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The role of MHC haplotypes $H2^d/H2^b$ in mouse resistance/susceptibility to cyst formation is influenced by the lineage of infective *Toxoplasma gondii* strain

MARIANNE G. RESENDE[1], BLIMA FUX[1,4], BRÁULIA C. CAETANO[1,2], ERICA A. MENDES[2], NEIDE M. SILVA[5], ADRIANA M. FERREIRA[3], MARIA NORMA MELO[3], RICARDO W.A. VITOR[3] and RICARDO T. GAZZINELLI[1,2,6]

[1]Laboratório de Imunopatologia, Instituto René Rachou, Av. Augusto de Lima, 1715, Centro
30190-002 Belo Horizonte, MG, Brasil
[2]Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais
Av. Antônio Carlos, 6627, Pampulha, 31270-901 Belo Horizonte, MG, Brasil
[3]Departamento de Parasitologia, Universidade Federal de Minas Gerais
Av. Antônio Carlos, 6627, Pampulha, 31270-901 Belo Horizonte, MG, Brasil
[4]Department of Molecular Microbiology, Washington University School of Medicine
660 S. Euclid Ave., 63110 Saint Louis, MO, U.S.A.
[5]Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia
Av. João Pinheiro, 565, Centro, 38400-902 Uberlândia, MG, Brasil
[6]Division of Infectious Diseases and Immunology, University of Massachusetts Medical School
364 Plantation Street, 01605 Worcester, MA, U.S.A.

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contributed by RICARDO T. GAZZINELLI*

ABSTRACT

Toxoplasma gondii strains displaying the Type I/III genotype are associated with acquired ocular toxoplasmosis in humans. Here, we used a mice model to characterize some immunological mechanisms involved in host resistance to infection with such strains. We have chosen the Type I/III strains D8, G2 and P-Br, which cause a chronic infection in mice that resembles human toxoplamosis. Mice deficient of molecules MyD88, IFN-γ, and IL-12 were susceptible to all three parasite strains. This finding indicates the importance of innate mechanisms in controlling infection. On the other hand, MHC haplotype did not influenced resistance/susceptibility; since mice lineages displaying a same genetic background but different MHC haplotypes ($H2^b$ or $H2^d$) developed similar mortality and cyst numbers after infection with those strains. In contrast, the C57BL/6 genetic background, and not MHC haplotype, was critical for development of intestinal inflammation caused by any of the studied strains. Finally, regarding effector mechanisms, we observed that B and CD8+ T lymphocytes controlled survival, whereas the inducible nitric oxide synthase influenced cyst numbers in brains of mice infected with Type I/III strains. These findings are relevant to further understanding of the immunologic mechanisms involved in host protection and pathogenesis during infection with *T. gondii*.

Key words: *Toxoplasma gondii* strains, innate immunity, acquired immunity, TLR and MHC.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular coccidian belonging to the phylum Apicomplexa. The parasite is globally distributed and has been described to infect more than 30 species of birds and 300 species of mammals, including humans (Dubey et al. 2002). Possibly one-third of the world's human population is infected with *T. gondii* (Dubey 1998, Tenter et al. 2000). Infection with the parasite occurs via three main routes: con-

*Member Academia Brasileira de Ciências
Correspondence to: Ricardo T. Gazzinelli
E-mail: ritoga@cpqrr.fiocruz.br

genitally by transplacental transmission of tachyzoites; ingestion of food or water contaminated with oocysts shed in feces of infected cats (Bahia-Oliveira et al. 2003, de Moura et al. 2006); or by ingestion of raw or undercooked meat containing tissue cysts (Dubey 1996). Infection is asymptomatic in most individuals, whereas severe pathology and lethality due to toxoplasmosis is a common finding in congenitally infected or immunodeficient patients (Desmonts and Couvreur 1974). In addition, toxoplasmosis is one of the most common causes of infectious uveitis in both immunocompetent and immunocompromised persons (Holland 1999, Colombo et al. 2005). Importantly, some studies indicate that toxoplasmic retinocoroiditis and ocular disease is frequently found in cases of acquired toxoplasmosis (Glasner et al. 1992). Variation in the clinical presentation and severity of disease in susceptible persons has been attributed to several factors, including the genetic heterogeneity of the host and the genotype of the infective parasite (Sibley and Boothroyd 1992, Howe and Sibley 1995, Holland 1999).

Studies performed with strains of *T. gondii* isolated in North America and Europe showed that they were morphologically similar yet could be grouped into three distinct clonal lineages by isoenzymes or DNA restriction fragment length polymorphisms (Darde et al. 1992, Sibley and Boothroyd 1992, Sibley et al. 1992, Howe et al. 1997). The structure of *T. gondii* population in those geographic areas is clonal, and most strains fall into one of the three categories denominated Type I, Type II and Type III lineages (Sibley and Boothroyd 1992, Howe and Sibley 1995). The three clonal types are apparently not minor or random polymorphic states of any phenotypic consequence: Type I lineage strains are highly virulent in outbred mice and perhaps humans (Grigg et al. 2001a), whereas type II and III lineage strains are relatively less virulent (Sibley et al. 1992, Howe et al. 1996, 1997). A small percentage of strains are recombinant between two of three parasite lineages, and vary in terms of their virulence phenotype in mice (Grigg et al. 2001a). In North America and Europe, Type II strains are most common in human toxoplasmosis and chronic infections in food animals (Howe and Sibley 1995). Importantly, while type I strains are relatively rare in animals, they occur with increasing frequency in human congenital toxoplasmosis (Fuentes et al. 2001) and ocular disease (Grigg et al. 2001a), whereas recombinant Type I/III strain are more often found in patients with ocular toxoplasmosis (Grigg et al. 2001b). In Brazil, *T. gondii* isolates present high genetic variability, as demonstrated by recent studies on multilocus PCR RFLP, and the parasite population has an epidemic structure with a few expanded clonal lineages (Ferreira Ade et al. 2006, Pena et al., in press).

