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ORIGINAL INVESTIGATION

Th1 and Th2 immune response to P30 and ROP18 peptides in human toxoplasmosis

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Abstract We determined the specific lymphocyte proliferative response and cytokine profile production regarding *Toxoplasma* P30 (2017 from virulent and non-virulent strain) and ROP18 protein-derived peptides (from clonal lineages I, II and III) in 19 patients having ocular toxoplasmosis, five suffering chronic asymptomatic infection, nine with congenital toxoplasmosis and eight *Toxoplasma* negative people. A Beckman Coulter FC500 flow cytometer was used for determining antigen-specific T cells (CD3+ CD4+ or CD3+ CD8+ cells) in peripheral blood culture. IFN γ and IL10 levels were determined in culture supernatants. Specific CD4+ and CD8+ T cell response to total antigen and P30- and ROP18-derived peptides was observed in infected people. Ocular toxoplasmosis patients had a preferential Th2 response after antigenic stimulation. Non-virulent peptide 2017 was able to shift response toward Th1 in congenitally infected children and virulent peptide 2017 induced a Th2 response in chronically infected, asymptomatic people. An immune response in human toxoplasmosis after ex vivo antigenic stimulation was Th1- or Th2-skewed, depending on a patient's clinical condition. Colombian ocular toxoplasmosis patients' immune response was Th2-skewed, regardless of the nature of antigen stimulus.

Keywords *Toxoplasma* · P30 protein · ROP18 · Interferon gamma · IL10 · Th1 · Th2 · Peptides · Vaccine

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Introduction

Toxoplasma gondii is a widely occurring intracellular parasite in humans [1]. The FAO and WHO have recently established toxoplasmosis as a foodborne parasite infection arousing global concern [2] and congenital infection leads to considerable public health problems in many countries [3]. Preexisting infection of a pregnant woman by *T. gondii* prevents parasite transmission to her fetus and such protection appears to be essentially mediated by cellular immunity to *Toxoplasma* antigens [4]. These considerations are compelling arguments for developing a vaccine against toxoplasmosis. The literature abounds with reports of subunit-based recombinant proteins, peptides or DNA vaccine candidates in animal models [5–10]; however, only two recent works have reported a cellular immune response by human lymphocytes determining protective T cell induction [10, 11]. Therefore, most knowledge about how the immune response occurs against *Toxoplasma* peptides has been derived from the mouse experimental model, but little has been gleaned regarding human lymphocytes. Our group has been working in an endemic region having a

Table 1 Demographic and clinical data regarding patients and asymptomatic *Toxoplasma*-infected and uninfected individuals

Group	*N*	Mean age in years [range] -except for the congenital group (months)	Gender (female) *N* (%)	Mean UI/ml IgG anti-*Toxoplasma* [range]	Individuals having retinal inflammatory activity at sampling (%)
Control (*Toxoplasma* seronegative people)	8	32.5 [24–46]	4 (50 %)	0 [0–0]	–
Ocular toxoplasmosis	19	19 [6–43]	9 (47.4 %)	157 [11–335]	11 (57.9 %)
Congenital toxoplasmosis	9	3 [2–10] (months)	3 (33.3 %)	269 [20–650]	–
Chronic asymptomatic infection	5	33 [28–52]	4 (80 %)	174 [50–276]	–

high prevalence of congenital [3] and ocular toxoplasmosis [13]; we also demonstrated a protective response in mice [6] and human antibody response to P30-derived peptides [14]. Interest has also been shown in the *Toxoplasma* protein ROP18 which has been linked to virulence [15]. Consequently, the aim of this work was to analyze the cellular immune response to peptides derived from these proteins and ascertains whether they were able to induce CD4+ and CD8+ responses and Th1/Th2 cytokine production.

Materials and methods

Bioethical aspects

Informed written consent, according to Colombian Ministry of Health regulation 008430/1993, was obtained from all the people who agreed to participate in the study. The Universidad del Quindio's Institutional Review Board (minute number 22, October 11, 2010) and the Universidad Tecnológica de Pereira's Ethics Committee approved the study.

