Effect of Sex Steroids on *Babesia microti* Infection in Mice

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Abstract. Sex-based differences are known to affect susceptibility to protozoan infections, but their effects on parasitemia and clinical symptoms in *Babesia* infections remain unclear. We examined the sex-based susceptibility of various mouse strains to *Babesia microti* Munich strain infection. In all strains, male mice exhibited significantly higher peak parasitemia and more severe anemia than female mice. Testosterone and estradiol-17 β treatment caused an increase in parasitemia and aggravation of anemia. Orchidectomized male mice receiving testosterone exhibited smaller splenic macrophage populations three days after infection, smaller B cell populations 10 days after infection, and reduced splenic tumor necrosis factor- α and interferon- γ mRNA expression than mice that did not receive testosterone. Mice receiving estradiol-17 β did not exhibit immunosuppressive effects. Thus, a weakened and delayed innate immunity response may lead to acquired immunity failure. The results suggested that testosterone directly affects T or B cells, leading to delayed acquired immunity, dramatically increased parasitemia, and severe anemia.

INTRODUCTION

Babesiosis is caused by a hemoproteozoan parasite of the genus *Babesia*. *Babesia* parasites are apicomplexan parasites transmitted to the hosts by ticks. These parasites invade erythrocytes of a wide range of domestic and wild animals and cause hemolytic anemia, fever, jaundice, and splenomegaly in infected animals. Babesiosis has been reported worldwide and is responsible for great economic losses in the animal industry.

Some epidemiologic studies have reported no significant differences in prevalence rates between males and females,^{1,2} but others have reported higher rates of *Babesia* infection in male animals.^{3,4} Any correlations of between *Babesia* infection in animals with pregnancy, estrus, or gonadectomy are also unknown. In addition, babesiosis is regarded as an emergent zoonosis between rodent hosts and humans. In humans, babesiosis may produce only minor symptoms or no symptoms. However, deaths have been reported in the older persons, asplenic patients, and immunocompromised patients.⁵ A correlation between pregnancy and human babesiosis has also been reported.⁶

Males are more susceptible than females to infections with some protozoans such as *Plasmodium berghei*,⁷ *P. chabaudi*,^{8,9} *P. falciparum*,¹⁰ *Leishmania major*, *Leishmania mexicana*,¹¹ *Trypanosoma cruzi*,^{12,13} and *Giardia muris*.^{14,15} Testosterone appears to increase this susceptibility of male animals to *P. berghei*,⁷ *P. chabaudi*,^{16–18} *L. major*,¹⁹ and *T. cruzi*.¹³ Female animals are more susceptible than males to *Toxoplasma gondii* infection, and estrogen causes this effect.^{20–22} Experimental infections and studies of immunity against *Babesia* infection have been performed using *Babesia microti*, *Babesia* sp. strain WA1, and susceptible mouse models.^{23–29} Aguilar-Delfin and others reported a sex-related influence on resistance to acute *Babesia* sp. strain WA1 infection in C57BL/6 mice, with males being more resistant than females.²³ Conversely, Hughes and Randolph reported that testosterone causes prolonged and more intense *B. microti* infection.²⁷ Thus, these apparently conflicting studies have failed to clearly define the impact of sex-based differences on parasitemia in and clinical symptoms of *B. microti* infection.

We used various mouse strains to test whether sex differences in host animals affected susceptibility to *B. microti* infection. In addition, we evaluated the effect of testosterone and estradiol-17 β on *B. microti* infection in mice.

MATERIALS AND METHODS

Mice and parasites. Six-week-old male and female BALB/c, C57BL/6, C3H/He, and ICR mice (Charles River, Yokohama, Japan) were used for experimental infections. The maintenance and care of experimental animals complied with the National Institutes of Health guidelines for the humane use of laboratory animals. The *B. microti* Munich strain was maintained by blood passages in female ICR mice. Each experimental infection was performed using six mice in each group.

Surgical procedure and sex steroid treatment. Male and female mice were anesthetized with pentobarbital and underwent orchidectomy or ovariectomy, respectively, or underwent a sham operation. The mice were then kept under standard conditions for 10 days before further experimental use. To assess the effect of testosterone on *Babesia* infection, the orchidectomized male mice received subcutaneous injections of 100 μ L of sesame oil (Wako, Osaka, Japan) containing 0.9 mg of testosterone propionate (Wako), twice a week for three weeks. The mice that underwent sham-operation and received sesame oil have been hereafter referred to as sham-operated mice. Orchidectomized male mice treated with sesame oil alone were considered control mice.

