

## Innate Immunity to *Toxoplasma gondii* Is Influenced by Gender and Is Associated with Differences in Interleukin-12 and Gamma Interferon Production

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**Given that differences between the sexes in relative susceptibility to parasitic infections have been noted, this study further elucidates the mechanisms responsible by demonstrating that male SCID mice are more resistant than female mice to infection with *Toxoplasma gondii* and that this difference correlates with enhanced innate immune responses in these animals. Male SCID mice exhibited longer survival times, lower parasite burdens, and less severe pathological changes postinfection. An immunological basis for these differences is demonstrated in that these animals produced interleukin-12 more rapidly and exhibited higher levels of gamma interferon earlier postinfection.**

Gender and sex hormones are known to influence the nature of the immune response (20), and in particular, susceptibility to infections with protozoan parasites has been shown to be determined by sex hormone-influenced immunological mechanisms (1). Previous studies with *Toxoplasma gondii* have shown that mortality rates are higher in females than in males (14), and a hormonal basis for this difference was suggested by studies showing that estrogen treatment of infected male and female gonadectomized animals resulted in increased mortality (13). An immunological basis for these differences has been suggested by more recent studies from our laboratory, where it was shown that male inbred mice produced gamma interferon (IFN- $\gamma$ ) and tumor necrosis factor alpha more rapidly in response to infection than their female counterparts (18). However, given previous studies showing that macrophages and natural killer (NK) cells, in the absence of T lymphocytes, mediate resistance to *T. gondii* infection via an interleukin-12 (IL-12)-, IFN- $\gamma$ -dependent mechanism (9, 12, 21), it is possible that the observed sex differences are due to the modulation of the innate immune response by sex hormones. This possibility is indeed suggested by other studies showing that sex hormones such as estrogen can influence macrophage (7) and NK cell (8) activity and modulate IFN- $\gamma$  levels (11). Therefore, to test this hypothesis, we monitored the course of infection in SCID mice, which are lymphocyte deficient, allowing a clearer analysis of the innate immune response to infection. Specifically, we measured mortality, severity of pathology, and parasite burdens in the brains of male and female SCID mice after oral infection with *T. gondii* tissue cysts and compared the results with innate immune activation as determined by circulating IL-12 and IFN- $\gamma$  plasma levels.

Eight- to ten-week-old C.B-17 *scid/scid* (SCID) mice (*H-2<sup>d</sup>*) were bred and maintained under pathogen-free conditions without the administration of antibiotics. These animals did not possess any circulating plasma immunoglobulins as determined by enzyme-linked immunosorbent assaying. Mice were

infected orally by gavage with 20 cysts (RRA/Beverley strain) as previously described (15, 18). To assess parasite burden and to determine parasite-induced pathology, brains were removed from infected SCID mice and subjected to histopathological analysis as previously described (19). Determination of the number and type of lesions was based on examining two longitudinal sections through brain tissue, and the relative area occupied by the lesions was calculated by Chalkley counting (5). For the determination of innate plasma cytokine levels, animals were bled every 2 days as previously described (22), and IFN- $\gamma$  and IL-12 plasma levels were determined by capture enzyme-linked immunosorbent assaying using appropriate antibody pairs at predetermined concentrations. Rat anti-mouse IFN- $\gamma$  antibodies and recombinant IFN- $\gamma$  for the standard curve were obtained from Cambridge BioScience, Cambridge, United Kingdom. Anti-IL-12 antibodies and recombinant IL-12 were a kind gift from Horst Bluethmann, F. Hoffman-La Roche Ltd., Basel, Switzerland. Briefly, the wells of microtiter plates were coated with capture antibody at 2.0  $\mu$ g/ml (IFN- $\gamma$ ) or 5  $\mu$ g/ml (IL-12 p75) in 20 mM Tris-HCl buffer (pH 9.0) by overnight incubation at 4°C. Wells were then washed with phosphate-buffered saline–0.05% Tween 20 and blocked by adding 1% bovine serum albumin in 0.15 M phosphate-buffered saline–0.05% Tween 20 (1 h, 37°C). Serial dilutions of plasma samples and appropriate recombinant standards were then incubated (triplicate wells) for 2 h at 37°C. Following further washing, captured cytokine was detected by incubation with either biotinylated rat anti-mouse IFN- $\gamma$  (1  $\mu$ g/ml) or peroxidase-conjugated anti-IL-12 p40 (500 ng/ml). Bound biotin conjugate was detected with streptavidin-alkaline phosphatase and subsequently visualized with *p*-nitrophenyl phosphate (1 mg/ml) in 0.25 M glycine buffer. Bound peroxidase-conjugated anti-IL-12 p40 was visualized with tetramethyl benzidine (0.06 mg/ml) in 0.1 M sodium acetate buffer (pH 5.5) containing 0.03% hydrogen peroxide, with 10% sulfuric acid added (50  $\mu$ l/well) to stop the color reaction. Absorbance was measured at 405 nm (alkaline phosphatase) and 450 nm (peroxidase).

