

Lack of evidence for superantigen activity of *Toxoplasma gondii* towards human T cells

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Abstract

Toxoplasma gondii is an obligatory intracellular parasite whose life cycle may include man as an intermediate host. More than 500 million people are infected with this parasite worldwide. It has been previously reported that *T. gondii* contains a superantigen activity. The purpose of the present study was to determine if the putative superantigen activity of *T. gondii* would manifest towards human T cells. Peripheral blood mononuclear cells (PBMC) from individuals with no previous contact with the parasite were evaluated for proliferation as well as specific V β expansion after exposure to *Toxoplasma* antigens. Likewise, PBMC from individuals with the congenital infection were evaluated for putative V β family deletions in their T cell repertoire. We also evaluated, over a period of one year, the PBMC proliferation pattern in response to *Toxoplasma* antigens in patients with recently acquired infection. Some degree of proliferation in response to *T. gondii* was observed in the PBMC from individuals never exposed to the parasite, accompanied by specific V β expansion, suggesting a superantigen effect. However, we found no specific deletion of V β (or V α) families in the blood of congenitally infected individuals. Furthermore, PBMC from recently infected individuals followed up over a period of one year did not present a reduction of the V β families that were originally expanded in response to the parasite antigens. Taken together, our data suggest that *T. gondii* does not have a strong superantigen activity on human T cells.

Key words

- *Toxoplasma*
- Tolerance
- Superantigen
- T cell receptor

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Introduction

Toxoplasmosis is caused by the intracellular protozoan, *Toxoplasma gondii*. Acquired disease may result in lymphadenopathy, low-grade fever, and sore throat. The disease often progresses to a symptomless

state in immunocompetent individuals (1,2). In the immunocompromised host, encephalitis caused by the parasite may be fatal (3-6). Infection during pregnancy occurs in two of 1000 cases in the United States with up to a 50% transplacental infection rate, with a higher rate in other parts of the world (2,7,8).

Acquired disease was thought to be rare. However, several studies indicate that ocular lesions may be caused by *T. gondii* infection after birth (9-11).

Superantigens are molecules from microorganisms that can activate large numbers of T cells. These molecules stimulate T cells due to their ability to interact with the T cell receptor (TCR) V β framework regions (12) and possibly with V α elements (13). As a consequence of such promiscuous interactions, 2 to 30% of all T cells from one individual may be stimulated by some superantigens. The *in vivo* effects of such massive T cell stimulation are often deleterious (i.e., toxic shock syndrome and food poisoning in humans). Unlike conventional antigens, which are bound to the antigen-binding groove on MHC molecules, superantigens interact with MHC molecules outside the peptide-binding groove and do not require processing. Kappler and colleagues (14,15) were the first to report superantigen activity. They described a strain-specific deletion of T cells expressing a specific V β in mice. The deletion was related to the presence of certain alleles of the minor histocompatibility loci. Minor histocompatibility locus products have been known for their ability to stimulate specific V β -bearing T cells (16-18). The cloning of these loci revealed that they were endogenous retroviruses of the mouse mammary tumor virus family and that various family members bind to different V β domains (12,19,20). Others have reported that staphylococcal enterotoxins (21) and products from *Yersinia pseudotuberculosis* (22), *Streptococcus* (23), and *Mycoplasma* also displayed superantigen-like activities (24). In addition, viruses such as rabies (25), Epstein Barr virus, and cytomegalovirus (26) express superantigens. Protozoans (*Plasmodium falciparum*) were also reported to contain components with superantigen properties (27).

Recently, *T. gondii* was also suggested to contain a component with superantigen ac-

tivity (28,29). These authors showed that naive CD8⁺ cells bearing the V β 5.1 were stimulated in the presence of the whole parasite. This finding is of particular importance since it has also been suggested that superantigens might be involved in triggering autoimmune diseases. There is an ongoing discussion in the ophthalmic literature regarding a possible role for autoimmunity in patients with recurrent ocular lesions following infection with *T. gondii*. Therefore, we set out to establish whether in humans the most common effects of a superantigen were detected following exposure to *T. gondii* or its antigens. We were able to demonstrate that T cells from some non-exposed individuals did proliferate in response to antigens of the parasite. Furthermore, there were expanded specific V β families. In addition, when the peripheral blood mononuclear cells (PBMC) from individuals with congenital disease were analyzed some deletions were observed in the T cell repertoire. These data suggest that indeed *T. gondii* has a superantigen that does activate and delete human T cells. On the other hand, when T cells from seronegative individuals were stimulated *in vitro* with *T. gondii* antigens, expansion was not followed by deletion of specific V β families. Furthermore, the deletions of TCR V β families observed in patients with congenital toxoplasmosis were shared among their kindred, even those that were not infected by the parasite. Since deletion of responding cells is a hallmark of superantigen activity, we suggest that, although there is some evidence for a superantigen activity in *T. gondii*, it is not likely that such activity affects human cells.

