Influence of Activating and Inhibitory Killer Immunoglobulin-Like Receptors (KIR) genes on the recurrence rate of ocular toxoplasmosis in Brazil

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25 Abstract

26 Background

27 Recurrence is a hallmark of ocular toxoplasmosis (OT), and conditions that influence its
 28 occurrence remain a challenge. Natural killer cells (NK) are effectors cells whose primary
 29 function is the cytotoxic activity against many parasites, including *Toxoplasma gondii*. Among
 30 the NK cell receptors, immunoglobulin-like receptors (KIR) deserve attention due to their high
 31 polymorphism. This study aimed to analyze the influence of KIR gene polymorphism in the
 32 course of OT infection and its association with recurrences after an active episode.

33 Methods

34 Ninety-six patients from the Ophthalmologic Clinic of the National Institute of Infectology
 35 Evandro Chagas (INI/Fiocruz/RJ, Brazil) were followed for up to five years. After DNA
 36 extraction, genotyping of the patients was performed by PCR-SSO utilizing Luminex
 37 equipment for reading. During follow-up, 57.4% had a recurrence.

38 Results

39 We identified 25 KIR genotypes and found a higher frequency of genotypes 1 (31.7%) with
 40 worldwide distribution. We note that the *KIR2DL2* inhibitor gene and the gene activator
 41 *KIR2DS2* were more frequent in patients without recurrence ($P = 0.03$ and $P = 0.02$,
 42 respectively). Additionally, we observed one activating gene, *KIR2DS1*, associated with more
 43 than four times faster progression to the development of recurrent ocular toxoplasmosis than
 44 individuals without this gene (aRR = 4.6, $P = 0.04$).

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47 **Conclusion**

48 The KIR2DL2 and KIR2DS2 are associated as possible protection markers and the KIR2DS1
 49 acting as a possible susceptibility marker. Additionally, the lower proportion of activating genes
 50 observed in individuals with recurrence corroborating with the hypothesis that these individuals
 51 are more susceptible to ocular toxoplasmosis recurrence (OTR).

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66 Introduction

67 *Toxoplasma gondii* is an obligate intracellular protozoan parasite that belongs to the
68 phylum apicomplexa, subclass coccidia. The parasite has a worldwide distribution with a high
69 prevalence that infects humans, birds, rodents, and other animals (intermediate hosts) and felids
70 (definitive hosts) on all continents. The toxoplasmosis can be classified as congenital or
71 acquired. Congenital toxoplasmosis may be exclusively ocular or accompanied by systemic or
72 central nervous system changes [1]. In recent decades, advances show that toxoplasmosis is one
73 of the most important causes of posterior uveitis in the world, representing up to 85% of all
74 cases [1-3]. The ocular lesions are characterized by necrotizing retinitis with oval or circular
75 lesions. Besides it, the lesion can remain active for weeks, and even after healing, it may contain
76 *T. gondii* cysts, so the protozoan remains viable in tissues for years [4].

77 Ocular toxoplasmosis (OT) is a disease characterized by recurrence episodes. However,
78 the conditions associated with recurrence episodes have not been completely elucidated. After
79 infection, the ocular symptoms depend on complex and variable factors, such as socioeconomic
80 factors and the parasite genotype. In Brazil, both the prevalence (> 80%) and severity of OT
81 are higher than in many other parts of the world [2].

82 Concerning the immune system, in the eye, the parasite contrives to manipulate the
83 immune response in such a way as to favor its survival without causing too much damage to
84 the organ [2, 5]. In the early phase of *T. gondii* infection, innate immunity cells are recruited to
85 the site of infection. Natural Killer (NK) are important lymphocytes acting in the acute phase
86 of the toxoplasmosis [6]. *In vivo* studies revealed that control of *T. gondii* requires the early
87 production of the pro-inflammatory cytokine IL-12, which stimulates NK, CD4⁺, and CD8⁺
88 lymphocytes to release IFN- γ [6-8].

Control of NK cell action is through membrane receptors, including Killer Immunoglobulin-Like Receptors (KIR), which recognize Human leukocyte antigen class I molecules (HLA class I) expressed by most cells in the body. The extensive genetic polymorphism of KIR receptors and the regulation of their expression in different NK cell clones are essential factors that delineate each individual's innate and adaptive immune response.

