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ORIGINAL PAPER

Modulation of immunity in mice with latent toxoplasmosis—the experimental support for the immunosuppression hypothesis of *Toxoplasma*-induced changes in reproduction of mice and humans

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Abstract The immunosuppression hypothesis suggests that the increased sex ratio in mice and women with latent toxoplasmosis, retarded embryonic growth in the early phases of pregnancy, prolonged pregnancy of *Toxoplasma*-infected women, and increased prevalence of toxoplasmosis in mothers of children with Down syndrome can be explained by the presumed immunosuppressive effects of latent toxoplasmosis. Here, we searched for indices of immunosuppression in mice experimentally infected with *Toxoplasma gondii*. Our results showed that mice in the early phase of latent infection exhibited temporarily increased production of interleukin (IL)-12 and decreased production of IL-10. In accordance with the immunosuppression hypothesis, the mice showed decreased production of IL-2 and nitric oxide and decreased proliferation reaction (synthesis of DNA) in the mixed lymphocyte culture in the early and also in the late phases of latent toxoplasmosis. Since about 30% of the world population are latently infected by *T. gondii*, the toxoplasmosis-associated immunosuppression might have serious public health consequences.

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Introduction

Toxoplasma gondii (Apicomplexa) is one of the most common parasitic protozoa in humans. The prevalence of *Toxoplasma* infection varies mostly from 20% to 80% in different territories (Tenter et al. 2000). The main source of infection for humans is the consumption of raw or undercooked meat of an intermediate host (particularly pig, sheep, and rabbit) harboring tissue cysts and foods or water contaminated with soil containing oocysts excreted by cats (Tenter et al. 2000; Beatie 1982). Acquired toxoplasmosis progresses in two clinical phases. The initial phase of infection, acute toxoplasmosis, is elicited by the tachyzoites as the predominant stage of *T. gondii* and can cause more or less serious clinical symptoms and, in pregnant woman, transplacental infection in the fetus. In immunocompetent subjects, acute toxoplasmosis spontaneously turns into the second stage, i.e., latent toxoplasmosis. Latent toxoplasmosis is clinically asymptomatic, but usually life-long infection, characterized by the presence of *Toxoplasma* bradyzoite cysts, typically in the nervous and muscular tissues, and by lifelong protective (both humoral and cellular) immunity to reinfection, manifested by the presence of low levels of anti-*Toxoplasma* IgG in the serum of infected individuals. There is no risk of transplacental infection from pregnant women with latent toxoplasmosis to the fetuses.

Latent toxoplasmosis is known to influence human pregnancy. Pregnant women with latent toxoplasmosis have been reported to have seemingly younger fetuses at pregnancy week 16 (Flegr et al. 2005; Kaňková and Flegr 2007). Two different immunological hypotheses were suggested to explain this effect of toxoplasmosis on pregnancy. The first one assumes that the changes in the immune system could delay the implantation of the blastocyst in multiparous women with toxoplasmosis. The other hypothesis is that *Toxoplasma* could weaken or switch off the mechanism of spontaneous abortion, normally responsible for the removal of embryos with developmental defects (and also with a slower fetal growth rate). The latter hypothesis was recently supported by the observed increase in the secondary sex ratio in children of women with latent toxoplasmosis (Kaňková et al. 2007a). The probability of the birth of a boy increased up to 0.71, which means that about 250 boys were born for every 100 girls, in women with moderate concentrations of anti-*Toxoplasma* antibodies (and therefore probably with recent but already latent infection). This effect of latent toxoplasmosis was later confirmed in experimental infection of mice (Kaňková et al. 2007b). Mice with toxoplasmosis produced a higher sex ratio (expressed as the proportion of males in all offspring) than controls, in the early phase of latent infection. The authors suggested that the increased sex ratio both in *Toxoplasma*-infected mice and humans might be just a nonadaptive side effect of *Toxoplasma*-induced immunosuppression—protection of more immunogenic male embryos against selective abortion.