Human clinical samples and definition of clinical condition

Forty-one samples from human cases having different clinical conditions regarding *Toxoplasma* infection were included in the study (infected, but asymptomatic, individuals and uninfected controls). A diagnosis of congenital toxoplasmosis was confirmed for babies ($n = 9$), as described previously [3]. Patients having ocular toxoplasmosis ($n = 19$) were recruited following ophthalmological consultation at the Universidad del Quindio. A clinical diagnosis of ocular toxoplasmosis was based on previously described criteria [14]. Active ocular toxoplasmosis was defined by the presence of an active creamy-white focal retinal lesion eventually resulting in hyper-pigmented retinochoroidal scars in either eye. Asymptomatic *Toxoplasma*-infected people ($n = 5$) were requested to participate when their serological status was "chronic infection" (IgG anti-*Toxoplasma* positive and IgM anti-*Toxoplasma* negative) and a fundoscopic eye examination proved negative for ocular lesions. Seronegative, controls ($n = 8$) were recruited at the Universidad del Quindío. Table 1 summarizes the demographic and clinical characteristics of the individuals involved in the study.

Preparing *Toxoplasma* soluble total antigen (STAg)

Soluble total antigen was prepared as described previously [17]. In brief, RH strain tachyzoites were maintained by in vitro passage in human foreskin fibroblasts at 37 °C. Antigen preparation involved tachyzoites being harvested from fibroblast cultures, passed through a 27-gauge needle, suspended in saline solution and submitted to freeze–thawing four times and then disrupted by sonication (4 × 20 W for 20 s) using a microprobe. After centrifugation (5,000 rpm × 20 min), the supernatant was used as antigen in the in vitro assays.

Peptides

Peptide sequences were chemically synthesized using the solid-phase peptide synthesis (SPPS) technique, involving MBHA resin (0.49 meq/g), terbutyloxycarbonile-Boc and low–high cleavage. The peptides were then extracted with 10 % acetic acid and water and purified by HPLC. The peptides used in this study were Rh strain type I virulent 2017 peptide (2017vir) (FAGAAGSAKS**A**AGTASHVSI), ME49 strain type II non-virulent 2017 peptide (2017avir) (FAGAAGSAKS**S**AGTASHVSI), ROP18 peptides derived from the protein's polymorphic region from the three clonal lineages (ROP18 I: PPERPFQ**A**TGITYTF**P**TDA; ROP18 II: PPERPFQ**T**T**D**ITYTFTTDA and ROP18 III: PPE**Q**PF**HSY**G**V**TYTF**A**TDA). The amino acid changes between strains are underlined and shown in bold. Additionally, in order to control for specificity, we use an irrelevant peptide of identical length. This control consisted of a randomized sequence of the ROP18 peptide (scrambled peptide: FRTTDPTEAYPGIPQTFPA). The peptides were synthesized at the "Fundacion Instituto de Inmunologia" (FIDIC, Bogota, Colombia) at 90 % purity in lyophilized form. A commercial Limulus test (E Toxate, Sigma, USA) was used, following the manufacturer's recommendations, to discard endotoxin presence in peptide preparation.

Lymphocyte antigen response analysis

Fifty microliters of whole blood were incubated during 5 days in sterile polypropylene tubes with Concanavalin A (10 μg/mL), *Toxoplasma* STAg (2.5 μg/mL), 2017vir (10 μg/mL) and 2017avir (10 μg/mL) P30- or ROP18-derived peptides from clonal lineages I, II or III. Phosphate-buffered saline (PBS) was used as control, according to previous reports [16, 17]. Cells were collected from each tube and a Beckman Coulter FC500 was used for flow cytometry. The percentage of antigen-specific T cells (CD3+/CD4+ or CD3+/CD8+ cells) was estimated using trichrome commercial antibodies (Beckmann Coulter, USA). The percentage of responders was calculated by determining whether the percentage of CD4+ or CD8+ cells from each individual was above CD4+ or CD8+ cut-off level (mean percentage of CD4+ or CD8+ in un-stimulated wells containing only culture medium +2 SD).

Cytokine measurement

IFN γ, IL10, IL13 and TNF α levels were determined by commercial ELISA test (Invitrogen, USA or BD Biosciences, USA) in blood culture supernatant for each stimulus. The results were expressed as pg/mL of target cytokine. IFN γ (Th1): IL10 (Th2) cytokine ratios were calculated and compared between groups to determine Th1 compared to Th2 cytokine bias in response to peptide stimulus.