To assess the effect of estrogen, female mice underwent intraperitoneal (IP) implantation of a silastic tube containing 10 μ g of estradiol-17 β (Sigma Aldrich, St. Louis, MO) dissolved in sesame oil, after the ovariectomy procedure. The female sham-operated mice underwent IP implantation of a silastic tube containing sesame oil alone have been hereafter referred to as sham-operated mice. Ovariectomized female mice that underwent IP implantation of a silastic tube containing sesame oil alone were considered control mice.

Infections with *B. microti*. Male and female BALB/c, C57BL/6, C3H/He, and ICR mice were infected by IP injection of 10⁴ *B. microti*-infected erythrocytes. The mice that were used to investigate the effects of orchidectomy or ovariectomy were infected by IP injection with 10⁶ *B. microti*-infected erythrocytes. The mice that were used to investigate sex steroids were infected by IP injection with 10⁶ *B. microti*-infected

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erythrocytes after one week of sex steroid or sesame oil treatment. Peripheral blood samples (10 μ L) were taken from the tail vein every other day. Parasitemia was evaluated by using Giemsa-stained blood smears. The packed cell volume (PCV) and erythrocyte count were monitored by using Celltac α (Nihon Kohden, Tokyo, Japan).

In vitro culture of *B. microti*. *In vitro* cultivation of *B. microti* was performed as described.³⁰ In brief, parasitized erythrocytes obtained from infected mice were washed with RPMI 1640 medium (Sigma Aldrich), and the parasitemia level was adjusted to approximately 1% with uninfected erythrocytes. The parasitized erythrocytes were resuspended in RPMI 1640 medium supplemented with 40% untreated fetal bovine serum and testosterone or estradiol-17 β at a PCV of 10%. Testosterone propionate was supplemented at concentrations of 0, 2, 20, or 200 ng/mL. Estradiol-17 β was added at concentrations of 0, 5, or 50 pg/mL. A 1-mL aliquot of this suspension per well was placed in a 24-well culture plate and incubated in a humidified atmosphere of 5% CO₂ in air at 37°C. Parasitemia was evaluated by using Giemsa-stained blood smears at 0, 12, and 24 hours after cultivation.

Flow cytometry. Spleens obtained from mice on days 0, 3, 5, 10, 13, and 15 postinfection were homogenized by using a 70- μ m cell strainer (BD Bioscience, Franklin Lakes, NJ). Erythrocytes were then removed by treatment with 0.83% NH₄Cl solution, and washed with RPMI 1640 medium three times. To examine the CD4⁺, CD8⁺, CD11b⁺, and IgG⁺ cell populations, 10⁶ cells in 200-fold diluted fluorescein isothiocyanate conjugated-rat anti-mouse CD4, CD8, CD11b, and IgG (Beckman Coulter, Brea, CA) were incubated for 30 minutes on ice, washed with phosphate-buffered saline (PBS), suspended in IsoFlow (Beckman Coulter), and analyzed by using a Cytomics FC500 (Beckman Coulter). Data analysis was performed by using the FlowJo software (Tree Star, Ashland, OR).

Quantitative real-time polymerase chain reaction. Total RNA was isolated from spleens of infected mice by an extraction method using TRIzol Reagent (Invitrogen, Carlsbad, CA). In brief, 0.1 g of spleen tissue was disrupted in 1 mL of TRIzol, and 0.2 mL of chloroform was added. The aqueous phase was recovered after 15 minutes of centrifugation at 2,500 $\times$ g. RNA was precipitated with isopropanol, washed with 75% ethanol, and re-dissolved in RNase-free water. Reverse transcription of 1.5 μ L of total RNA from each individual mouse sample was conducted with oligo (dT)₁₆ primers according to the manufacturer's protocol for the SuperScript III First-Strand Synthesis System for real-time reverse transcription-polymerase chain reaction (RT-PCR) (Invitrogen).