Many studies, including several reviews, have covered the changes in the immune system associated with acute toxoplasmosis (Remington and Krahenbuhl 1982; Alexander and Hunter 1998; Blader and Saeij 2009; Costa da Silva and Langoni 2009). In contrast to acute toxoplasmosis, there is a lack of information about immunomodulatory and especially immunosuppressive effects of *Toxoplasma* infection in the latent phase. It is known that in the latent phase of toxoplasmosis, *Toxoplasma*-infected cells can be killed by CD8+ T lymphocytes and activated macrophages and the pivotal role of interferon (IFN)-γ producers is adopted by CD4+ T cells (Alexander et al. 2000). In mice infected with an avirulent *Toxoplasma* strain, the interleukin (IL)-10 level increased after the acute phase of infection and correlated with susceptibility to reinfection with a different *T. gondii* strain (Brandao et al. 2009). Ulku et al. (2008) have found significantly higher nitric oxide (NO) levels in humans with latent toxoplasmosis compared with seronegative controls and Dzitko et al. (2008) have found increased prevalence of latent toxoplasmosis in women with an aberrant level of a strong immunomodulator, the prolactin.

To confirm the critical premise of the immunosuppression hypotheses of latent toxoplasmosis-based embryonic growth retardation, sex ratio shift, and decrease in the stringency of quality control of embryos, we searched for indices of immunosuppression in mice with latent toxoplasmosis by monitoring the production of NO and various cytokines, as well as by testing the proliferative activity of stimulated spleen cells.

Materials and methods

Experimental animals and infection

In the experiment, a total of 80 BALB/c female mice at the age of 5–6 weeks (mean weight 17.5 g) were used. One half of the mice were orally infected with brain homogenate from mice infected with a relatively avirulent cystogenic strain HIF of *T. gondii* (Kodym et al. 2002). To eliminate a possible confounding effect of body weight, pairs of mice of the same weight were selected first, and then one animal of each pair was included in the "infected" group and the other was placed in the control group. Each mouse of the "infected" group was given orally 75 μl of brain homogenate containing approximately ten tissue cysts. The controls were given the same amount of isotonic saline (0.8% NaCl). The mice were maintained in groups of five.

The course of the acute infection was monitored by visual inspection and regular measurement of body weight. The symptoms of acute toxoplasmosis (i.e., lethargy and ruffled fur) were observed on days 6–9. The collection of samples for immunological analysis began after the end of acute toxoplasmosis, i.e., 2 months after the infection (Kodym et al. 2002). The mice were regularly weighed (at least twice a week) during the entire experiment and always before blood sample collection.

The mice were divided into four groups of 20 (10 infected animals and 10 controls). Blood and macrophage samples were collected 9, 11, 13, and 15 weeks after the infection. Approximately 0.5–1 ml of blood was obtained from the tail vein of both infected and control mice. The collection of peritoneal macrophages was performed in Narcotan-anesthetized mice. At the end of the experiment (17 weeks after the infection), the mice were killed and the spleens of eight infected and six control mice were removed and homogenized for in vitro tests.

The efficiency of the experimental *Toxoplasma* infection in mice was confirmed by the complement fixation test for detection of specific anti-*Toxoplasma* antibodies (Ondriska et al. 2003), and mice with undetected or very low concentrations of specific antibodies were excluded from the analysis. The final set of experimental animals included 73 mice, i.e., 35 infected females and 38 control females.

Cytokine production and determination

Peritoneal macrophages obtained by washing the peritoneal cavity with 10 ml of cell culture medium (RPMI 1640 with 10% fetal calf serum (FCS)) were cultivated at a concentration of 1×10^6 cells/ml in a volume of 0.4 ml of RPMI 1640 medium (Sigma, St. Louis, MO, USA) containing 10% FCS (Sigma), antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin), 10 mM HEPES buffer, and 5×10^{-5} M 2-mercaptoethanol (thereafter called as complete RPMI 1640 medium) for 48 h. Macrophages were either unstimulated or stimulated with 1.5 μg/ml of bacterial lipopolysaccharide (LPS, Difco Laboratories, Detroit, MI, USA) and 200 U/ml of mouse IFN-γ (PeproTech, Rocky Hill, NJ, USA). The cell culture supernatants were collected after a 48-h incubation period for IL-10, IL-12, and NO determination. The macrophages were tested only in groups 1 and 4, i.e., 9 and 15 weeks after the infection.