Immunogenetic studies provide powerful tools for understanding protective and pathogenic mechanisms in such infectious disease (McLeod et al. 1995, 1996, Mack et al. 1999). Specifically, in the case of *T. gondii* infection, genes located in different regions of the host genome have been implicated in resistance to infection. Particularly, major histocompatibility (MHC) alleles are important determinants of resistance and susceptibility to early infection, as well as controllers of cyst numbers and encephalitis at later stages of infection with *T. gondii*, both in mice and humans (McLeod et al. 1989a, Brown and McLeod 1990, Brown et al. 1994, Blackwell et al. 1993, Johnson et al. 2002a, b). These studies are confirmed by the critical role of CD8+ T as well as CD4+ T cells in host resistance to toxoplasmosis (Brown and McLeod 1990, Suzuki et al. 1991, Gazzinelli et al. 1992). In addition, various cytokines have shown to be critical in host resistance to *T. gondii* infection (Denkers and Gazzinelli 1998). In the acute phase of toxoplasmosis, *T. gondii* tachyzoites trigger the synthesis of IL-12 and other co-stimulatory cytokines (e.g. TNF-α), which initiate the synthesis of IFN-γ by "natural killer" (NK) cells and CD4+ $\alpha\beta$ T lymphocytes (Gazzinelli et al. 1993b, 1994b, Hunter et al. 1994, Cai et al. 2000). IFN-γ and TNF-α remain critical to host resistance to chronic toxoplasmosis, probably by their ability to activate the inducible nitric oxide synthase (iNOS) and production of reactive nitrogen intermediates (RNI) that exert both microbicidal and microbiostatic effects.

We have recently reported a non-clonal distribution of natural recombinant Type I/III strains, in different geographic areas of Brazil, where acquired ocular toxoplasmosis (up to 20% of the infected population) is commonly found among patients with chronic toxoplasmosis (Glasner et al. 1992, Dubey et al. 2002, Fux et al. 2003, Ferreira Ade et al. 2004, 2006, Portela et al. 2004). Importantly, we and others were unable to iso-

late the avirulent Type II clonotype *T. gondii* strains in Brazilian territory, where approximately 80% of all isolated strains were virulent (Dubey et al. 2002, Ferreira Ade et al. 2004, 2006). However, most of the immunological studies concerning *T. gondii* infections have been performed with the ME49, a Type II strain. In the present study, we evaluated the role of different immunological compartments on host resistance to three different recombinant Type I/III strains of *T. gondii*, which presents low virulence during acute infection and are cystogenic during chronic phase, thus resembling human toxoplasmosis. Our results indicate that components of innate immunity are critical in a similar manner in infections with Type II or the Type I/III strains of *T. gondii* studied here. In contrast, MHC haplotypes $H2^b$ and $H2^d$ were not determinants of mice susceptibility and resistance to Type I/III strains, as previously shown for Type II strains of *T. gondii* (Brown and McLeod 1990).

MATERIALS AND METHODS

ANIMALS

BALB/c, C57BL/6, MyD88-/-, TLR2-/-, TLR4-/-, TLR9-/-, IL-12-/-, IFN-γ-/-, iNOS-/-, CD8-/-, Igμ-chain-/-, congenic CB10H2 and C57BL/KsJ and outbreed Swiss Webster mice were housed at the animal facilities of Institute of Biological Sciences (ICB), of the Federal University of Minas Gerais (UFMG), Brazil. Congenic and knockout mice were bred as homozygotes, and knockout mice were backcrossed to at least 8 generations into the genetic background of C57BL/6. All animals were 8 weeks old, and were managed according to institutional standard guidelines.

PARASITE STRAINS

Strains BV, EGS, EFP (virulent), D8, G2 and P-Br (avirulent) were used as representative of Type I/III isolates, whereas strain ME49 was used as a representative of a Type II strain. Strains P-Br and D8 were isolated from dogs, BV and G2 were isolated from goats, and EGS and EFP were isolated from congenitally infected humans (Jamra and Vieira 1991, Fux et al. 2003, Ferreira Ade et al. 2004). ME49 was isolated from a sheep (Lunde and Jacobs 1983). Each strain was maintained by successive inoculation of free tachyzoite and/or tissue cysts in female Swiss Webster mice. Cyst and/or tachyzoites obtained from reservoir mice were used to infect experimental animals.

EXPERIMENTAL INFECTIONS

Experimental infections with tachyzoites from BV, EGS, EFP, D8, G2 and P-Br were performed as described: free tachyzoites were harvested from Swiss Webster reservoirs, 5 to 7 days after infection, by peritoneal washing with PBS. Tachyzoite inoculums were adjusted to 1, 10, 100 and 1,000 cells per 100μL PBS and administered to mice by i.p. injection. Survival was followed thereafter in groups submitted to each tachyzoite dose. Experimental infections with cysts of strains D8, G2, P-Br and ME49 were performed as follows: brains were collected from Swiss Webster reservoirs, 60 days after infection, and homogenized in 1mL PBS. Cysts numbers were determined by microscopic analysis of 10μL aliquots of brain homogenates, in duplicates. Inoculums were adjusted to 4, 20 or 100 cysts per 200μL and administered orally. Survivors were killed 60 days after infection for determination of brain cyst loads, as described above.