The investigated mRNAs were quantified by real-time RT-PCR by using a 7300 Fast Real-Time PCR System (Applied Biosystems, Warrington, United Kingdom), and the PCR product was detected with Power SYBR Green PCR Master Mix (Applied Biosystems). Each PCR reaction mixture (25 μ L) contained 12.5 μ L of SYBR Green, 900 nM forward primer,

900 nM reverse primer, and 1 μ L of RT products in total reaction mixture of 50 μ L. Thermal cycling conditions were 50°C for 2 minutes; 95°C for 10 minutes; and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Primer sequences for the genes studied are shown in Table 1. The cycle threshold values for cDNA amplified from mRNA were determined for each gene studied and for the housekeeping gene β -actin. For each sample, the mRNA copy numbers of the genes studied and the housekeeping gene were determined from a standard curve. A normalized mRNA value was then calculated by dividing the mRNA copy number for the gene studied by that for β -actin.

Enzyme-linked immunosorbent assay. Antibody production against *B. microti* was assessed by enzyme-linked immunosorbent assay every other day after infection. *Babesia microti* native proteins were used as antigens. *Babesia microti*-infected erythrocytes were hemolyzed with 0.1% saponin-PBS, and washed twice with PBS. The pellet was sonicated in PBS and centrifuged at 11,000 $\times$ g for 5 minutes, and the supernatant was used as antigen. Ninety-six wells of microtitration plates (Nunc, Roskilde, Denmark) were coated with *B. microti* antigen (0.5 μ g/ μ L) diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6, overnight at 4°C. The wells were subsequently washed three times with PBS containing 0.05% Tween-20 and blocked with 100 μ L of PBS containing 3% skim milk for 1 hour at 37°C to reduce nonspecific binding. The blocking agent was removed, and 50 μ L of individual mouse serum diluted to 1:100 in PBS containing 3% skim milk was added to each well and then incubated for 1 hour at 37°C. After washing 3 times with PBS containing 0.05% Tween-20, 50 μ L of horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Cappel, Cochranville, PA) diluted to 1:10,000 in PBS containing 3% skim milk was added to each well and incubated for 1 hour at 37°C. The plates were washed, and 100 μ L of substrate solution (0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H₂O₂, and 0.3 mg/mL of 2,2'-azide-bis [3-ethylbenzthiazoline-6-sulfonic acid]) was added to each well. The absorbance at 405 nm was measured after 1 hour of incubation at 37°C by using the Ultrospec Visible Plate Reader II 96 (Amersham Bioscience, Uppsala, Sweden).

Statistical analyses. Parasitemia and PCV data are reported as mean $\pm$ SD. Comparative analyses were performed by using Student's *t*-test. All differences were considered significant at *P* < 0.05.

RESULTS

In all mouse strains, an increase in parasitemia was observed during days 10–12 after infection, and this increase peaked during days 14–20 after infection (Figure 1A). The peak parasitemia levels in male BALB/c, C57BL/6, C3H/He, and ICR mice were 35.5%, 42.9%, 60.9%, and 65.0%, respectively. In male BALB/c, C57BL/6, and C3H/He mice, peak parasitemia was observed on day 18 after infection, whereas

TABLE 1
Sequences of the primers used for quantitative RT-PCR for studying the effect of sex steroids on *Babesia microti* infection in mice*

Gene	GenBank accession no.	Sense primer, 5'→3'	Antisense primer, 5'→3'
TNF- α	NM_013693	CTCATTCCTGCTTGTGGCAGGGG	GGGAGGCCATTGTGGAACTTCTCA
IL-12	NM_008352	CGCAGCAAAGCAAGATGTGTCTCTCA	GGCATCGGGAGTCCAGTCCACC
IFN- γ	NM_008337	GACAATCAGGCCATCAGCAAC	CTCGGATGAGCTCATTGAATGC
β -actin	NM_007393	GCCACCAGTTCGCCATGGATG	GGGAATACAGCCCGGGGAGCA

*RT-PCR = reverse transcription-polymerase chain reaction; TNF- α = tumor necrosis factor- α ; IL-12 = interleukin-12; IFN- γ = interferon- γ .

