Single nucleotide polymorphisms in the interferon gamma gene are associated with distinct types of retinochoroidal scar lesions presumably caused by *Toxoplasma gondii* infection

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*The association of single nucleotide polymorphisms (SNPs) in the interferon (IFN)-γ gene (*IFNG*) with different types of retinal scar lesions presumably caused by toxoplasmosis were investigated in a cross-sectional population-based genetic study. Ten SNPs were investigated and after Bonferroni correction, only the associations between SNPs* rs2069718 *and* rs3181035 *with retinal/retinochoroidal scar lesions type A (most severe scar lesions) and C (least severe scar lesions), respectively, remained significant. The associations of two different* IFNG *SNPs with two different types of retinal lesions attributable to toxoplasmosis support the hypothesis that different inflammatory mechanisms underlie the development of these lesions. The in vitro analysis of IFN-γ secretion by peripheral blood mononuclear cells stimulated with* Toxoplasma gondii *antigens was also investigated. The association between SNP* rs2069718 *and type A scar lesions revealed that differential IFN-γ levels are correlated with distinct genotypes. However, no correlation was observed with IFN-γ secretion levels and the SNP* rs3181035*, which was significantly associated with type C scar lesions. Our findings strongly suggest that immunogenetic studies of individuals with congenital or postnatally acquired infection are needed to better understand the role of IFN-γ and its polymorphisms in the pathogenesis of ocular toxoplasmosis.*

Key words: single-nucleotide polymorphisms - IFN-γ - *Toxoplasma gondii* - uveitis - retinochoroiditis

Ocular toxoplasmosis is one of the most important clinical manifestations in immunocompetent individuals infected by the intracellular obligatory parasite *Toxoplasma gondii* and is considered a major cause of visual impairment (Holland 2003, 2004). Ocular toxoplasmosis may be the result of congenital as well as postnatally acquired infections (Silveira et al. 1988, Holland et al. 1999). The disease outcome depends on numerous factors including the frequency of exposure, the route of infection, the parasitic load and patient age at infection as well as immunological and unidentified cofactors (McLeod et al. 1996, Garweg & Candolfi 2009). Type 1 cytokines are of fundamental importance in the immune response to *T. gondii* in competent hosts (Denkers & Gazzinelli 1998). However, a pro-inflammatory specific T-helper (Th)1-oriented response is observed mainly in patients exhibiting retinochoroidal scars from the healing of severe lesions, which suggests that the exacerbation of the immune response can be related to tissue damage. The central role of interferon (IFN)-γ as the major mediator of resistance against *T. gondii* was described by Suzuki et al. (1988). This cytokine seems to be important in both cases, namely, in the exacerbated and in the regulated context of the in vitro cellular immune response, suggesting that the cellular immune responses against *T. gondii* in the eye should be suitably tailored (Bahia-Oliveira et al. 2009, 2012, Garweg & Candolfi 2009). Single nucleotide polymorphisms (SNPs), such as +874T>A (*rs2430561*) in the IFN-γ gene (*IFNG*) that determine high (TT), low (AA) and intermediate (TA) responder phenotypes have been associated with the susceptibility to and the outcomes of infectious and chronic inflammatory diseases (Popadic et al. 2012), including retinochoroiditis toxoplasmic susceptibility (de Albuquerque et al. 2009) and the persistence of symptoms during the acute phase of infection (Neves et al. 2012).

The present study was undertaken to investigate *IFNG* polymorphisms and IFN-γ production in vitro by peripheral blood mononuclear cells (PBMCs) specifically stimulated with parasitic antigens in patients with retinal scars presumably caused by *T. gondii* infection. Patients were grouped according to the severity of retinal damage (Bahia-Oliveira et al. 2012). Ten SNPs of the *IFNG* were genotyped and their possible relationship with the diversified clinical sets of retinochoroidal scars was investigated. The SNPs *IFNG_rs2069718* and *IFNG_rs3181035* were significantly associated with type A and type C scar lesions, respectively. However, the phenotype of IFN-γ production level was not associated

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with any IFN-γ SNP or the type of scar lesion. Our data exemplify the multifactorial nature of ocular toxoplasmosis and suggest that studies on cytokine gene polymorphisms and their products (cytokine secretion) are valuable to help elucidate the complex network of genes influencing the manifestations of ocular toxoplasmosis.

SUBJECTS, MATERIALS AND METHODS

Study population - The present population-based study included individuals living in the city of Campos dos Goytacazes located in northern of the state of Rio de Janeiro (RJ), Brazil and has a population of approximately 400,000 persons. Study subjects were recruited from the county blood bank (n = 213) and from households located in areas previously studied (n = 198). Their serologic status for toxoplasmosis was unknown on entry into the study and was later determined using commercial kit for IgM and IgG anti-*Toxoplasma* antibodies (ELFA VIDAS, bioMérieux). All study subjects were examined by binocular indirect ophthalmoscopy (Welch Allyn) by two ophthalmologists. Photographical registrations of retinal scars or retinochoroidal lesions were made in a retinographer (Zeiss-Visucam). The study objectives were explained to all patients (or their guardians) and voluntarily written informed consent was obtained from those who agreed to participate. The present study was approved by the Ethical Committee of the Oswaldo Cruz Foundation in RJ (protocol 347/06) and the National Commission for Ethics in Research, Ministry of Health (protocol 013/2007).