HISTOPATHOLOGY

Histological examination was carried out in small intestine samples obtained 7 days after infection with different *T. gondii* strains. Small intestine samples were embedded in paraffin and cut in 4μm-width sections, which were stained with Hematoxylin-Eosin dye. Slides containing 2 sections of each intestinal sample were analyzed under light microscope for presence of inflammatory infiltrates, and scored from 1 (+) to 4 (++++) based on the intensity of pathological changes.

FLOW CYTOMETRY

Spleen homogenates were obtained individually from infected or non-infected mice (3 animals per group) and red blood cells were disrupted with ACK lysis buffer. A total of 2×10^6 purified splenocytes were stained with 15μl of optimal concentrations of FITC-conjugated anti-Vβ 8, 6 or 5 specific monoclonal antibodies (BD Pharmingen, San Diego, CA) in combination with PE-conjugated anti-CD4+ or CD8+ antibodies (BD Pharmingen). Analysis of stained cells was performed in FACScan® (BD Biosciences, San Jose, CA) with Cell Quest® software (BD Biosciences).

Statistical Analysis

To compare brain cyst numbers obtained in different mice lineages infected with different *T. gondii* strains we used ANOVA test, followed by Tukey multiple comparisons, when appropriate. Otherwise, comparisons were performed by Kruskal-Wallis test, followed by Dunn's multiple comparisons. To compare data between mice of the same lineage infected with different doses (4 or 20 cysts) of one specific *T. gondii* strain we performed Student's T (for parametric data) or Mann-Whitney's test (for non-parametric data). Comparison of survival data was performed by Kaplan-Meir test. All data were tested for significance in MINITAB software, version 13.0, and tests with $p < 0.05$ were considered statistically significant.

RESULTS

Different Virulence of Natural Recombinant Type I/III strains of *T. gondii*

Strains BV, EGS, EFP, D8, G2 and P-Br were isolated in Minas Gerais and São Paulo States, in Brazil, and characterized as recombinant Type I/III strains (Fux et al. 2003, Ferreira Ade et al. 2004). Results of experimental infections of BALB/c with different tachyzoite doses of each strain are shown in Figure 1. All animals infected with BV, EGS and EFP died, independently from inoculum dose. On the other hand, all mice infected with low dose (1 tachyzoite) of EFP survived. In this case, infection was confirmed by detection of specific anti *T. gondii* antibodies in ELISA and Western Blot (data not shown). Thus, BV, EGS and EFP were classified as highly virulent, virulent and of intermediate virulence, respectively. In contrast, all animals infected with D8, G2, and P-Br survived (Fig. 1), and those strains were considered to be avirulent.

Role of Toll-Like Receptors (TLRs) on Innate Immune Response and Host Resistance to Infection with Type I/III Strains of *T. gondii*

Initially, we infected MyD88-/-, TLR2-/-, TLR4-/- and TLR9-/- mice with either of the Type I/III strains of *T. gondii*. In addition, we infected the mice devoid of functional CD14, a co-receptor required for optimal TLR2 and TLR4 functions. Mice received an oral dose of 20 cysts of P-Br, D8 and G2 and, after 45 days, survivors were sacrificed for determination of brain cysts numbers as an indicator of host resistance to infection. As previously shown for strain ME49 (Type II) (Scanga et al. 2002), MyD88-/- mice were highly susceptible to all Type I/III strains tested (not shown). In contrast, we were unable to detect any change in host resistance/susceptibility to infection with Type I/III strains in single TLR (TLR2, TLR4 or TLR9) or CD14 knockout mice (not shown). An early study suggested that increasing infective doses of ME49 could lead to a susceptible phenotype in TLR2-/- (Mun et al. 2003). Thus, we decided to use 100 cysts of P-Br as inoculums for different knockout mice. Results were identical to those obtained with 20 cysts, i.e., enhanced susceptibility of MyD88-/- mice, but not of TLR2-/-, TLR4-/-, TLR9-/- or CD14-/- mice (Table I).

TABLE I
Number of brain cysts and survival of mice after per-oral infection with P-Br strain of *T. gondii*.

P-Br Strain (100 cysts)		
Mice strain	Survival	Number of brain cysts
C57BL/6 a	91,6% f	444 ± 186 f
CD14 b	100% f	170 ± 20 f
TLR2 c	100% f	233 ± 33 f
TLR4 d	90,9% f	255 ± 95 f
TLR9 e	83.3% f	260 ± 248 f
MyD88 f	27,2% abcde	2633 ± 752, 2 abcde

Brain cyst loads were evaluated 40 days after infection. Small letters (a-f) indicate statistically significant difference between mice strains. Data from one representative experiment. Experiment was repeated two times with similar results.

MyD88 is an adaptor of all TLR (except TLR3) functions, and is critical for induction of pro-inflammatory cytokines, including IL-12. Importantly, IL-12 is the initiator for IFN-γ synthesis by NK cells and T lymphocytes, and thus to host resistance to *T. gondii*. To further analyze the importance of these pro-inflammatory cytokines during initial stages of infection, we inoculated IL-12-/- and INF-γ-/- mice with either 04 or 20 cysts of P-Br, D8 and G2 strains and the survival was monitored over 45 days period. Infection of IL-12-/- and INF-γ-/- mice, even with the lower dose (4 cysts), resulted in 100% mortality around 10-20 days post-infection (Fig. 2).