The concentrations of IL-12 were also analyzed in the serum. Given the limitation by the amount of blood samples obtained and other technical obstacles, the concentrations of some cytokines were determined only in some groups.

The spleens from eight infected and six control mice were homogenized and the isolated spleen cells were incubated at a concentration of 0.5×10^6 cells/ml either unstimulated or stimulated with Concanavalin A (Con A, Sigma) (1.5 μg/ml), or 0.75×10^6 spleen cells/ml were stimulated with irradiated 1×10^6/ml spleen cells from C57BL/10 mice. The cell supernatants were harvested after 24 h/48 h (IL-2 determination), 48 h/72 h (IFN-γ determination), and 72 h/96 h (IL-4 and IL-10 determination) of incubation.

The presence of cytokines in the supernatants (and sera) was measured by enzyme-linked immunosorbent assay (ELISA) using sets of cytokine-specific capture and detection monoclonal antibodies (mAb) purchased from PharMingen (San Diego, CA, USA) (IL-2, IFN-γ, IL-4, and IL-10) and from R&D Systems (Minneapolis, MN, USA) (IL-12). For the quantification of cytokine levels, standards for IL-2, IFN-γ, IL-4, IL-6, IL-10, and IL-12 were included in all ELISA determinations.

Nitrite assay

The nitrite concentrations were measured using the Griess reaction (Green et al. 1982). Nitrite was quantified by spectrophotometry at 540 nm using sodium nitrite as a standard.

Cell proliferation analysis

Spleen cells from controlled and infected mice were cultured in the complete RPMI 1640 medium at a concentration of 0.75×10^6 cells/ml alone or were stimulated with Con A (1.5 μg/ml) or LPS (1.5 μg/ml), or at a concentration of 1.5×10^6 cells/ml with irradiated spleen cells from C57BL/10 mice (1×10^6 cells/ml) in a final volume of 200 μl of culture medium in 96-well plates (Corning Co., Corning, NY, USA). To determine cell proliferation, 0.5 μCi/well of ^{3}H-thymidine (Nuclear Research Institute, Rez, Czech Republic) was added to the cultures for the last 6 h of the 72-h cultivation period (Con A or LPS stimulation) or of the 96-h incubation period. The radioactivity incorporated in the spleen cells was measured using a Tri-Carb 2900TR scintillation counter (Packard, Meridien, CT, USA).

Statistical analysis

The data were statistically analyzed using the program Statistica® 6.0. Five infected mice without detectable level of anti-*Toxoplasma* antibodies and two control females for which a sufficient amount of sera was not available were excluded from the analysis.

Since the levels of interleukin, NO, and other immune markers were not normally distributed even after log-transformation, nonparametric Kendall correlation with variables TOXO (positive/negative) and level of interleukin (or other immune markers) as factors was used to assess the influence of toxoplasmosis on individual interleukin levels both in the supernatants from spleen cells (blood) and in the macrophages. To control for the confounding effect of time from the infection, the partial Kendall tau correlation test was used in all analyses (Siegel and Castelan 1988; Sheskin 2003); the spreadsheet for the computation of the partial Kendall tau correlation test is available at http://natur.cuni.cz/flegr/programy.php.

Results

Difference in immunological reactivity between infected and control mice

The partial Kendall correlation (with time from the infection as a covariate) showed that latent toxoplasmosis strongly affects the immune system of infected mice from 9 to 15 weeks after the infection.