NK cells have great importance in the control of *T. gondii* infection however, the role of *KIR* genes that encode the immune receptors of NK cells and can trigger local inflammation in the eye has not been elucidated in ocular toxoplasmosis yet. *KIR* genes have been described as risk or protective factors in different types of inflammatory ocular diseases [9-11] and are also associated with many other infectious diseases [12-19].

In ocular toxoplasmosis, no study has evaluated the association of KIR receptors with ocular toxoplasmosis involving recurrence events. Thus, the characterization of these receptors in individuals with ocular toxoplasmosis may help to understand their role in regulating the immune response, clinical evolution of the disease, as well as their relationship with faster or lower recurrences. Besides it, the identification of predisposal individuals may help in their clinical management. However, others studies should be performed such as histological analyses of the ocular tissue affected by *T. gondii* and NK cytotoxicity assays to better understanding the role of NK cells and the expression of KIR in the immunopathogenesis of ocular toxoplasmosis.

112 **Material and methods**

113 **Ethics statement**

114 The Research Ethics Committee of the National Institute of Infectology Evandro Chagas
115 (INI/Fiocruz) has approved this study protocol as a subproject under the register CAAE
116 0075.0.009.000-11. All the volunteers gave written informed consent after being informed
117 about the study's nature, including the objectives, laboratory procedures that would be
118 performed. They allowed the store and future use in research of their samples.

119 **Patients**

120 This study was conducted using 96 blood and serum samples stored at the
121 Toxoplasmosis Laboratory at IOC-Fiocruz. The patients were attended by the same
122 ophthalmologist between January 2010 and January 2014, and follow-up until July 2015 at the
123 outpatient unit of the Infectious Ophthalmology Laboratory of the National Institute of
124 Infectology Evandro Chagas at Fiocruz [20].

125 For the purpose of this study, the patients were classified according to the recurrence of
126 ocular toxoplasmosis. As previously described in Aleixo *et. al* [20, 21], recurrent cases were
127 defined as an active retinochoroiditis associated with retinal scar in either eye [22]. It's
128 important to highlight that episodes of inflammation of the anterior segment in eyes with
129 retinochoroiditis scars were not considered recurrence [23]. Creamy-white focal
130 retinochoroidal lesions in the absence of other retinochoroidal scars were considered as primary
131 lesions. Primary retinochoroidal lesion cases with no recurrence were considered to be highly
132 probable of ocular toxoplasmosis and thus included in the follow-up [21]. The follow-up criteria
133 adopted for the inclusion of patients in the non-recurrence group are described in more detail
134 in Aleixo *et al.* [20]. It has been demonstrated that the risk of OT recurrence is higher in the
135 year following the first infection than in future years [20, 24]. However, to avoid erroneous

136 associations we included in the non-recurrence group only patients who were followed up for
137 at least two years.

138 The patients' exclusion criteria's were pregnant during any recurrent episodes,
139 genetically related, having co-morbidities (e.g., chronic renal failure), systemic infections (e.g.,
140 AIDS, syphilis, and tuberculosis), autoimmune diseases, history of intravenous drugs use,
141 single and unilateral or multiple exudative lesions of retinochoroiditis, and history of cancer
142 chemotherapy or immunosuppressive drug or peri- and/or intraocular steroids use. All exams
143 were performed in the Laboratory of Immunology and Immunogenetics (INI/Fiocruz).

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145 **Genomic DNA extraction**

146 According to the manufacturer's instructions, genetic DNA was isolated from peripheral
147 blood samples collected in EDTA by using a QIAamp® DNA Blood Midi/Maxi Kit (Qiagen,
148 Valencia, CA). The DNA concentration was determined using a Qubit fluorimeter (Life
149 Technologies, Carlsbad, CA), and the filtrates containing the isolated DNA were stored at -20°
150 C until time of use.