Statistical analysis

The results were expressed as means [min–max] for continuous variables and percentages (*N*) for categorical variables. Differences in percentages were analyzed using the chi-squared test. Differences in means were compared by a two-tailed nonparametric test. One-way ANOVA was used for evaluating differences between groups from quantitative clinical or laboratory variables. Values below $p < 0.05$ were considered statistically significant. SPSS software (version 14.0, Lead Technologies Inc, USA) was used for analyzing the statistical tests.

Results

P30- and ROP18-derived peptides induced specific CD4+ and CD8+ T cell responses, but differently for each clinical group

Figure 1 gives the percentage of CD4+ cells after total antigen or peptide stimulation. STAg induced a significant increase in CD4+ T cell response in all groups, except for the seronegative controls. The virulent 2017 peptide induced a specific CD4+ T cell response in congenital and ocular toxoplasmosis, but not in chronically infected people. The non-virulent 2017 peptide only induced a specific CD4+ response in the congenitally infected group. When comparing individual response to peptides in chronic asymptomatic individuals, 60 % were responders to the virulent 2017 peptide but not to the non-virulent 2017 peptide. ROP18 I, II and III peptides induced significant increases in CD4+ T cells in ocular toxoplasmosis. Only ROP18 I induced a significant increase of CD4+ T cells in the congenitally infected group. Mean increase regarding chronic asymptomatic toxoplasmosis was statistically significant concerning ROP18 I and III, but not ROP18 II; however, 60 % of patients were responders to this peptide. Statistically significant CD8+ T cell response was also induced by STAg in all groups, except for seronegative people. Similarly, as with CD4+ T cells, the virulent 2017 peptide induced a specific CD8+ T cell response in congenital and ocular toxoplasmosis but not in chronically infected people. The non-virulent 2017 peptide only induced a specific CD8+ T cell response in the congenitally infected group. When comparing individual response to peptides in chronically infected asymptomatic people, 60 % were responders to the 2017 virulent peptide, but none responded to the non-virulent 2017 peptide. ROP18 I, II and III peptides induced a significant increase in the percentage of CD8+ in ocular toxoplasmosis, but not in the congenital toxoplasmosis group. The mean increase to ROP18 I and III was statistically significant regarding chronic asymptomatic toxoplasmosis, but not to ROP18 II stimulus; however, 60 % of patients were responders to this peptide. Nobody in the chronically infected asymptomatic group had increased CD8+ T cells in response to the virulent and non-virulent 2017 peptides. The endotoxin (Limulus) test was negative for all peptide preparations. Also, the response to an irrelevant peptide of identical length were not statistically significant for CD4+ (median of % of CD4+ cells was in asymptomatic group of 4.8 in medium alone wells versus 1.0 in wells with scrambled peptide, $p = 0.1$; in ocular toxoplasmosis group was of 5.3 in medium alone wells vs. 5.2 in wells with scrambled peptide, $p = 0.6$; in congenital toxoplasmosis group of 2.6 in wells with medium alone vs. 3.4 in wells with scrambled peptide, $p = 0.2$) or CD8+ (median of % of CD8+ cells was in asymptomatic group of 2.3 in medium alone wells vs. 0.5 in wells with scrambled peptide, $p = 0.9$; in ocular toxoplasmosis group was of 0.8 in medium alone wells vs. 0.4 in wells with scrambled peptide, $p = 0.6$; in congenital toxoplasmosis group of 0.1 in wells with medium alone vs. 0.5 in wells with scrambled peptide, $p = 0.8$) (Fig. 2).