Latent toxoplasmosis significantly increased the level of serum IL-12. While the median concentration of IL-12 in the serum of control mice was 8.6 pg/ml, serum from infected mice contained 670 pg/ml ($n=52$, tau=0.695, $z=7.271$, $P<0.001$). Since IL-12 is produced mainly by macrophages and other antigen-presenting cells, we determined IL-12 production by nonstimulated and LPS-stimulated peritoneal macrophages from control and infected mice. As demonstrated in Fig. 1, unstimulated macrophages from infected mice produced spontaneously significantly

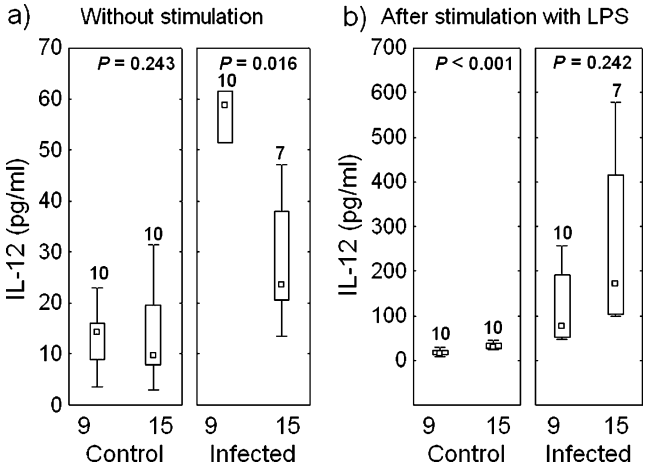

Fig. 1 Differences in IL-12 levels **a** in supernatants from non-stimulated macrophages and **b** in supernatants from LPS-stimulated macrophages between *Toxoplasma*-infected and control mice at 9 and 15 weeks after infection (*x* axis). The *y* axis shows the level of IL-12 in control (*left part of each panel*) and *Toxoplasma*-infected (*right part of each panel*) mice 9 and 15 weeks after infection. The *boxes* and *spreads* show median and 25–75% range, respectively. The *numbers above the boxes* show the number of mice in a particular category

more IL-12 than macrophages from control mice (n=37, tau=0.617, z=5.372, P<0.001), and this difference was even more profound after stimulation with LPS (n=37, tau=0.750, z=6.534, P<0.001).

On the contrary to IL-12, both unstimulated (n=37, tau=−0.387, z=−3.375, P<0.001) and stimulated (n=37, tau=−0.441, z=−3.845, P<0.001) macrophages from infected mice produced significantly less IL-10 than macrophages from infected mice (Fig. 2). Similarly, production of IL-6 by unstimulated macrophages from infected mice was significantly decreased (n=17, tau=−0.508, z=−2.846, P=0.004). The negative influence of toxoplasmosis on the level of IL-6 after stimulation with LPS approached the level of significance (n=17, tau=−0.339, z=−1.901, P=0.057; median 666 pg/ml in controls and 633 pg/ml in infected mice).

There was no evidence of a significant influence of latent toxoplasmosis on the level of NO produced by nonstimulated macrophages (n=37, tau=0.128, z=0.117, P=0.264); however, macrophages of *Toxoplasma*-positive mice produced less NO after stimulation with LPS than macrophages from control mice (n=37, tau=−0.286, z=−2.487, P=0.013) (Fig. 3).

The analysis of the reactivity of spleen cells included 14 mice (eight infected animals and six controls). Splenocytes from the infected mice produced significantly more IFN-γ (n=14, tau=0.726, z=3.618, P=0.003) (Fig. 4a) and significantly less IL-4 (n=14, tau=−0.424, z=−2.111, P=0.035) after stimulation with Con A (Fig. 4b). After stimulation with irradiated allogeneic cells, the splenocytes from *Toxoplasma*-infected mice produced significantly less IL-2 (n=14, tau=−0.428, z=−2.134, P=0.033) (Fig. 4c) and incorporated significantly less radioactive thymidine, i.e., they had a decreased proliferative response (n=14, tau=−0.454, z=−2.261, P=0.024) (Fig. 4d) than splenocytes from control mice. The concentrations of other monitored cytokines (IL-6, IL-10, and IFN-γ) in supernatants after stimulation with irradiated allogeneic splenocytes and proliferation of unstimulated Con A or LPS-stimulated splenocytes did not significantly differ between infected and control mice.

