Int. J. Exp. Path. (2007), 88, 325–335
doi: 10.1111/j.1365-2613.2007.00534.x

ORIGINAL ARTICLE

BALB/c mice resistant to *Toxoplasma gondii* infection proved to be highly susceptible when previously infected with *Myocoptes musculinus* fur mites

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INTERNATIONAL JOURNAL OF EXPERIMENTAL PATHOLOGY

Received for publication:
2 January 2007
Accepted for publication:
22 March 2007

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Summary

The immune response induced by *Toxoplasma gondii* is characterized by Th1 immune mechanisms. We previously demonstrated that C57BL/6 mice infested with *Myocoptes musculinus* and infected with *T. gondii* by intraperitoneal route undergo accelerated mortality according to Th2 immune mechanisms induced by the acarian. To evaluate whether infection with *M. musculinus* influences *T. gondii*-induced Th1 response in a resistant mouse lineage, BALB/c, which develops latent chronic toxoplasmosis in a way similar to that observed in immunocompetent humans, this study was done. The animals were infected with *T. gondii* ME-49 strain 1 month after *M. musculinus* infestation, being the survival and the immune response monitored. The double-infected displayed higher mortality rate if compared with the mono-infected mice. In addition, infection with *M. musculinus* changed the *T. gondii*-specific immune response, converting BALB/c host to a susceptible phenotype. Spleen cells had increased the levels of IL-4 in double-infected mice. This alteration was associated with severe pneumonia, encephalitis and wasting condition. In addition, a higher tissue parasitism was observed in double-infected animals. It can be concluded that infection with these two contrasting parasites, *M. musculinus* and *T. gondii*, may convert an immunocompetent host into a susceptible one, and such a host will develop severe toxoplasmosis.

Keywords
BALB/c mice, coinfection, *Myocoptes musculinus*, *Toxoplasma gondii*

The Infestation of mice with mites is relatively common in research laboratories, where mice are kept under conventional conditions (Jungmann *et al.* 1996a; Matsuda *et al.* 1997). Mite-associated ulcerative dermatitis (MAUD) has been described in C57BL/6 mice and has been associated with *Myobia musculi* infestation (Dawson *et al.* 1986). A related disease in BALB/c mice is caused by infestation with the mite *Myocoptes musculinus*, and it is accompanied by a T-helper-2 (Th-2) type response (Jungmann *et al.* 1996b) and allergic manifestations, such as erythematous and

pruritic skin lesions, widespread hair loss, lymphadenopathy, lymphocytopenia, granulocytosis and wasting, in addition to high plasma levels of specific-immunoglobulin E (IgE) (Jungmann *et al.* 1996a,b). This condition is similar to scabies in humans, and is associated with high plasma levels of total immunoglobulin E (IgE) and IgG, as well as eosinophilia and elevated IL-4 production (Leung 2000; Walton *et al.* 2004).

Toxoplasma gondii infection is asymptomatic in most adult animals and humans due to the protective immunity developed by competent hosts (Frenkel 1988). In cases of immunodeficiency or during congenital infection, toxoplasmosis may emerge as a severe disease, which can cause host death (Denkers & Gazzinelli 1998).

Murine models of *T. gondii* infection have highlighted the important role performed by certain cytokines, such as interleukin-12 (IL-12), tumour necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), as well as by the generation of reactive nitrogen intermediates (RNI) in the host resistance to early infection (Denkers & Gazzinelli 1998). After *T. gondii* infection, mice from virtually all strains develop a strong Th1 immune response, regardless of the fact that they have MHC haplotypes associated with susceptibility or resistance to toxoplasmosis (Gazzinelli *et al.* 1991, 1992). The resistance phenomenon in both acute and chronic infection with *T. gondii* in the murine model is highly dependent on endogenous IFN-γ (Suzuki *et al.* 1988, 2000; Scharton-Kersten *et al.* 1996; Denkers & Gazzinelli 1998). In the murine model of toxoplasmosis, C57BL/6 are considered more susceptible to *T. gondii* infection than BALB/c mice when inoculated with either the type I (C56) or the type II (ME-49) strain of the parasite (Suzuki *et al.* 1995, 2000). Both C57BL/6 (H-2^b), and CBA (H-2^k) mice are genetically susceptible to the development of toxoplasmic encephalitis (TE) (Suzuki *et al.* 1991, 1994; Brown *et al.* 1995). In contrast, genetically resistant strains [e.g. BALB/c (H-2^d) mice] are able to control *T. gondii* infection in their brains, and they develop a latent chronic infection, as in the case of immunocompetent humans (Suzuki *et al.* 1991, 1994; Brown *et al.* 1995).

Considering that murine models greatly contribute to elucidating the immune mechanisms involved in *T. gondii* infection and the widespread prevalence of coinfections among human and animal hosts, there is growing interest in evaluating the influence of coinfection in the course of experimental toxoplasmosis. The interest is still greater when the coinfection is determined by an organism that triggers an immune response opposite to the Th1 type one induced by *T. gondii*, since type 1 and type 2 cytokines present cross-regulatory properties (Abbas *et al.* 1996). The influence of the Th2 immune response in proinflammatory immune mechanisms induced by *T. gondii* has been consistently studied in the susceptible lineage of mouse, C57BL/6 (Marshall *et al.* 1999; Araujo *et al.* 2001; Liesenfeld *et al.* 2004) and in all of the experimental procedures, the Th2 immune response altered the Th1 response induced by the protozoan. Concerning to this matter, recently, we have observed that C57BL/6 mice that had been previously infested with *M. musculinus* develop more severe manifestations of *T. gondii* infection than mono-infected mice (Welter *et al.* 2006). Related to BALB/c, in contrast to C57BL/6 mice, the Th2 immune response was not modified by the Th1 response induced by *T. gondii* infection (Liesenfeld *et al.* 2004).