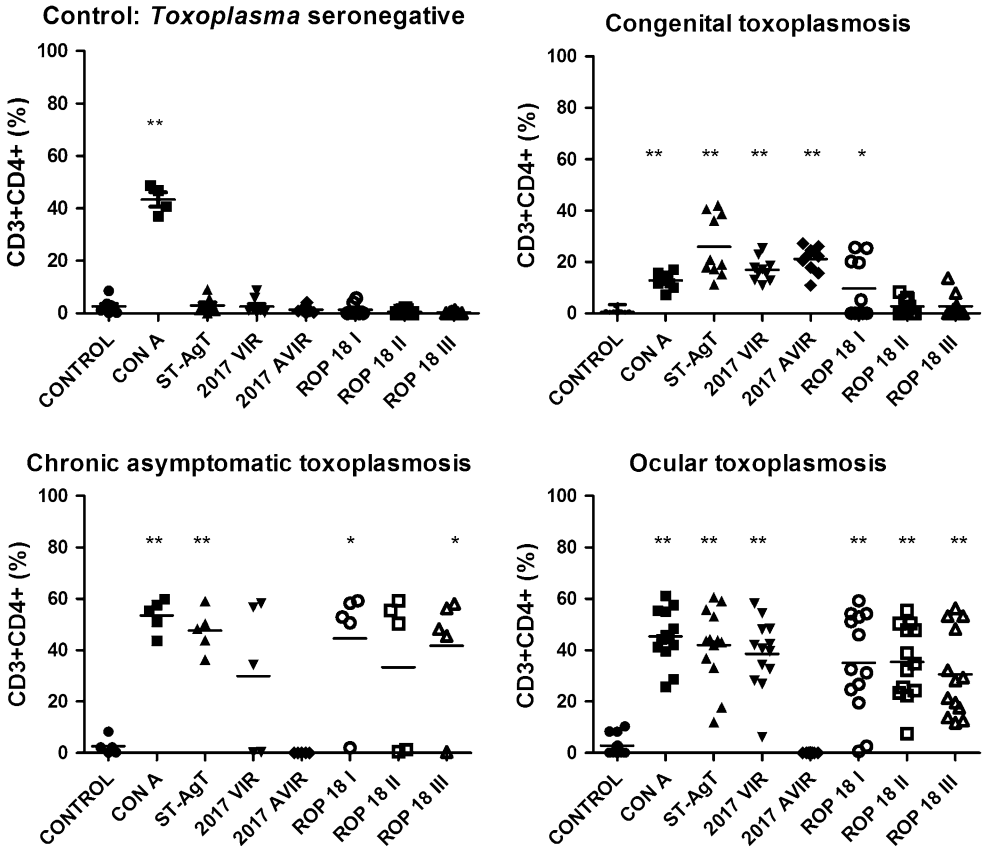

Fig. 1 Antigen-induced stimulation of CD4 + CD3 + T cells from uninfected individuals ($n = 8$), babies with congenital toxoplasmosis ($n = 9$), patients with ocular toxoplasmosis ($n = 19$) or asymptomatic chronically infected individuals ($n = 5$), after stimulation with Concanavalin A (ConA), *Toxoplasma* soluble total antigen (STAg) and the peptides derived from the carboxy terminal region of the P30 surface protein from the *Toxoplasma* virulent (2017vir) and non-virulent strain (2017avir) and from the ROP18 protein from clonal lineages type I (ROP18 I), type II (ROP18 II) and type III (ROP18 III). *p values between 0.05 and 0.0011; **p values $\leq$ 0.001 versus control wells containing medium for the same group

IFN γ response induced by ROP18 and P30 peptides was different in each clinical condition

IFN γ production in culture supernatant is shown in Fig. 3. IFN γ levels were presented on a scale reaching 1,500 pg, except for the congenital toxoplasmosis group, represented on a lower scale (up to 150 pg/ml). This was because a lower amount of cytokine was obtained in the newborn compared to other groups (consisting of adults). An important observation was that statistically significant IFN γ levels ($p = 0.02$ nonparametric two-tailed t test) in un-stimulated control wells were higher in ocular patients (mean 14.1: range 3.3–61) compared to other groups (mean in uninfected or infected people 0.1: range 0.1–35.9). IFN γ levels were significantly higher in active ocular toxoplasmosis (mean 19, range 12.8–61) than in patients having inactive lesion (mean 12.5, range 3.3–17.1; $p = 0.005$). Interestingly, ConA stimulation induced an increase in IFN γ in all groups, except for the newborn having congenital toxoplasmosis. STAg enhanced IFN γ levels in congenital and ocular toxoplasmosis groups but did not do so in chronically infected asymptomatic people. Only virulent and non-virulent 2017 and ROP18 II peptides induced a significant IFN γ increase in the newborn suffering congenital toxoplasmosis. A significant increase was only obtained with ROP18 I and II peptides in ocular toxoplasmosis. No significant increase was observed in chronically infected asymptomatic people regarding STAg or peptides.

