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6  
7 **Serotyping and cytokines response in human ocular toxoplasmosis**

8  
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32  
33 **Running Title: Host genes and serotype in ocular toxoplasmosis**

34

35

36 **Abstract**

37

38 In human ocular toxoplasmosis, serotype I is related with greater severity. We analyze *Toxoplasma*  
39 GRA6 serotype in 23 patients with ocular toxoplasmosis (13 confirmed, two co-infections- and eight  
40 unconfirmed cases) and 20 individuals chronically infected with *Toxoplasma* but without ocular  
41 involvement. In patients with ocular toxoplasmosis, we also studied host gene polymorphisms related  
42 to immune response (IL-1 $\beta$ ; IL-1 $\alpha$ ; IL-10; IFN- $\gamma$ ; TNF- $\alpha$ , IL-12), IL-17R, TLR-9, and P2RX7.  
43 Additionally, eight patients were studied for the production of TNF $\alpha$ , IL1- $\beta$ , IFN- $\gamma$  and IL-10 by  
44 their peripheral leukocytes after *ex vivo* stimulation with soluble *Toxoplasma* antigens. There were  
45 no differences in the distribution of serotypes (GRA6-I versus GRA6 non-I) between infected  
46 individuals with- or without ocular involvement. Seropositivity for GRA6-I was associated with  
47 higher number of retinal lesions and higher levels of IL-1 $\beta$ . Two polymorphisms were associated  
48 with specific clinical manifestations of OT: IL-10 -819 C/T with bilateral lesions, and IL-12 +169774  
49 A/C, with synechia. Higher levels of IL-10 were found in patients with the allele G/G at the  
50 polymorphic region IL-10 -1082. People with a GRA6 I serotype and possessing the allele G/G at the  
51 polymorphic region TNF $\alpha$ -857 suffered from an increased number of retinal lesions. We found a  
52 positive association between host cytokine genes polymorphisms and GRA6 serotypes correlated  
53 with specific clinical manifestations and immune response in ocular toxoplasmosis.

54

55 **Keywords:** serotype, cytokines, genetic polymorphisms, *Toxoplasma gondii*, ocular toxoplasmosis

56

57

58 **1. Introduction**

59

60 Although toxoplasmosis is due to a single genus and a single species of protozoan parasite,  
61 *Toxoplasma gondii*, many different genotypes related to specific parasite virulence and clinical  
62 severity have been identified in infected hosts including human beings [1]. One manner to identify  
63 infection by specific genotypes is the serotyping assay. Previous reports in Germany [2] and the  
64 United States [3] have described specific serotypes (related to specific genotypes) in some patients  
65 with OT, compared to people without ocular manifestations. However, until now the existing serotype  
66 techniques in human toxoplasmosis have not had practical applications and have only shown that  
67 there exist geographical differences in serotypes distribution [4–6]. In addition to parasite factors, the  
68 host genotype, particularly related to the specific immune response, plays an important role in  
69 determining the final clinical outcome. In human toxoplasmosis, many cytokines genotypes and  
70 promoter sequences of the cytokines genes have been associated with the development of ocular  
71 toxoplasmosis, that has been estimated occurs in ~10% of people infected with *Toxoplasma*  
72 postnatally in Colombia [7]. Many host genes cytokines polymorphisms have been more frequently  
73 found in people with ocular lesions compared to controls within the same geographical setting [8–  
74 12]. It has been suggested that the development of ocular lesions is a result of host gene susceptibility  
75 in addition to exposure to more virulent strains [13].

76

77 This work aimed to evaluate a modified serotype method based on the use of polypeptides derived  
78 from the GRA6 protein [14] and to determine the cytokine gene polymorphisms, compared to the  
79 clinical characteristics (number of lesions, the severity of inflammation) and with cytokine production  
80 *ex-vivo*.

81

82 **2. Material and Methods**

83

84 **2.1. Population sample and setting**

85

86 A descriptive cross-sectional study was performed in 23 patients with suspected OT at the Uveitis  
87 Service of the Ophthalmology Reference Center, *Clinica Barraquer*, in Bogotá, Colombia, from July  
88 2014 to April 2016. Data collected included ophthalmic findings: the intraocular pressure, the grade  
89 of inflammation in the anterior chamber and in the vitreous according to the SUN (Standardization  
90 of Uveitis Nomenclature) guidelines, fundus examination findings (a description of the  
91 retinochoroidal lesions, their size in disk diameters, their number and their localization, if present),

92 and evaluation of ocular complications such as synechia, cataract and cystoid macular edema (CME).  
93 Twenty serum samples of voluntary individuals with positive *Toxoplasma* IgG titers but negative  
94 specific IgMs and with eye funduscopy negative for lesions were used as controls for the comparative  
95 analysis of the serotype distribution.