Material and Methods

Patients

Blood samples were collected from 36 individuals who had negative antibody (IgM and IgG) titers for *T. gondii* (less than 1:4),

48 seropositive individuals (IgG titers above 1:16), and 11 patients with congenital toxoplasmosis. Antibody titers were determined with a test kit from Sigma Chemical Co. (St. Louis, MO, USA). The patients studied were 19 and 56 years old and came from Erechim or São Paulo, Brazil. All gave informed consent to participate in the study. The clinical status (congenital ocular toxoplasmosis or absence of ocular lesions) of the individuals evaluated was determined as previously described (9,11,30). Briefly, patients were considered to have congenital toxoplasmosis when their mother had a positive IgM response to *Toxoplasma* antigens that developed during pregnancy and when ocular lesions were visualized by fundoscopy on the occasion of their first visit to the ophthalmologist. None of the individuals studied were under treatment with any immunosuppressive or immunomodulatory drug. Women using oral anticonceptives were not included in this study since previous unpublished results from our group indicate that they may alter the antigen-specific response patterns of T cells. Other drugs being used by the patients during the study were antibiotics (2 patients), aspirin (2 patients), acetaminophen (3 patients) and antihistamine (5 patients). Patients with acute toxoplasmosis were diagnosed by their clinical features (malaise, lymphadenopathy and sore throat) and based on positive IgM titers to *Toxoplasma* antigens. All procedures were approved by the Ethics Committee of the institutions where the patients were being followed up (UNIFESP, USP).

Proliferation assays

Proliferation assays were performed as described previously (31). Briefly, PBMC obtained by gradient centrifugation were diluted to 10^6 cells/ml and added to 96-flat-bottom well microtiter plates (Falcon). Cultures were stimulated with 2.5 $\mu\text{g/ml}$ of phytohemagglutinin (PHA) or 5 $\mu\text{g/ml}$ of soluble

toxoplasma tachyzoite antigen (STAg) or control antigens (tuberculin purified protein derivative (PPD) or tetanus toxoid), in a final volume of 200 μl of RPMI 1640 per well containing 5% AB+ human serum pooled from blood donors. PBMC were cultured for 96 h and then pulsed with 0.5 μCi [^3H]-thymidine per well and processed accordingly for standard gaseous or liquid scintillography. Results are presented as stimulation index units that represent the mean proliferation in cpm in response to a given stimulus divided by the mean background proliferation of unstimulated cells. Stimulation indices above two were considered positive. PBMC were obtained from blood drawn at least one month after the last active episode of disease and before two months had elapsed from that episode.

Soluble tachyzoite antigen preparation

STAg was prepared as described previously (32). Briefly, tachyzoites of the RH strain were maintained by *in vitro* passage in human foreskin fibroblasts at 37°C. For antigen preparation, tachyzoites were harvested from fibroblast cultures, passed through a 27-gauge needle, centrifuged at 70 g for 5 min and pelleted at 590 g for 10 min. Tachyzoites were then sonicated 4 times for 20 s each round and centrifuged at 10,000 g for 30 min. The supernatant preparation termed STAg was used as antigen in the *in vitro* assays.

Flow cytometry

Standard flow cytometry analysis was performed using antibodies against human V α and V β chain from Endogen (Cambridge, MA, USA) or from PharMingen (San Diego, CA, USA). One million cells were stained directly in a final volume of 100 μl PBS according to manufacturer instructions and read with a FACScan (Becton-Dickinson Immunocytometry Systems, San Jose, CA,

USA) using the Lysis II program or a FACScallibur instrument (Becton-Dickinson) using the CellQuest program.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of the V β TCR chain

TCR analysis by RT-PCR was performed as described previously (33). RNA from PBMC was obtained using the RNAzol B method, reverse transcribed and amplified using primers described elsewhere (33). PCR products were separated on 1.5% agarose gels and analyzed by Southern blot hybridization with fluorescein-labeled internal probes using the ECL-3' oligolabeling and detection system and Hyperfilm-ECL (Amersham Pharmacia Biotech, Little Chalfont, UK).

