Virulent *Toxoplasma gondii* strain RH promotes T-cell-independent overproduction of proinflammatory cytokines IL12 and γ-interferon


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The aim of this study was the analysis of the cytokine response in BALB/c mice infected with the highly virulent RH or the weakly virulent Beverley strains of *Toxoplasma gondii*. Analysis of cytokine messages showed increased expression of IL12, IFN-γ and TNF-α, but not IL4 mRNAs in spleen cells after infection with the *T. gondii* strains RH and Beverley. High levels of circulating IL12 and IFN-γ were detected in the serum of mice infected with strain RH, although TNF-α levels remained low. In contrast, the same cytokines were detected at only low levels in the serum of mice infected with the Beverley strain. Administration of antibody against IL12 or IFN-γ significantly delayed time to death of mice infected with strain RH compared to controls. T-Cell-deficient as well as normal mice were equally infected by strain RH, suggesting that T lymphocytes do not contribute to the response. Depletion of natural killer cells from the splenocyte population abolished the *in vitro* production of IFN-γ. Together, our data suggest that the virulent strain RH induces in BALB/c mice a type 1 cytokine pattern with T-cell-independent overproduction of IL12 and IFN-γ that may be involved in the pathogenesis of this micro-organism.

**INTRODUCTION**

In mice, the current paradigm is that CD4+ T cells can be separated into subsets on the basis of their cytokine production, and that the distinct cytokine profile produced by these cells determines their function. This model includes two major CD4+ subsets: Th1 cells produce IL2, IL12 and IFN-γ, and control the production of IgG2a antibodies, whereas Th2 cells produce IL4, IL5 and IL10, and control the production of IgG1 and IgE (Fiorentino *et al.*, 1989; Mosmann *et al.*, 1986). These subsets cross-regulate each other. Which subset predominates may determine the outcome of an infection (Afonso *et al.*, 1994).

The weakly virulent Beverley (Bev) strain of *Toxoplasma gondii* promotes a T-helper cell type 1 response by inducing persistent expression of IFN-γ and IL12 (Gazzinelli *et al.*, 1991, 1992, 1993a, 1994; Nguyen *et al.*, 1998; Yap *et al.*, 2000) and triggers high levels of IgG2a in serum (Nguyen *et al.*, 1998; Burke *et al.*, 1994; Suzuki *et al.*, 1996; Villard *et al.*, 1995). IFN-γ has been shown to play a major role both in acquired immunity to acute infection, and in control of parasite growth in chronically infected animals (Gazzinelli *et al.*, 1991, 1996; Suzuki *et al.*, 1989). More recently, gene knockout mice have established the importance of type 1 cytokines, such as IFN-γ, IL12 and TNF-α, in the control of experimental toxoplasmosis. Absence of any one of these proinflammatory mediators results in increased mortality during infection as a result of uncontrolled tachyzoite growth (Gazzinelli *et al.*, 1994; Sher *et al.*, 1993). Similarly, in *Leishmania* infection, a Th1 response, characterized by the production of proinflammatory cytokines, is protective, whereas a Th2 response with secretion of anti-inflammatory cytokines is pathogenic (Scott *et al.*, 1988; Scott, 1991).

Abbreviations: i.p., intraperitoneal(ly); NK, natural killer; p.i., post-infection; SCID, severe combined immunodeficiency.
Despite the protective role of type 1 cytokines during experimental T. gondii infection, it is nevertheless well known that overproduction of these same factors can also underlie host pathology (Araujo et al., 2001). For example, cerebral malaria is thought to be mediated by parasite-induced TNF-α (Grau et al., 1987). More recently, it has been shown that TNFRp55-deficient mice die after infection with Mycobacterium avium due to high levels of IL12 (Ehlers et al., 2000).