IL10 production was significantly different in each clinical condition (including un-stimulated blood cells)

IL10 response to antigenic stimulation is presented in Fig. 4. Importantly, IL10 levels (pg/ml) were significantly higher in un-stimulated control wells for congenitally infected newborn (mean 20: range 5–86) compared to other groups (mean 0.1: range 0.1–35.9; nonparametric two-tailed t test $p = 0.021$) and these levels became significantly reduced after stimulation with ConA ($p = 0.01$) and ROP18 I ($p = 0.03$). IL10 became significantly increased

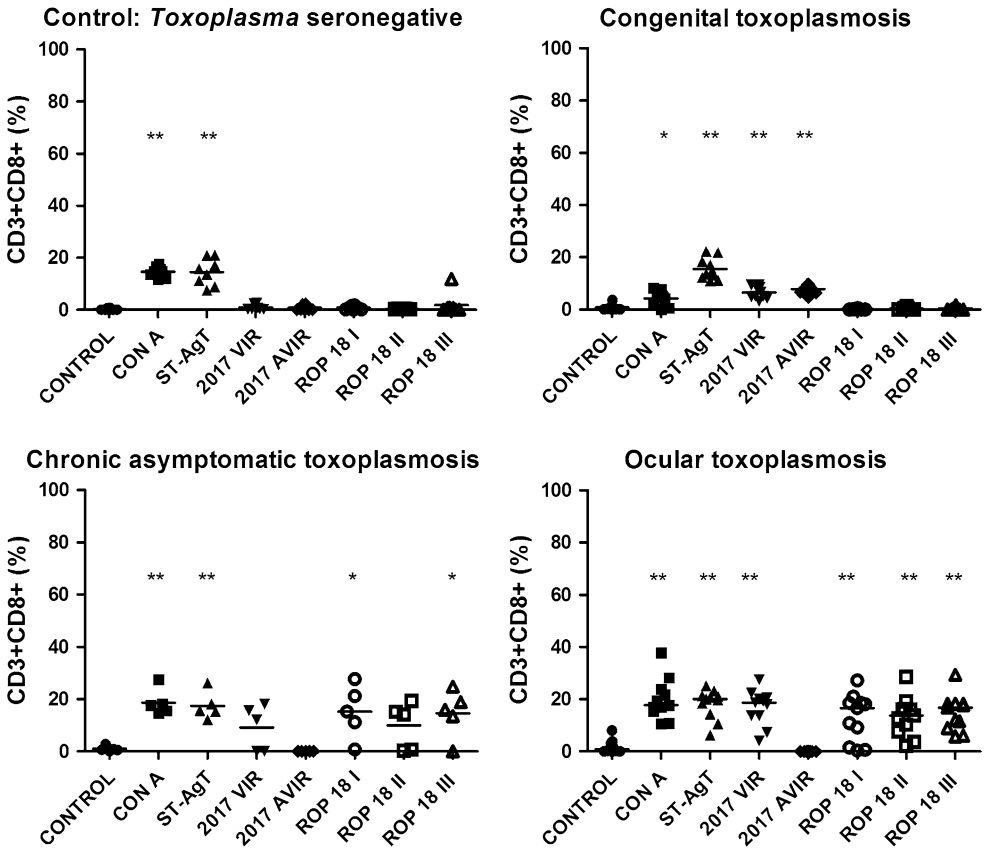

Fig. 2 Antigen-induced stimulation of CD8+ CD3+ T cells from uninfected individuals ($n = 8$), babies with congenital toxoplasmosis ($n = 9$), patients with ocular toxoplasmosis ($n = 19$) or asymptomatic chronically infected individuals ($n = 5$), after stimulation with Concanavalin A (ConA), Toxoplasma soluble total antigen (STAg), peptides derived from the carboxy terminal region of the P30 surface protein from Toxoplasma virulent (2017vir) and non-virulent strain (2017avir) and from the ROP18 protein from clonal lineages type I (ROP18 I), type II (ROP18 II) and type III (ROP18 III). *p values between 0.05 and 0.0011; **p values $\leq$0.001 versus control wells containing medium from the same group