Reagents

Isolymph was purchased from Gallard-Schlesinger (Cale Place, NY, USA). PHA-p was obtained from Difco Laboratories (Detroit, MI, USA). Interleukin (IL)-2 was purchased from Boehringer-Mannheim Corp. (Mannheim, Germany), and human IL-4 and IFN- α were purchased from Genzyme Diag-

nostics (Cambridge, MA, USA). Human IL-1, IL-12 and TNF- α were obtained from R&D Systems (Minneapolis, MN, USA), and human IL-10 and IL-5 were from PharMingen.

Statistical analysis

Data were analyzed by ANOVA Fisher PLSD and by the Kruskal-Wallis test for nonparametric data. Results were considered statistically different when a 95% confidence level was achieved.

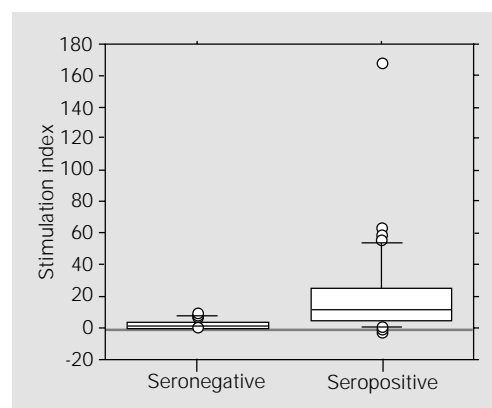
Results

In a recent population-based household survey performed in Erechim, Southern Brazil, 184 (17.7%) of 1042 individuals examined were considered to have ocular toxoplasmosis (9,11). Because of the high frequency of the disease, its occurrence in multiple siblings, and its low prevalence in children as compared to the adult population, many of the ocular toxoplasmosis cases in Erechim are thought to be sequelae of post-natal infection (10).

Here we studied the immune response against *Toxoplasma* antigens from patients that have been followed by some of us (Silveira C and Belfort Jr R, unpublished data). Patients were divided into three groups: a) normal controls with negative serology for *T. gondii*, matched to the other groups for sex and age, b) patients with positive serology for *T. gondii* but without ocular lesions, and c) patients with congenital toxoplasmosis. In addition, we studied six patients who had recently acquired toxoplasmosis and followed their lymphocyte proliferative response to *T. gondii* antigens and the pattern of V α and V β expression of their lymphocytes following stimulation with STAg.

Figure 1 shows the stimulation indices (SI) obtained when PBMC from seronegative and seropositive individuals were cultured in the presence of STAg. Although

Figure 1. PBMC proliferation in response to STAg. The box plot presents the proliferation of PBMC from 36 seronegative individuals and 48 individuals with previous contact with *Toxoplasma gondii* (seropositive). The data are expressed as stimulation index (SI). The seronegative individuals had a median (\pm SD) proliferation of 1.1 ± 2.4 . The median SI for the seropositive group was 11.5 ± 28.7 . The mean SI of STAg for the two groups was 2.3 and 20.9, respectively. The average SI of PHA in the groups was 29.8 and 30.1, respectively (proliferation was measured at 48 h and antigen-specific proliferation at 96 h). The average SI of PPD in the groups was 10.6 and 12.1, respectively. The average SI of tetanus toxoid in the groups was 9.9 and 10.0, respectively. PBMC, peripheral blood mononuclear cells; STAg, soluble toxoplasma tachyzoite antigen; PHA, phytohemagglutinin; PPD, tuberculin purified protein derivative.



some seronegative individuals had positive proliferative responses (= 2 SI), most of them did not respond to *Toxoplasma* antigens. In contrast, PBMC from seropositive individuals proliferated significantly in response to STAg irrespective of the presence of ocular lesions. There was no difference between groups in response to PHA or unrelated antigens (PPD and tetanus toxoid).

Because PBMC from some individuals in the seronegative group showed an *in vitro* response to *T. gondii* antigens, it was suggested that a superantigen activity was at play. Therefore, we decided to analyze PBMC from patients with congenital toxoplasmosis for deletions of specific V β -bearing lymphocytes. Those individuals were more likely to carry deletions since they had been exposed to the parasite antigens for the longest period of time. The presence of V β families was tested by flow cytometry when antibodies were available or by RT-PCR for the V β families against which antibodies were not available. Deletions of V β 3, V β 12, V β 14 and V β 15 were observed (Figure 2). One patient had a deletion of V β 5.1 cells, less than 0.5% by flow cytometry (data not shown). However, most patients did not show any gaps in their V β repertoire. The V β deletions found in patients with congenital toxoplasmosis were often shared by noninfected members of the same family, suggesting that these were familial variations not due to the nature of *Toxoplasma* infection. However, it is still possible that deletion of STAg-specific T cells does occur and the methods used to evaluate TCR V α and V β families are not sensitive to detect minor changes in these families.