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152 ***KIR* genotyping**

153 The reverse sequence-specific oligonucleotide technique (One Lambda Inc., Canoga
154 Park, CA) with Luminex xMap technology (Luminex Corp., Austin, TX) was used for the
155 typing of the 14 *KIR* genes and two *KIR* pseudogenes namely *KIR2DL1*, *KIR2DL2*, *KIR2DL3*,
156 *KIR2DL4*, *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5*, *KIR3DL1*,
157 *KIR3DL2*, *KIR3DL3*, *KIR3DS1*, *KIR2DPI*, and *KIR3DP1*, according to the manufacturer's
158 instructions. The carrier frequency was calculated through the direct counting of individuals
159 owning at least one copy of the gene. It was necessary to consider that all frequencies were in

Hardy-Weinberg equilibrium (HWE). In contrast, individuals heterozygous for a KIR gene could not be distinguished from those that were homozygous without family members. The genotypic frequency (GF) was calculated by the formula $GF=1-\sqrt{1-CF}$, where CF indicates the carrier frequency, calculated by $CF=\text{frequency}(F)\%/100$ [25]. The survey data were recorded and entered into an Epi Info 2007 (Centers for Disease Control and Prevention, Atlanta, GA) database.

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167 **Statistical analysis**

Mann-Whitney U tests were used to compare clinical-demographic features for continuous numerical variables between recurrent and non-recurrent groups. In contrast, for categorical nominal variables, Fisher's exact tests were used to evaluate frequencies independence among these features and the groups. Proportional tests based on the chi-square distribution with one degree-of-freedom were used to test if proportions of the non-recurrent group were equal to that of the recurrent group. For each participant, person-years (py) at risk were calculated between the discharge of a previous episode and the discharge date of the last episode of ocular toxoplasmosis or the last follow-up visit, which occurred first. Recurrence rates of ocular toxoplasmosis per 100 py and its ninety-five percent confidence intervals (CI95%) were estimated according to asymptotic standard errors calculated from a Gamma distribution [26]. The effects of potential hazard factors on OTR were assessed using relative risks (RR) and its CI95% estimated by the fit of Simple and Multiple (aRR) Poisson Generalized Linear Mixed Effect Models (P-GLMM) [27] optimized by maximum likelihood. Repeated measurements were treated as random effects. Degrees of freedom for estimated effects were approximated by the Satterthwaite method [28]. Confounding variables were selected by Simple/Bivariate P-GLMM and included in multivariate models if any p-value <

0.2 to eliminate sampling bias. All statistical analyses were performed using R version 3.6.1 [29].

Results

General features of the studied population

The clinical-demographic data summarized in Table 1 show that patients with ocular toxoplasmosis were followed up for different periods. In our studied population, most individuals were adults, residents of the state of Rio de Janeiro, and they were followed up over two years. The age range was 14–67 years, with an average of 32 years, and the proportion of men was higher (56.2%) than for women (43.8%). The average follow-up time between recurrence group and non-recurrence group was very similar (1338 and 1370 days, respectively).

The number of recurrences varied between 1 and 5 episodes, and, according to this variation, 33 individuals (34.4%) had one recurrence episode, 18 (18.7%) had two episodes, 5 (5.2%) presented three episodes and 2 (2.1%) had five recurrence episodes during the follow-up (Table 1).

Table 1. Clinical-demographic features of patients consulted at the outpatient unit of the Infectious Ophthalmology Laboratory of the National Institute of Infectology Evandro Chagas, Fiocruz, RJ (N =96)

Gender	Male (N/%)	54/56.2
	Female (N/%)	42/43.6
	Total (N)	96
Age ($\bar{x} \pm SD$)		32 $\pm$ 11
Follow-up period	≤ 2 years (N/%)	4/4.2
	> 2 and ≤ 3 years (N/%)	18/18.7
	> 3 and ≤ 4 years (N/%)	28/29.2
	> 4 and ≤ 5 years (N/%)	45/46.9
	> 5 and ≤ 6 years (N/%)	1/1
N recurrence	0 (N/%)	38/39.6
	1 (N/%)	33/32.7
	2 (N/%)	18/17.8
	3 (N/%)	5/5.2
	5 (N/%)	2/2.1
Follow-up in days ($\bar{x} \pm SD$)	Recurrence group	1338 $\pm$ 324
	Non-recurrence group	1370 $\pm$ 321
State	Rio de Janeiro (N/%)	87/90.6
	Others* (N/%)	9/9.4

$\bar{x}$ mean; N, number of individuals; SD, standard deviation.; *Alagoas, Espírito Santo, Mato Grosso, Minas Gerais, Paraíba, and Rio Grande do Sul.