FIGURE 1. Sex differences in parasitemia and packed cell volume (PCV) in mice infected with *Babesia microti*. **A**, Parasitemia in males (closed circles) and females (open circles) of various mouse strains at different times after intraperitoneal inoculation of erythrocytes infected with *B. microti* Munich strain ($n = 6$). One male ICR mouse died on day 19 after infection. **B**, PCV in males (closed circles) and females (open circles) of various mouse strains at different times after intraperitoneal inoculation of erythrocytes infected with *B. microti* Munich strain ($n = 6$). Error bars indicate mean $\pm$ SD. * $P < 0.05$ vs. male mice.

in male ICR mice, it was observed on day 14 after infection. One male ICR mouse showed a peak parasitemia level of 73.1% on day 14, and died on day 19 after infection. The peak parasitemia levels in female BALB/c, C57BL/6, C3H/He, and ICR mice were 27.0%, 25.1%, 46.9%, and 21.0%, respectively. Peak parasitemia was observed on day 18 in female BALB/c and C3H/He mice, on day 16 in C57BL/6 mice, and on day 14 in ICR mice. In C3H/He and ICR mice, the peak parasitemia levels were significantly higher than those of BALB/c and C57BL/6 mice ($P < 0.05$). In all mouse strains, males showed significantly higher peak parasitemia levels than females ($P < 0.05$). The difference between males and females was greatest in ICR mice. Concurrent with the increase in parasitemia, PCV and erythrocyte counts decreased, and anemia was more severe in male mice than in female mice, as shown by the lowest PCV (Figure 1B).

In male mice, peak parasitemia was observed on day 11 in sham-operated mice and on day 10 in orchidectomized mice (Figure 2A). The peak parasitemia levels of the sham-operated male mice and the orchidectomized male mice were 66.0% and 43.0%, respectively (Figure 2A). The orchidectomized mice showed significantly lower peak parasitemia levels than the sham-operated mice ($P < 0.05$). In addition, anemia was aggravated on day 12 after infection, and the PCV values of the sham-operated mice and orchidectomized mice were 14.1% and 21.5%, respectively (Figure 2B). The sham-operated male mice showed a body weight loss of 4.4 g after peak parasitemia, but the orchidectomized mice showed no weight loss (Figure 2C). The sham-operated male mice also exhibited severe symptoms such as rough fur and depression. However, these symptoms were milder in orchidectomized mice.

In female mice, peak parasitemia was observed on day 11 in sham-operated mice and on day 12 in ovariectomized mice (Figure 2D). The peak parasitemia levels of the sham-operated female mice and ovariectomized female mice were 55.3% and 37.2%, respectively (Figure 2D). The ovariectomized mice showed significantly lower peak parasitemia levels than sham-operated mice ($P < 0.05$). In addition, the anemia was aggravated on day 12 after infection, and the PCV values of the sham-operated mice and ovariectomized mice were 17.8% and 24.3%, respectively (Figure 2E). Sham-operated female mice and ovariectomized mice showed no weight loss after peak parasitemia (Figure 2F). Symptoms such as rough fur and depression were milder in ovariectomized mice than in sham-operated mice.

We investigated the effect of testosterone and estradiol-17 β on *B. microti* infection in ICR mice. The orchidectomized male mice that received sesame oil showed significantly lower peak parasitemia levels than sham-operated and testosterone-treated orchidectomized mice (Table 2). The anemia was aggravated on day 12 after infection, and the PCV values of orchidectomized mice that received sesame oil were higher than those of the sham-operated and testosterone-treated orchidectomized mice (Table 2). In female mice, the ovariectomized mice that received sesame oil showed significantly lower peak parasitemia levels than sham-operated and estradiol-17 β -treated ovariectomized mice (Table 2). These results suggest that testosterone and estradiol-17 β contribute to aggravating *B. microti* infection in mice.

In parasite cultures, the growth rates of parasites cultured in the medium to which testosterone was added were similar to those cultured in the control medium (Figure 3A). We also

FIGURE 2. Effects of orchidectomy in male mice and ovariectomy in female mice on *Babesia microti* infection. Parasitemia (A and D), packed cell volume (PCV) (B and E), and changes in body weight (C and F) of male (A–C) and female (D–F) mice at different times after intraperitoneal inoculation of erythrocytes infected with *B. microti* Munich strain (n = 6). Closed circles = sham-operated mice; open circles = orchidectomized male mice (A–C); open circles = ovariectomized female mice (D–F). Error bars indicate mean $\pm$ SD. * $P < 0.05$ vs. sham-operated mice.

performed parasite cultures with media containing 5 or 50 pg/mL of estradiol-17 β , and the growth rates of parasites cultured in the medium to which estradiol-17 β was added were similar to those cultured in control medium (Figure 3B). Testosterone and estradiol-17 β did not appear to directly affect parasite growth *in vitro*. Cell divisions of parasites have been reported in parasitized erythrocytes.³⁰ However, our results indicated that cell division of parasites in media containing added sex steroids occurred at the same level as was observed in the control medium.

