gene-gene interactions could enhance the power and accuracy of predicting disease outcomes of a complex disorder. For a better description of the genetic architecture of disease susceptibility and unambiguous identification of factors responsible for both causality and predisposition to a disease, functional appraisal of disease-associated polymorphisms is essential (30, 32, 81).

MATERIALS AND METHODS

Subjects. The Ethics Committee of the Universidad Tecnológica de Pereira approved this study's protocol. The group of patients was composed of 61 cases (mean age \pm standard deviation [SD], 37.37 \pm 17.27 years), with a male/female ratio of 1.44. Patients from the Bogotá and Quindío regions in Colombia with OT were diagnosed as previously described (5, 8). These patients had diagnostic confirmation by sampling of aqueous humor. The control group was divided in two subgroups. Subgroup 1 was composed of 22 Colombian patients with symptoms of uveitis for whom ocular toxoplasmosis was excluded as the cause of disease, and subgroup 2 was composed of 94 healthy individuals aged 36.29 \pm 13.81 years (male/female ratio, 0.84).

Biological samples and diagnostic assays for ocular toxoplasmosis. Both patients ($n = 83$) and healthy controls ($n = 94$) included in the study consented to provide blood samples. In addition, patients

TABLE 7 Cytokine candidate genes and gene polymorphisms evaluated in a Colombian population^a

Gene	Locus	SNP	rs ID	Location	Predicted functional effect(s)
IL-1 α	2q13	-889G>A	rs1800587	Promoter	TFBS, splicing (ESE or ESS)
IL-1 β	2q13	+3954G>A	rs1143634	Exon 4	sSNP, splicing (ESE or ESS)
		-31G>A	rs1143627	Promoter	TFBS
		-511G>A	rs16944	Promoter	TFBS
IL-10	1q31	-819G>A	rs1800871	Promoter	TFBS
		-1082A>G	rs1800896	Promoter	TFBS
IFN- γ	12q24.1	+874T>A	rs2430561	Intron 1	NF- κ B binding site
TNF- α	6p21.3	-308G>A	rs1800629	Promoter	TFBS
		-238C>T	rs361525	Promoter	TFBS
		-863C>A	rs1800630	Promoter	TFBS
		-857G>A	rs1799724	Promoter	TFBS
		-1031T>C	rs1799964	Promoter	TFBS

^ars ID, reference SNP ID number; TFBS, transcription factor binding site; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; sSNP synonymous SNP.

with ocular diseases (ocular toxoplasmosis [$n = 61$] or other causes of uveitis [$n = 22$]) provided ocular fluid samples. Seven patients did not have aqueous humor sampling because they had only a single functional eye, for which the sampling risk was considered too high. Blood samples were used to obtain serum and peripheral blood mononuclear cells (PBMCs) for further DNA extraction and PCR tests. AH samples (0.1 to 0.2 ml) were obtained at the Ophthalmological Center, Clínica Barraquer, under sterile conditions after topical anesthesia and sent to a laboratory for analysis. All serum samples were analyzed for anti-*Toxoplasma* IgG and IgM antibody titers by using commercial enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's recommendations (Vidas Toxo IgG II [reference number 30210] and Toxo IgM [reference number 30202]; bioMérieux, France). Those with positive IgG results underwent local ocular antibody production testing. In order to detect intraocular anti-*Toxoplasma* antibodies, the Goldmann-Witmer coefficient (GWC) was calculated as follows: (anti-*Toxoplasma* IgG in aqueous humor/total IgG in aqueous humor)/(anti-*Toxoplasma* IgG in serum/total IgG in serum) (8, 82). The specific anti-*Toxoplasma* IgG antibody titers in aqueous humor samples were determined by an ELISA as described previously (83). An index of <2 was considered a positive result for ocular *Toxoplasma* infection.

DNA extraction from blood samples. A tube with heparin was used to collect blood samples, which were then processed for DNA extraction. The Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) was used, and procedures were performed as recommended by the manufacturer. Briefly, DNA from white blood cells was obtained by incubating samples with a cell lysis solution for 10 min at room temperature. After centrifugation at $13,000 \times g$ for 20 s at room temperature, the supernatant was discarded, and the pellet was recovered. The cellular proteins were then removed by a salt precipitation step, and genomic DNA was concentrated and desalted by isopropanol precipitation.

Genotyping. (i) Primer design and multiplex PCR amplification. The polymorphisms to be studied were selected based on data from previous studies carried out with patients with ocular toxoplasmosis or other human diseases (21, 23–25). Genes and SNPs were selected based on their functional significance and previous reports on an association with any disease condition. A list of cytokine candidate genes and their selected polymorphisms indicating the SNPs, reference SNP ID numbers (rs IDs), chromosomal positions, locations, and predicted functional effects is shown in Table 7. Functional-effect predictions of SNPs were done by using the FuncPred (Functional SNP Prediction) tool (<https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.html>). Functional prediction of the deleterious effect, if any, of the associated SNPs with respect to functional categories such as protein coding, splicing regulation, transcriptional regulation, and posttranslation was assessed by using the F-SNP program (<http://compbio.cs.queensu.ca/F-SNP/>). We selected polymorphisms in the following genes: TNF- α (rs1799964, rs1800629, rs1799724, rs1800630, and rs361525), IL-1 β (rs16944, rs1143634, and rs1143627), IL-1 α (rs1800587), IFN- γ (rs2430561), and IL-10 (rs1800896 and rs1800871). Each primer set was designed by using Primer3 software (<http://frodo.wi.mit.edu/primer3/>) to generate amplicons (including each SNP) of <150 bp by setting each primer binding site closer to the SNP. Each primer was checked for potential structures of the self-dimer by using AutoDimer software (<http://www.cstl.nist.gov/strbase/AutoDimerHomepage/AutoDimerProgramHomepage.htm>). PCR was carried out by using a Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA) for a final volume of 10 μ l containing 1 to 10 ng genomic DNA, $1 \times$ Qiagen Multiplex PCR master mix (Qiagen, Hilden, Germany), and 0.2 to 0.6 μ M each primer (Table 8) under following conditions: an initial denaturation step at 95°C for 10 min and 35 cycles of 94°C for 1 min, 60°C for 90 s, and 72°C for 50 s, followed by a final extension step at 72°C for 7 min.

(ii) Multiplex SBE reaction and electrophoresis. The PCR product was cleaned with 1 μ l of ExoSAP-IT (Affymetrix, Santa Clara, CA, USA). The product was incubated at 37°C for 45 min and then heated at 85°C for 15 min to inactivate the enzyme. By using this preamplification product as the template, reactions were carried out to detect SNP variants using a minisequencing method (SBE

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