To investigate whether the interference of the immune response in *T. gondii* infection is restricted to susceptible mice or whether it is extended to resistant animal strains, in this work, we have evaluated the outcome of toxoplasmosis in BALB/c mice that had been previously infested with *M. musculinus*. The infestation itself induced the production of Th2 cytokines, and coinfection with *T. gondii* was followed by accelerated mortality rates and high tissue protozoan burden, contrasting with what was observed for the BALB/c mice infected with *T. gondii* only. These results indicate that mice from a *T. gondii*-resistant lineage turned out to be highly susceptible hosts when previously infested with *M. musculinus*.

Materials and methods

Animals

Females BALB/c (H-2^d) and Swiss (outbred) mice aged 8–12 weeks were bred as homozygotes and kept in the Bioterism Center of the Animal Experimentation Laboratory, Biomedical Sciences Institute, Federal University of Uberlândia, Brazil. The mice were housed under standard laboratory conditions. Animal experiments were conducted according to institutional guidelines for animal ethics.

Parasites and antigen preparations

The low-virulent ME-49 strain of *T. gondii* was maintained by intraperitoneal inoculation of the Swiss mice with brain homogenate. *Myocoptes musculinus* was obtained from and maintained in the Swiss mice infested by this ectoparasite in an isolated clean room with air filtration.

For *T. gondii* soluble antigen (STAg) preparation, tachyzoites of the RH strain were harvested from the peritoneal cavities of the Swiss mice that had been injected with 10^7 organisms 2 days earlier. The tachyzoites were sonicated

and centrifuged, and the supernatant was collected and prepared as previously described (Gazzinelli *et al.* 1991).

For *M. musculinus* soluble antigen (SMAg) preparation, furs from infested mice were cut down and skin-scrapings were taken and placed in Petri dishes. The scrapings and furs were examined under stereomicroscope, and living mites of different stages were collected with a fine needle, placed in PBS and stored at −20 °C. The preparation of SMAg was carried out as described in (Kumar *et al.* 1998), with some modifications. After centrifugation of the collected material, the pellet was suspended in 5 mM borate buffered saline (pH 8.0) containing protease inhibitors, triturated in liquid nitrogen for mite disruption and extracted overnight at 4 °C under gentle shaking. After centrifugation at 45,000 × ***g*** for 30 min at 4 °C, the supernatant was concentrated and the protein concentration was determined.

Experimental procedure and tissue processing

Myocoptes musculinus infestations were accomplished by keeping the infested Swiss mice together with BALB/c mice. The infestation was monitored by periodically, taking skin scrapes, which were then examined under a microscope, as well as by clinical examination of the animals. BALB/c mice infested or not with *M. musculinus* 1 month earlier, were infected with 10 *T. gondii* cysts by intraperitoneal route, and groups of three mice were killed by cervical dislocation on days 0, 7, 14, 21 and 30 postinfection (p.i.). Blood samples were collected for serological analysis. Tissue samples, such as the central nervous system (CNS), lung, liver and spleen, were collected, fixed in 10% buffered formalin and processed routinely for paraffin embedding and sectioning. Tissue sections with 4 μm thickness (40 μm distance between sections) of each mouse were obtained in microtome and mounted on slides for histopathological and immunocytochemical studies. To verify the histological changes, tissue sections were stained with haematoxylin and eosin. In addition, the tissue sections were stained by the immunoperoxidase method for identification and quantification of tissue parasitism.

Quantification of tissue parasitism by immunocytochemistry

Tissue parasitism was quantified by immunocytochemistry, as described previously (Silva *et al.* 2002a). Deparaffinized sections were incubated for 30 min at 37 °C in 1% BSA and then overnight at 4 °C with polyclonal rabbit antibody, against antigens of the *T. gondii* ME-49 strain. Alternatively, tissue sections were also incubated with non-immune rabbit serum, which was used as negative control. After incubation with biotinylated sheep anti-rabbit antibodies, the sensitivity was improved with avidin–biotin complex (ABC kit, PK-4000; Vector Laboratories, Inc., Burlingame, CA, USA). The reaction was developed with H_2O_2 plus 3,3′-diaminobenzidine tetrahydrochloride (Amresco, Solon, OH, USA) for 5 min, and the slides were examined under optical microscopy.

Tissue parasitism was scored by counting the number of parasitophorous vacuoles per section in the CNS, or from 40 microscopic fields in the lung, liver and spleen (1 × 400), from two histological sections of each mouse and from three mice per group.

Blood eosinophil numbers and serum T. gondii or M. musculinus-specific IgE levels

Blood smears were air–dried on glass slides, stained by May Grunwald Giemsa (Merck, Darmstadt, Germany), and analysed for numbers of eosinophils. The relative numbers of eosinophils were expressed as percentage of white cells (eosinophils/100 white cells), and at least 300 cells were counted by blood smears.