There are few studies on the immune response of mice infected with T. gondii strain RH. Inoculation with tachyzoites of this strain kills all inoculated mice in 3–7 days (Hisaeda et al., 1997; Nguyen et al., 1996). In vitro, splenic adherent cells produce IFN-γ and low levels of TNF-α when exposed to either live tachyzoites of T. gondii or a soluble parasite extract (Sher et al., 1993). Wille et al. (2001) recently reported that IL10 is not required for the pathogenesis of strain RH.

In the present study, we wanted to determine which cytokines are produced after BALB/c mouse infection with strain RH. We also compared the expression and production of IFN-γ, IL12, TNF-α and IL4, and isotypic pattern of IgG antibodies in mice infected with strain RH in comparison with mice infected with the weakly virulent T. gondii strain BEV.

**METHODS**

**Experimental infection with T. gondii.** Female BALB/c and BALB/c SCID (severe combined immunodeficiency) mice were bred in isolators at the Ludwig Institute for Cancer Research (Brussels, Belgium) and were used for experiments at the age of 8–10 weeks. Their microbiological status was described previously (Coutelier et al., 1986a). Six- to eight-week-old female NMRI (Naval Medical Research Institute) mice were used for maintenance of strain RH. Mice were obtained from the animal facility of the Catholic University of Louvain, Brussels, Belgium. Sera were tested by ELISA (Nguyen et al., 1996) to confirm the absence of anti-T. gondii antibodies before the experiments. The weakly virulent T. gondii strain BEV was isolated by Beverley (1959) and kindly provided by G. Desmonts from the Institut de Pédiatrie, Paris, France, in 1977. Cysts of strain BEV were obtained from chronically infected female NMRI and BALB/c mice, which were killed with ether overdose, and their brains were removed and homogenized in 3 ml sterile PBS (pH 7.2). For each brain suspension, the mean number of cysts from 10 samples (10 µl each) was then determined by light microscopy under ×10 magnification. After appropriate dilution in PBS, each mouse was inoculated intraperitoneally (i.p.) with 20 cysts. The virulent strain of T. gondii, strain RH, was maintained by continuous passage in female NMRI mice, injected i.p. with about 104 tachyzoites in 0.5 ml PBS (Nguyen et al., 1996). The research project was approved by the local ethical review committee.

**Preparation of samples.** Blood samples were collected on EDTA or heparin (under light ether anaesthesia) by cardiac puncture or from the tail vein (Nguyen et al., 1996). Plasma was stored at −80 °C until assay. Because of the short survival of the infected host (7 days) after inoculation of the strain RH, the mice were treated with Eusaprim (GlaxoWellcome) immediately after infection: this treatment was continued for 30 days. For practical reasons, the drugs were added to the drinking water at the following concentrations: 800 µg sulfamethoxazole ml⁻¹ and 160 µg trimethoprim ml⁻¹ (Nguyen & Stadtsbaeder, 1983).

**Cell preparation.** Spleens were removed aseptically and cell suspensions were prepared in culture medium as described previously (Gazzinelli et al., 1996; Sher et al., 1993). Briefly, 2×10⁶ cells were distributed into 96-well microtitre plates and brought to a final volume of 0.2 ml medium with the different culture additions. Escherichia coli LPS (E. coli 026 : B6) was obtained from Difco and used at 100 µg ml⁻¹. Stimulation with T. gondii strain RH was performed with 10⁷ tachyzoites ml⁻¹. After incubation for 6 h at 37 °C in a 5% CO₂ atmosphere, 50 µl supernatant was removed and assayed for TNF-α in ELISA assay. IFN-γ and IL12 were measured in 48 h supernatants of spleen cells. In some experiments, the spleen donors were injected i.p. 24 h before use with 300 µl rabbit anti-asialo-GM1 polyclonal antibody, as described previously (Markine-Goriaynoff et al., 2002).