In the CD4⁺ and CD8⁺ cell populations, no difference was observed between the testosterone-injected mice and control mice after infection (Figure 4). The IgG⁺ cell population of orchidectomized mice that received testosterone injections was lower than that of control mice on days 10 and 15 after infection (Figure 4). In addition, B cells reduced on testosterone injection, and this effect was observed 10 days postinfection. In contrast, the spleen CD11b⁺ population of estradiol-17 β -treated mice appeared to increase on days 5 and 15 postinfection, but there

was no significant difference between control female mice and estradiol-17 β -treated mice on these days postinfection. In addition, no significant differences were observed between control female mice and estradiol-17 β -treated mice with respect to CD4⁺, CD8⁺, and IgG⁺ cell populations (Figure 5).

On day 3 after infection, tumor necrosis factor- α (TNF- α) mRNA expression increased in control mice, but its expression level in testosterone-injected mice was significantly lower than that in control mice (Figure 6A). In addition, interleukin-12 (IL-12) mRNA expression levels of testosterone-treated mice were also slightly lower than those of control mice (Figure 6B), suggesting that testosterone suppresses the production of cytokines such as TNF- α and IL-12 by splenic macrophages. In addition, splenic interferon- γ (IFN- γ) mRNA expression was

TABLE 2
Effect of testosterone or estradiol-17 β on parasitemia and anemia in *Babesia microti* infection

Sex	Mice treatment	Mean $\pm$ SD peak parasitemia	Mean $\pm$ SD packed cell volume of day 12 postinfection
M	Sham operated	70.0 $\pm$ 9.9*	16.3 $\pm$ 3.4*
	Control (orchidectomized)	56.3 $\pm$ 5.4	23.2 $\pm$ 4.6
	Testosterone-treated (orchidectomized)	73.8 $\pm$ 9.7*	14.3 $\pm$ 1.6*
F	Sham operated	76.1 $\pm$ 3.5*	14.3 $\pm$ 1.7*
	Control (ovariectomized)	63.5 $\pm$ 5.2	22.8 $\pm$ 2.5
	Estradiol-17 β -treated (ovariectomized)	74.2 $\pm$ 3.0*	15.7 $\pm$ 8.9*

*Versus control ($P < 0.05$).

FIGURE 3. Effect of sex steroids on parasite growth *in vitro* in mice. Parasitemia at different times of cultivation *in vitro* using medium to which different concentrations of testosterone (A) or estradiol-17 β (B) have been added. Error bars indicate mean $\pm$ SD.

FIGURE 4. Effect of testosterone on the CD11b⁺, CD4⁺, CD8⁺, and IgG⁺ cell populations in splenic cells of mice after *Babesia microti* infection. White bars = control mice (orchidectomized); black bars = testosterone-treated mice (orchidectomized). Error bars indicate mean $\pm$ SD. * $P < 0.05$ vs. control mice.

suppressed by testosterone treatment (Figure 6C), suggesting reduced IFN- γ production by splenic T cells in testosterone-injected mice. In contrast, estradiol-17 β did not affect spleen TNF- α , IL-12, and IFN- γ mRNA expression (Figure 6D-F).

Orchidectomized male mice that received testosterone had significantly lower antibody increases than control mice on Day 13 after infection (Figure 7A). In contrast, ovariectomized female mice that received estradiol-17 β exhibited sig-

nificantly higher antibody increase than control mice on day 13 after infection (Figure 7B).

DISCUSSION

A previous study using *Babesia* sp. strain WA1 and moderately susceptible mouse strains C57BL/6, AKR, and 129/J²² showed a sex-related influence on resistance to babesiosis in

FIGURE 5. Effect of estradiol-17 β on the CD11b⁺, CD4⁺, CD8⁺, and IgG⁺ cell populations in the splenic cells of mice after *Babesia microti* infection. White bars = control mice (ovariectomized); black bars = estradiol-17 β -treated mice (ovariectomized). * $P < 0.05$ vs. control mice.