Immunoglobulin E antibodies to *T. gondii* or *M. musculinus* were also measured by using an ELISA. Plates were coated with *T. gondii* (10 μg/ml) or *M. musculinus* (20 μg/ml) soluble antigens and subsequently incubated with serum samples diluted 1:2. Specific IgE antibodies were detected by incubation with biotinylated goat anti-mouse IgE (Caltag, Burlingame, CA, USA), followed by streptavidin-peroxidase (Sigma Co., St Louis, MO, USA). The reactions were revealed by adding the enzyme substrate consisting of 0.03% H_2O_2 and 0.01 M ABTS in 0.07 M citrate-phosphate buffer (pH 4.2).

Antibody levels were expressed as ELISA index (EI) as previously described (Ferro *et al.* 2002), and according to the following formula: EI = mean optical density (OD) of the sample/cutoff, where the cut off is calculated as the OD mean values of negative control sera plus three standard deviations. EI values > 1.0 were considered positive.

Cytokine quantification

BALB/c mice infested or not with *M. musculinus* and infected with *T. gondii* were killed on the 0th, 7th, 14th, and 21st days p.i. and their spleens were aseptically removed. The control groups consisted of one group of non-infected mice and another group infested with *M. musculinus* alone (30 days of *M. musculinus* infestation that corresponds to day zero of *T. gondii* infection). Cell suspensions were

washed in RPMI medium, and erythrocytes were eliminated by hypotonic shock, then washed three times and adjusted to 5×10^6 cells/ml in RPMI medium supplemented by 10% heat-inactivated foetal calf serum. Cell suspensions were added (200 μl/well) to a 96-well tissue culture plate and incubated at 37 °C in 5% CO_2 in the presence or absence of STAg (5 μg/ml) or concanavalin A (con A) (5 μg/ml) (Sigma). Cell-free supernatants were harvested and assayed for IFN-γ, following 72-h culture; IL-4, IL-5, and IL-10, following 48-h culture.

Interleukin-4, IL-5, IL-10 and IFN-γ were quantified by using an ELISA kit according to instructions from the manufacturer (BD PharMingen, San Diego, CA, USA, for IL-4, IL-5 and IL-10; and PeProtech, Veracruz, México, for IFN-γ). Cytokines levels were calculated by comparison with a standard curve constructed with recombinant murine cytokines [all from PharMingen except for recombinant IFN-γ (PeProtech)]. The detection sensitivity in these immunoassays was 200 pg/ml (IFN-γ), 7 pg/ml (IL-4), 15 pg/ml (IL-5) and 30 pg/ml (IL-10).

ELISA for measurement of T. gondii or M. musculinus-specific IgG1 and IgG2a antibodies

Plates were coated with *T. gondii* (10 μg/ml) or *M. musculinus* (20 μg/ml) soluble antigens and incubated with 1:10 dilutions of serum samples from individual animals. Peroxidase-labelled goat IgG anti-mouse IgG1 or IgG2a were subsequently added to the plates. The assay was developed by adding the enzyme substrate consisting of 0.03% H_2O_2 and 1,2-phenylendiamin (OPD; Merck, Schuchardt, Germany), and the OD was determined at 492 nm (Titertek Multiskan; Flow Laboratories, McLean, VA, USA). The antibody levels were arbitrarily expressed as EI as previously described. EI values >1.0 were considered positive.

Statistical analysis

The Kaplan–Meier method was used to compare the survival rates of the studied groups, as described by Fletcher *et al.* (1996). The survival curves were compared using logrank and chi-square tests that generate a two-tail *P*-value, to test the null hypothesis that they are identical in the overall groups of animals. Antibody and cytokine levels, as well as tissue parasitism and the numbers of eosinophils produced by different groups of animals, were compared by using Mann–Whitney *U* or Kruskal Wallis *H* tests, when appropriate. Statistical analyses and graphs were performed using GraphPad prism version 4.0 (GraphPad Software, San Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Myocoptes musculinus infestation turned out BALB/c mice highly susceptible to T. gondii infection

BALB/c mice, known as a lineage that develops a chronic activation of Th2 lymphocytes in response to infestation with *M. musculinus*, became highly susceptible to *T. gondii* infection. Mice infested with *M. musculinus* only displayed progressive weight loss, achieving 18.3% on day 30 after infestation, in addition to erythematous and pruritic skin lesions as well as widespread fur loss. Weight values for adults started at 24 g and declined progressively to 11 g at the final phases of mange disease that occurred at around 13–15 months of age. Despite the wasting condition, BALB/c mice infested with *M. musculinus* only, presented a 100% survival rate during all experimental observation period (data not shown).

BALB/c mice, a commonly *T. gondii*-resistant lineage, presented a 100% survival rate at least 95 days after the *T. gondii* infection alone. However, when BALB/c mice were previously infested with *M. musculinus* and 30 days after were infected with *T. gondii*, these animals became highly susceptible to infection with the protozoan ($\chi^2 = 10.87$; $P = 0.001$; d.f. = 1), presenting 80% mortality 28 days post-*T. gondii* inoculation (Figure 1a). Additionally, double-infection worsened weight loss comparing with animals mono-infested with *M. musculinus* alone.

Concomitant M. musculinus infestation and T. gondii infection causes higher tissue parasitism and pathology if compared with mono-infected animals

In *T. gondii* mono-infected mice, major histopathological changes were observed in the lung on days 7 and 14, and in the CNS on day 21 p.i. In BALB/c double-infected mice, the major histopathological alterations were observed in the skin, lung and CNS, which presented more severe lesions.