SCID mice of either sex were found to be highly susceptible to oral infection with *T. gondii*, with no survivors after 22 days postinfection (Fig. 1). However, female SCID mice were found

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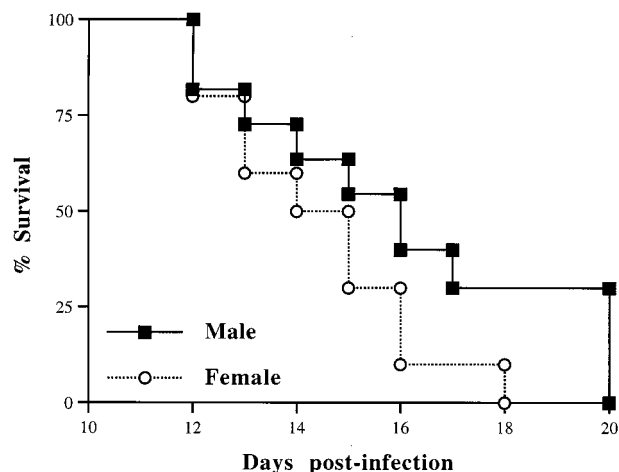


FIG. 1. Comparison of mortality between male and female SCID mice infected with *T. gondii*. Age-matched male and female SCID mice ( $n = 20$  for each sex) were infected orally with 20 *T. gondii* tissue cysts (RRA strain) and monitored for survival. Male SCID mice were found to survive significantly longer than their female counterparts ( $P = 0.0006$ , Mann-Whitney test). This pattern was observed in two subsequent experiments.

to be more susceptible to infection than males, as determined by their higher mortality levels ( $P = 0.0006$ ). To further characterize these observed differences between males and females, histopathological analysis was performed on brain tissue obtained from infected animals at 15 days postinfection. This analysis showed that females possessed larger numbers of brain tissue cysts (Table 1) and displayed more severe pathological changes, particularly in terms of increased numbers of large necrotic lesions which contained numerous proliferating parasites (not shown). Furthermore, Chalkley counting to estimate the area of brain tissue containing lesions revealed a significant difference between the sexes, with female SCID mice having a far greater lesion area (0.527%) than their male counterparts (0.177%;  $P < 0.01$ , Student's  $t$  test).

To determine whether an immunological mechanism could account for these observed differences in the severity of disease between males and females, we examined plasma samples from infected animals for circulating levels of cytokines associated with the innate immune response in these animals, namely, IL-12 and IFN- $\gamma$ . Levels of these cytokines were found to fluctuate throughout the course of infection in both sexes (Fig. 2). However, plasma levels of IL-12 were found to peak more

TABLE 1. Pathological changes in the brains of *T. gondii*-infected SCID mice

SCID mouse	No. exhibiting:		
	Large necrotic lesions	Small lesions ( $\pm$ necrosis)	Clusters of small cysts
<b>Males</b>			
1	1	10	8
2	1	8	3
3	1	3	8
Avg	1	7	6.3
<b>Females</b>			
1	6	10	14
2	6	14	13
3	7	9	6
Avg	6.3	11	11