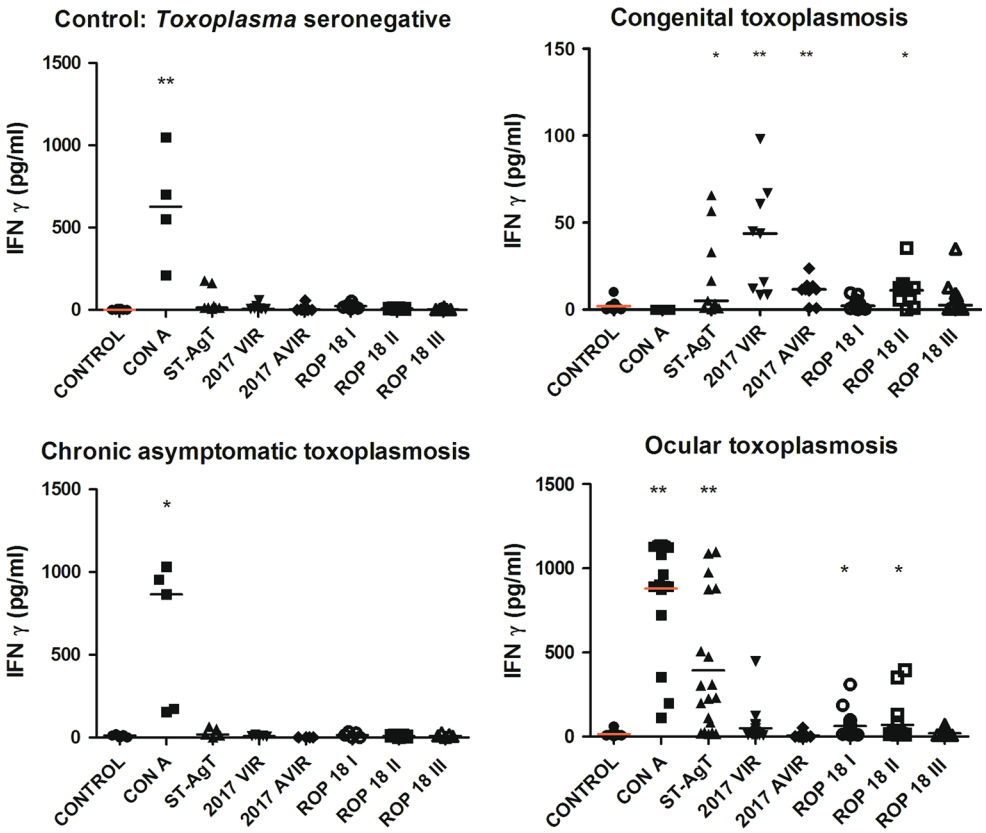

Fig. 3 Interferon gamma (IFN γ) levels (pg/mL) in culture supernatants from whole blood samples from uninfected individuals ($n = 8$), babies with congenital toxoplasmosis ($n = 9$), ocular toxoplasmosis patients ($n = 19$) or asymptomatic chronically infected individuals ($n = 5$), after stimulation with Concanavalin A (ConA), *Toxoplasma* soluble total antigen (STAg), peptides derived from the carboxy terminal region of the P30 surface protein from the *Toxoplasma* virulent (2017vir) and non-virulent strain (2017avir) and from the ROP18 protein from clonal lineages type I (ROP18 I), type II (ROP18 II) and type III (ROP18 III). *p values between 0.05 and 0.0011; **p values $\leq$0.001 versus control wells containing medium from the same group