The skin of *M. musculinus*-infested mice revealed an increased number of dermal infiltrates of mononuclear and polymorphonuclear leukocytes. In some areas, demarcated regions of epithelial ulcerations were observed (Figure 1b). Furthermore, cross-sections of parasites could be identified in the keratin layers of some animals.

The lung of *T. gondii*-mono-infected mice presented interstitial pneumonia with mononuclear inflammatory infiltration in the alveolar septa. When coinfected, a severe mononuclear inflammatory infiltrate (Figure 1c) was observed in addition to a large number of polymorphonuclear cells. In some animals the granular leukocytes were

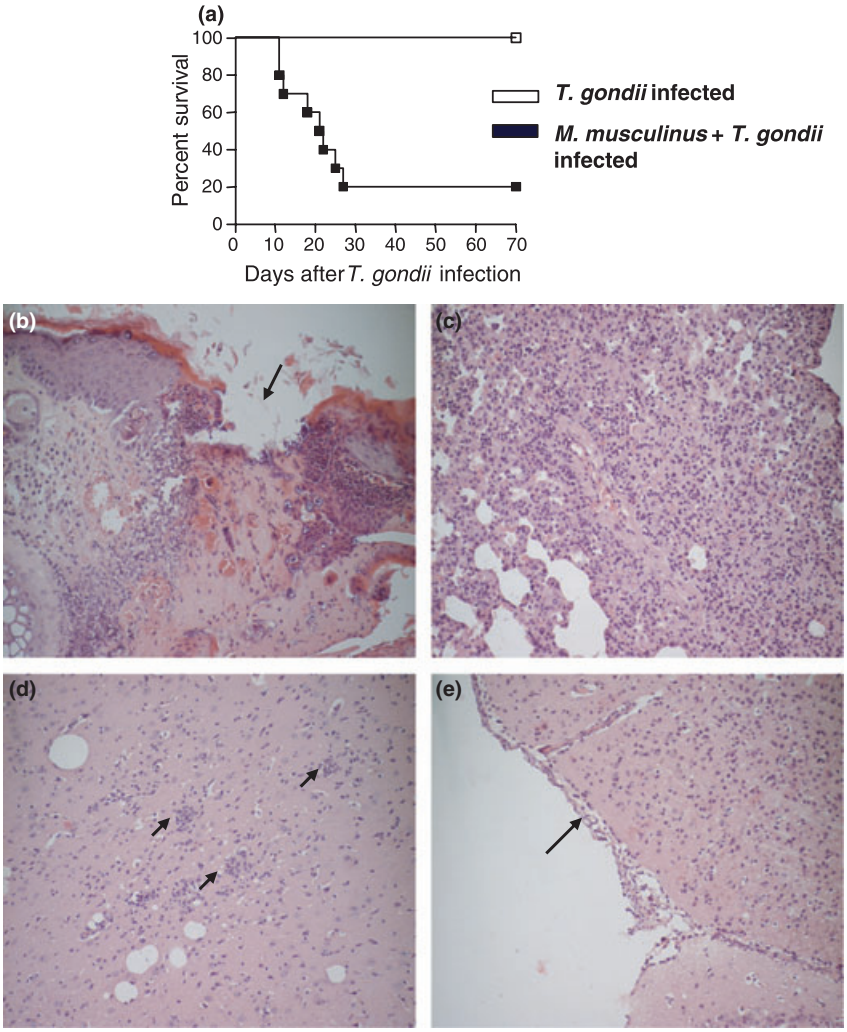

Figure 1 Mortality rates and histological changes visualized by H&E staining in organs of BALB/c mice infected with *Toxoplasma gondii* plus *Myocoptes musculinus* or *T. gondii* alone. The survival of BALB/c mice (a) pre-infested with *M. musculinus* 30 days earlier and then infected by intraperitoneal route with 10 cysts of the ME-49 strain of *T. gondii* were monitored daily (10 animals per experimental group). The survival of coinfected mice was significantly reduced ($\chi^2 = 10.87$; $P = 0.001$; d.f. = 1) whether compared with mono-infected animals. Data are representative of at least two independent experiments that provided similar results. Skin sections from coinfected animals (day 15 of *T. gondii* infection) had an increased number of dermal infiltrates of inflammatory cells and an epithelial ulceration (arrow) (b). In the pulmonary tissue (day 15 of *T. gondii* infection), a severe mononuclear inflammatory infiltrate was seen in the septa (c). In the CNS on day 21 of *T. gondii* infection, glial nodules (arrows) (d) and infiltrates of inflammatory cells in the meninges (arrow) (e) were observed. Original magnification, ×20.

associated with necrotic areas, leading to a focal suppurative interstitial inflammation. In parallel with the gravity of inflammatory changes, double-infected mice were more susceptible to higher *T. gondii* burden in the lung, mainly on day 7 p.i. (Figure 2a).

In the CNS, the lesions were characterized by diffuse infiltrates of mononuclear cells, glial nodules (Figure 1d), vascular cuffing by lymphocytes, and focal infiltrates of inflammatory cells in the meninges (Figure 1e) on day 21 p.i.. Also in the CNS, pre-infestation with *M. musculinus* contributed to higher tissue parasitism by *T. gondii* whether compared with mono-infected mice (Figure 2b).