96

## 97 **2.2. Laboratory diagnostic test of *Toxoplasma* infection**

98

99 Aqueous humor (AH) and/or vitreous fluid, blood and serum samples were collected from patients  
100 suspected of having OT. All serum samples were analyzed for anti-*Toxoplasma* IgG and IgM  
101 antibody titers by using ELISA commercial assays and following the manufacturer's (Human,  
102 Gesellschaft für Biochemica und Diagnostica mbH Max-Planck-Ring 21, 65205 Wiesbaden,  
103 Germany) recommendation. Blood samples were used to isolate serum and peripheral blood  
104 mononuclear cells (PBMC) for further DNA extraction and PCR tests. The diagnosis of OT was  
105 confirmed by 1) measurement of IgG serum titers and quantification of antibodies with the  
106 Goldmann–Witmer coefficient (GWC) and 2) detection of *Toxoplasma* DNA in aqueous humor by a  
107 real-time PCR TaqMan-based assay, as described previously [15,16]. Presence of the human  
108 herpesvirus 3 (Varicella Zoster) IE62 gene (VZV), the human HSV type 2 UL36 region (HVS2), the  
109 human herpesvirus 5 (Cytomegalovirus) glycoprotein B gene (CMV), the glycoprotein B gene, the  
110 Epstein–Barr virus (human herpesvirus 4) non-glycosylated membrane protein (BNRF1) gene (EBV)  
111 and the *M. tuberculosis* MPB64/IS6110 repeated genomic sequence were tested using the Genesig  
112 Advanced Kit (Primerdesign Ltd, York House, School Lane, Chandler's Ford, United Kingdom) and  
113 following the instructions of the manufacturer. The GWC index was calculated by measurement of  
114 the levels of intraocular anti-*Toxoplasma* antibodies, as described previously [17] (anti-*Toxoplasma*  
115 IgG in aqueous humor/total IgG in aqueous humor)/(anti-*Toxoplasma* IgG in serum/total IgG in  
116 serum). If local IgG production was detected (GWC index less than 2) or if PCR analysis in the  
117 aqueous humor detected parasite DNA, then the clinical diagnosis was considered as being  
118 “confirmed OT.” If local specific IgG production failed to be detected (GWC index  $\geq 2$ ) or if the PCR  
119 remained negative or if an aqueous humor sample was not available, but the eye lesions were  
120 characteristic of toxoplasmosis, and anti-*Toxoplasma* IgG antibodies were positive in the serum, the  
121 diagnosis was considered as being “undetermined OT.” If anti-*Toxoplasma* IgG antibodies were  
122 negative in the serum or if the aqueous humor sample was not available, but PCR results were positive  
123 in aqueous humor for other etiologies, the diagnosis was considered as being “other uveitis,” and such  
124 patients were excluded from the study. If two or more PCR were positive for different pathogens in  
125 the same AH sample, then the clinical case was considered as resulting from a co-infection.

126

127 **2.3.ELISA quantification of specific antibodies against GRA6 from the *Toxoplasma* clonal**  
128 **lineages I, II and III**

129

130 The ELISA assay was based on the use of recombinant polypeptides derived from the carboxy-  
131 terminal sequence of GRA6 from the three major *Toxoplasma* clonal lineages, respectively type I  
132 (RH, amino acids 174-230), type II (76K, amino acids 174-224) and type III (C56, amino acids 174-  
133 230), all expressed as soluble Hisx6-tagged polypeptides in *Escherichia coli* using the expression  
134 vector pUET1 (DNA-GdanskII sc. Gdansk, Poland). The recombinant polypeptides were  
135 concentrated by the Proteogenix company (Schiltigheim, France), such as to reach the final  
136 concentrations of 1.2 mg/mL (RH polypeptide), 1.25 mg/mL (76K polypeptide) and 3.2 mg/mL (C56  
137 polypeptide).

138

139 Indirect ELISAs were performed by coating 100  $\mu$ L of each recombinant polypeptide ( $4.2 \times 10^{-2}$   
140 nmol) diluted in 0.1 M carbonate buffer pH 9.6 (0.159 g/100 mL  $\text{Na}_2\text{CO}_3$ , 0.293 g/100 mL  $\text{NaHCO}_3$ ),  
141 for 24 h, at 4°C, in Maxisorp micro titer plates. The unspecific binding sites were saturated for 1 h, at  
142 37°C, with 200  $\mu$ L of 1% Bovine Serum Albumin (BSA) diluted in 0.01 M PBS, pH 7.4, mixed with  
143 0.05% Tween 20 (Merck) (PBS-Tween buffer). Plates were washed 3 times with PBS-Tween buffer  
144 and 100  $\mu$ L of serum diluted 1:50 in 0.5% BSA-PBS-Tween buffer were added to each well and  
145 incubated for 1 h, at 37°C. After 3 washes in PBS-Tween buffer, 100  $\mu$ L of horseradish peroxidase-  
146 conjugated anti-mouse IgG diluted 1:6,000 in PBS-Tween buffer were added for 1 h, at 37°C. Finally,  
147 after 5 washes in PBS-Tween buffer, the horseradish peroxidase activity was developed for 15 min,  
148 at 37°C, after addition of 3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate. The reaction was  
149 stopped by addition of a 5%  $\text{H}_2\text{SO}_4$  solution. Duplicates from each serum were read at 450 nm using  
150 an Epoch 2 spectrophotometer (Bio Tek Instruments, USA).