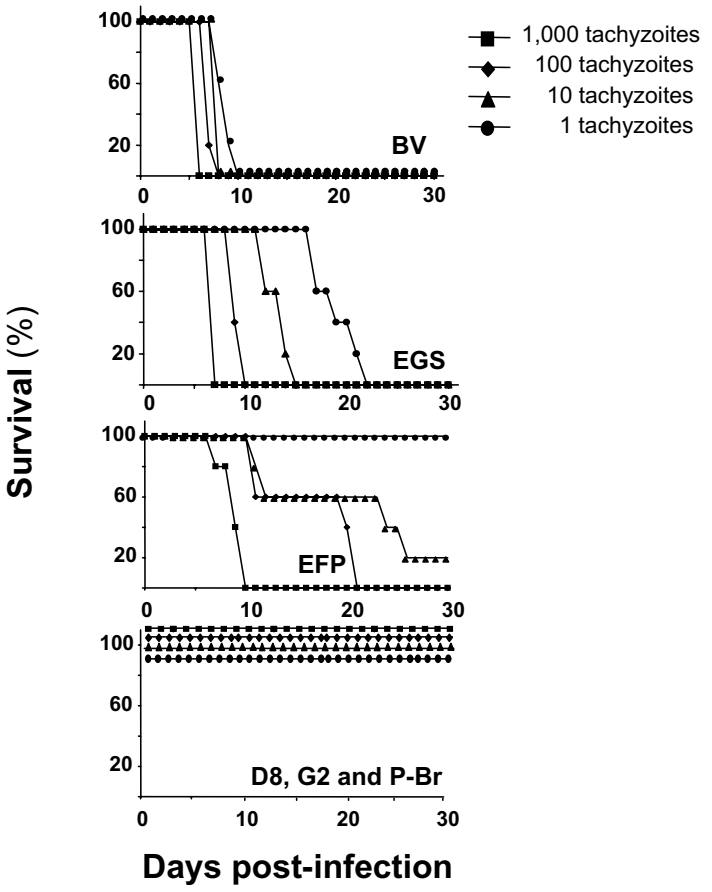

Fig. 1 – Survival of BALB/c mice infected with intra-peritoneal injections of 1, 10, 10^2, or 10^3 tachyzoites from highly virulent (BV), virulent (EGS), intermediate virulent (EFP) or avirulent (D8, G2 and P-Br) Type I/III strains of *T. gondii*. Data from one representative experiment. Experiment was repeated two times with similar results.

Influence of MHC Haplotype in Cyst Numbers of Mice Infected with Type I/III Strains of *T. gondii*

We have investigated the influence of MHC haplotype in controlling cyst numbers and mouse resistance to Type I/III strains of *T. gondii*. To achieve this purpose, we selected mice lineages which display H2 haplotypes "b" and "d", which were previously demonstrated as markers of, respectively, susceptibility and resistance to infection with *T. gondii*. Mice lineages involved in this experiment were parental BALB/c ($H2^d$) and C57BL/6 ($H2^b$) or the derivative congenic strains CB10-H2 (BALB/c genetic background with $H2^b$ haplotype) and C57BL/KsJ (C57BL/6 genetic background with $H2^d$ haplotype). Results (Table II) confirm previous observations that mice strains expressing haplotype "d" are more resistant to ME49 than mice that express "b" haplotype, since BALB/c and C57BL/KsJ developed fewer brain cysts after 45 days of infection. Nevertheless, this feature was not observed in infections with D8 and G2, which induced similar cyst burdens and low mortality in the different mouse congenic strains analyzed. It is noteworthy, that the C57BL/6 genetic background, regardless of the MHC haplotype, conferred resistance to cyst formation, when infected with P-Br strain (Table II).

To depict the immune mechanisms involved in susceptibility/resistance of different mice to *T. gondii*, we