TABLE I

The characteristics of individuals according to *Toxoplasma gondii* serology with respect to age, gender and the presence or absence of retinochoroidal scar lesions

Toxoplasmosis serology	Clinical profile	Lesion type	Gender (male/female)	Sample size
Seronegative	No lesion	-	67/30[a]	97
Seropositive	No lesion	-	130/84[b]	214
	Lesion	A	23/13[c]	36
		B	17/13[d]	30
		C	16/18[e]	34
Total	-	-	-	411

a: the seronegative group was not included in the genetic analysis; *b*: the seropositive group without lesions was considered as a control group for the genetic analysis; *c*: the group of type A scar lesions includes individuals with single type A scar lesions (n = 17) and individuals with multiple type AB (n = 9), ABC (n = 8) and AC (n = 2) scar lesions, totalling the 36 individuals presented in the Table; *d*: the group of type B scar lesions includes individuals with single type B lesions (n = 23) and individuals with multiple type BC scar lesions (n = 7), totalling the 30 individuals presented in the Table; *e*: the group of type C scar lesions includes only individuals with type C scar lesions.

Subjects answered a standardised questionnaire on demographic characteristics and risk factors for toxoplasmosis. Previous studies in this region have identified three general levels of toxoplasmosis risk based on socioeconomic status and responses to exposures listed in the questionnaire: low (termed population 1 - P1), middle (termed population 2 - P2) and upper (termed population 3 - P3) (Bahia-Oliveira et al. 2003, 2012). All study belong to P1 and P2 (Bahia-Oliveira et al. 2003).

Toxoplasmosis status was determined by: (i) toxoplasmosis seropositivity, defined by the presence of anti-*T. gondii* IgG antibodies, (ii) the presence or absence of retinal lesions due to infection by *T. gondii*, assessed by ophthalmoscopy and (iii) the type of retinal lesions according to the classification of severity of retinal destruction described by Aleixo et al. (2009), Bahia-Oliveira et al. (2009, 2012) and Dutra et al. (2013). In this classification, retinal scar lesions are rated as exhibiting high, medium or low degrees of severity, which are identified as types A, B and C, respectively. In the present study, the individuals exhibiting scar lesions with multiple degrees of severity were allocated to the group corresponding to the most severe type. Therefore, patients with type A, B and C scar lesions were allocated to the group with type A scar lesions, the patients with types B and C scar lesions were allocated to the group with type B lesions and the group with type C lesions comprised patients with type C lesions only.

A total of 411 individuals were subjected to serologic and ophthalmoscopic exams; 97 individuals had no evidence of infection by *T. gondii*, 100 of the 314 seropositive individuals exhibited scar lesions attributable to toxoplasmosis and the remaining 214 seropositive individuals exhibited no lesions (Table I). The average age was 36 years-old (standard deviation: 12.8 years).

Genomic DNA extraction and SNP selection - DNA samples from 314 patients infected by *T. gondii*, including both those with (n = 100) and without retinal lesions (n = 214) and from 97 non-infected individuals (whose sample analyses were not included in the present genetic study, but only in the immunological evaluations to measure IFN-γ secretion) were extracted using the commercial DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. The DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

Ten *IFNG* SNPs were selected (Table II) using the online HapMap database (hapmap.ncbi.nlm.nih.gov/) and were genotyped by the KBioscience Co. Haploview software, v.4.2 was used to estimate the linkage disequilibrium (LD) between the selected SNPs. Those with a minor allele frequency (MAF) below 0.05 were not included in the LD map.

Antigens - Antigenic preparations of *T. gondii* soluble tachyzoites antigen (STAg) were obtained from fibroblast cultures prepared as described previously (Gazzinelli et al. 1991) and were used at a final concentration of 2 μg/mL.

Cell culture and cytokine measurement - PBMCs were isolated by Ficoll-diatrizoate density gradient centrifugation (Organon Teknica, Charleston, SC, USA) as described previously (Gazzinelli et al. 1983). The culture medium consisted of 90.4% RPMI-1640, 1.6% L-glutamine, 3% antibiotic-antimycotic mixture stock (10,000 U penicillin, 5 µg streptomycin, 25 µg Fungizone per mL, GIBCO) and 5% heat-inactivated normal human serum AB, Rh+ (Sigma). For cytokine analysis, supernatants were collected from the cultures of 1 x 10^6 cells per well in flat-bottom 24-well tissue plates. The IFN-γ levels in the supernatants of these PBMCs were assessed using the Human Th1/Th2 Cytokine Kit, BD™ Cytometric Bead Array (CBA) following the manufacturer's instructions. Readings were performed using a BD FACScan™ flow cytometer with the BD CellQuest™ and BD™ CBA Analysis software for data acquisition and analysis, respectively.