151

152 The positive cut-off reaction index was obtained by dividing each patient serum absorbance by the  
153 mean absorbance plus 3 standard deviations (SD) calculated from 20 serums from *Toxoplasma*  
154 negative individuals. Values  $\geq 1.0$  were considered as significant when positive reactivity against each  
155 peptide was detected. As a cross-reaction between type I and type III antibodies had been reported  
156 when using similar ELISA protocols [5,18], we establish a ratio between both types of antibodies: the  
157 absorbance values obtained with GRA6 type I as coating antigen divided by the absorbance values  
158 obtained with GRA6 type III as coating antigen, and reciprocally.

159

#### 160 **2.4. Host polymorphisms in genes related to the immune response**

161

162 The following Single Nucleotide Polymorphisms (SNPs) of cytokines' and cytokine receptor's genes  
163 were evaluated, namely IL-1 $\beta$  (+3954G/A, -511G/A, -31G/A); IL-1 $\alpha$  (-889G/A); IL-10 (-819G/A, -  
164 1082A/G); IFN- $\gamma$  (+874T/A); TNF- $\alpha$  (-1031T/C, -308G/A, -238C/T, -857G/A,); IL-12  
165 (+169774G/T); and IL-17R (+18661C/T). Analysis of polymorphisms of both the gene encoding the  
166 purinoceptor for ATP P2RX7 (rs 1718119 G/A; rs 1621388 C/T; rs 2230912 C/T) and the Toll-like  
167 receptor TLR-9 (rs 352140 C/T) were included, as reported previously [8]. The amplifications  
168 products were analyzed by capillary electrophoresis. The mini-sequencing technique, also called  
169 "ddNTP primer extension," was used as previously reported [8]. Briefly, after genomic DNA isolation  
170 from blood cells using the Qiagen DNA mini kit (QIAGEN), regions containing the SNPs to be  
171 studied were amplified using initiation primers. The PCR was carried out in a total volume of 10  $\mu$ L  
172 containing 1 to 10 ng of genomic DNA, 1X Qiagen Multiplex PCR Master Mix (Qiagen), 1X Q-  
173 solution and 0.2 to 0.6  $\mu$ M of each specific primer. Amplification of the fragments was performed in  
174 a Veriti Thermal Cycler (Applied Biosystems, USA). The data were analyzed according to the color  
175 of the peaks and the size of the amplified fragment using the GeneMapper v3.2 software (Applied  
176 Biosystems, USA).

177

#### 178 **2.5. Cytokine response of peripheral blood mononuclear cells (PBMC) stimulated *ex-vivo* with** 179 **soluble *Toxoplasma* antigens**

180

181 Fifty microliters of peripheral blood containing approximately  $5 \times 10^5$  PBMC were incubated for five  
182 days in sterile polypropylene tubes containing either 10  $\mu$ g/mL concanavalin A, 2,5  $\mu$ g/mL soluble  
183 *Toxoplasma* antigens (STAg) or  $10^4$  tachyzoites of the *Toxoplasma* RH strain. PBS was used as a  
184 mock incubation control. Supernatants were collected, and cytokines measurements were performed.  
185 The levels of interferon-gamma (IFN- $\gamma$ ), interleukin-10 (IL-10), IL-1 $\beta$  and tumor necrosis factor  
186 alpha (TNF- $\alpha$ ) were determined by a commercial ELISA test (Invitrogen, USA or BD Biosciences,  
187 USA) according to the recommendations of the suppliers. The results were expressed as pg/mL of  
188 each cytokine.

189

#### 190 **2.6. Statistical analyses**

191

192 Differences in proportions among groups were compared using Fisher's exact test. Mean differences  
193 in each group were compared by One-way ANOVA, once the normality of the distribution of data



194 had been tested by the Kolmogorov Smirnov test. The interaction between the parasite serotype and  
195 specific host genes polymorphisms and their effect on the final clinical outcome was analyzed by  
196 univariate analysis of variance. The statistical significance of the relationship between the clinical  
197 features and the serotype or the cytokine levels was studied by a Spearman's non-parametric  
198 correlation-test.  $p$  values  $<0.05$  were considered significant after Bonferroni correction  
199 ([http://alexandercoppock.com/statistical\\_comparisons.html](http://alexandercoppock.com/statistical_comparisons.html)) via an adaptive 2-stage linear step-up  
200 procedure for control of false discovery rate. The SPSS software (version 14.0, Lead Technologies  
201 Inc, USA) was used for analyzing the statistical tests.