KIR genes frequencies

The distribution of KIR gene frequencies ($F\%$) in patients consulted at the outpatient unit of the Infectious Ophthalmology Laboratory from INI is illustrated in Table 2. All 16 KIR genes investigated (14 genes and two pseudogenes, including framework loci) were detected in the study population, and the framework loci *KIR2DL4*, *KIR3DL2*, *KIR3DL3*, and *KIR3DP1* were present in all individuals. In general, the frequencies of inhibitory KIR genes were higher than 90%, except for the *KIR2DL2* and *KIR2DL5* genes (54.2% and 54.5%, respectively). Also, the proportion of patients carrying any activating KIR gene ranged from 18.7% to 51%, except for the *KIR2DS4*, with a 96.9% frequency.

Table 2. Distribution of KIR gene frequencies in patients consulted at the of the Infectious Ophthalmology Clinic of the National Institute of Infectology Evandro Chagas, Fiocruz, RJ.

<i>KIR Gene</i>	(N)	F%	GF
<i>Inhibitory</i>			
<i>2DL1</i>	94	97.9	0.856
<i>2DL2</i>	52	54.2	0.324
<i>2DL3</i>	88	91.7	0.712
<i>2DL5</i>	53	55.2	0.331
<i>3DL1</i>	95	98.9	0.896
<i>Activating</i>			
<i>2DS1</i>	40	41.7	0.237
<i>2DS2</i>	49	51	0.3
<i>2DS3</i>	18	18.7	0.099
<i>2DS4</i>	93	96.9	0.824
<i>2DS5</i>	41	42.7	0.244
<i>3DS1</i>	34	35.4	0.197
<i>Framework</i>			
<i>2DL4</i>	96	100	1
<i>3DL2</i>	96	100	1
<i>3DL3</i>	96	100	1
<i>^A3DP1</i>	96	100	1
<i>Pseudogene</i>			
<i>2DP1</i>	93	96.9	0.824

F%, KIR genes frequencies; GF, genotypic frequency; KIR, killer cell immunoglobulin-like receptors; N, number of individuals; A, framework, and pseudogene.

239 **KIR Genotypes**

240 A total of 24 different KIR genotypes were identified in the studied population (Fig 1)
 241 based on the presence and absence of 16 KIR genes. All of these were previously described in
 242 the KIR genotype database (www.allelefreuencies.net) and contained between 8 and 16 KIR
 243 genes per individual. The genotype 1, the most common, accounted for 31.3% of all genotypes
 244 in our studied population, and genotypes 4, 2 and 3 were detected in 12.5%, 11.5%, and 8.3%
 245 of the studied population respectively (Figure 1). Among the 24 genotypes, more than half (14
 246 genotypes) were in low frequency, presenting only in 1% of the population. The two most
 247 common genotypes (1 and 4) observed in our population were present at a similar frequency to
 248 that of North, Central, and South American populations (www.allelefreuencies.net) ($P > 0.05$
 249 for all). The population was classified according to the haplogroups (A and B) existing for the
 250 KIR genes. Haplogroup B was named Bx because of the lack of distinction between the
 251 haplotype AB and BB. The haplogroup Bx was the most frequent (68.7%), while the
 252 haplogroup AA was present in 31.3% of our population.

253

254 ***Comparison of KIR genes frequencies between the recurrence and non-recurrence group***

255 In order to evaluate the possible influence of KIR genes on the recurrence of ocular
 256 toxoplasmosis after active episode, the recurrence group (58/60.4%) was compared to the non-
 257 recurrence group (38/39.6%). About the follow-up time, more than half (32/55%) of the
 258 individuals in the recurrence group were followed for more than four years. In the non-
 259 recurrence group, the majority (26/68.5%) of the individuals were followed for more than three
 260 years. When we evaluated if the difference between KIR gene frequencies among groups were
 261 significant, we observed that between the 16 genes KIR evaluated, the inhibitory *KIR2DL2* and

the activating *KIR2DS2* were more frequent in the non-recurrence group than in the recurrence group ($P = 0.03$ and $P = 0.02$, respectively) (Table 3).

Table 3. Distribution of KIR gene frequencies among recurrence and non-recurrence group from an outpatient unit of the Infectious Ophthalmology Laboratory of the National Institute of Infectology Evandro Chagas, Fiocruz, RJ.