Statistical analysis - Genotypes in the control group (*T. gondii*-seropositive individuals without ocular lesions) were used to test the Hardy-Weinberg equilibrium (HWE) for each SNP, calculated with a customised spreadsheet in Microsoft Excel. Deviation from equilibrium was considered when p values were < 0.05. The allele and genotype frequencies between groups were compared using Fisher's exact test. The following comparisons were performed: (i) *T. gondii*-seropositive individuals without ocular lesions vs. *T. gondii*-seropositive individuals with retinochoroidal scars lesions type A, (ii) *T. gondii*-seropositive individuals without ocular lesions vs. *T. gondii*-seropositive individuals with retinochoroidal scars lesions type B and (iii) *T. gondii*-seropositive individuals without ocular lesions vs. *T. gondii*-seropositive individuals with retinochoroidal scars lesions type C. This analysis was performed by the website vassarstats.net/. GraphPad Prism 4 software was further used to calculate the odds ratio (OR) within a 95% confidence interval (CI) among the groups of individuals with different types of ocular scar lesions. Because we performed multiple tests (10 SNPs) in the same group of samples, the Bonferroni method was used to correct the p-value (each p was multiplied by 10). In the immunological study, statistical analyses regarding the evaluation of culture supernatant cytokine levels were performed using the Kruskal-Wallis test followed by Dunn's test with GraphPad Prism 4 software. A 95% CI was considered for all tests.

RESULTS

The allele and genotype frequencies of the groups seropositive for toxoplasmosis who exhibited type A, B or C scar lesions presumably caused by toxoplasmosis were compared with these frequencies in the "control" group (individuals seropositive for *T. gondii*, but without ocular lesions suggestive of toxoplasmosis) to assess the association between the 10 investigated SNPs and the severity of the retinal/retinochoroidal scar lesions. The allele and genotype frequencies of the SNP *rs2069718* differed significantly (p = 0.01 and p = 0.04/p = 0.002, respectively) between seropositive subjects with type A lesions and those with no eye lesions (Table III), although only the difference in genotype frequency AG vs. GG remained significant after Bonferroni correction ($P_{corrected}$ – multiplied by 10 = 0.02). The frequency of the G allele of the SNP *rs2069718* was higher in the group with type A scar lesions compared with the con-

TABLE II

Single nucleotide polymorphisms (SNP) selected for interferon-γ gene (*IFNG*)

SNP	Chromosome position[a]	Allele 1	Allele 2
IFNG_rs3181035	68546396	A	G
IFNG_rs2069723	68548594	A	G
IFNG_rs2069718	68550162	C	T
IFNG_rs2069713	68552476	A	G
IFNG_rs2069715	68550986	C	T
IFNG_rs2069716	68550815	A	G
IFNG_rs2069727	68548223	A	G
IFNG_rs2069720	68549710	A	G
IFNG_rs1861494	68551409	C	T
IFNG_rs1861493	68551196	A	G

a: data retrieving from the Ensembl databases, National Center for Biotechnology Information reference sequence NC_000012.11.

TABLE III

Allele and genotype distribution in subject with type A lesions and seropositive persons with no eye lesions (controls) relative to the single nucleotide polymorphisms interferon-γ gene *rs2069718*

Genotypes/ alleles	Type A n (%)	Controls n (%)	p[a]	$P_{corrected}$[b]	OR (95% CI)
AA	6 (17)	47 (22)	0.0434[c]	0.434	0.36 (0.13-0.97)
AG	11 (31)	110 (52)	0.0019[c]	0.019[c]	0.28 (0.12-0.63)
GG	19 (53)	53 (25)			1
A	23 (32)	204 (49)	0.0103[c]	0.103	0.497 (0.292-0.845)
G	49 (68)	216 (51)			
GG + AG	30 (83)	163 (78)	0.517	5.17	1.442 (0.566-3.672)
AA	6 (27)	47 (22)			0.694 (0.272-1.767)
GG	19 (53)	53 (25)	0.0014[c]	0.014[c]	3.31 (1.604-6.835)
AA + AG	17 (47)	157 (75)			0.302 (0.146-0.623)

a: Fisher's exact test; *b:* Bonferroni method; *c*: statistically significant; CI: confidence interval; OR: odds ratio.

trol group (0.68 vs. 0.51, respectively, p = 0.01). The GG genotype was associated with a 3.3-fold higher risk of type A scar lesion compared with the allele A positive genotypes (OR = 3.31). Conversely, the presence of the A allele in either homozygosity or heterozygosity seems to be protective. The likelihood of type A scar lesions in individuals carrying the A allele was 70% less than for individuals with the GG genotype (AA vs. GG: OR = 0.36; AG vs. GG: OR = 0.28).

The protective effect of the A allele is dominant, whereas the "increased susceptibility" phenotype of the G allele is recessive (AA + AG vs. GG: $P_{corrected\ -}$ multiplied by 10 = 0.014) (Table III).