202

## 203 **2.7. Bioethical aspects**

204

205 This study was conducted according to the tenets of the Declaration of Helsinki, strictly following  
206 the Guide for Good Laboratory Procedures. The protocol was approved by the institutional ethical  
207 committee (Universidad Tecnológica de Pereira, Act 06 of May 14, 2012). All the patients and  
208 volunteers accepted to participate in the study and signed the informed consent. Clinical relevant  
209 results were returned to both the involved patients and their physicians.

210

## 211 **3. Results**

212

### 213 **3.1. Population included in the study**

214

215 In total, 23 patients with suspected OT were included in this study, from which 13 had confirmed OT,  
216 two suffered from a co-infection (one with tuberculosis and the other with varicella zoster virus), and  
217 eight were OT unconfirmed (typical retinal lesions, IgG *Toxoplasma* positive in serum but AH  
218 examinations negative). Table 1.

219

### 220 **3.2. Distribution of GRA6 serotypes in individuals with toxoplasmosis with- or without ocular 221 lesions (work hypothesis 1)**

222

223 The serotyping assay allowed distinguishing the analyzed samples between serotype GRA6-I, GRA6-  
224 II, GRA6-III or GRA6-undetermined, this former if no positivity was obtained for any polypeptide  
225 (Table 2). Cross-reactions were observed in 16 serum samples. While this was observed in only 6  
226 out of 23 (26%) samples from OT patients, it was more frequent in control samples: 10 out of 20  
227 samples (50%). Cross-reactions were observed between GRA6-I and -III in 14 samples (9 samples

228 from controls and 5 from OT) and between GRA6-I and GRA6-II in one sample from controls group  
229 and one sample from OT patients. After calculation of the ratios between the absorbance values for  
230 all recombinant peptides, all 16 samples that exhibited cross-reactions were finally assigned to the  
231 GRA6-I serotype (Table 2).

232

233 No statistically significant differences in the distribution of serotypes were found between patients  
234 with OT and those without ocular lesions. Nor were differences found relating to the group of  
235 diagnosis (confirmed *versus* undetermined) or the socioeconomical strata (Median in cases was of 2,  
236 range 1 to 4 and in controls of 2.5, range 1 to 5; t test  $p=0.52$ ). However, as the co-infection group  
237 was constituted of only two samples, we did not compare its results to those obtained for the two  
238 other groups. Importantly, together, these results allowed the distinction of the analyzed serums  
239 between serotype GRA6-I *versus* GRA6 non-I (II, III or undetermined).

240

### 241 **3.3. Influence of the GRA6 serotype I *versus* GRA6 non-I on the clinical characteristics of ocular** 242 **toxoplasmosis and production of cytokines (work hypothesis 2)**

243

244 Comparison of the qualitative eye manifestations developed in the ten patients diagnosed with OT  
245 and serotyped as GRA6-I to those developed in the 13 patients diagnosed with OT and serotyped as  
246 GRA6 non-I revealed no significant differences (Table 3). In contrast, comparison of the quantitative  
247 eye clinical manifestations between OT patients serotyped GRA6-I *versus* GRA6 non-I revealed a  
248 significantly higher number of retinal lesions in OT patients serotyped GRA6-I (Table 4).

249

250 We then evaluated the levels of cytokines produced by the PBMCs isolated from patients with OT  
251 (confirmed cases, co-infections or undetermined OT) and stimulated *ex vivo* with STAGs: no  
252 differences were observed (Supplementary material 1). In contrast, when mean values of cytokines  
253 were evaluated according to the serotype, significantly higher mean levels of IL1- $\beta$  ( $p=0.021$ ) were  
254 found in patients serotyped GRA6-I than in patients serotyped GRA6 non-I. No significant  
255 differences were observed for levels of IL-10 ( $p=0.4$ ), TNF- $\alpha$  ( $p=0.64$ ) or IFN- $\gamma$  ( $p=0.492$ ) according  
256 to the serotype or between cases and controls for levels of IL-10 ( $p=0.133$ ), TNF- $\alpha$  ( $p=0.057$ ) or IFN-  
257  $\gamma$  ( $p=0.217$ ). A Spearman correlation test between the results of the GRA6 serotyping, the levels of  
258 cytokines and quantitative clinical manifestations showed that high levels of anti-GRA6-I antibodies  
259 in OT patients were positively correlated with a high number of retinal lesions (Table 5). This analysis  
260 also showed that 1) production of higher levels of IL-10 after *ex-vivo* stimulation of PBMCs with  
261 STAGs was positively correlated with a higher number of recurrences and retinal lesions of higher

262 diameter and 2) higher levels of both IFN- $\gamma$  and TNF- $\alpha$  correlate positively with a higher number of  
263 cells in the vitreous. In contrast, the age was inversely correlated with the production of both IFN- $\gamma$   
264 and TNF- $\alpha$  (Table 5).