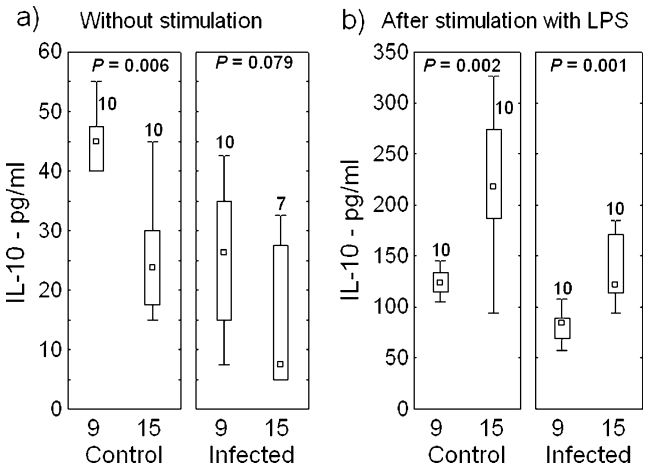

Fig. 2 Differences in IL-10 levels **a** in supernatants from non-stimulated macrophages and **b** in supernatants from LPS-stimulated macrophages between *Toxoplasma*-infected and control mice at 9 and 15 weeks after infection (*x* axis). The *y* axis shows the level of IL-10 in control (*left part of each panel*) and *Toxoplasma*-infected (*right part of each panel*) mice at 9 and 15 weeks after infection. The *boxes* and *spreads* show median and 25–75% range, respectively. The *numbers above the boxes* show the number of mice in a particular category

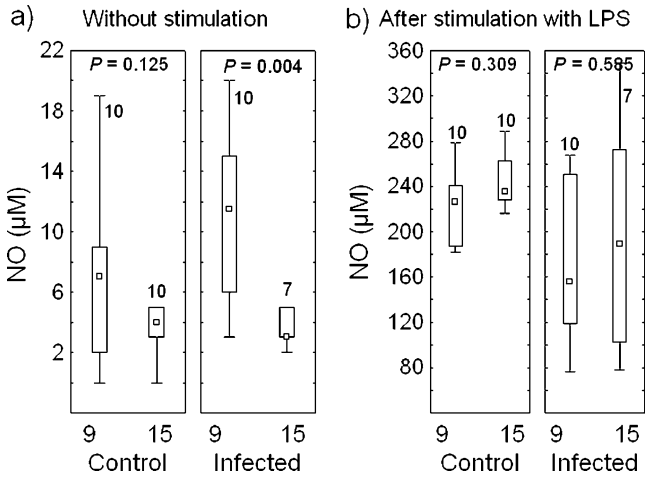

Fig. 3 Differences in NO levels **a** in supernatants from nonstimulated macrophages and **b** in supernatants from LPS-stimulated macrophages between *Toxoplasma*-infected and control mice at 9 and 15 weeks after infection (*x* axis). The *y* axis shows the level of NO in control (*left part of each panel*) and *Toxoplasma*-infected (*right part of each panel*) mice at 9 and 15 weeks after infection. The *boxes* and *spreads* show median and 25–75% range, respectively. The *numbers above the boxes* show the number of mice in a particular category