Myocoptes musculinus infestation interferes with T. gondii-specific IgG1 and IgG2a isotypes

In serum samples from both mono and double-infected mice, *T. gondii*-specific IgG1 and IgG2a antibodies were detected after the 14th and 7th day p.i., respectively, showing an increasing seropositivity profile (Figure 3a,b). *T. gondii*-specific IgG1 and IgG2a levels were higher in mono- than in double-infected mice on day 30 p.i. (Mann–Whitney $U = 0.0$; $P = 0.0022$; d.f. = 2) (Figures 3a,b).

The detection of *M. musculinus*-specific IgG1 and IgG2a antibodies revealed a significant production of both isotypes after 30 days of infestation by the mite alone (day 0 of *T. gondii* infection) and IgG1 levels were not affected by *T. gondii* infection (Figure 3c). The highest level of *M. musculinus*-specific IgG2a was observed on day 14 after *T. gondii* infection (Figure 3d).

Production of cytokine by spleen cells from mono- or double-infected mice

To verify the influence of *M. musculinus* infestation in the Th1 immune response normally induced by toxoplasmosis,

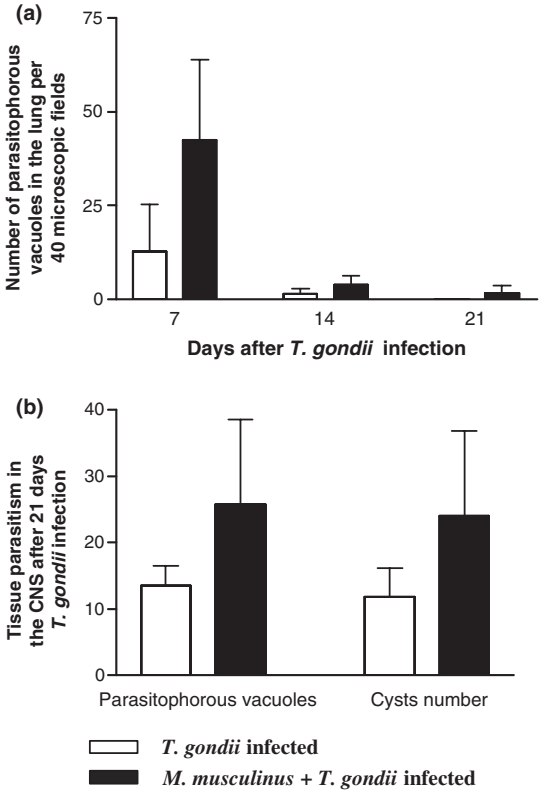

Figure 2 Parasite load in the lung and CNS of BALB/c mice infected with *Toxoplasma gondii* alone or coinfected with *Myocoptes musculinus*. Tissue parasitism was detected by immunocytochemistry staining and scored by counting the number of parasitophorous vacuoles and cysts per section in the CNS and in 40 microscopic fields in the lung. The coinfected animals were more susceptible to higher parasite burden in the lung (a) and in the CNS (b) whether compared with mono-infected animals. Similar results were obtained in two independent experiments and data are expressed as mean and standard deviation.

the cytokine production was measured in the supernatant of spleen cells from mono- and double-infected mice. *T. gondii* infection induced rising IFN-γ levels from spleen cells stimulated *in vitro* with STAg until 21 days p.i. (Figure 4b). After *in vitro* stimulation with Con A, similar IFN-γ concentrations were obtained (Figure 4a). When coinfected animals were analysed, the levels of IFN-γ production were lower on day 14 p.i. (Figure 4a, b).

Type 2 cytokines, IL-4, IL-5 and IL-10 in single and double-infections were also analysed (Figure 4c–f). Important IL-4 production in the supernatant of spleen cells stimulated with Con A was observed in mono-infected mice (day 0 of *T. gondii* infection) (Figure 4c). However, consistently with the Th1 response induced by *T. gondii* when this mouse lineage was infected with the protozoan, a significant drop in the production of this cytokine was verified, particularly on day 21 p.i. (Kruskal–Wallis test, $H = 9.667$; $P = 0.0216$; d.f. = 2) (Figure 4c). Furthermore, when mice were coinfected, *M. musculinus* induced significantly higher levels of IL-4 production on day 14 p.i. than those found in animals infected with *T. gondii* alone (Kruskal–Wallis test, $H = 10.38$; $P = 0.0156$; d.f. = 2).

Concerning IL-5, *M. musculinus* infestation for 30 days induced significant levels of this cytokine (day 0 of *T. gondii* infection). In addition, double-infection also induced increased levels of this cytokine (Figure 4f). Stimulation with STAg did not induce detectable IL-4 and IL-5 levels in either mono- or double-infected mice (data not shown), showing that the production of these type 2 cytokines was probably induced by the acarian and not by the protozoan.

Interleukin-10 was detected in both mono- and double-infected mice, showing that the double-infection did not alter IL-10 production (Figure 4d,e). *T. gondii* infection induced higher IL-10 levels in spleen cells on day 7 p.i., in mono- and double-infected mice under STAg stimulation (Figure 4e).

Our results showed that *M. musculinus* infestation decreased IFN-γ production on day 14 after *T. gondii* infection, which corresponds to the peak of IL-4 levels. Conversely, *M. musculinus* infestation significantly increased the levels of IL-4 and IL-5 cytokines after infection with the protozoan, probably impairing the effective mechanisms that control *T. gondii* replication.