265

### 266 **3.4. Influence of the polymorphisms in genes related to the immune response on the clinical** 267 **characteristics of ocular toxoplasmosis (work hypothesis 3)**

268

269 We investigated whether gene polymorphisms are related to clinical characteristics of OT. Table 6  
270 presents the *p* values of the statistical tests after Bonferroni correction that compared the frequency  
271 of each qualitative clinical manifestation of OT to each genetic polymorphism. Only two  
272 polymorphisms were associated with specific qualitative clinical characteristics of OT, namely IL-10  
273 -819 G/A with bilateral lesions and IL-12 +169774 G/T with synechia. Only one allele was found to  
274 positively correlate with a specific quantitative clinical manifestation of OT, namely the G/G allele  
275 of the TNF- $\alpha$  -857 G/A gene that was significantly associated with a higher number of retinal lesions  
276 (Table 7).

277

### 278 **3.5. Production of cytokines by PBMCs from OT patients after *ex vivo* stimulation with soluble** 279 ***Toxoplasma* antigens according to gene polymorphisms in 9 genes of the immune response** 280 **(work hypothesis 4)**

281

282 When the levels of cytokines were analyzed according to the host gene polymorphisms, significantly  
283 higher levels of IL-10 were observed in patients with the allele G/G at the polymorphic region IL-10  
284 -1082 (*p*= 0.032), the allele C/A at the polymorphic region TNF- $\alpha$ -863 C/A (*p*= 0.022), the allele  
285 G/G at the polymorphic region IL-1 $\beta$  +3954 G/A (*p*=0.045) and the allele A/A at the polymorphic  
286 region P2RX7 1718119 G/A (*p*=0.000). Higher levels of TNF- $\alpha$  were also observed when the allele  
287 T/T was present at the polymorphic region P2RX7-1621388 C/T (*p*=0.00000).

288

### 289 **3.6. Interaction between the parasite serotype and specific host gene polymorphisms**

290

291 As the only OT quantitative clinical characteristic simultaneously associated with the GRA6 serotype  
292 and the host gene polymorphism, namely TNF $\alpha$ - $\alpha$  -857, was the number of retinal scars, we  
293 performed an analysis of interaction by univariate analysis of variance to test the effects between  
294 these factors. OT people with a GRA6-I serotype and possessing the G/G allele at the polymorphic  
295 region TNF $\alpha$ -857 exhibited an increased number of retinal lesions, this allele functioning as an

296 additive to the GRA6-I serotype. The median number of lesions in people exhibiting both the GRA6-  
297 I serotype and the G/A allele at TNF $\alpha$ -857 was 2.5 but increased to 5.5 with the G/G allele (corrected  
298 model:  $p=0.002$ ).

299

#### 300 **4. Discussion**

301

302 The results of prevalence of serotype GRA6-I were similar to those obtained in previous studies in  
303 the Colombian population [4,14]. They showed that, in Colombia, most people exhibit antibodies  
304 against the type I GRA6 protein or against undetermined GRA6 and that such prevalence was similar  
305 between people with or without ocular lesions. Such studies, however, did not analyze the severity of  
306 the ocular lesions. In the present study, we analyzed the effect of serotyping GRA6-I on clinical  
307 characteristics obtained after specialized ophthalmological examination. We resolved the problem of  
308 cross-reactions by including additional amino acids of the polymorphic region and by calculating a  
309 ratio between the absorbance values for each serotype of anti-GRA6, which enabled us to ascribe a  
310 serum to one particular serotype. This study, therefore, provided further insight into the significance  
311 of the infection with serotype GRA6-I and found that the serotype is related to a higher production of  
312 IL-1 $\beta$  and a higher number of lesions. It was also found that patients positive for GRA6 I had a higher  
313 number of retinal lesions and higher levels of IL-1 $\beta$ , suggesting that this technique could be a good  
314 marker to identify people infected with *Toxoplasma* virulent strains.