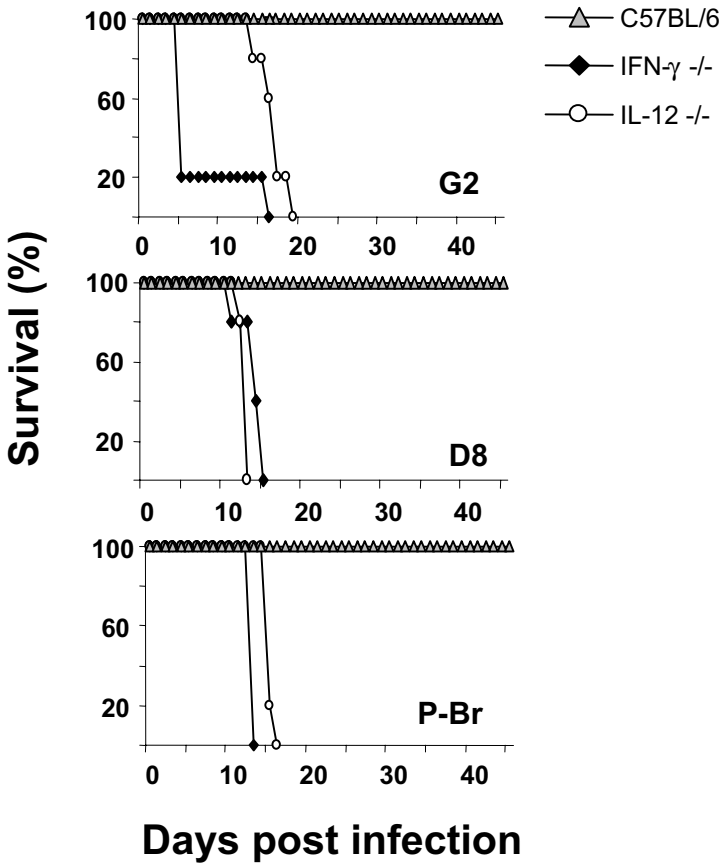

Fig. 2 – Survival of IFN-γ-/-, IL-12-/- and C57BL/6 mice after per-oral infection with 4 cysts of Type I/III strains D8, G2 and P-Br of *T. gondii*. Data from one representative experiment. Experiment was repeated two times with similar results.

studied the pattern of antibody response, and compared IgG1 versus IgG2a levels in infected animals. We observed that, independently of the *T. gondii* strain used in infection, antibody responses were consistent within a similar mouse genetic background. BALB/c and CB10-H2 produced high IgG1:IgG2a ratios, whereas the same parameter was smaller in the C57BL/6 and C57BL/KsJ, indicating a stronger Th1 response in the latter mice (data not shown). Consistently, the intense intestinal inflammatory process associated to acute toxoplasmosis – which is dependent on strong Th1 responses, with high levels of IFN-γ and RNI – was only observed in mice of the C57BL/6 genetic background (Table III). The $H2^d$ haplotype expressed by the C57BL/KsJ strain resulted in increased intestinal inflammation in mice infected with D8 and P-Br strains when compared to C57BL/6 mice that express the $H2^b$ haplotype.

Usage of specific TCR Vβ8 chain was previously indicated as a mechanism of resistance to severe Toxoplasmic Encephalitis in BALB/c. On the other hand, susceptible CBA mice were demonstrated to activate higher levels of Vβ6 bearing T cells after infection with *T. gondii*, and develop greater inflammation and brain cysts than BALB/c (Wang et al. 2005). We then decided to investigate the frequency of CD4+ T and CD8+ T cells expressing Vβ8, Vβ6 and Vβ5, in BALB/c and C57BL/6 mice infected with P-Br or ME49. According to results shown in Figure 3 and Table IV, a higher frequency of CD4+ and CD8+ T lymphocytes expressing Vβ8 is naturally found in BALB/c and C57BL/6 mice. However,

TABLE II
Number of brain cysts and survival of mice after a per-oral infection with different strains of *T. gondii*.

		Toxoplasma gondii strain							
		G2 H		D8 I		P-Br J		ME49 K	
Mice strain	Inoculum (cysts)	Survival	Brain cysts	Survival	Brain cysts	Survival	Brain cysts	Survival	Brain cysts
C57BL/6 genetic background									
$H2^b$ a	4	100% K	1220±665 Jb	100% K	311±154	100% K	170±155 HKd	16,6% HIJbc	7200±4808 Jc
(C57BL/6)	20	100% K	2290±765 Jb	100% K	1666±709 Jcd	100% K	220±92 HIbd	0% HIJbd	*
$H2^d$ c	4	70%	771±513 JK	100%	319±169 J	100%	92,8±18,8 Hd	81,8% a	111±86 HIad
(C57BL/Ks)	20	60%	1316±466 JK	70%	643±425 a	80%	200±136 Hbd	50%	240±114 Hbd
BALB/c genetic background									
$H2^b$ d	4	88,8%	950±446 J	87,5%	362±324 JK	100%	2762±1001 HIac	60%	1750±352 Ic
(CB10-H2)	20	60%	1700±1361	100%	800±594 JKa	90%	3211±1879 Iac	60% a	3583±1860 Ibc
$H2^d$ b	4	90%	477±281 a	100%	825±710	100%	580±501	66,6% a	237±106
(BALB/c)	20	90%	966±260 Ka	90%	1177±821 K	100%	1810±1321 Kac	80% a	250±107 HIJd

Brain cyst numbers were evaluated at 45 days after infection. Small letters (a-d) indicate statistically significant difference between mice strains. Capital letters (H-K) indicate statistically significant difference between *T. gondii* strains. (*) Indicate that all animals succumbed to infection.

TABLE III
Inflammatory lesions on small intestine of mice infected with different strains of *T. gondii*.

	Toxoplasma gondii strain (20 cysts)			
Mice strain	G2	D8	P-Br	ME49
	Inflammatory score – C57BL/6 genetic background			
$H2^b$ (C57BL/6)	++	+	+	++++
$H2^d$ (C57BL/Ks)	++	+++	++	+++
	Inflammatory score – BALB/c genetic background			
$H2^b$ (CB10-H2)	+	+	+	+
$H2^d$ (BALB/c)	+	+	+	+

Two samples from each mouse were examined and scored for pathologic changes. Scores: (+), mild; (++), moderate; (+++), severe; (++++), very severe.

no alteration of frequency of any specific Vβ family was detected after infection with either *T. gondii* strain.