KIR Receptor	Recurrence group (N= 58) N/%	Non-recurrence group (N= 38) N/%	P-Value
<i>Inhibitory</i>			
<i>KIR2DL1</i>	57/98.3	37/97.3	1
<i>KIR2DL2</i>	26/45	26/68.4	0.03
<i>KIR 2DL3</i>	53/91.4	35/92.1	1
<i>KIR 2DL5</i>	33/57	20/53	0,8
<i>KIR 3DL1</i>	58/100	37/97.3	0,3
<i>Activating</i>			
<i>KIR2DS1</i>	26/45	14/37	0,5
<i>KIR2DS2</i>	24/41.4	25/66	0.02
<i>KIR2DS3</i>	18/31	8/21	0.3
<i>KIR2DS4</i>	57/ 98.3	36/95	0.5
<i>KIR2DS5</i>	26/45	15/39	0.6
<i>KIR3DS1</i>	21/36.2	13/34.2	1
<i>Framework</i>			
<i>KIR2DL4</i>	58/100	38/100	-
<i>KIR3DL2</i>	58/100	38/100	-
<i>KIR3DL3</i>	58/100	38/100	-
<i>^aKIR3DP1</i>	58/100	38/100	-
<i>Pseudogene</i>			
<i>KIR2DP1</i>	56/96.5	37/97.4	1

N, number of individuals; %, KIR genes frequencies; ^a, framework, and pseudogene.

When we evaluated a possible association between KIR genes and the acceleration of recurrence of ocular toxoplasmosis after an active episode, we used the multiple analysis to estimate the relative-risks of recurrence. We observed that recurrence was 4.6 times in individuals with KIR2DS1 compared with individuals without this gene (Table 4). Besides, it is interesting to mention that recurrence was 1.2 faster in individuals between 20 and 40 years old.

Table 4. Poisson Generalized Linear Mixed Effect Models (P-GLMM) for adjusted incidence ratios between KIR gene carriers/non carriers and the time until the recurrence of ocular toxoplasmosis.

Features	Levels	Outcome	pY	Incidence/100 pY (CI95%)	aRR (CI95%); p-value
Overall		94	359,2087611	26.17 (21.15-32.02)	
Gender	M	58	196,36	29.54 (22.43-38.18)	Reference
	F	36	162,85	22.11 (15.48-30.6)	2.145 (1.6526-3.1879); 0.2052
Age	(20,40]	69	252,87	27.29 (21.23-34.53)	Reference
	(0,20]	2	34,67	5.77 (0.7-20.84)	1.2534 (1.0565-2.5306); 0.0391
	(40,67]	23	71,68	32.09 (20.34-48.15)	3.0026 (1.9814-5.8579); 0.6956
KIR2DL1	1	93	351,98	26.42 (21.33-32.37)	Reference
	0	1	7,23	13.84 (0.35-77.11)	2.8038 (1.1485-2161.2466); 0.9762
KIR2DL2	1	39	195,39	19.96 (14.19-27.29)	Reference
	0	55	163,82	33.57 (25.29-43.7)	2.4239 (1.3767-11.6106); 0.8148
KIR2DL3	1	89	334,18	26.63 (21.39-32.77)	Reference
	0	5	25,03	19.97 (6.49-46.61)	2.6406 (1.4562-12.2912); 0.9516
KIR2DL5	1	56	202,75	27.62 (20.86-35.87)	Reference
	0	38	156,46	24.29 (17.19-33.34)	1.9611 (1.5333-2.8899); 0.0884
KIR2DP1	1	92	347,51	26.47 (21.34-32.47)	Reference
	0	2	11,7	17.09 (2.07-61.74)	2.5209 (1.245-49.4396); 0.915
KIR2DS1	0	49	209,58	23.38 (17.3-30.91)	Reference

	1	45	149,63	30.07 (21.94-40.24)	4.6253 (2.7393-10.2543); 0.0459
KIR2DS2	1	35	183,39	19.08 (13.29-26.54)	Reference
	0	59	175,81	33.56 (25.55-43.29)	5.8398 (1.8667-146.8155); 0.2841
KIR2DS3	0	80	295,18	27.1 (21.49-33.73)	Reference
	1	14	64,03	21.87 (11.95-36.69)	3.209 (1.8048-9.9995); 0.6583
KIR2DS4	1	93	347,99	26.72 (21.57-32.74)	Reference
	0	1	11,21	8.92 (0.23-49.68)	1.5681 (1.0642-25.8153); 0.4286
KIR2DS5	0	48	203,24	23.62 (17.41-31.31)	Reference
	1	46	155,97	29.49 (21.59-39.34)	3.854 (2.4221-7.8259); 0.1643
KIR3DS1	0	56	233,58	23.97 (18.11-31.13)	Reference
	1	38	125,63	30.25 (21.4-41.52)	3.9636 (2.4694-8.1504); 0.1363