Myocoptes musculinus *infestation increases markers of Th2 response*

Blood eosinophilia was also analysed under the influence of *M. musculinus* infestation. As shown in Table 1, BALB/c mice infested 30 days earlier with *M. musculinus* only (day 0 of *T. gondii* infection) showed a significantly larger number of blood eosinophils when compared with non-infested mice (Mann–Whitney $U = 4.0$; $P = 0.0140$; d.f. = 2). Coinfected animals, however, presented a remarkable reduction in the number of blood eosinophils when compared with mice that were infested with the acarian only. Animals that were mono-infected with *T. gondii* only showed a small number of eosinophils throughout the period of observation. Thus, despite being disturbed by *M. musculinus* infestation, the immune response induced by *T. gondii* interfered with the number of this cell phenotype.

Toxoplasma gondii-specific IgE antibodies were analysed during *T. gondii* infection in mono- or double-infected animals. The IgE isotype was detected in low levels starting from day 21 after infection with the protozoan, and there

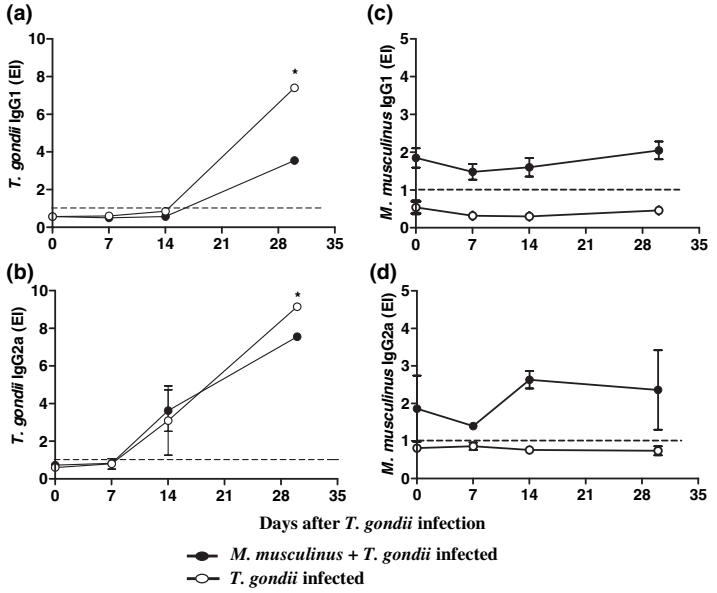

Figure 3 Kinetics of *Toxoplasma gondii*- or *Myocoptes musculinus*-specific IgG1 and IgG2a isotypes in serum samples from mono- or double-infected BALB/c mice. The animals were experimentally infested or not with *M. musculinus* 30 days prior infection with 10 cysts of the *T. gondii* ME-49 strain. On days 0, 7, 14 and 30 after *T. gondii* infection, serum levels of *T. gondii*-specific IgG1 (a), IgG2a (b), or *M. musculinus*-specific IgG1 (c) and IgG2a (d) were measured by ELISA. Mice infected with *T. gondii* alone presented significantly higher specific IgG1 and IgG2a levels on day 30 after *T. gondii* inoculation if compared with coinfected mice. Results are expressed as ELISA index (EI), where values of EI > 1.0 (horizontal dashed line) were considered positive. Data represent the mean and standard deviation obtained from three mice per each time point and are representative of at least two independent experiments that provided similar results. *Significantly different from values obtained for double-infected mice (Mann–Whitney $U = 0.0$; $P = 0.0022$; d.f. $= 2$).

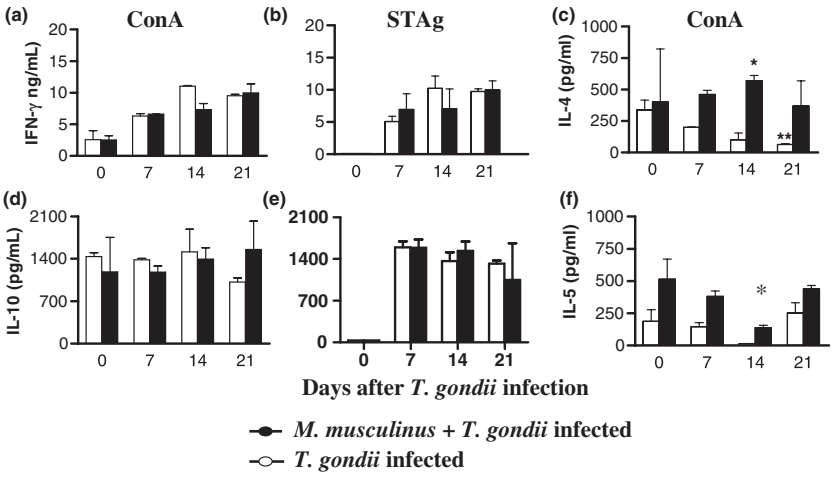

Figure 4 Concentration of interferon-γ (IFN-γ), interleukin (IL)-4, IL-5 and, IL-10 in the supernatants of spleen cells taken from BALB/c mice mono- or double-infected with *Myocoptes musculinus* and *Toxoplasma gondii*. Spleen cells from mice infested or not with *M. musculinus* 30 days prior infection with 10 cysts of *T. gondii* were harvested on days 0, 7, 14 and 21 after *T. gondii* infection and cultured in the presence of soluble tachyzoite antigen (STAg; 5 μg/ml) (b,e) or Concanavalin A (Con A; 5 μg/ml) (a,c,d,f). Supernatants were collected and assayed by ELISA. Data represent the mean and standard deviation of three mice per time point and are representative of at least two independent experiments that provided similar results. *Significantly different from values obtained for animals mono-infected with *T. gondii* (Kruskal–Wallis test, $H = 10.38$; $P = 0.0156$; d.f. $= 2$). **Significantly different from values obtained for non-infected mice (Kruskal–Wallis test, $H = 9.667$; $P = 0.0216$; d.f. $= 2$).