Effector Mechanisms in Control of Cyst Numbers and Host Resistance to Infection with Type I/III Strains of *T. gondii*

Finally, we decided to investigate the role of different effector mechanisms in control of cyst numbers and hosts resistance to Type I/III strains of *T. gondii*. We infected CD8-/-, Igμ-chain-/- (B-/-) and iNOS-/- mice with 4 (not shown) and 20 cysts of D8, G2 and P-Br strains (Table V). Our results indicate that CD8+ T cells and, in a lesser extent, B lymphocytes were important components controlling survival. However, regarding cyst formation, it appears that CD8+ T and B cells, individually, are not the major mediators of protection. Infection of iNOS -/- mice resulted in 40%, 80% and 40% survival with G2, D8 and P-Br strains, respectively. Further, the remaining iNOS-/- survivors developed high cyst burdens (Table V). These findings suggest that IFN-γ produced by CD4+ T cells and production of RNI is indeed a key pathway for controlling cyst numbers in the CNS of mice infected with Type I/III strains.

DISCUSSION

Although *T. gondii* is regarded as the only species in genus Toxoplasma, genetically different strains of the parasite have been described. The genetic variance was already extensively assessed in samples of *T. gondii* found mainly in Europe and North America. According to genetic markers and virulence during acute infection in mice those strains have been divided into Type I, Type II and Type III groups (Sibley and Boothroyd 1992, Howe and Sibley 1995). Different studies have illustrated the high frequency of Type I and III, and the absence of Type II, isolates in Brazil (Dubey et al. 2002, 2003a, b, 2004). A previous study of our group revealed an unusually high frequency in Brazil (100% of isolates analyzed) of strains that shared Type I and III genetic markers (Fux et al. 2003). Such strains were separately classified as natural recombinant Type I/III, and amongst them, 85% displayed some degree of virulence and only 15% were avirulent. The highly virulent strains were more closely related to Type I genotype (Ferreira Ade et al. 2006). These findings contrast with those from North America and Europe, where most isolates associated to human infections are avirulent Type II (Sibley and Boothroyd 1992, Howe and Sibley 1995). Importantly, Grigg and colleagues (Grigg et al. 2001b) also found in North America an unexpected high occurrence of Type I, and recombinant Type I/III strains in patients with ocular toxoplasmosis. A high prevalence of ocular toxoplasmosis is observed in Brazilian populations, and most cases are attributed to acquired infection (Glasner

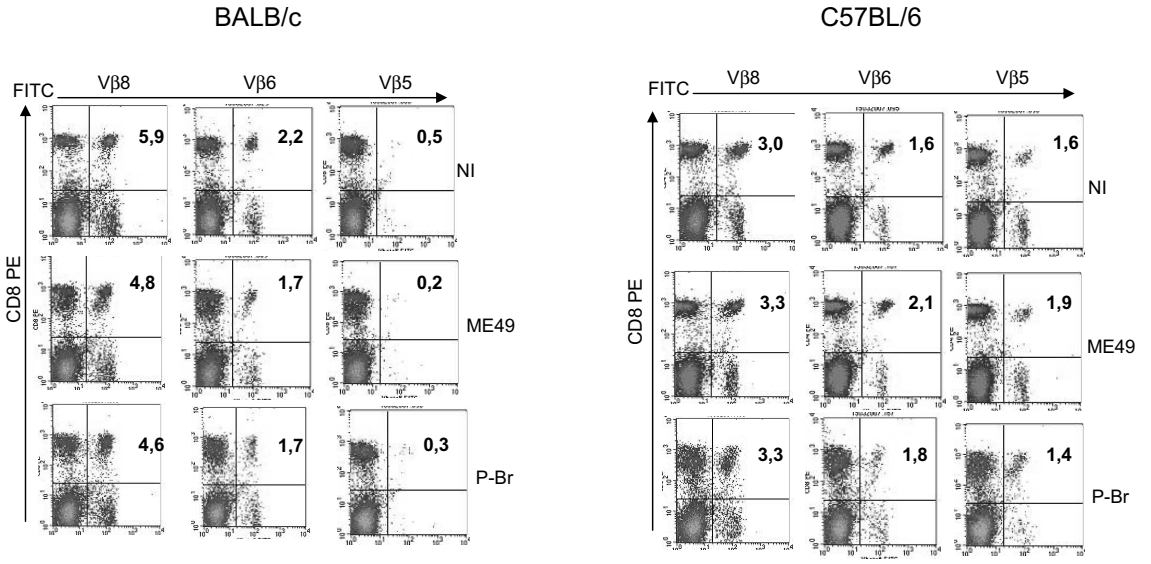

Fig. 3 – Frequency of different TCR Vβ chains in CD8+ T cells from animals infected with *T. gondii*. Splenocytes were obtained 2-4 weeks after oral infection with tissue cysts. Cells were double-stained with monoclonal antibodies specific for $CD8^+$ molecule and different Vβ chains. NI, non-infected mice. Results of one representative experiment. Experiments were repeated independently four times.

et al. 1992, Holland 1999, Silveira et al. 2001, Portela et al. 2004). Although speculative, we suggest that these occurrences could be attributed in part to the dominance of Type I/III virulent strains in our territory (Glasner et al. 1992, Silveira et al. 2001, Portela et al. 2004).