315

316 We previously demonstrated that Colombian patients have more severe clinical characteristics than  
317 European patients, including greater inflammation, higher number, and size of lesions [19], which  
318 has been linked to a Th2 cytokine profile and infection by type I/III strains, including higher levels  
319 of IL-1 $\beta$  [20]. The present results confirm and provide additional information related to the use of the  
320 ELISA assay to distinguish between anti-GRA6 type- I and type- III antibodies, and show that the  
321 increase in IL-1 $\beta$  is due to infection with serotype GRA6-I. Moreover, we previously linked ROP18  
322 virulent allele with more intense vitreitis [21] that can be explained by higher levels of the  
323 proinflammatory cytokine IL-1 $\beta$  [20]. ROP18 protein is a virulent factor in toxoplasmosis first  
324 identified by studying clonal lineages on a mouse model of virulence [22]. The presence of virulent  
325 alleles such as ROP18 or ROP16 is not limited to clonal lineages as originally described, but they can  
326 be present more frequently in atypical strains from South America, as a consequence of the  
327 predominance of chromosome 1a among strains in anthropized environments [23,24]. Overall,  
328 present results of serotyping give additional support to the hypothesis that the more severe cases of

329 ocular toxoplasmosis, as reported in Brazil [25] and Colombia [19], are related to virulent alleles that  
330 are associated with the manipulation of protective immune response [13].

331

332 Although the strain hypothesis can explain the geographical differences in clinical presentation and  
333 immune response in ocular toxoplasmosis, this cannot completely explain the diversity in clinical  
334 presentation. For this reason, we previously analyzed the association between host gene  
335 polymorphisms and the risk of OT in a Colombian. In that study, we found that the IL-10 -1082A/G,  
336 the IFN  $\gamma$  +874A/T polymorphisms and the haplotype “GAG” of the IL-1 $\beta$  gene promoter  
337 polymorphisms were significantly more prevalent in OT patients than in control infected people  
338 without ocular lesions [8]. Here we confirm that these polymorphisms were related to a phenotype  
339 of higher production of IL10. A previous study found that IL10 was characteristically more produced  
340 by OT patients than people with chronic asymptomatic infection [26]. Present results confirm those  
341 findings as well as their genetic basis. Additionally, the IL-10 -1082A/G polymorphism was related  
342 to the presence of a bilateral lesion, that can reflect uncontrolled migration of parasites and  
343 polymorphism in IL12 gene was related with synechia, indicating an exaggerated inflammatory  
344 response.

345

346 It has been well established by many authors using different methods of analysis that the cytokine  
347 profile of response in Colombian individuals infected with *Toxoplasma* is strikingly different to that  
348 of infected individuals from the United States and Europe [20,27]. The results of this work explain  
349 those findings, indicating that the immune response is driven by the infecting strains but also  
350 conditioned by the host cytokine gene polymorphisms. This is demonstrated by the interaction  
351 between the polymorphic site TNF- $\alpha$  -857G/A and infection by type I strain. The number of lesions  
352 was duplicated in individuals that had the polymorphisms and were infected with serotype GRA6-I  
353 strain compared to those with the same polymorphisms but infected by serotypes of GRA6 non-type  
354 I.

355

356 The diversity in clinical expression – such as the intensity of inflammation in the eye that results in  
357 production of synechia, papillitis, vasculitis and control of the migration of parasites (that impedes or  
358 permit increase or reduction in size or number of lesions) – can be explained by how the diverse array  
359 of cytokines is- produced, as well as by how the parasite controls that response. The results presented  
360 in this study in a population exposed to virulent strains of *Toxoplasma* offer an unprecedented view  
361 of how diversity in cytokine response can explain the diverse consequences observed in human ocular

362 toxoplasmosis and offer perspectives for immunomodulatory strategies for the treatment of this  
363 parasitic infection with high impact on ocular health.

364

### 365 **Compliance with Ethical Standards**

366

367 **Protection of human and animal subjects.** The authors declare that no experiments were performed  
368 on humans or animals for this investigation

369 **Confidentiality of data.** The authors declare that no patient data appears in this article.

370 **Right to privacy and informed consent.** The authors declare that no data that enables identification  
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382

383

384

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- 499  
500

501 **Table 1.** Groups of patients and controls for each work hypothesis

502

Work hypothesis	Number of patients with ocular toxoplasmosis	Number of controls
1. To test if the serotype is in agreement with the diagnosis of ocular toxoplasmosis	23	20
2. To test if the serotype is in agreement with clinical characteristics	23	The hypothesis tested only within the group of patients
3. To test if the host cytokine gene's polymorphisms and the serotype determine the clinical characteristics of the disease	23	The hypothesis tested only within a group of patients
4. To test if the levels of cytokines quantified after PBMC* <i>ex vivo</i> stimulation are in agreement with the serotype or cytokine gene polymorphisms	8	4

503

504 \*PBMC: Peripheral blood mononuclear cells

505

506 **Table 2.** Distributions of GRA6 serotypes between individuals with ocular toxoplasmosis (clinical  
 507 cases) and those with asymptomatic chronic ocular toxoplasmosis (controls) after cross-reactions  
 508 were resolved  
 509