The allele and genotype frequency of the *IFNG* SNP *rs3181035* differed significantly (p = 0.002 and p = 0.01, respectively) between the persons with type C eye lesions and seropositive persons with no eye lesions (Table IV), although only the difference in allele frequency remained significant after Bonferroni correction ($P_{corrected-}$ multiplied by 10 = 0.02). The frequency of the C allele was significantly lower in the group with type C scar lesions in comparison with the control group (0.69 vs. 0.85, respectively, $P_{corrected-}$ multiplied by 10 = 0.02), thus pointing to a protective role of this allele. The homozygous or heterozygous T allele was associated with increased susceptibility (TT vs. CC: OR = 4.8; CT vs. CC: OR = 2.8), whereas the presence of the homozygous C allele seemed to be protective (OR = 0.33) i.e, the likelihood of disease in individuals with a CC genotype developing type C retinal lesions was 70% less than for individuals carrying the T allele. The protective effect of the C allele is recessive, whereas the increased susceptibility phenotype of the T allele is dominant (CC vs. CT + TT: $P_{corrected\ -}$ multiplied by 10 = 0.034) (Table IV). The presence of the T allele in either homozygosity (TT) or heterozygosity (CT) was associated with a three-fold higher risk for type C lesions.

No individual in the present sample exhibited the GG genotype in the SNP *rs2069718* and the TT or CT genotype in the SNP *rs3181035* concomitantly (data not shown).

The allele frequency of the *IFNG* SNP *rs1861493* exhibited a significant difference (p = 0.047) between *T. gondii*-seropositive individuals without ocular lesions (controls) vs. *T. gondii*-seropositive individuals with retinochoroidal scars lesions type C (Table V). Specifically, the frequency of the G allele was higher in the control group, whereas the frequency of the A allele was higher in the group with type C scar lesions. However, this difference did not remain significant after Bonferroni correction ($P_{corrected\ -}$ multiplied by 10 = 0.47).

No statistically significant differences were observed in the remaining SNPs assessed in the present study (*rs1861494*, *rs2069723*, *rs2069713*, *rs2069715*, *rs2069716*, *rs2069727* and *rs2069720*) (Table V). The genotype frequencies of all 10 SNPs in the control group did not differ from those predicted by the HWE (p > 0.05) (Table V).

The production of IFN-γ in *T. gondii* STAg-stimulated PBMC cultures from 43 genotyped patients was assessed and the results are presented in Figs 1, 2. With respect to the phenotypic analyses (IFN-γ secretion), the secretion levels of IFN-γ were compared for the groups with ocular lesions vs. the group with no lesions as well as the group of *T. gondii* seronegative individuals vs. the remainder of the groups with positive serology to *T. gondii*. In all *T. gondii* seropositive groups, independent of the presence of retinal scar lesions, the levels of STAg-stimulated IFN-γ production were significantly higher than in the group of *T. gondii* seronegative individuals (data not shown). In Fig. 1A (corresponding to the SNP *rs2069718*), the individuals are clustered according to their genotypes independently of the presence of or type of retinal scar lesions. Although none of the results depicted in Fig.1A-C exhibited statistically significant differences, we observed that IFN-γ levels in the supernatants of PBMC cultures from individuals with AA and AG genotypes were similar and were higher than those of individuals with the GG genotype (Fig. 1A). However, when the individuals were clustered according to the presence (Fig. 1B) and type (Fig. 1C) of scar lesions, IFN-γ levels were higher in the heterozygous AG genotype of the control group (without lesions) as well as in the AA and GG homozygous genotypes of the group with lesions (Fig. 1B). This trend was confirmed by the stratification of the group with lesions (Fig. 1C). Specifically, the highest levels of IFN-γ production corresponded to the individuals with homozygous AA and GG genotypes, which exhibited the most severe scar lesions (type A). Interestingly, individuals with type B scar lesions (intermediate severity) exhibited a correlation between the

TABLE IV

Allele and phenotype distribution in subject with type C lesions and seropositive persons with no eye lesions ("controls") relative to the single nucleotide polymorphisms interferon-γ gene *rs3181035*

Genotypes/ alleles	Type C n (%)	Controls n (%)	p[a]	$P_{corrected}$[b]	OR (95% CI)
CC	16 (47)	155 (73)			1
CT	15 (44)	52 (24)	0.0102[c]	0.102	2.79 (1.29-6.04)
TT	3 (9)	6 (3)	0.0559	0.559	4.84 (1.10-21.24)
C	47 (69)	362 (85)	0.0021[c]	0.021[c]	0.396 (0.222-0.706)
T	21 (31)	64 (15)			
CC + CT	31 (91)	207 (97)	0.1118	1.118	0.299 (0.071-1.260)
TT	3 (9)	6 (3)			3.339 (0.7936-14.04)
CC	16 (47)	155 (73)	0.0034[c]	0.034[c]	0.3326 (0.159-0.696)
CT + TT	18 (53)	58 (27)			3.006 (1.437-6.289)

a: Fisher's exact test; *b:* Bonferroni method; *c*: statistically significant; CI: confidence interval; OR: odds ratio.