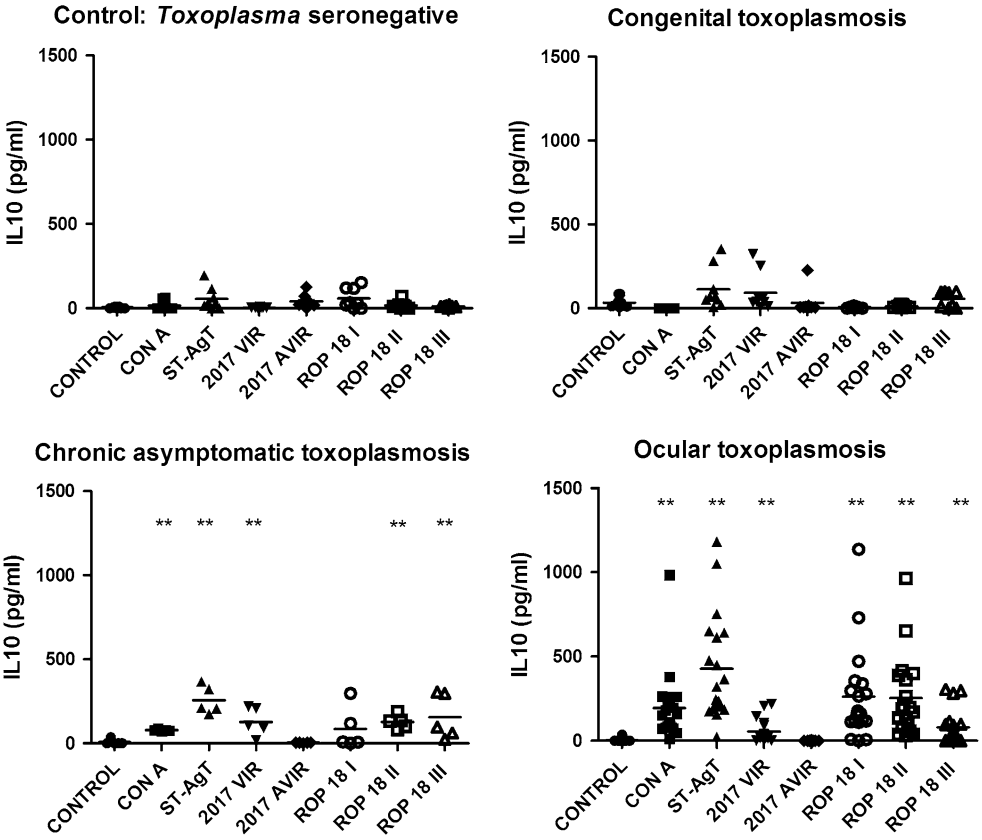

Fig. 4 Interleukin 10 (IL10) levels (pg/ml) in culture supernatant from whole blood samples from uninfected individuals ($n = 8$), babies with congenital toxoplasmosis ($n = 9$), ocular toxoplasmosis patients ($n = 19$) or asymptomatic chronically infected individuals ($n = 5$), after stimulation with Concanavalin A (ConA), *Toxoplasma* soluble total antigen (STAg), peptides derived from the carboxy terminal region of the P30 surface protein of *Toxoplasma* virulent (2017vir) and non-virulent strain (2017avir) and from the ROP18 protein from clonal lineages type I (ROP18 I), type II (ROP18 II) and type III (ROP18 III). *p values between 0.05 and 0.0011; **p values $\leq$0.001 versus control wells containing medium from the same group

in ocular toxoplasmosis when blood cells were stimulated with STAg, virulent 2017, ROP18 I, II and III peptides. All stimuli in chronically infected asymptomatic people significantly increased IL10 level, except for non-virulent 2017 and ROP18 I.

Th1/Th2 ratios were significantly different in each clinical group and modified by antigenic stimulation

Table 2 shows the Th1/Th2 ratios for each clinical group. Th1/Th2 ratios were significantly modified by most stimuli; however, a shift toward Th1 or Th2 was not dependent on antigen stimulation but rather on clinical condition. Mean Th1/Th2 ratios after ex vivo antigen stimulation became skewed toward Th1 in congenital toxoplasmosis and toward Th2 in ocular toxoplasmosis. However, as variation was heterogeneous inside each group for some stimuli and depended on clinical condition, the statistical tests indicated when stimuli were strong enough to change responses in all patients. This would have signaled when antigen stimulus was predominant over an intrinsic individual response. Importantly, a significant skewing toward Th1 was only found in congenital toxoplasmosis for all patients with the non-virulent 2017 peptide; inversely, skewing toward Th2 with the virulent 2017 peptide was found in chronically infected asymptomatic people. All responses in ocular toxoplasmosis were skewed toward Th2 regardless of stimuli, indicating that irrespective of antigenic stimulus, final intrinsic response was always skewed toward Th2.

Discussion

Our work involved the first simultaneous characterization of ex vivo immune response to toxoplasmosis in different human clinical conditions, concerning total *Toxoplasma* soluble antigen preparation or peptides as candidates to be included in a multimer vaccine. Our initial aim was to examine whether peptides were antigenic enough and if they were recognized by naturally infected humans. It was also important to determine whether the peptides induced a significant IFN γ or IL10 response. However, cytokine production analysis revealed that ocular toxoplasmosis patients' peripheral blood cells spontaneously secreted IFN γ. Such secretion occurred in patients lacking active ocular lesions, yet IFN γ levels were higher when they had active ocular lesions. This confirmed that such immune response was systemic and not only local during ocular reactivation of lesions [17]. The most interesting finding was that all patients having toxoplasmic ocular lesions had a Th2-skewed response after antigenic stimulation. Our group has recently found that toxoplasmic ocular lesion patients had