Table 1 Number of eosinophils in peripheral blood smears of mono- or double infected BALB/c mice[a]

	Number of eosinophils/100 white cells[b]			
	Day 0	Day 7	Day 14	Day 21
Naive	0.6 ± 0.3	–	–	–
Mono-infected with *T. gondii*	–	0.8 ± 0.7	0.8 ± 0.9	0.7 ± 0.3
Mono-infected with *M. musculinus*	2.5 ± 0.6*	–	–	–
Double infected with *M. musculinus* and*T. gondii*	–	1.2 ± 0.2	1.0 ± 0.5	1.4 ± 0.3

[a]The animals were infested or not with *M. musculinus* 30 days prior infection with 10 cysts of *T. gondii*. On days 0, 7, 14, and 21 after *T. gondii* infection, blood was obtained and blood smears were prepared and analysed following May Grünwald staining.
[b]A total of 300 white cells were counted per mouse at a magnification of ×400 and the number of eosinophils per 100 white cells was determined. Data represent the mean and standard deviation from four mice per group and are representative of at least two independent experiments that provided similar results.
*Significantly different from values obtained for mono-infected mice with *T. gondii* (Mann–Whitney $U = 4.0$; $P = 0.0140$; d.f. = 2).

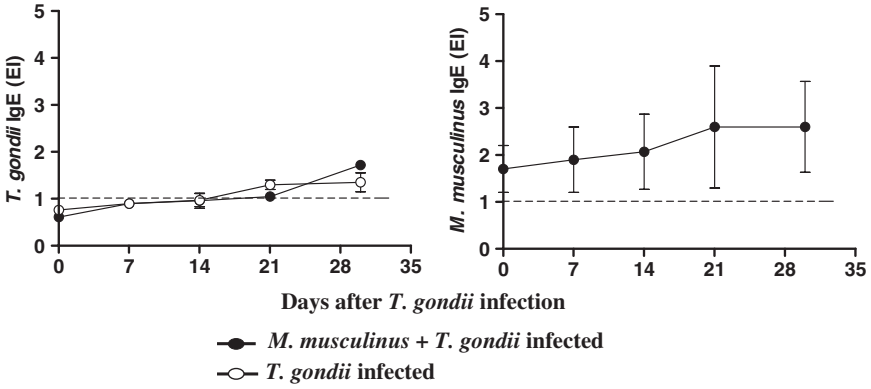

Figure 5 Kinetics of *Toxoplasma gondii*- or *Myocoptes musculinus*-specific IgE isotype in serum samples from mono- or double-infected BALB/c mice. The animals were experimentally infested or not with *M. musculinus* 30 days prior infection with 10 cysts of the *T. gondii* ME-49 strain. On days 0, 7, 14 and 30 after *T. gondii* infection, serum levels of *T. gondii*-specific IgE (a) or *M. musculinus*-specific IgE (b) were measured by ELISA. Results are expressed as ELISA index (EI), where values of EI > 1.0 (horizontal dashed line) were considered positive. Data represent the mean and standard deviation obtained from three mice per each time point and are representative of at least two independent experiments that provided similar results.

was no difference in the levels of this isotype between mono- and double-infected mice (Figure 5a).

To verify whether *T. gondii* infection interferes with the levels of *M. musculinus*-specific IgE, we measured the levels of this isotype during *T. gondii* infection. Mice infested with *M. musculinus* showed increases in a typical Th2 marker, elevated serum IgE levels (Figure 5b) that were not significantly affected by the subsequent *T. gondii* infection. In addition, *M. musculinus*-specific IgE levels were higher than *T. gondii*-specific IgE levels.

Discussion

In the present study, we have shown that BALB/c mice, a lineage previously resistant to *T. gondii* infection, turned out to be highly susceptible to this protozoan when pre-infested with the acarian parasite *M. musculinus*. Accelerated death and high rates of parasite burden in the lung and CNS were observed in BALB/c mice infested with *M. musculinus* and infected with *T. gondii*. Thus, animals with mange disease have their immune response altered, and it worsened the outcome of *T. gondii* infection. Disease due to *M. musculinus* started 1 month after the acarian infestation, and it was characterized by reddening of the skin, pruritus, ruffled fur, hair loss and progressive weight loss. Our own observations support the role of the parasite itself as a primary cause of lesions. As previously reported, the fact that the mites do not invade the tissue also supports a hypersensitive reaction to parasite-derived antigens, which may enter the body through skin lesions (Jungmann *et al.* 1996a). The early