**Treatment with anti-cytokine antibodies.** Rat mAb to E. coli β-galactosidase (GL117; rat IgG2a, courtesy of G. Abrams, DNAX, Palo Alto, CA, USA) served as an isotype control and anti-IFN-γ neutralizing mAb (F3; rat IgG2a, prepared and purified as previously described by Heremans et al. (1990)) was administered at the dose of 1 mg on days 5 and 7 post-infection (p.i.). Anti-IL12 neutralizing mAb (C17.8; rat IgG2a, cell line kindly provided by G. Trinchieri, Wistar Institute, Philadelphia, PA, USA) was administered at the dose of 1 mg on day 5, then 0.5 mg on days 8 and 10 p.i.

**ELISA.** Subclasses of anti-T. gondii antibodies were assayed by ELISA in microplates (Immu-no plate Maxisorp P96; Nunc). Wells were coated by overnight incubation at 4 °C with 100 µl of a lysate of T. gondii (6.5 µg protein ml⁻¹ in PBS) (Nguyen et al., 1998). The plates were washed three times in PBS, and saturated with 5% (v/v) fetal calf serum (Gibco) in PBS for 15 min. Then, 100 µl mouse plasma, serially diluted in PBS containing 0.5% (v/v) Tween 20 (PBS-T) were added and incubated for 30 min. After three washings in PBS, 100 µl rabbit anti-mouse IgG subclass antibody, labelled with peroxidase (Serotec), diluted 1 : 1000 in PBS-T, were added and incubated for 30 min. The plates were washed again three times before addition of 100 µl chromogen solution (27 g tetramethylbenzidine 1⁻¹, 0.1 ml hydrogen peroxide 3⁻¹; Sorin Biomedica). The reaction was stopped with 100 µl 0.5 M H₂SO₄. The absorbance of each sample was read at 450 nm with a Sorin spectrophotometer. Results, expressed in µg ml⁻¹, were calculated from standard curves obtained after incubation of selected anti-DNP mAbs on DNP-coated plates (Coutelier et al., 1986b).

**Cytokine assays.** IL12 and TNF-α plasma levels were determined by sandwich ELISA. Rat anti-mouse IL12 (P40/P70) mAbs and biotinylated rat anti-mouse IL12 (P40/P70) mAbs were obtained from Pharmingen (San Diego, CA, USA). Murine recombinant IL12 (rIL12) was used for the standard curve (Wolf et al., 1991). Rat anti-mouse TNF-α mAbs and recombinant TNF-α were obtained from ICN. Rabbit anti-mouse TNF-α antibodies were obtained from Calbiochem. Briefly, wells of microplates (Immu-no plate Maxisorp P96; Nunc) were coated with capture antibody (5 µg ml⁻¹) in PBS by overnight incubation at 4 °C. The plates were washed with PBS. Wells were saturated with 5% (v/v) fetal calf serum (Gibco) in PBS for 15 min. Serial dilutions of plasma samples and appropriate recombinant standards diluted in PBS-T were added and incubated overnight at 4 °C. Following further PBS washing, the captured cytokine was detected by incubation with either biotinylated rat anti-mouse IL12 (1 µg ml⁻¹) or rabbit anti-mouse TNF-α (9.6 µg ml⁻¹), followed with streptavidin–alkaline phosphatase or alkaline-phosphatase-conjugated rat anti-rabbit immunoglobulin and p-nitrophenyl phosphate (1 µg ml⁻¹) in 0.25 M glycine buffer. The reaction was stopped by adding 30 µl of 3 M NaOH to each well and absorbance was measured at 405 nm. IFN-γ levels were measured by using a mouse specific IFN-γ kit according to the instructions of the manufacturer (Gibco).

**Reverse transcriptase-PCR (RT-PCR).** Unfractionated spleen cells
from BALB/c mice were resuspended in TRIzol (Gibco) and frozen at −80°C. Then, the cells were homogenized and processed for RNA isolation, as described previously (Coutelier et al., 1995). Briefly, after a separation with chloroform and precipitation with 2-propanol as recommended by the manufacturer, cDNA was prepared with Moloney murine leukaemia virus reverse transcriptase (Gibco) and amplified by PCR with a gene Amp kit (Perkin-Elmer Cetus) in a Techne PHC 3 programmable Dri-block (New Brunswick Scientific). Nucleotide sequences of primers for actine, IL12 (p40), IFN-γ, TNF-α, IL4 and experimental conditions were the same as those we described previously (Coutelier et al., 1995; Gazzinelli et al., 1993b; Monteyne et al., 1997).