TABLE V

The analyses of the genetic association between the single nucleotide polymorphisms (SNP) selected in interferon-γ gene and ocular manifestation of toxoplasmosis

		p[c]		$P_{corrected}$[d]		
SNP	Cases[a] vs. controls[b]	Allele	Genotype	Allele	Genotype	p HWE (controls[b])
rs2069718	Type A	0.010[e]	0.005[e]	0.100	0.050[e]	0.49
	Type B	0.681	0.871	6.810	8.710	
	Type C	0.696	0.267	6.960	2.670	
rs3181035	Type A	0.146	0.478	1.460	4.780	0.52
	Type B	0.847	0.187	8.470	1.870	
	Type C	0.002[e]	0.006[e]	0.020[e]	0.060	
rs1861493	Type A	0.295	0.580	2.950	5.800	0.12
	Type B	0.363	0.348	3.630	3.480	
	Type C	0.047[e]	0.201	0.470	2.010	
rs1861494	Type A	0.312	0.548	3.120	5.480	0.52
	Type B	0.214	0.207	2.140	2.070	
	Type C	0.078	0.267	0.780	2.670	
rs2069727	Type A	0.892	0.660	8.920	6.600	0.49
	Type B	0.770	0.961	7.700	9.610	
	Type C	0.567	0.359	5.670	3.590	
rs2069723	Type A	1.000	1.000	10.000	10.000	0.94
	Type B	0.076	0.076	0.760	0.760	
	Type C	0.093	0.092	0.930	0.920	
rs2069713	Type A	1.000	1.000	10.000	10.000	0.78
	Type B	0.620	0.356	6.200	3.560	
	Type C	1.000	0.999	10.000	9.990	
rs2069715	Type A	1.000	0.999	10.000	9.990	0.86
	Type B	0.211	0.210	2.110	2.100	
	Type C	0.250	0.250	2.500	2.500	
rs2069716	Type A	0.334	0.556	3.340	5.560	0.3
	Type B	1.000	0.999	10.000	9.990	
	Type C	0.340	0.552	3.400	5.520	
rs2069720	Type A	0.398	0.598	3.980	5.980	0.81
	Type B	0.605	0.601	6.050	6.010	
	Type C	1.000	0.999	10.000	9.990	

a: Toxoplasma gondii-seropositive individuals with retinochoroidal scars lesions categorised respectively as class type A, B or C; *b*: *T. gondii*-seropositive group without eye lesions; *c:* Fisher's exact test; *d:* Bonferroni method; *e*: statistically significant; HWE: Hardy-Weinberg equilibrium test.

levels of IFN-γ and the *rs2069718* genotypes intermediate between the group with type A and the group with type C lesions, even though the levels of IFN-γ production were lower in the group with type C lesions. Specifically, among the individuals in the group with type B lesions, those with AA genotype exhibited the highest levels of IFN-γ secretion (as in the group with type A lesions), followed by the individuals with the heterozygous AG genotype (as in the group with type C lesions). Fig. 1C further demonstrates that the correlation between the genotypic profile (homozygosity or heterozygosity of the SNP *rs2069718*) and the phenotypic profile (IFN-γ production) in the group of patients with type C lesions follows the same trend as that of the control group (*T. gondii* seropositive without retinal lesions). In this case, the highest levels of IFN-γ production correspond to the individuals with a heterozygous AG genotype. However, the correlation between the GG genotype and the presence of type C lesions could not be assessed because no individual with this pattern of lesions exhibited the GG genotype (Fig. 1C). Nonetheless it is important to mention that all the interpretation for the results referent to subgroups of analysis (Fig. 1C) is purely speculative due to the very small number of patients in these subgroups and will require that more patients are evaluated for consolidation of these hypotheses.