Studies on mice have suggested that *T. gondii* contains a superantigen-like activity capable of selective induction of T cells bearing the V β 5 chain both *in vitro* and *in vivo* (28,29). Because superantigen stimulation causes expansion, followed by deletion of the T cells bearing the TCR with which they interact, we hypothesized that if *T. gondii*

expresses a superantigen, exposure to STAg would result in the expansion of T cells bearing specific V β in seronegative individuals. Therefore, we stimulated PBMC from seronegative individuals with STAg. Five days after stimulation, the V α and V β repertoire in culture was evaluated by flow cytometry in comparison to the V α and V β repertoire expressed by unstimulated cells. A V α or V β was considered expanded if the percentage of positive cells after stimulation was at least two times higher than its frequency in the unstimulated population. Nine out of 26 seronegative individuals showed an expansion of some T cells bearing the V β 5.1 receptor (Table 1). Increases in the percentage of T cells expressing V β 5.2/V β 5.3, V β 6.7, V β 7.1, V β 8, V β 9 and V β 23 were also found. One patient showed a significant expansion of V α 12.1-bearing cells. The fact that T cells from unexposed individuals proliferate in response to STAg does support the hypothesis that a superantigen expressed by *T. gondii* is capable of stimulating human T cells. This hypothesis is further supported by the finding that such proliferation occurs through the expansion of specific V β -bearing T cells. Interestingly, one of the V β families that is found to be expanded in patients after stimulation with

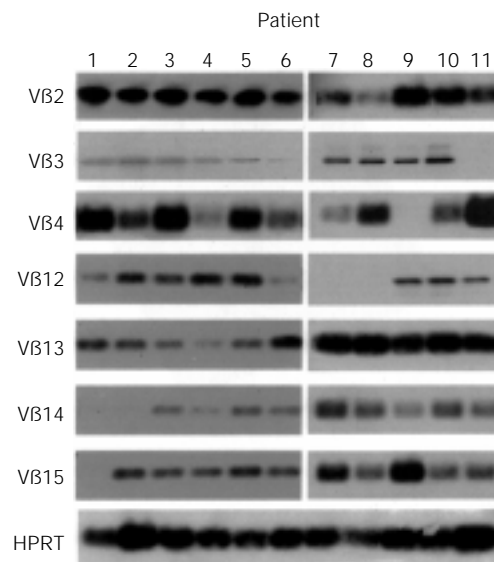


Figure 2. Peripheral blood mononuclear cell expression of V β families as measured by RT-PCR. Patients with congenital ocular toxoplasmosis (1 to 11) were evaluated for the presence of the V β families shown. Hypoxanthine phosphoribosyltransferase (HPRT) was used as a housekeeping gene control.

Table 1. V β expression in peripheral blood mononuclear cells from seronegative individuals after stimulation with soluble toxoplasma tachyzoite antigen (STAg).

Patient #	V β enhanced
1	V β 5.1 (50%)
2	V β 5.2/V β 5.3 and V β 9 (150 and 148%, respectively)
3	none
4	none
5	V β 5.1 (200%)
6	none
7	V β 5.1 and V β 8 (210 and 167%, respectively)
8	none
9	V β 5.1 and V β 7 (202 and 135%, respectively)
10	V β 5.1 and V β 6.7 (154 and 195%, respectively)
11	V α 12.1 (149%)
12	none
13	V β 5.1 and V β 5.2/V β 5.3 (102, 183 and 190%, respectively)
14	V β 9 (163%)
15	V β 23 (102%)
16	none
17	V β 7.1 (111%)
18	none
19	V β 6.7 and V β 9 (123 and 200%, respectively)
20	V β 5.1 (157%)
21	V β 5.1 (132%)
22	V β 5.1 and V β 6.7 (173 and 229%, respectively)
23	none
24	V β 5.1 and V β 7.1 (139 and 213%, respectively)
25	V β 5.1 and V β 9 (166 and 191%, respectively)
26	none

V α or V β was considered to be enhanced if the number of positive cells after exposure to STAg was at least double the number of cells bearing the same V α or V β in cultures without stimulation. Percent increase in expression of V α or V β over expression in unstimulated cells is shown in parentheses.