In addition to intrinsic parasite virulence factors, susceptibility to Toxoplasma is determined by host genetic characteristics and environmental factors, such as co-infections, including HIV, that affect the immunological status of the host. It was observed that humans infected with *T. gondii* have a parasite-specific Th1-polarized cell response, characterized by strong IFN-γ production when peripheral blood mononuclear cells (PBMCs) are stimulated with parasite antigens (Gazzinelli et al. 1995) and high levels of anti-parasite specific IgG1 and IgG3 (Giraldo et al. 2000, Portela et al. 2004). Further, presence of primed CD4+ T helper and CD8+ cytotoxic T cells have been demonstrated in PBMCs from patients with chronic toxoplasmosis (Hunter et al. 1996). However, decreased cellular responses to *T. gondii* antigens, including lower IFN-γ and IL-2 secretion, occur in congenitally infected patients (McLeod et al. 1985, 1990, Yamamoto et al. 2000), and individuals co-infected with HIV (Hunter et al. 1996). In latter case, weaker responses were associated with development of toxoplasmic encephalitis. Regarding the influence of host genetics over toxoplasmosis, it is known the MHC contribution to disease development. It was reported, for example, an increased frequency of MHC HLA-DQ3 in infants with hydrocephalus lesions (Mack et al. 1999). A lower than expected homozygosity also suggested that this allele increased susceptibility to toxoplasmosis. Another study indicated that AIDS patients with HLA-DQ3 haplotype more often experienced reactivation of chronic toxoplasmosis (Suzuki et al. 1996). Studies with mice expressing transgenic human class II MHC alleles showed that HLA-DQ1 was associated with lower parasite burden and pathology comparing to mice with HLA-DQ3 (Mack et al. 1999). Further, mice expressing human class I molecules HLA-DB27 or HLA-Cw3 had enhanced resistance to infection (Brown et al. 1994). Here we employed a murine model of toxoplasmosis to characterize the role of different immunological compartments on resistance to natural recombinant Type I/III strains of *T. gondii*, whose infection in mice resembles human toxoplasmosis.

In mice, cytokines such as IL-12, TNF-α and IFN-γ and reactive nitrogen intermediates (RNI) are mediators

TABLE IV
Analysis of the frequency of different TCR Vβ chains in T cells from mice infected with P-Br or ME49 strains of *T. gondii*.

	BALB/c						C57BL/6					
	Non-infected		ME49		P-Br		Non-Infected		ME49		P-Br	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
Vβ8	8,2 (1,94)	5,9 (0,66)	4,3 (0,58)	4,8 (0,47)	5,6 (1,12)	4,6 (0,45)	4,2 (0,93)	3,0 (0,44)	4,1 (0,92)	3,3 (1,19)	3,4 (0,55)	3,3 (1,21)
Vβ6	3,7 (0,42)	2,2 (0,34)	2,3 (0,79)	1,7 (0,78)	2,2 (1,06)	1,7 (0,45)	1,6 (0,17)	1,6 (1,02)	1,6 (0,30)	2,1 (0,97)	1,5 (0,28)	1,8 (0,43)
Vβ5	0,5 (0,33)	0,5 (0,37)	0,2 (0,14)	0,2 (0,19)	0,2 (0,14)	0,3 (0,51)	0,7 (0,17)	1,6 (0,25)	0,8 (0,16)	1,9 (0,50)	0,7 (0,25)	1,4 (0,22)

Values correspond to mean ($\pm$ standard deviation) percentage of T cells showing positive staining with monoclonal antibodies specific to each Vβ. Results of one representative experiment. Experiment was repeated independently four times, with similar results.

TABLE V
Brain cyst numbers and survival of mice infected with different Type I/III strains of *T. gondii*.

	T. gondii strain (20 cysts)					
	G2 H		D8 I		P-Br J	
Mice strain	Survival	Brain cysts	Survival	Brain cysts	Survival	Brain cysts
C57BL/6 a	100% bd	2290$\pm$765 Jc	100% bc	1666$\pm$ 709 Jbc	100% bd	220$\pm$ 92 HId
CD8-/- b	0% Jacd	*	20% ad	550$\pm$ 71 ad	58,3% Ha	414$\pm$ 254 d
B-/- c	60% b	833$\pm$ 427 IJad	20% ad	250$\pm$ 141 Had	62,5%	175$\pm$ 155 d H
iNOS-/- d	40% Iab	2500$\pm$1273 c	80% HJbc	1300$\pm$594 Jbc	40% Ia	4100$\pm$0 Iabc

Number of brain cysts was evaluated 45 day after infection. Small letters (a-d) indicate statistically significant difference between mice strains. Capital letters (H-J) indicate statistically significant difference between *T. gondii* strains. (*) Indicate that all animals succumbed at 45 days. Data from one representative experiment. Experiments were repeated two times with similar results.

of resistance to *T. gondii* (Suzuki et al. 1989, Gazzinelli et al. 1991, 1992, 1993a, b, 1994a, Hayashi et al. 1996a, b, Scharton-Kersten et al. 1996, 1997, Denkers and Gazzinelli 1998). Nevertheless, pathology associated with excessive stimulation of those pro-inflammatory cytokines also occur during acute toxoplasmosis in mice lacking IL-10 (Gazzinelli et al. 1996, Liesenfeld et al. 1996, Neyer et al. 1997). Results presented here show that components of innate immunity, i.e., MyD88, IL-12 and IFN-γ are also critical for resistance during early stages of infection with Type I/III strains. Indeed, MyD88 and TLR11 have been shown to be critical in eliciting pro-inflammatory cytokines in mice, and development of Th1 lymphocytes during toxoplasmosis (Scanga et al. 2002, Yarovinsky et al. 2005). In addition, we have recently demonstrated that *T. gondii* derived glycosylphosphatidylinositol (GPI) anchors activate TLR2 and TLR4, and that mice lacking both receptors have a small increment in susceptibility. However, by testing mice deficient in a single TLR (i.e. TLR2, TLR4, or TLR9) or the related co-receptor (CD14) we were unable to define a single innate immune receptor that is critical for resistance. Together, our findings with natural recombinant Type I/III strains indicate that *T. gondii* parasites may engage various TLRs during infection of the intermediate host.