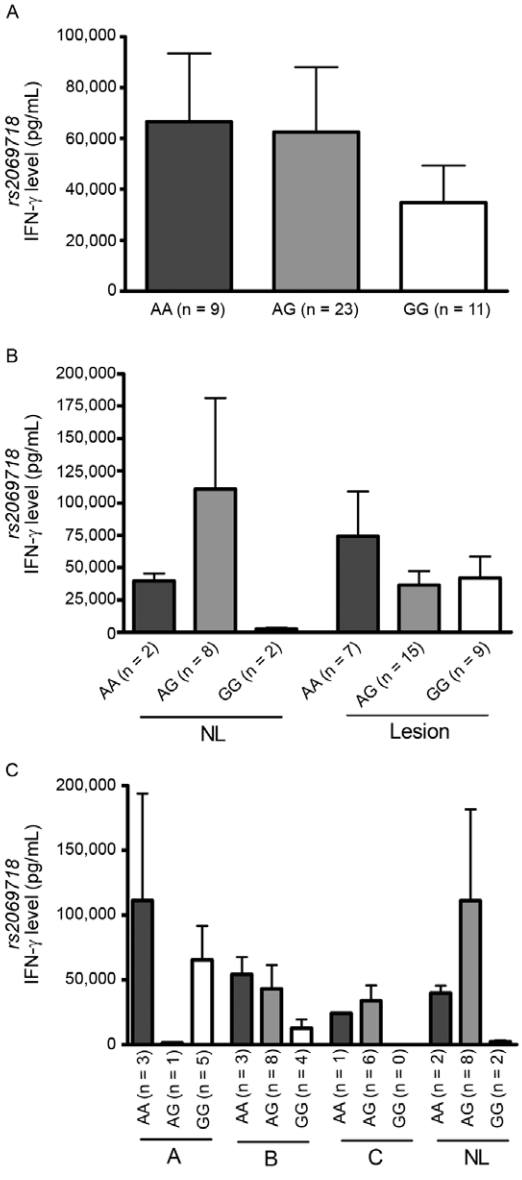

Fig. 1: correlation between the levels of interferon (IFN)-γ gene production and the different genotypes of the single nucleotide polymorphisms (SNP) *rs2069718*. IFN-γ production by soluble tachyzoites antigen-stimulated peripheral blood mononuclear cells from individuals seropositive for *Toxoplasma gondii* (A) independent of the presence of retinal lesions (B), with or without retinal lesions or (C) with different types of retinal lesion (types A, B and C) or no lesion (NL). The results are clustered according to the three possible genotypes of the SNP *rs2069718*.

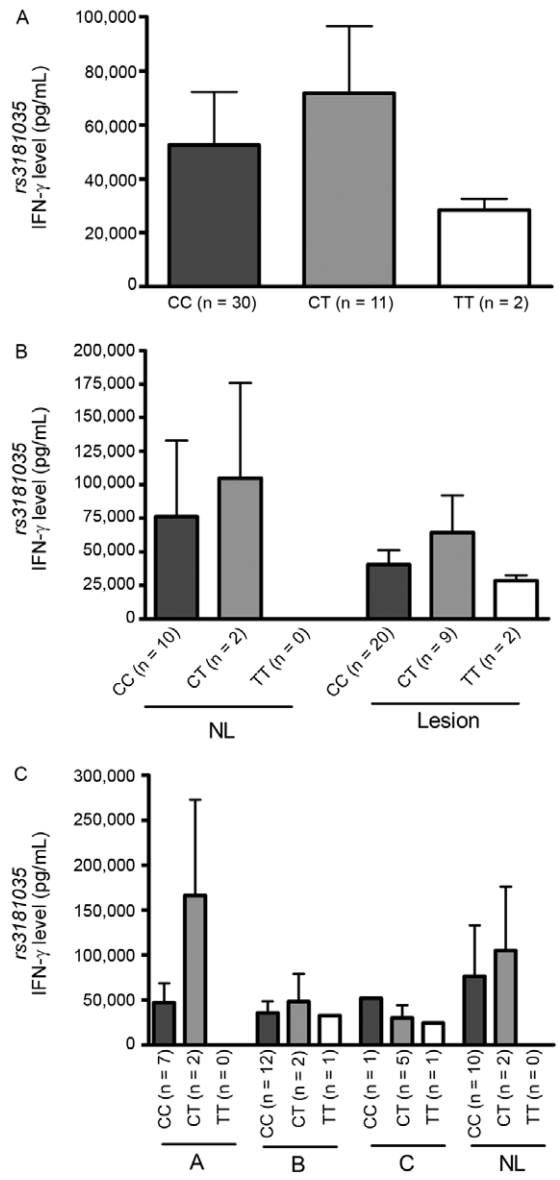

Fig. 2: correlation between the levels of interferon (IFN)-γ gene production and the different genotypes of the single nucleotide polymorphisms *rs3181035*. IFN-γ production by soluble tachyzoites antigen-stimulated peripheral blood mononuclear cells from individuals seropositive for *Toxoplasma gondii* (A) independent of the presence of retinal lesions (B), with or without retinal lesions or (C) with different types of retinal lesions (types A, B and C) or no lesion (NL). The results are clustered according to the three possible genotypes of the SNP *rs3181035*.

In Fig. 2A (corresponding to SNP *rs3181035*), the individuals were clustered according to their genotype only, irrespective of the presence or type of retinal scar lesions. Although none of the results depicted in this Fig. 1A-C exhibited statistically significant differences, we observed a similar pattern of IFN-γ secretion in the supernatants of STAg-stimulated PBMC cultures in all comparisons, i.e., the CC and CT genotypes exhibited higher levels than the TT genotype, therefore exhibiting no correlation with the severity of the retinal scar lesions. In the same way as previously considered, for the interpretations referring to the Fig. 2C it is important to mention that they are purely speculative due to the very small number of patients in these subgroups.

DISCUSSION

We have recently proposed that immunological and immunogenetic parameters are important contributors to the diversity of ocular lesions presumably caused by toxoplasmic infection observed in epidemiologic surveys (Bahia-Oliveira et al. 2012). This assumption is further supported by the results of the present study, in

which we investigated whether *IFNG* SNPs are associated with the risk of or protection against retinochoroiditis presumably caused by toxoplasmosis. Two *IFNG* SNPs (*rs2069718* and *rs3181035*) out of 10 evaluated were found to exhibit a statistically significant association with the presence of retinal/retinochoroidal scar lesions with different degrees of severity, after correction for multiple testing (n = 10) of independent SNPs with MAF > 0.1. The fact that we found two different SNPs in *IFNG* associated with two different forms of retinal disease attributable to toxoplasmosis lends further support to the hypothesis that different inflammatory mechanisms underlie the development of the diversified retinal lesions observed in the studied area.