FIGURE 6. Effect of testosterone (A–C) or estradiol-17β (D–F) on splenic cytokine mRNA expression in *B. microti*-infected mice. Relative expression levels of tumor necrosis factor-α (A and D), interleukin-2 (B and E), and interferon-γ (C and F). White bars = control mice (orchidectomized or ovariectomized); black bars = testosterone or estradiol-17β-treated mice (orchidectomized or ovariectomized), black bars. * $P < 0.05$ vs. control mice.

mice, with the males of these mouse strains being more resistant than the females. Conversely, our study indicated that in BALB/c, C3H/He, C57BL/6, and ICR mouse strains, males were more susceptible than females. The parasite species or strain may affect host immunity, resulting in sex and strain susceptibility in mice. In this study, particularly significant differences were observed between male and female ICR mice. ICR mice mature earlier than the other three strains

(<http://www.criver.com>), which may explain the sex difference that was observed.

There are known sex differences in some infections caused by bacteria, viruses, fungi, and parasites.^{31–36} Males generally exhibit lower immune responses than females.^{22,31,37} The phagocytic activity of macrophages and production of inflammatory cytokines are higher in females than in males.^{38,39} Antibody production by B cells is also greater in females than in males.³⁸ However, in *Leishmania* infection, IgG1, IgE, and TNF-α production is increased in males,^{11,19} and in *Toxoplasma* infection, IL-12, TNF-α, and IFN-γ production is higher in males than in females.^{22,40} Thus, these sex differences in host immunity might vary among species of parasites. We observed differences in parasitemia between males and females in the acute phase of infection, and these differences were noteworthy for peak parasitemia. In addition, peak parasitemia occurred earlier (in days) in male mice. These data suggest that innate immunity or subsequent cell-mediated immunity is suppressed in male mice in the acute phase of *B. microti* infection. Immunologic differences between sexes are considered to be caused by sex hormones such as testosterone, estradiol-17β, and progesterone.³¹

Our study demonstrated that orchidectomy and ovariectomy either control parasite growth or enhance immunity against *B. microti* infection in male and female mice. When infected with *P. chabaudi*, a hemoprotozoan parasite, female mice and castrated male mice are capable of self-healing.¹⁶ Viselli and others reported that castrated male mice showed thymic hypertrophy and splenic enlargement because of increases in B cell populations, and that culture supernatants

FIGURE 7. Effect of testosterone (A) and estradiol-17β (B) on serum antibody against *Babesia microti* in infected mice. A, Open circles = control male mice (orchidectomized); closed circles = testosterone-treated mice (orchidectomized). B, Open circles = control female mice (ovariectomized); closed circles = estradiol-17β-treated mice (ovariectomized). O.D. = optical density. Error bars indicate mean $\pm$ SD. * $P < 0.05$ vs. control mice.

of castrated mouse splenic cells produced higher levels of IL-2 and IFN- γ than control splenic cells.⁴¹ Mayr and others reported that major histocompatibility class II expression by macrophages in male mice was suppressed after trauma-induced hemorrhage, and that this suppression was prevented by castration.⁴² In male mice, these alterations in immune function, which may be caused by the lack of testosterone after orchidectomy, might control parasitemia increases and aggravation of anemia in infected orchidectomized mice.

Similarly, ovariectomy has been reported to reduce susceptibility to infection with bacteria, viruses, fungi, and parasites.^{33,43–45} In *P. chabaudi* infection, ovariectomized female mice exhibit reduced production of the cytokines IL-12 and IFN- γ , leading to increased susceptibility.²⁴ However, in our study, ovariectomy in female mice caused low peak parasitemia and contributed to resistance against *B. microti* infection.

The transmission potential of *B. microti* was shown to increase in hosts with high rather than low testosterone levels because testosterone causes increased locomotor activity and reduced innate and acquired resistance to tick feeding.²⁷ In this study, we also demonstrated that *B. microti*-infected mice showed high parasitemia levels and severe anemia when exposed to testosterone and estradiol-17 β . However, results of *in vitro* testing did not indicate a direct influence of testosterone or estradiol-17 β on *B. microti* growth. In general, androgens (testosterone and 5 α -dihydrotestosterone) inhibit humoral and cell-mediated immune responses.⁴⁶ A study by Angele and others indicated that androgens are responsible for immunosuppression after trauma-induced hemorrhage in males.⁴⁷ In contrast, estrogen generally promotes cytokine production or antibody production, and contributes to resistance against infection.³⁷ Therefore, we investigated the effect of testosterone and estradiol-17 β on the immune systems of *B. microti*-infected mice.