Table 2 Th1:Th2 cytokine ratios in culture supernatants from peripheral blood samples obtained from uninfected people ($n = 8$), babies having congenital toxoplasmosis ($n = 9$), patients with ocular toxoplasmosis ($n = 19$) or asymptomatic, chronically *Toxoplasma*-infected people ($n = 5$), after stimulation with STAg or peptides (2017vir, 2017avir, ROP I, ROP18 II, ROP18 III)

Group/stimulus	Th2 Index*	*p* value vs. control Th2**	Th1 Index*	*p* vs. control Th1**
Congenital/control wells	202.00		0.16	
ST Ag	0.15	0.819	1.77	0.280
2017vir	0.00	0.201	2.80	0.123
2017avir	0.00	0.200	2.54	**0.048**
ROP18 II	0.13	0.727	1.73	0.051
Chronically infected, asymptomatic /control wells	0.37		52.31	
ST Ag	25.83	0.249	0.00	0.177
2017vir	9.36	**0.024**	0.00	0.176
ROP18 I	409.66	0.227	0.28	0.976
ROP18 II	15.96	0.140	0.00	0.176
ROP18III	10.55	0.052	0.00	0.177
Ocular/control wells	0.08		119.14	
ST Ag	14.78	**0.019**	0.01	**0.000**
2017vir	3.14	**0.001**	0.03	**0.000**
ROP18 I Oc	20.73	**0.000**	0.00	**0.000**
ROP18 II Oc	18.57	**0.026**	0.00	**0.000**
ROP18III Oc	5.37	**0.001**	0.11	**0.000**

***** Th2 index for each group (congenital, ocular or chronic) was calculated as the mean of Th2 ratios (IL10 each stimulus/IL10 control wells for each patient), and Th1 Index for each group (congenital, ocular or chronic) was calculated as the mean of Th1 ratios (IFN γ each stimulus/IFN γ control well for each patient). Th1:Th2 ratios were calculated only for peptides which induced statistically significant cytokine secretion related to control wells containing just culture medium

** *p* values were obtained by comparing means in each group of Th1 or Th2 ratio for stimulated wells versus control wells for each patient, using a nonparametric paired two-tailed T test (statistically significant difference if $p < 0.05$ are indicated in bold)

significantly higher Th2 cytokine levels in aqueous humor [18]; this suggested that this group of patients' intrinsic immune response was predetermined to be Th2. The predominance of virulent type I or non-type II strains infection in Colombian patients having ocular toxoplasmosis could explain this immunological clinical observation [19–21]. Virulent type I strains (such as RH) inhibit IL12 production, a major determinant of Th1 response, as has been reported for human fibroblast cell lines [22].

Congenitally infected newborns' un-stimulated blood cells have a significant Th2-biased index, as described previously [23, 24]. Moreover, ConA stimulation was unable to induce significant IFN γ secretion in congenitally infected children, high IL10 levels, observed in un-stimulated wells, could have explained the non-induction of IFN γ by ConA [25]. One peptide induced a significant ex vivo change to Th1 in all patients (non-virulent 2017); therefore, contrarily to ocular toxoplasmosis patients, a Th1 cytokine response might thus be induced in congenitally infected children.

The different cytokine outcome after antigenic stimulation with virulent 2017 (inducing Th2 response in asymptomatic patients) and non-virulent 2017 peptide (inducing a Th1 response in congenitally infected newborn) confirmed a previous report in the mouse model [26]. The 2017 peptide covers amino acids 301–319 from the P30 major surface protein. There is a Ser 311 (non-virulent strain) → Ala 311 (virulent strain) polymorphism conferring a higher probability of a β-turn for the non-virulent strain and higher hydrophobicity [26]. The 2017 virulent peptide induced a Th2-skewed response in the mouse model, whereas the non-virulent 2017 peptide induced a Th1-skewed response [26].

Based on CD4+ and CD8+ T cell induction, as well as IFN γ and IL10 production, the peptides tested here were not optimum candidates to be included in a multimer vaccine, given that not one alone was able to induce a strong IFN γ response for all groups; however, the effect of mixed stimulation should be examined.

The results given above have thus highlighted how an intrinsic immune response can be induced according to clinical condition regarding human toxoplasmosis and have provided new data which should help in understanding this infection's immunopathogenesis and in designing vaccine candidates.

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