RESULTS

Mortality, cytokine mRNA expression by spleen cells and production, and IgG subclass distribution of anti-parasite antibody responses during acute infection with T. gondii strains Bev and RH

All mice infected by T. gondii strain Bev survived the acute phase of the infection, whereas all mice inoculated with strain RH died of acute toxoplasmosis up to day 5 p.i. (Fig. 1a). To assess how the immune response differed between mice infected with strain RH or strain Bev, IL12, IFN-γ and TNF-α were measured by ELISA in sera collected from five mice inoculated with either parasite strain. High levels of circulating IL12 and IFN-γ were detected in BALB/c mice infected with strain RH, while the same cytokines were only slightly increased in sera of BALB/c mice infected with strain Bev (Fig. 1b, c). At the same times, circulating TNF-α was detected at low levels in the sera of both groups (Fig. 1d). In addition, splenocytes were collected at five time-points after i.p. infection and the expression of cytokine mRNA was analysed by RT-PCR. Increases in expression of IL12, IFN-γ and TNF-α were detected in spleens as early as 1 day p.i. with strain RH, but only at 2 days p.i. with strain Bev. For both strains, the cytokine gene expression persisted up to 5 days p.i. We could not detect expression of IL4 in mice infected with either of these strains (Fig. 2). Finally, the IgG subclasses of anti-T. gondii antibodies were determined by ELISA in individual mouse plasma at 30 days p.i. Strain Bev induced in BALB/c mice higher levels of IgG2a> IgG2b> IgG3 and IgG1 (Fig. 3). This IgG2a antibody response was significantly higher than in strain RH, which also induced a specific antibody response ranked as IgG2a> IgG2b> IgG3 and IgG1 (Fig. 3; difference between response for Bev and RH: IgG2a, P < 0.0001; IgG1, P = 0.2709; IgG2b, P = 0.3542; IgG3, P = 0.8015).

Proinflammatory cytokine production and role in mortality after infection with T. gondii strain RH

After i.p. infection with 10^2 or 10^6 parasites of strain RH, all mice developed fatal toxoplasmosis. However, mice infected with 10^2 parasites survived longer than mice infected with

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**Fig. 1.** (a) Cumulative mortality in groups of 10 BALB/c mice inoculated with 10^6 virulent T. gondii RH parasites (closed squares) or with 20 cysts of the Beverley strain (open squares) and in control mice (triangles). (b–d) Levels of IL12 (b), IFN-γ (c) and TNF-α (d) in sera obtained at different times post-infection. Cytokine results are shown as means ± SE for groups of five animals.
the sera of mice infected with 10^2 or 10^6 parasites was observed earlier and was marginally increased in the sera of BALB/c mice infected with 10^6 parasites (Fig. 4b, c). At the same time, circulating TNF-α was marginally increased in the sera of both groups (Fig. 6d). These results suggested that proinflammatory cytokine production elicited by infection with RH did not originate from lymphocytes and pointed to natural killer (NK) cells as a possible source for at least some of these molecules.