The SNP *rs2069718,* which was assessed in the present study, exhibits strong LD relative to the SNPs *rs2430561* (also known as +874T>A) (r^2 = 0.98) and *rs2069705* (Cooke et al. 2006) (r^2 = 0.84)*,* which are associated with differential levels of IFN-γ production and all three SNPs are located in intronic regions of the *IFNG* gene. Several studies have reported an association between the polymorphism in position +874T>A (*rs2430561*) and the level of IFN-γ production. Specifically, the TT, TA and AA genotypes have been associated with high, medium and low levels of IFN-γ production, respectively (Pravica et al. 2000, López-Maderuelo et al. 2003, Dai et al. 2006, Henao et al. 2006). de Albuquerque et al. (2009) have suggested that the AA genotype of *rs2430561* is associated with susceptibility to ocular toxoplasmosis, although this correlation was not statistically significant. Neves et al. (2009) identified the A allele in most of the individuals with toxoplasmosis-related retinochoroiditis (albeit with no statistical significance), also suggesting an association between the A allele in homozygosis and ocular toxoplasmosis. In addition, Neves et al. (2012) identified an association between the AA genotype in the SNP +874T>A (*rs2430561*) and a longer duration of symptoms in the acute stage of disease; however, the association failed to reach statistical significance, which the authors attributed to the relatively small sample size.

In the present study, the presence of the GG genotype in the SNP *rs2069718* was associated with the most severe form of retinal scar lesion (type A). Individuals with the GG genotype in SNP *rs2069718* also exhibited the lowest IFN-γ production. The risk of ocular disease with type A scar lesions was 3.3-fold higher among the carriers of the GG genotype compared with the carriers of the A allele in either homozygosis (AA) or heterozygosis (AG). Thus, we can conclude that the profile of association of the G allele of the SNP *rs2069718* in homozygosis (GG) with ocular toxoplasmosis is the same as the one previously described for the A allele in the SNP *rs2430561* (+874T>A) in homozygosis (AA) (de Albuquerque et al. 2009). This finding is consistent with the fact that both the *rs2069718* and +874T>A SNPs are intronic and exhibit a strong mutual LD (r^2 = 0.98).

Nevertheless, it seems that the mere association of given *IFNG* SNPs with a predisposition to higher or lower levels of IFN-γ production does not suffice to explain the manifestation of the ocular disease in the patients who carry these SNPs. Our analysis of the correlation between the profile of IFN-γ production and the GG genotype (associated with ocular toxoplasmosis) as well as the AA and AG genotypes (associated with protection against ocular toxoplasmosis) (Table III) of the SNP *rs2069718* revealed that the levels of IFN-γ production were similar in the control group and the group with the most severe pattern of retinochoroidal scar lesions (type A) (Fig. 1C). To the best of our knowledge, no other study has assessed the polymorphisms in *IFNG* concomitantly with the levels of IFN-γ production in patients with retinal/retinochoroidal scar lesions that are presumably caused by toxoplasmosis.

Type A retinochoroidal scar lesions are universally acknowledged as being related to toxoplasmosis (Aleixo et al. 2009, Bahia-Oliveira et al. 2009, 2012, Dutra et al. 2013) and can arise from either congenital or postnatally acquired infection (Glasner et al. 1992). However, the mode of acquisition (congenital or postnatally acquired) cannot be established except in the cases with confirmed congenital infection and where the lesion has been monitored since birth or the first years of the child's life. We have previously suggested that type A lesions might be the result of exacerbated, IFN-γ-rich inflammatory processes, not necessarily requiring a large number of parasites to be triggered (Bahia-Oliveira et al. 2009, 2012). Nonetheless, such lesions also might result from intensive parasite replication in the retina within a context of ineffective immune inhibition of parasite replication because of low IFN-γ production. This possibility illustrates the double-edged sword of IFN-γ production described in some diseases (Bahia-Oliveira et al. 1998, Zaidi & Merlino 2011).

When interpreting population-based genetic studies on ocular toxoplasmosis, it is necessary to keep in mind that it is not always possible to determine with absolute certainty if a case of ocular disease results from an intrauterine or a postnatal infection. Epidemiological studies in Brazil have reported that the majority of cases of ocular toxoplasmosis are from postnatally acquired infection (Glasner et al. 1992, Melamed 2009).