279 *Analysis with the weighted average of Inhibitory and Activating KIR between recurrence and* 280 *non-recurrence group*

281 In order to evaluate if the weighted mean of inhibitory and activating genes present in
282 the genotypes found in our population would be associated with recurrence of ocular
283 toxoplasmosis, we used the formula $WM = [(ATIV * 5) - (INIB * 3)]$, where WM is the
284 weighted mean of inhibitory and activating genes, ATIV is the number of activating KIR genes,
285 and INIB is the number of inhibitory KIR genes of an individual. With this calculation, we
286 verified that the negative weighted mean belonged to individuals with a lower proportion of
287 activating genes (between 1 and 4 genes, N = 75). In comparison, the positive weighted mean
288 was present in individuals with a higher proportion of activating genes (between 4 to 6 genes,
289 N = 21).

290 We observed in our population that the majority of individuals had a lower number of
291 activating KIR genes (1 to 4 activator genes) (78%; $P < 0.01$). Among these individuals, the
292 majority belonged to the group of individuals with recurrence of ocular toxoplasmosis (62.7%,
293 $P = 0.003$) (Table 5).

Table 5. The weighted mean of inhibitory and activating KIR between recurrence and non-recurrence groups from an outpatient unit of the Infectious Ophthalmology Laboratory of the National Institute of Infectology Evandro Chagas (INI), Fiocruz, RJ.

Weighted mean	Individuals		P-Value
	Recurrence group	Non-recurrence group	
	(N/%)	(N/%)	
Negative (1 to 4 activating KIR) (N= 75)	47/62.7	28/37.3	0.003
Positive (4 to 6 inhibitory KIR) (N=21)	11/52.4	10/47.6	1

N, number of individuals; %, KIR genes frequencies;

310 Discussion

311 Because of KIR's role in the immune response and its extensive genomic diversity, it is
312 known that variation in KIR genes affects the resistance and/or susceptibility to the
313 pathogenesis of various diseases. In this context, the current study characterized the gene
314 frequency profile of the 16 genes encoding KIR receptors and evaluated their influence in
315 patients with recurrence and non- recurrence ocular toxoplasmosis after active episodes.

316 In our study, 91% of the 101 participants were followed for at least two years. The
317 number of recurrences varied from 1 to 5 episodes. About the follow-up time, more than half
318 (55%) of the individuals in the recurrence group were followed for more than four years. In the
319 non-recurrence group, the majority (68.5%) of the individuals were followed for more than
320 three years.

321 Concerning the profile of the gene frequencies of KIR receptors in the study population,
322 we observed that the genes KIR3DL1, KIR2DL1, KIR2DS4, and KIR2DP1 were the most
323 prevalent (above 97%). These high frequencies are similar to those found in other Brazilian
324 populations, including Rio de Janeiro [19, 25, 30-33].

325 When we evaluated whether there were significant differences in the frequencies of the
326 KIR genes between the recurrence and non-recurrence group, we observed that the inhibitory
327 gene KIR2DL2 and the activator gene KIR2DS2 were more frequent in the non-recurrence
328 group than in recurrence group ($P = 0.03$ and $P = 0.02$, respectively). According to the literature,
329 the KIR2DL2 and KIR2DS2 genes are in strong linkage disequilibrium in most populations
330 worldwide [25, 34, 35]. Due to this strong linkage disequilibrium between the two loci, it is
331 difficult to determine which one mediates the effect [36].

The inhibitor gene KIR2DL2 and its corresponding activator KIR2DS2 have already been associated with other diseases. They have been associated with protection against HIV-1 infection [37], and KIR2DL2+HLA-C1 was associated with a decreased risk of chronic HBV [13]. In contrast, a previous study that determined and compared the frequencies of the KIR genes of children with severe or uncomplicated malaria with healthy controls in the same area found that the frequencies of both genes were significantly higher in malaria cases (severe or uncomplicated) than in controls. These results suggest that KIR2DL2 + C1 and/or KIR2DS2 + C1 carriers were more at risk of being infected with malaria parasites than those without any of these genotypes [36]. As in our population, both genes were more frequent in the non-recurrence group. We can suggest that the inhibitory gene KIR2DL2, together with the activator KIR2DS2, may be acting as protective markers, slowing the recurrence of ocular toxoplasmosis.