The ability of T. gondii strain RH to induce in vitro production by spleen cells of IFN-γ, IL12 and TNF-α was then compared to that of the classical immune activator, bacterial LPS. A 10-fold higher TNF-α production was triggered by LPS stimulation than by stimulation with T. gondii strain RH (15·22 ± 2·51 vs 1·22 ± 0·47 ng TNF ml^-1; P < 0·0001 by Student’s t-test). In contrast, levels of IL12 and IFN-γ were not significantly different after stimulation with LPS or with T. gondii strain RH (17·84 ± 2·40 vs 12·39 ± 2·54 ng IFN ml^-1 and 10·11 ± 3·52 vs 8·47 ± 2·66 ng IL12 ml^-1). When this experiment was performed with spleen cells from BALB/c SCID mice, a similar induction of IFN-γ secretion by T. gondii RH parasites was observed (10·24 ± 3·63 ng IFN ml^-1), confirming that lymphocytes were not involved in this phenomenon. The putative role of NK cells in the IFN-γ response was then tested by treating BALB/c SCID mice with one injection of rabbit anti-asialo-GM1 antibody, a procedure previously shown to eliminate NK cells (Markine-Goriaynoff et al., 2002). Spleen cells from such NK cell-depleted mice failed to mount a significant IFN-γ response to strain RH, as compared with infected mice receiving normal rabbit immunoglobulin (0·61 ± 0·21 vs 0·17 ± 0·07 ng ml^-1; P < 0·0001 by Student’s t-test).

**DISCUSSION**

Our data indicate that infection with T. gondii triggers expression and production of proinflammatory cytokines, including IL12 (p40) and IFN-γ, a finding in accordance with previous reports (Burke et al., 1994; Gazzinelli et al., 1994; Yap et al., 2000). It has been demonstrated that these proinflammatory cytokines, and especially IFN-γ, play a major role in the protection of the infected host, since they control tachyzoite growth (Suzuki et al., 1989; Gazzinelli...
et al., 1991, 1994, 1996; Sher et al., 1993). Moreover, this proinflammatory context may lead to a modulation of immune responses, either directed against parasite or unrelated antigens that develop in the host concomitantly with the infection. Since IFN-γ has been shown to be a potent inhibitor of Th2 cell proliferation (Gajewski & Fitch, 1988), T. gondii infection may be expected to result in a preferential differentiation of T helper cells towards the Th1 type. The observation by Gazzinelli et al. (1994) of increased levels of IL4 and IL10 synthesis in anti-IL12-treated mice, supports this hypothesis. In addition, antibodies raised against the parasite predominantly belonged to the IgG2a subclass, an isotype of which production is usually mostly controlled by Th1 cytokines, and especially IFN-γ. This isotypic restriction of anti-T. gondii antibodies that confirms previous reports (Burke et al., 1994; Villard et al., 1995; Suzuki et al., 1996; Nguyen et al., 1998), was maintained up to 325 days p.i. (not shown), and was independent from the mouse strain and the mode of infection. Interestingly, although in addition to this anti-parasite IgG2a antibody response, T. gondii also triggers an IgG2a-restricted polyclonal hypergammaglobulinaemia, this early production of total IgG2a was found to differ from the specific anti-parasite response by its control by IL6 rather than by IFN-γ (Markine-Goriaynoff et al., 2000, 2001).

In contrast to mice infected with T. gondii strain Bev, where all survived, mice infected with the virulent strain RH quickly died. The early mortality of these mice infected with strain RH was paralleled by serum levels of IL12 and IFN-γ higher than in mice infected with strain Bev. Moreover, the time-course of mortality, and the IFN-γ and IL12 serum levels were correlated with the dose of strain RH administered to mice. More importantly, protection of infected mice, although incomplete, was achieved by administration of anti-IL12 or anti-IFN-γ antibodies. These results strongly suggest that cytokine-mediated pathology can occur during disease progression. This is consistent with previous findings showing that IL10-deficient mice die from infection with the weakly virulent T. gondii strain ME49, due to high production of IL12 and IFN-γ (Gazzinelli et al., 1996; Neyer et al., 1997; Suzuki et al., 2000). Administration of anti-IL12 or anti-IFN-γ antibodies in IL10-deficient mice resulted in delayed time to death. Moreover, SCID mice die from infection with strain ME49, which triggers prolonged overproduction of IL12 and IFN-γ (Walker et al., 1997). Histopathological evaluation of infected IL10-deficient mice has indicated that the livers were necrotic. In addition, the highly susceptible C57BL/6 mice die from peroral infection with strain ME49, developing severe necrosis of the small intestine.