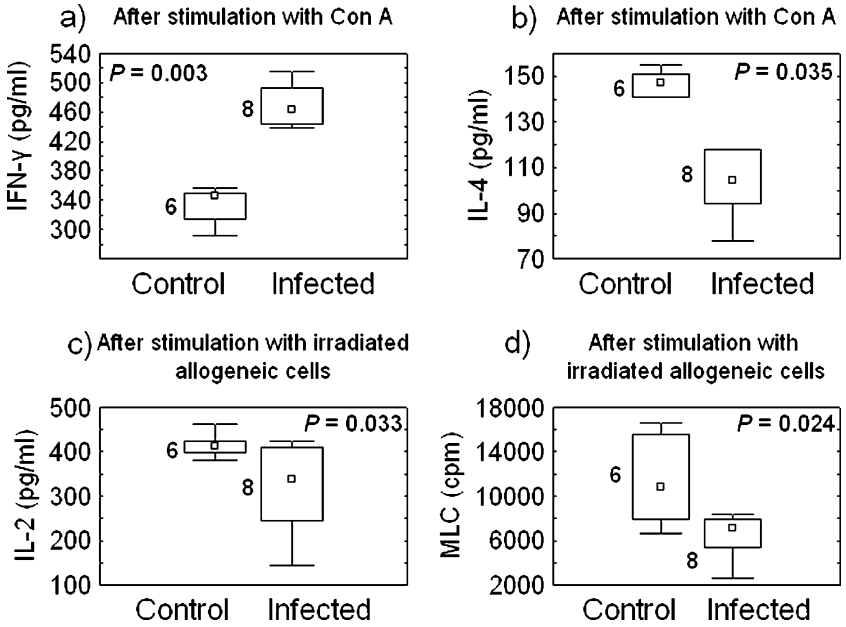

Fig. 4 The analysis of the reactivity of spleen cells from control and infected mice that were killed 17 weeks after the infection. **a** Production of IFN-γ after stimulation with Con A, **b** production of IL-4 after stimulation with the Con A, **c** production of IL-2 after stimulation with irradiated allogeneic cells, **d** cell proliferation in MLC after stimulation with irradiated allogeneic cells determined by ^{3}H-thymidine incorporation, between *Toxoplasma*-infected and *Toxoplasma*-free mice. *Toxoplasma*-free mice are in the *left part* and *Toxoplasma*-infected mice are in the *right part of each panel*. The *boxes* and *spreads* show median and 25–75% range, respectively. The *numbers above the boxes* show the number of mice in a particular category

Difference between immunoreactivity of infected mice in the 9th and 15th weeks of the study

In further analyses, we studied the differences in the levels of interleukins and NO produced by macrophages of infected mice in two stages of latent infection: 9 weeks after the infection (n=10) and 15 weeks after the infection (n=7). The binary variable STAGE (either 9 weeks or 15 weeks after the infection) was used as an independent variable and the levels of the NO, IL-12, or IL-10 served as dependent variables in separate analyses. The macrophages of infected mice produced significantly higher levels of NO (n=17, tau=−0.519, z=−2.908, P=0.004) (Fig. 3) and IL-12 (n=17, tau=−0.429, z=−2.404, P=0.016) (Fig. 1) without stimulation and significantly lower levels of IL-10 after stimulation with LPS (n=17, tau=0.567, z=3.175, P=0.002) (Fig. 2) at 9 weeks than at 15 weeks after the infection. There were no significant differences in other immunological markers (NO after LPS stimulation, IL-12 after LPS stimulation, or IL-10 without stimulation).

Difference between immunoreactivity of control mice in the 9th and 15th weeks of the study

The same analyses were repeated for the control group of mice. We studied the differences in the levels of interleukins and NO produced by macrophages in control mice at the same two age stages as in the infected mice: 9 weeks after the infection (n=10) and 15 weeks after the infection (n=10). The younger control mice had significantly higher IL-10 levels without stimulation (n=20, tau=−0.444, z=−2.735, P=0.003) (Fig. 2) and after LPS stimulation (n=20, tau=0.5032, z=3.102, P=0.001) (Fig. 2) and significantly lower levels of IL-12 after stimulation with LPS (n=20, tau=0.564, z=3.474, P<0.001) (Fig. 1) than older control mice. There were no significant differences in other immunological markers (NO production after LPS stimulation and IL-12 production without stimulation) between younger and older control mice. The influence of the animal's age on the NO production without stimulation approached the level of significance (n=20, tau=−0.2486, z=−1.532, P=0.063) (Fig. 3), with younger control mice producing higher NO levels without stimulation.