Regarding the time until the recurrence of ocular toxoplasmosis, we found that the KIR2DS1 activating gene accelerated recurrences of ocular toxoplasmosis. Previous studies have shown the involvement of the KIR2DS1 activating gene in inflammatory bowel disease, like Crohn's disease and ulcerative colitis [38, 39]. On the other hand, Ayo et al. [40] demonstrated an association between others activating KIR and their HLA ligands (KIR3DS1-Bw4-80Ile and KIR2DS1+/C2++ KIR3DS1+/Bw4-80Ile+) and increased susceptibility for ocular toxoplasmosis and its clinical manifestations.

NK cells can express from one to all possible KIR genes present in their genotype on their surfaces. Considering that the KIR repertoire depends directly on the inherited KIR genes, the genotype and allelic diversities of KIR genes can affect the function and the levels of specificity and sensitivity of NK cells [41]. In this context, we evaluated whether the variation

in the proportion between inhibitory and activating KIR genes, present in the genotypes of our population, could be associated with OTR.

In the analysis of the frequencies of the activating genes in the studied population, we observed that the majority of individuals (78%) have a lower proportion of activating KIR genes (between 1 to 4 activating genes) ($P < 0.01$). As for the distribution of individuals who presented a lower proportion of activating genes, we observed that the majority belonged to individuals with recurrence (62.7%; $P = 0.003$). This finding is in agreement with the foundation for the balance of the immune response. The balance between the inhibition and activation signals regulate NK cells and is responsible for protecting or predisposing an individual to diseases [42-44]. It allows us to suggest that the lower proportion of activating KIR genes is associated with accelerating OTR episodes.

In the present study, we characterize KIR genes' profile in patients with ocular toxoplasmosis after an active episode. We observed two genes (KIR2DL2 and KIR2DS2), acting together as possible protection markers. Additionally, we founded one activating gene (KIR2DS1) acting as a possible susceptibility marker and the lower proportion of activating genes observed in individuals with recurrence corroborating with the hypothesis that these individuals are more susceptible to OTR. As far as we know, no literature relates to the polymorphism of KIR and the follow up of recurrence of ocular toxoplasmosis for a prolonged period (up to 5 years). Thus, the characterization of KIR genes makes this study a pioneer in searching for an association between the KIR genes polymorphisms and the time until recurrence of ocular toxoplasmosis.

378 **Author Contributions Conceptualization:**

379 **Conceived and designed the experiments:** DSPS, MRRA, DMB, JOF, LCMS

380 **Performed the experiments:** DSPS, TEJ, ALQC, JM, LCMSP

381 **Analyzed the data:** DSPS, TEJ, MRA, JOF, DMB, LCMSP, MRRA

382 **Contributed reagents/materials/analysis:** MRRA, ALQC, LCMS

383 **Wrote the paper:** DSPS, TEJ, DMB, MRRA

384

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ID Genotype	Inhibitory KIR								Activating KIR						Pseudogenes		Haplotypes	Total	
	2DL					3DL			2DS					3DS	2DP	3DP		N	%
	1	2	3	4	5	1	2	3	1	2	3	4	5	1	1	1			
1																	AA	30	31.3
2																	Bx	11	11.5
3																	Bx	8	8.3
4																	Bx	12	12.5
5																	Bx	5	5.2
6																	Bx	4	4.2
7																	Bx	4	4.2
9																	Bx	2	2.2
18																	Bx	1	1
20																	Bx	2	2.2
21																	Bx	3	3.2
24																	Bx	1	1
28																	Bx	1	1
32																	Bx	1	1
72																	Bx	1	1
73																	Bx	1	1
79																	Bx	1	1
86																	Bx	1	1
90																	Bx	2	2.2
92																	Bx	1	1
106																	Bx	1	1
112																	Bx	1	1
184																	Bx	1	1
237																	Bx	1	1
N	94	52	88	96	53	95	96	96	40	49	18	93	41	34	93	96		96	100
%	97.9	54.2	91.7	100	55.2	98.9	100	100	41.7	51	18.7	96.9	42.7	35.4	96.9	100			

Figure