Fig. 4. (a) Cumulative mortality in groups of 10 mice inoculated with 10^6 T. gondii RH (closed squares), 10^2 T. gondii RH (open squares) and in control mice (triangles). Levels of IL12 (b), IFN-γ (c) and TNF-α (d) in sera at different times post-infection. Cytokine results are shown as means ± SE for groups of five animals.
and the liver with overproduction of IL12 and IFN-γ. When mice were treated with anti-IFN-γ or anti-IL12 antibodies, the development of the small intestine and liver necrosis was prevented and the time before death was prolonged (Scott et al., 1988; Liesenfeld et al., 1997; Suzuki et al., 2000). Finally, Mordue et al. (2001) reported similar findings of acute toxoplasmosis leading to lethal overproduction of Th1 cytokines, including IL12, IL18 and IFN-γ, by using the virulent strain RH and a different T. gondii strain (PTG), which is of lower virulence. However, they also found an increase of TNF-α, especially in mice submitted to the virulent parasite or to high doses of strain PTG. Moreover, they could prolong the survival of mice with an anti-IL18 antibody, but not with anti-IFN-γ antibody, which contrasted with our results. These differences might be due to the genetic background of the animals, since they used CD1 mice, when we infected BALB/c mice. Similarly, Ehlers et al. (2000) reported that the TNFRp55-deficient mice succumb to infection with Mycobacterium avium due to high levels of IL12, with necrotic foci in the livers. Gram-negative bacterial septicemia, with accompanying endogenous endotoxinaemia induces high levels of IL12, IFN-γ and TNF-α production, contributing much to the pathological

Fig. 5. Effect of anti-IL12 and anti-IFN-γ mAbs on survival of mice infected with T. gondii strain RH. Groups of five BALB/c mice were infected i.p. with 10⁵ virulent RH parasites. Five days later, anti-IL12 antibody, anti-IFN-γ antibody or control anti-β-galactosidase antibody were administered (1 mg anti-IL12 or control antibody on day 5 p.i., followed by 0.5 mg on day 8 and 0.5 mg on day 10; 1 mg anti-IFN-γ antibody on day 5 and 7). Results are expressed as percentage of survival.

Fig. 6. (a) Cumulative mortality in groups of 10 BALB/c (closed squares) and BALB/c SCID (open squares) mice inoculated with 10⁶ virulent RH parasites. Levels of IL12 (b), IFN-γ (c) and TNF-α (d) in sera collected at different times post-infection. Results are shown as means ± SE for groups of 3–5 animals.
findings and lethality observed in experimental toxic shock (Heinzel et al., 1994; Ozmen et al., 1994; Wysocka et al., 1995).

In contrast to IL12 and IFN-γ, TNF-α was not detected in the serum of IL10-deficient mice infected with *T. gondii*, as it did not increase in our experimental infection with the virulent RH strain, indicating that this cytokine is probably not involved in the lethality induced by the parasite.

Two peaks of IFN-γ overproduction were found in mice infected with *T. gondii* strain RH, suggesting that two different cell populations were involved. Because SCID mice produced levels of IL12 and IFN-γ as high as immunocompetent mice, we may postulate that lymphocytes do not play any role in the early overproduction of proinflammatory cytokines induced by *T. gondii* infection. The finding that *T. gondii* strain RH triggers *in vitro* production of IL12 and IFN-γ by spleen cells, including those derived from SCID mice, pointed to a possible role for NK cells. This hypothesis was supported by treatment of mice with anti-asialo-GM1 antibody that eliminated the *in vitro* IFN-γ production subsequently induced by the parasite in their spleen cells, and is in agreement with previous reports by Sher et al. (1993). Thus, inflammatory mediators including IL12 and IFN-γ, but not TNF-α, secreted by cells of the innate immune system, such as NK cells, and required to stop tachyzoite growth and multiplication, and thus to prevent host death from massive parasitaemia, also potentially induce detrimental host pathology when produced at excessive levels.

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