Discussion

The results showed that during latent toxoplasmosis, significant modifications of cytokine production and modulation of some parameters of the immune response occurred. The most remarkable were the changes in the in vitro production and in the in vivo serum levels of IL-12. The mice infected 15 weeks earlier with no clinical signs of disease had significantly increased levels of serum IL-12. Since IL-12 is produced by antigen-presenting cells, we measured the production of IL-12 by macrophages

obtained from the peritoneal cavity of control and infected mice. We observed that macrophages from infected mice spontaneously produced more IL-12 than those from control mice, and the difference was more pronounced after macrophage stimulation with LPS.

Macrophages from the infected mice produced significantly less IL-12 at 15 weeks than at 9 weeks after infection; however, they still produced more IL-12 than uninfected controls. The decrease in the IL-12 production in the later phases of the infection was associated with the increase in the production of IL-10 that has a suppressive effect on the immune system and with the overall decrease in the immunological reactivity. The comparison of the immunoreactivity of the control (uninfected) and infected mice at 9 and 15 weeks after the infection, however, showed that most of the observed effects of length of infection, generally the decrease in the immunoreactivity between the 9th and 15th weeks, were actually the effects of animal age.

After the stimulation with irradiated allogeneic cells, the splenocytes from infected mice produced less IL-2 than those from controls and their proliferative response after stimulation with alloantigens was significantly lower in comparison with the cells from controls (uninfected mice). It can be concluded that despite the indices of certain immunostimulation (increased production of IL-12 and IFN-gamma), the important components of the effector arm of the immune system (IL-2 production and proliferative activity of splenocytes after stimulation with allogeneic cells) were downregulated in mice during the latent phase of *Toxoplasma* infection. While the production of certain cytokines returned to a normal level between weeks 9 and 15 of infection, the production of NO by LPS-stimulated macrophages and proliferative activity of splenocytes stimulated with allogeneic cells remained suppressed. Therefore, unlike the transient immunostimulation, the observed immunosuppression is probably a specific effect of latent toxoplasmosis rather than a nonspecific carryout effect of the acute phase of the past infection.

The results for the infected mice are in accordance with the hypothesis that the increased probability of birth of male offspring in *Toxoplasma*-infected mice (Kaňková et al. 2007b) and humans (Kaňková et al. 2007a) might be just a nonadaptive side effect of *Toxoplasma*-induced immunosuppression. Similarly, the immunosuppression could also be responsible for the observed longer pregnancy of mothers with latent toxoplasmosis (Kaňková and Flegr 2007), either due to reduced implantation potential of the fertilized ovum in immunosuppressed females (Krackow 1995) or due to higher probability of survival of fetuses with genetic or developmental variations (Flegr et al. 2005; Kaňková and Flegr 2007) including chromosomal aberrations (which can also explain the extremely high prevalence (about 84%) of latent toxoplasmosis in mothers of children with Down syndrome (Hostomská et al. 1957)). It can only be speculated whether the observed immunosuppression could be also responsible for the strong effect of latent toxoplasmosis on weight gain during pregnancy in Rh-negative women (Kaňková et al. 2010). Our results, namely, the decreased production of NO by stimulated macrophages and decreased production of IL-2 and IL-4 and a lower proliferative activity of splenocytes suggest that the immunosuppression might play an important role in the observed physiological effects of latent toxoplasmosis.

Between 20% and 80% of the population in various countries have life-long "asymptomatic" latent toxoplasmosis. The toxoplasmosis-associated immunosuppression might influence the risk and course of various infectious and autoimmune diseases and therefore might have serious public health consequences.

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