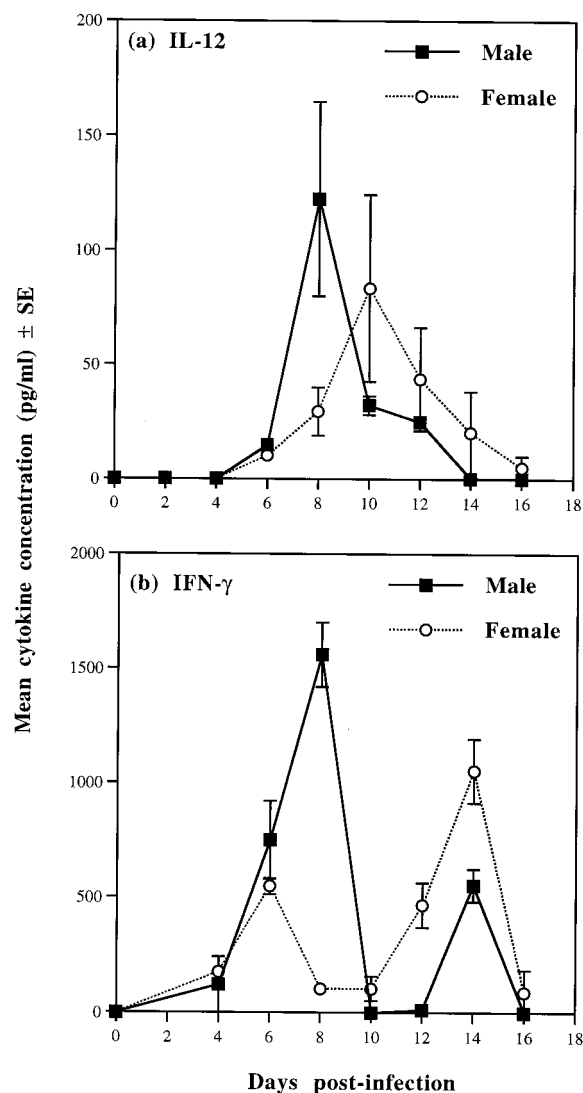


FIG. 2. Comparison of innate immunity associated cytokines in male and female SCID mice infected with *T. gondii*. (a) Analysis of plasma samples from infected animals ( $n = 3$  for each sex at all time points) for IL-12 concentration revealed significant differences between males and females. IL-12 plasma concentrations peaked at day 8 postinfection in males and at day 10 in females. The level of IL-12 in male samples was significantly higher in males at day 8 postinfection than in females ( $P < 0.03$ ). (b) Similar measurements of IFN- $\gamma$  levels revealed that male SCID mice produced significantly higher amounts of this cytokine early after infection compared with females ( $P < 0.001$ ). This pattern of cytokine concentration was consistently observed in two subsequent experiments. Statistical comparisons were done by Student's  $t$  test.

rapidly in male SCID mice after infection with *T. gondii* (Fig. 2a). IL-12 concentrations peaked at day 8 postinfection in males ( $122.3 \pm 42.2$  pg/ml). In female animals, this peak of IL-12 production was not detected until day 10 postinfection ( $83.3 \pm 40.8$  pg/ml). The IL-12 levels measured in males at day 8 postinfection were significantly higher than female IL-12 levels at this time ( $P < 0.03$ ). After these peaks of IL-12 production, the levels of this cytokine in both sexes rapidly declined and were not found to be significantly different between males and females at subsequent time points. Similar analysis of IFN- $\gamma$  levels also revealed significant differences between males and females (Fig. 2b). By day 8 postinfection, plasma samples from male SCID mice were found to contain

significantly higher levels of IFN- $\gamma$  compared with levels in plasma samples from female animals (males,  $1,560 \pm 140$  pg/ml; females,  $104 \pm 10.4$  pg/ml;  $P < 0.001$ ). A second peak of IFN- $\gamma$  production was also observed in both sexes at day 14 postinfection; this peak was significantly higher in females than in males at the same time point ( $P < 0.02$ ). However, this second peak was still significantly lower than the first peak observed in male SCID mice ( $P < 0.03$ ). Given that a second peak in IL-12 production was not observed in infected animals, this late IFN- $\gamma$  production may reflect IL-12-independent mechanisms of induction associated with the severe pathology observed at this time.

In conclusion, this novel study demonstrates that sex differences in the susceptibility to parasitic infection operate at the level of the innate immune response, as shown by significant differences in the in vivo kinetics of IL-12 and IFN- $\gamma$  production between males and females. Given that innate immunity is known to be highly dependent on the production of IFN- $\gamma$  by activated NK cells (2) and macrophage-derived IL-12 has been shown to be required for this process in *T. gondii*-infected SCID mice (10), our novel observations provide an explanation for the improved resistance of males over their female counterparts. Indeed, our data accord with other experimental data showing that both macrophage and NK cell function can be influenced by changes in their sex hormone environment. For example, the female sex steroids estradiol and progesterone have been demonstrated to influence IL-1 and prostaglandin E<sub>2</sub> production by macrophages, (3) and in vitro studies by Chao et al. have shown that estradiol can suppress macrophage activation, resulting in inhibited nitrite release (7) and lower tumor necrosis factor production (6). Similarly, estradiol has also been shown to suppress the cytotoxic activity of NK cells (8, 17) and down-regulate IFN- $\gamma$  production (11). Our findings strengthen these studies and suggest that they have relevance to physiological in vivo immune responses. Finally, our findings may have relevance to human *T. gondii* infections, where it has been shown that cases of infection-associated lymphadenopathy are significantly higher in adult females than in adult males (4), and the incidence of toxoplasmic encephalitis in AIDS patients is significantly higher in females than in males (16).

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