Resistance to chronic *T. gondii* infection is associated with an acquired Th1-type cellular response (Gazzinelli et al. 1991, 1992, 1994a). Indeed, both class I and class II MHC molecules have been shown to influence disease outcome after *T. gondii* infection in mice (McLeod et al. 1989b, Brown and McLeod 1990, Suzuki

et al. 1991, Blackwell et al. 1993, Johnson et al. 2002a). More precisely, H2 haplotypes "d" and "b" are, respectively, determinants of host resistance and susceptibility to infection with ME49 (Brown and McLeod 1990, Brown et al. 1995). Consistently, CD4+ and CD8+ T cells are essential components in host resistance to the parasite (Gazzinelli et al. 1991, Suzuki et al. 1991). Further, there is also evidence that a singular pattern of TCR Vβ chain usage by anti-*T. gondii* T cells could influence protection. Indeed, it was demonstrated that a higher frequency of Vβ8 chain in T lymphocytes was associated with BALB/c ($H2^d$) resistance against TE caused by ME49, whereas preferential Vβ6 chain usage correlated with susceptibility in CBA/Ca ($H2^k$). Adoptive transfer of BALB/c V$\beta8^+$, but not V$\beta6^+$, T cells was capable to prevent mortality and encephalitis in nude mice (Kang et al. 2003, Wang et al. 2005). Another evidence of the importance of cell-mediated effector mechanisms is that neutralization of IFN-γ or TNF-α and inhibition of NOS2 leads to reactivation of chronic infection with ME49 and results in encephalitis and uveitis (Gazzinelli et al. 1992, 1993a, 1994a, Hayashi et al. 1996a, b). Finally, B lymphocytes have also been shown to contribute to host resistance, as demonstrated in experiments with knockout mice (Johnson and Sayles 2002). However, most of the studies described above employed strain ME49 that belongs to Type II lineage of *T. gondii*.

Consistently, we found that Type I/III strains also elicited strong Th1 responses with high IFN-γ, and high IgG2/IgG1 ratio of specific antibodies (Fux et al. 2003). Indeed, IFN-γ was shown essential for survival of animals infected with these parasite isolates. However, in contrast to results obtained with congenic mice infected with ME49, MHC haplotype $H2^d$ was not an important determinant of resistance to Type I/III isolates. Further, we were unable to find any association between usage of specific TCR Vβ chains and resistance/susceptibility to Type I/III strains. While not essential, B lymphocytes, CD8+ T cells and NOS2 seamed to be involved in mechanisms controlling cyst numbers and host survival during infection with those natural recombinant strains of *T. gondii* isolated in Brazil.

In conclusion, the elements MyD88, IL-12 and IFN-γ from the cellular compartment of innate immunity are critical during early stages of infection with Type I/III strains of *T. gondii*. However, we found a discrepancy on the role of MHC haplotypes in host resistance/susceptibility to Type I/III Brazilian isolates, when comparing to the standard ME49, which is used in many laboratories for immunological studies. Results suggest that an immunodominant T cell epitope expressed by Type II strains, but not by Type I/III isolates, may be involved on induction of protective acquired immunity during *T. gondii* infection. Identification of parasite antigen that encodes this particular T cell epitope may contribute to better understanding the different biological behavior of Type II versus Type I/III strains of *T. gondii* and eventually the pathogenesis of ocular toxoplasmosis.

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RESUMO

Cepas de *Toxoplasma gondii* que apresentam o genótipo I/III são associadas a toxoplasmose ocular adquirida em humanos. No presente trabalho, nós utilizamos um modelo da doença em camundongos para caracterizar mecanismos imunológicos envolvidos na resistência do hospedeiro à infecção por aquelas cepas. Escolhemos as cepas D8, G2 e P-Br, que causam infecção crônica em camundongos, semelhante à toxoplasmose humana. Camundongos deficientes em MyD88, IFN-G e IL-12 foram susceptíveis a infecções com todas as três linhagens do parasita. Esses dados indicam a importância de mecanismos inatos no controle da infecção. Por outro lado, o haplótipo do MHC não influenciou na resistência/susceptibilidade, na medida em que linhagens de camundongos com um mesmo "background" genético, mas diferentes haplótipos de MHC ($H2^b$ e $H2^d$) apresentam o índice de mortalidade e número de cistos semelhantes após a infecção com aquelas cepas do parasita. Em contraste, o "background" genético de C57BL/6, mas não

o haplótipo de MHC, foi crítico para o desenvolvimento de inflamação intestinal causada pelas cepas estudadas. Finalmente, com relação aos mecanismos efetores, observamos que linfócitos B e T CD8+ controlam a sobrevivência após infecção. Por outro lado, a ativação da enzima óxido nítrico sintase induzida foi um fator importante para controle do número de cistos cerebrais em camundongos infectados com cepas do Tipo I/III. Esses achados são relevantes para o melhor entendimento dos mecanismos imunológicos envolvidos na proteção e patogênese durante infecção com *T. gondii*.

Palavras-chave: cepas de *Toxoplasma gondii*, imunidade inata, imunidade adquirida, TLR e MHC.

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