Individuals with intrauterine (congenital) infection who exhibit severe retinal lesions at birth or during the first years of life have been exposed to infection under unfavourable defense conditions due to the immaturity of the immune system at the time of infection compared with individuals infected after birth, when the immune system is more mature. Interestingly, the results of the present study regarding the genotype of the individuals with type A scar lesions, as well as the control group, revealed a higher frequency of individuals with the GG genotype for the SNP *rs2069718* in the group with type A scar lesions, as opposed to a predominantly heterozygous genotype (AG) in the control group. On the other hand, despite the similar frequency of the AA genotype among both groups, the two groups exhibited different profiles of IFN-γ production. Specifically, among the individuals with the AA genotype, the group with type A scar lesions produced more IFN-γ than the control group (Fig. 1C). In view of this finding, we hypothesise that in the group of patients with type A scar lesions the carriers of the homozygous AA genotype (high IFN-γ producers) suffered

a congenital infection at a time when the immaturity of their immune system prevented them from responding with a high production of IFN-γ and from benefitting from carrying this genotype as much as they could if they were only infected with *T. gondii* later in life, when the immune system had reached maturity. Another possible interpretation for the same findings is that patients who get infected *in utero* develop lesions because of the inability of their immune system to control the parasite replication and, furthermore, they lose or never develop the capacity to regulate IFN-γ production; in consequence, they exhibit a strong and destructive inflammatory reaction against parasites or parasite antigens.

In contrast, the individuals with the GG genotype of *rs2069718* (low IFN-γ producers) presumably developed type A scar lesions due to their inability to inhibit parasite replication in the retina. Indeed, the data reported by Neves et al. (2012) indicate that individuals with low-IFN-γ-producer genotypes tend to exhibit a longer duration of symptoms in the acute phase of toxoplasmosis, suggesting an inability to inhibit parasite replication. If this hypothesis is confirmed, i.e., if scar lesions exhibited by high-IFN-γ producers with genotype AA are of congenital origin, then the role we have attributed to IFN-γ in the aggravation of retinochoroidal lesions will have to be reevaluated in the context of ocular toxoplasmosis. For this reason, further studies addressing this question are needed. It remains to be determined why apparently the individuals with genotype AA for the SNP *rs2069718* in the control group produced lower levels of IFN-γ than those with the AG genotype of the same group. It is necessary to evaluate the IFN-γ secretion profile in a larger group of affected and non-affected patients genotyped for SNP *rs2069718*. Because of the strong protective character of the AG genotype in the seropositive group without eye lesions (Table III) and because of the low number of patients in some subgroups, we cannot yet rule out the hypothesis that a high and uncontrolled IFN-γ production is detrimental to the development of severe toxoplasmic ocular lesions.

The SNP *rs2069718* also exhibited LD with the SNP *rs2069705* ($r^2 = 0.84$), which is located in the *IFNG* promoter region. *rs2069705* has been suggested to be associated with differential levels of IFN-γ production, although its effect on actual IFN-γ expression has not been directly assessed (Kim et al. 2010). In addition, *rs2069718* also exhibits LD relative to *rs1861493* ($r^2 = 0.74$) and *rs1861494* ($r^2 = 0.80$), both of which were assessed in the present study, but *rs1861493* lost statistical significance in the association with scar phenotype after Bonferroni correction.

Further, the SNP *rs3181035* exhibited significant differences in genotype between affected and non-affected in the present study. The presence of the T allele in this SNP in either homozygosity or heterozygosity was significantly associated with the least severe form of retinal scar lesion (type C). With respect to the levels of IFN-γ production by PBMCs from individuals with the high-risk genotypes of this marker (CT and TT), there was no significant difference compared with the control group (Fig. 2A-C). We were not able to locate any study that has investigated the association of *rs3181035* genotype with the level of IFN-γ production and our data suggest that this SNP does not have a direct relationship with differential IFN-γ production (Fig. 2A-C).

The particular contribution of the present study relative to previous studies is that we investigated polymorphisms in *IFNG* using 10 SNPs (Table II) and found that two of them maintained statistical significance after Bonferroni correction. In addition to the high statistical significance exhibited by the SNP *rs2069718* in the comparison between affected and non-affected, we further found that (i) the association described in the present paper refers only to the most severe form of retinochoroidal scar lesions presumably caused by toxoplasmosis (type A) and does not apply to the milder forms of scar lesions (types B and C) and (ii) the association of the polymorphism in homozygosis (GG) with the low-IFN-γ-producer profile was confirmed (Fig. 1A-C).

With respect to the SNP *rs3181035*, we did not observe a clear relationship between the genotypes that exhibited a significant association with type C lesions (CT and TT) (Table IV) and the level of IFN-γ production by *T. gondii* STAg-stimulated PBMCs from the corresponding individuals compared with the controls (Fig. 2A-C).

Similar to other studies on cytokine gene polymorphisms, the present study shows the intrinsic limitations to this type of approach, especially relative to the number of affected (seropositive for *T. gondii* with different types of scar retinal/retinochoroidal lesions) and non-affected patients (seropositive for *T. gondii* without retinal/retinochoroidal lesions). Concomitant analysis of the phenotype (IFN-γ production by STAg-stimulated PBMCs) and genotype results of the same individuals at the two SNPs that exhibited statistical significance strengthens the hypothesis regarding the multifactorial nature of ocular toxoplasmosis and calls for larger cross-sectional and follow-up studies to advance the knowledge in this field. Such studies should ideally include patients in the acute and chronic stages of infection, which would significantly improve our understanding of the pathological mechanisms of the disease and consequently improve the management of ocular toxoplasmosis, particularly in endemic areas such as Brazil.

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