mortality observed in double-infected mice is probably due to the major pathological findings detected mainly in the pulmonary tissue and in the CNS as a result of the *T. gondii* infection, in addition to high parasite burden in these organs, associated with a wasting syndrome induced by the acarian infestation. According to our previous investigations using C57BL/6 mice (Silva *et al.* 2002a,b), the lung and CNS are important sites of *T. gondii* replication and morbidity in the acute and chronic phases of infection, respectively. In the present study, however, we have observed that this condition is worsened when BALB/c mice have their Th1 response impaired because of the previous infection with *M. musculinus*. In contrast with the induction of Th2-like immune mechanisms in BALB/c and C57BL/6 mice by *M. musculinus* (Jungmann *et al.* 1996b; Welter *et al.* 2006), a dominant Th1 immune response was induced in *T. gondii* infection (Denkers & Gazzinelli 1998). In previous studies using BALB/c-background IFN-γ-deficient (IFN-$\gamma^{-/-}$) mice, which are highly susceptible to *T. gondii* infection, it had been demonstrated that the protective activity to control the protozoan is mediated by non-cytolitic and IFN-γ dependent Th1 mechanisms in the CNS (Wang *et al.* 2004).

We have therefore hypothesized that the Th2 immune response developed during mite infestation could be detrimental to the resistant host to control *T. gondii* infection. In our experimental design, we observed that the immune mechanisms induced by *M. musculinus* slightly alter the levels of IFN-γ production, and lower levels of this cytokine were observed only on day 14 after *T. gondii* infection. In contrast, throughout the observation period, increased IL-4 levels were observed in the supernatants of spleen cells following stimulation with Con A in coinfected animals. The increase in IL-4 concentrations appeared to be *M. musculinus*-specific, since *in vitro* stimulation of spleen cells with STAg did not result in the production of this cytokine. In addition, these results were associated with increased eosinophilia and considerable production of specific IgE against *M. musculinus*, which is not changed due to *T. gondii* inoculation in coinfected animals. These data suggest that the production of Th2 cytokines induced by *M. musculinus* infestation in BALB/c mice could negatively interfere with the effector mechanisms that control *T. gondii* replication.

In vitro experiments have shown a crucial role for both IFN-γ and TNF-α in the induction of RNI and microbicidal activity displayed by murine macrophages against tachyzoites (Langermans *et al.* 1992). It is well known that IL-4 antagonizes the macrophage-activating effects of IFN-γ, thus inhibiting cell-mediated immune reaction (Gajewski & Fitch 1988). Therefore, the higher levels of IL-4 found in supernatants of spleen cells from coinfected BALB/c mice stimulated with Con A, suggest that these IL-4 levels *in vivo* could be impairing the macrophage activation state.

When we analysed *T. gondii*-specific IgG isotypes, we observed that the coinfected mice had lower *T. gondii*-specific IgG1 and IgG2a antibody levels than the mono-infected mice. Since released tachyzoites can be effectively killed, at least in part, through antibody-mediated phagocytosis by macrophages (Anderson *et al.* 1976), the lower antibody levels could be contributing to the higher parasite burden verified in coinfected mice. On the other hand, *T. gondii* infection did not alter the levels of *M. musculinus*-specific IgG isotypes.

Considering the high prevalence of *T. gondii* infection (Dubey 2004) and ectoparasite infestation (Walton *et al.* 2004) in many countries and that *M. musculinus* and *T. gondii* could infect hosts simultaneously, we investigated the influence of coinfection in the immune response of a *T. gondii*-resistant mouse lineage. Immunocompetent humans are usually resistant to the development of TE, the same happening to genetically resistant mouse strains. Thus, the results of the present study raised the hypothesis that if coinfection with these two unrelated parasites occurs naturally in humans, the outcome of this dual infection could impair control of the *T. gondii* infection in these hosts, in a similar way to the condition described herein for BALB/c mice.

It was previously demonstrated that the downregulation of established Th2 response by *T. gondii* infection was dependent on the genetic background of the mice. In this context, the Th2 immune response induced by previous infection with *Nippostrongylus brasiliensis* is modified by *T. gondii* infection in C57BL/6 but not in BALB/c mice (Liesenfeld *et al.* 2004). In contrast, in our present investigation using a resistant lineage of mouse to *T. gondii* infection the acarian itself was able to impair the Th1 immune response normally induced by *T. gondii*. In other coinfection experimental models using BALB/c mice, infection with *T. gondii* before infection with *Leishmania major* protected the *L. major* susceptible BALB/c mice (Santiago *et al.* 1999). Infection with *T. gondii* posterior infection with *Helicobacter felis* induced mucosal IFN-γ and IL-12 elevated levels and IL-10 substantially reduced becoming BALB/c mice susceptible to *T. gondii* infection associated with severe mucosal inflammation (Stoicov *et al.* 2004). In our present work, we demonstrated for the first time that the immune response to the acarian *M. musculinus* interferes with the Th1 response normally induced by the protozoan *T. gondii* in a resistant mouse lineage.

In conclusion, the results presented herein suggest that the immune response induced by the mange interferes with the type-1 immune response induced by *T. gondii* and with the effector mechanisms that control protozoan burden. Furthermore, we demonstrated that these alterations are detrimental

to a host that is normally known to be resistant to this parasite.

Acknowledgements

This work was supported by Conselho Nacional de Pesquisa Científica e Tecnológica (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). We thank Dr Ana Maria Aparecida Guaraldo, Laboratory of Parasitology from the University of Campinas, who helped us with her entomological expertise.

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