	<b>GRA6 I</b> n/N* (%)	<b>GRA6 II</b> n/N* (%)	<b>GRA6 III</b> n/N* (%)	<b>GRA6 UNDETERMINED</b> n/N* (%)
<b>Ocular toxoplasmosis</b> (N=23)	10/23 (43%)	2/23 (8.6%)	0/23 (0%)	11/23 (43%)
<b>Sub-groups of patients with ocular toxoplasmosis</b>				
Confirmed OT (n=13)	7/13 (53.8%)	1/13 (7.7%)	0/13 (0%)	5/13 (38.4%)
Co-infection (n=2)	0/2 (0%)	½ (50%)	0/2 (0%)	½ (50%)
Undetermined OT (n=8)	3/8 (37.5%)	0/8 (0%)	0/8 (0%)	5/8 (62.5%)
<b>Control subjects (chronically infected but without ocular disease) (N=20)</b>	10/20 (50%)	0/20 (0%)	2/20 (10%)	8/20 (40%)

510  
 511 \*n: number of samples positive for the test, N: the total number of analyzed samples. X<sup>2</sup> test: No  
 512 statistically significant differences between the clinical cases (including subgroups) and the  
 513 control subjects.

514  
 515

516 **Table 3.** Distribution of the qualitative eye clinical manifestations in patients serotyped GRA6-I  
 517 *versus* GRA6 non-I and diagnosed with ocular toxoplasmosis  
 518

<b>Clinical manifestations</b>	<b>Number of patients with particular eye clinical manifestation (n) out of those serotyped as GRA6 I and diagnosed with ocular toxoplasmosis (N) (%)</b>	<b>Number of patients with particular eye clinical manifestation (n) out of those serotyped as GRA6 non-I and diagnosed with ocular toxoplasmosis (N) (%)</b>	<b>X<sup>2</sup> test (p level of significance)</b>
Panuveitis	3/10 (30%)	5/13 (38.5%)	1.00
Bilateral lesions	1/10(10%)	3/13 (23.1%)	0.60
Cataract	3/10 (30%)	4/13 (30.8%)	1.00
Synechia	2/10 (20%)	2/13 (15.4%)	1.00
Papilitis	1/10 (10%)	3/13 (23.1%)	0.54
Vasculitis	0/10 (0%)	3/13 (23.1%)	0.26
Cystoid macular edema	2/10 (20%)	3/13 (23.1%)	0.95
Macular lesion	5/10 (50%)	9/13 (69.3%)	0.63
High intraocular pressure (>21 mm Hg)	3/10 (30%)	1/13 (7.69%)	0.34

519

520 **Table 4.** ANOVA analysis of quantitative clinical characteristics and *Toxoplasma* GRA6 serotype.  
 521 Statistically significant correlations are shown in bold ( $p < 0.05$ ).

522

Serotype		Number of recurrences	Number of cells in vitreous	Number of retinal lesions <sup>††</sup>	Size of the lesion (in disk diameters) <sup>††</sup>
<b>GRA6-I</b>	Mean	2	1.2	<b>4.1</b>	1.5
	SD*	2.5	1.1	1.8	1.2
	N <sup>†</sup>	10	10	8	8
<b>GRA6 non-I</b>	Mean	1.8	1.6	<b>1.2</b>	1.8
	SD	2.7	1.4	0.707	1.8
	N <sup>†</sup>	13	13	8	7
<i>p</i> -value		0.89	0.51	<b>0.001</b>	0.67

523

524 \*standard deviation; <sup>†</sup> number of patients diagnosed with ocular toxoplasmosis and serotyped as  
 525 GRA6-I *versus* GRA6 non-I. <sup>††</sup> Some patients have no data on the number of lesions or size because  
 526 inflammation does not permit to observe the lesion.

527

528 **Table 5.** Spearman analysis of the correlation between the levels in anti-**GRA6- I** antibodies  
 529 (absorbance), cytokines (pg/ml) and quantitative clinical characteristics. Statistically significant  
 530 correlations are shown in bold ( $p<0.05$ ).