STAg is the same that is found to be expanded in response to *Toxoplasma* stimulation in the mouse (32). However, we found no evidence of specific V β family expansion in *ex vivo* PBMC from individuals with congenital ocular toxoplasmosis, acquired ocular toxoplasmosis, or asymptomatic seropositive individuals (data not shown).

Because we found a degree of V β -specific T cell expansion in response to STAg in naive donors, we decided to investigate the evolution of the T cell response to *Toxoplasma* antigens in individuals recently infected with *T. gondii*. We studied six individuals who presented positive IgM titers to *T. gondii* with classic symptoms of acute infection (see Material and Methods). The first three patients have been followed up for over a year, three additional patients have been followed up for different periods of less than 240 days. Figure 3 shows that proliferation in response to STAg does not seem to decrease over the period of time the patients were evaluated. Furthermore, flow cytometry analysis of the V α and V β expanded upon STAg stimulation of the PBMC from these patients showed that no families were deleted over the study period (Table 2).

Figure 3. PBMC proliferation from six patients in response to PHA (filled line and open symbols), STAg (dotted line and closed symbols) and PPD (thick filled line and open symbols). Each symbol represents the proliferation over time of one given patient. The standard error is shown for each point. PHA-stimulated proliferation was measured at 48 h and antigen-specific proliferation at 96 h. Proliferation data are presented as stimulation index (mean cpm with the stimulus divided by the cpm obtained in culture without the stimulus). For abbreviations, see legend to Figure 1.

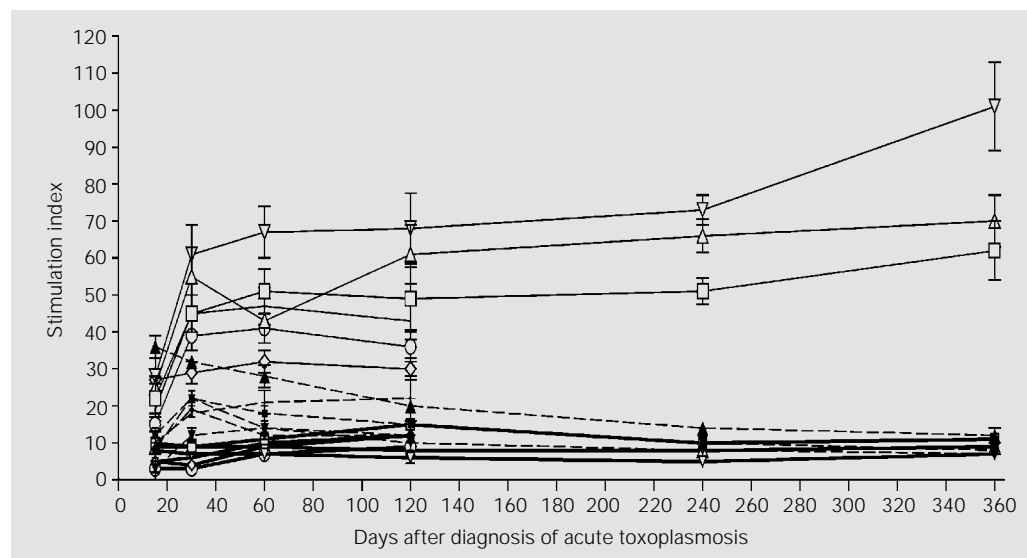


Table 2. Flow cytometry analysis of the V α and V β expression by peripheral blood mononuclear cells from patients with acute toxoplasmosis after stimulation with soluble toxoplasma tachyzoite antigen (STAg).