Before infection, splenic macrophage and CD4⁺ and CD8⁺ T cell populations of testosterone-treated mice were smaller than those of untreated mice. On day 3, splenic macrophage populations increased in untreated mice, but remained at preinfection levels in testosterone-treated mice. In addition, the results of qRT-PCR showed that TNF- α and IFN- γ mRNA expression in testosterone-treated orchidectomized mouse spleens was lower than that in orchidectomized mice on day 3 after infection. Testosterone has been reported to inhibit the expression of inducible nitric oxide synthase after lipopolysaccharide stimulation in RAW264.7 cells.⁴⁸ Testosterone has also been reported to decrease toll-like receptor 4 expression in these cells.⁴⁹ *Babesia* has not been reported to stimulate toll-like receptor 4, but expression of other receptors that sense *Babesia* antigens might be reduced by testosterone. Consequently, testosterone reduced macrophage and T cell populations, and after infection, early macrophage activation and subsequent T cell activation were suppressed by testosterone.

Splenic B cell populations were lower in testosterone-treated mice on days 10 and 15, and the increase in antibodies in serum of testosterone-treated mice was also lower on days 13–15 after infection. Testosterone is reported to inhibit IgG and IgM production by human peripheral blood mononuclear cells.⁵⁰ Testosterone may suppress B cell function directly or through reduction of cytokines such as IL-4 and IL-6 from helper T cells or macrophages. The effect of testosterone on parasitemia was observed during days 10–11

TABLE 3
Effect of estradiol-17 β on parasitized erythrocyte counts of mice infected with *Babesia microti*

Mice treatment	Mean erythrocyte count $\times 10^6$ cells/ μ L	Mean $\pm$ SD peak parasitemia, %	Mean parasitized erythrocyte count $\times 10^6$ cells/ μ L
Sham operated	367	76.1 $\pm$ 3.5*	244
Control (ovariectomized)	433	63.5 $\pm$ 5.2	236
Estradiol-17 β -treated (ovariectomized)	363	74.2 $\pm$ 3.0*	234

*Versus orchidectomized.

after infection, but antibody levels of testosterone-treated mice and control mice were similar in this infection stage. These results suggest that the suppressive effect of testosterone on antibody production does not contribute to the peak parasitemia burden.

In contrast to the effect of testosterone, estrogen promotes cytokine production or antibody production, and contributes to resistance against infection.³⁷ In this study, splenic macrophage population on day 5 increased slightly after estradiol-17 β treatment. In addition, antibody production also increased in estradiol-17 β -treated mice. It is suggested that estradiol-17 β does not have immunosuppressive effects like testosterone; rather, it seems to contribute to increased production of macrophage or antibodies. However, our data indicated that estradiol-17 β treatment promoted an increase in parasitemia and aggravated anemia. Estradiol-17 β treatment may promote these effects by a mechanism that differs that for from testosterone. Furthermore, estrogen has been shown to exert an inhibitory effect on hematopoiesis.⁵¹ In this study, estradiol-17 β -treated mice tend to have lower erythrocyte counts than the control mice both before and after the day that they reached the peak parasitemia, and it is possible that the number of apparent parasitemia decreased for this reason (Table 3). The corrected parasite count/microliter of estradiol-17 β -treated mice was similar to that of ovariectomized control mice (Table 3).

In conclusion, our results indicate that male mice were more susceptible than female mice to *B. microti* Munich strain infection. This sex difference was caused by testosterone, which apparently weakened and delayed innate immunity, including splenic macrophage activation, resulting in failure of subsequent acquired immunity. This further resulted in lower production of IFN- γ or antibodies, leading to a dramatic increase in parasitemia and severe symptoms. It is also possible that the effects of testosterone in babesiosis will be apparent in other hosts. Therefore, studies on sex differences in other animals (e.g., cattle, horses, and dogs) and investigation of the effects of testosterone on parasitemia and related symptoms is required. In this study, testosterone was shown to affect innate immunity against *B. microti*. However, the mechanism associated with this effect remains unknown. Additional research on the effects of testosterone on *Babesia* infection could reveal important functions of host immune systems for clearance of this parasite.

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