531

		<b>GRA6 I ELISA absorbance values</b>	<b>IL-10 (pg/mL)</b>	<b>TNF-<math>\alpha</math> (pg/mL)</b>	<b>IFN-<math>\gamma</math> (pg/mL)</b>	<b>IL-1<math>\beta</math> (pg/mL)</b>
<b>Total number of retinal lesions</b>	Correlation coefficient	0.7202	0.1581	0	0	0.4743
	Sig. (2-tailed)	<b>0.0016</b>	0.7995	1	1	0.4195
	N	16	5	5	5	5
<b>Age</b>	Correlation coefficient	-0.4648	0.4	-0.80	-0.952	-0.309
	Sig. (2-tailed)	<b>0.02541</b>	0.2861	<b>0.0165</b>	<b>0.00026</b>	0.4556
	N	23	9	8	8	8
<b>Number of recurrences</b>	Correlation coefficient	-0.0807	0.9023	0,0060	-0.060	0.5301
	Sig. (2-tailed)	0.71431	<b>0.0008</b>	0.9886	0.88731	0.1765
	N	23	9	8	8	8
<b>Number of cells in vitreous</b>	Correlation coefficient	-0.18566	-0.025	0.8687	0.80100	0.0250
	Sig. (2-tailed)	0.39634	0.9470	<b>0.0051</b>	<b>0.01687</b>	0.95
	N	23	9	8	8	8
<b>Size of lesion (in disk diameters)</b>	Correlation coefficient	-0.19982	0.9	-0.5	-0.5	0.8
	Sig. (2-tailed)	0.47520	<b>0.0373</b>	0.3910	0.39100	0.1040
	N	15	5	5	5	5

532

533

534 **Table 6.** *p* values after Bonferroni correction of a X<sup>2</sup> test performed to compare the results of the  
 535 analysis of 17 polymorphisms in 9 genes related to the immune response and the qualitative clinical  
 536 characteristics analyzed in 23 Colombian patients with ocular toxoplasmosis. Statistically significant  
 537 *p* values are shown in bold (*p*< 0.05).

538

	Panuveitis	Bilateral lesions	Cataract	Synechia	Papilitis	Vasculitis	Cystoid macular edema	Macular lesion	High intraocular pressure (>21 mm Hg)
TNF -1031 T/C	1	1	1	1	1	1	1	1	1
TNF -308 G/A	1	1	1	1	1	1	0,48	1	1
TNF -857 G/A	1	1	1	1	1	1	1	1	1
TNF -238 C/T	1	1	1	1	1	1	1	1	1
TNF-863 C/A	1	1	1	1	1	1	1	1	0,27
IL-10 -819 G/A	1	<b>0,028</b>	1	1	1	1	1	1	1
IL-10 -1082 A/G	1	1	1	1	1	1	1	1	1
IL-1B -511 G/A	1	1	1	1	1	1	1	0,39	1
IL-1B +3954 G/A	1	1	1	1	1	1	1	1	0,37
IL-1B -31 G/A	1	1	1	1	1	1	1	1	1
IL-1A -889 G/A	1	1	1	1	1	1	1	1	1
IL-17 +18661 C/T	1	1	1	1	1	1	1	1	1
IFN +874 T/A	1	1	1	1	1	1	1	1	1
IL-12 +169774 G/T	1	1	1	<b>0,04</b>	1	1	1	1	1
P2RX7 1718119 G/A	1	1	1	1	1	1	1	1	1
P2RX7 1621388 C/T	1	1	1	1	1	1	1	1	1
P2RX7 2230912 C/T	1	1	1	1	1	1	1	1	1
TLR-9 352140 C/T	1	1	1	1	1	1	1	1	1

539

540

541



542 **Table 7.** *p* values after Bonferroni correction of an ANOVA performed to compare the results of the  
 543 analysis of 17 polymorphisms in 9 genes related to the immune response and the quantitative clinical  
 544 characteristics analyzed in 23 Colombian patients with ocular toxoplasmosis. The statistically  
 545 significant *p*-value is shown in bold ( $p < 0.05$ ).

546

	<i>p</i> values of the ANOVA statistical analysis			
	Number of recurrences	Number of vitreous cells	Number of retinal lesions	Size of lesion (in disk diameters)
TNF -857 C/T	0,356	0,883	<b>0,005</b>	0,204
TNF -238 G/A	0,722	0,266	NA	NA
TNF-863 C/A	0,882	0,436	0,285	0,142
TNF -1031 T/C	0,665	0,539	0,774	0,088
TNF -308 G/A	0,771	0,935	0,342	0,299
IL-10 -819 C/T	0,832	0,558	0,253	0,092
IL-10 -1082 G/A	0,221	0,312	0,104	0,956
IL-1B -511 C/T	0,317	0,463	0,320	0,132
IL-1B 3954 C/T	0,152	0,919	0,292	0,237
IL-1B -31 T/C	0,990	0,580	0,842	0,184
IL-1A -889 C/T	0,469	0,885	0,182	0,864
IL-17 +18661 C/T	0,709	0,272	0,297	0,717
IFN +874 A/T	0,424	0,409	0,739	0,252
IL-12 +169774 A/C	0,132	0,729	0,331	0,717
P2RX7 1718119 G/A	0,309	0,076	0,518	0,408
P2RX7-1621388 C/T	0,632	0,076	0,518	0,523
P2RX7-2230912 C/T	0,292	0,079	0,619	0,628
TLR-9 C/T	0,660	0,155	0,669	0,876

547

548 \*NA: Not applicable due to low numbers (<3) in some alleles.

549