Patient	V α or V β expanded (15 days)	V α or V β expanded (60 days)	V α or V β expanded (120 days)	V α or V β expanded (360 days)
1	V β 9 (103%), V β 8 (101%), V β 5.1 (87%)	V β 9 (98%), V β 8 (105%), V β 5.1 (97%), V α 2 (55%)	V β 9 (100%), V β 8 (75%), V β 5.1 (105%), V α 2 (55%)	V β 9 (98%), V β 8 (105%), V β 5.1 (97%), V α 2 (55%)
2	V β 6.7 (107%), V β 9 (75%)	V β 6.7 (101%), V β 9 (85%), V β 8 (63%)	V β 6.7 (107%), V β 9 (75%), V β 8 (43%), V α 2 (45%)	V β 6.7 (97%), V β 9 (55%), V β 8 (73%)
3	V β 7.1 (112%)	V β 7.1 (103%), V β 5.2/V β 5.3 (95%)	V β 7.1 (105%), V β 5.2/V β 5.3 (101%)	V β 7.1 (98%), V β 5.2/V β 5.3 (109%)
4	V β 8 (120%), V β 5.1 (95%)	V β 8 (120%), V β 5.1 (95%)	V β 8 (150%), V β 5.1 (105%), V β 13 (57%)	NT
5	V β 3.1 (101%), V β 23 (75%)	V β 3.1 (101%), V β 23 (75%)	V β 3.1 (101%), V β 23 (75%), V β 3 (62%), V β 2 (43%)	NT
6	V β 13 (100%), V β 7.1 (65%)	V β 13 (98%), V β 7.1 (85%)	V β 13 (111%), V β 7.1 (33%)	NT

V α or V β was considered to be enhanced if the number of positive cells after exposure to STAg was at least double the number of cells bearing the same V α or V β in cultures without stimulation. Percent increase in expression of V α or V β over expression in unstimulated cells is shown in parentheses. NT: not tested; these patients were followed up for a shorter period of time.

Taken together, these data do not support the hypothesis that *T. gondii* bears a superantigen that acts on human lymphocytes.

Discussion

The possibility that *T. gondii* carries a superantigen has been extensively discussed in the literature (28,29,34). Aside from its scientific significance, this information also carries important clinical implications. If confirmed, these findings could shed light on a number of clinical idiosyncrasies observed in some patients with *Toxoplasma* chorioretinitis, such as the number of recurrences and the inconstant response to corticosteroids. It may also help to explain why patients with the congenital form of the disease have a decreased immune response to the parasite's antigens.

We have shown that PBMC from more than one third of the individuals with no previous contact with *T. gondii* proliferated in the presence of *Toxoplasma* antigens. These data would confirm that the parasite contains a superantigen capable of stimulat-

ing human T cells. However, when the same cells were analyzed for V β -specific deletions we were unable to detect any deletion of V β families. Since superantigen stimulation always results in the deletion of the proliferating T cells, these conflicting results suggest that proliferation induced by STAg in these "naive" individuals may be due to some other cause rather than superantigenic stimulation. To further evaluate the question of specific V β family deletions after stimulation with STAg, we studied the T cell repertoire of individuals with congenital toxoplasmosis. These studies revealed that 11 of 36 of these individuals presented specific V β deletions (gaps) in their T cell repertoire. However, their siblings as well as other members of the family also showed the same deletions irrespective of their status regarding the infection or contact with *T. gondii*. These results suggest that the inability to respond to *Toxoplasma* antigens by patients with congenital toxoplasmosis cannot be explained on the basis of T cell repertoire gaps caused by superantigenic stimulation over a prolonged period of time (35). Fi-

nally, when we analyzed the evolution of the T cell immune response to STAg in patients recently infected with *T. gondii*, we were unable to show any changes in the V β families they express. It is possible that the methods used to analyze T cell deletion (flow cytometry and PCR) were not sensitive enough to detect minor changes in the T cell repertoire. Nevertheless, the literature indicates that changes induced by a superantigen are usually easily detected by such methods (36,37).

Taken together, our data strongly suggest that *T. gondii* does not contain a superantigen capable of stimulating human lymphocytes. However, if a superantigen is not responsible for the stimulation induced by STAg in naive cells, why do they proliferate? A distinct possibility is the cross-reactivity between *Toxoplasma* antigens and a previously encountered antigen. T cells that react to *Toxoplasma* have been reported to recognize other parasites (35,38,39). It remains to be investigated which antigen or antigens

might be involved in the individuals evaluated in this study. The seronegative volunteers had an unremarkable clinical history, without any uncommon infectious episodes, suggesting that the antigen(s) is(are) of a common nature, either from another microorganism or from environmental sources such as food. It is of interest that, whatever the cross-reactive antigen might be, it does not stimulate a humoral response. This would not be a first since a similar situation has been reported for Chagas' disease (40). In Chagas' disease, lymphocytes from normal individuals used as controls responded with proliferation and cytokine production following stimulation with the *Trypanosoma cruzi* antigen protein B13.

We suggest that *T. gondii* does not have a superantigen that stimulates human T cells. We also suggest that cross-reactivity is responsible for the lymphocyte proliferation observed in response to *T. gondii* in seronegative patients.

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