Effect of Sex on *Coxiella burnetii* Infection: Protective Role of 17β-Estradiol

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Q fever is a zoonosis caused by *Coxiella burnetii* and recently has been recognized as a potential agent of bioterrorism. In Q fever, men are symptomatic more often than women, despite equal seroprevalence. We hypothesized that sex hormones play a role in the pathogenesis of *C. burnetii* infection. When C57/BL6 mice were injected with *C. burnetii,* bacteria load and granuloma numbers were lower in females than in males. Ovarectomized mice showed increased bacteria load in the spleen and the liver, similar to that found in males. The granuloma number was also increased in ovarectomized mice and reached the levels found in males. Tissue infection and granulomatous response are largely under the control of estrogens: treatment of ovarectomized mice with 17β-estradiol reduced both bacteria loads and granuloma numbers. These results show that sex hormones control host response to *C. burnetii* infection and may account for host-dependent clinical presentation of Q fever.

Q fever is a zoonosis caused by *Coxiella burnetii,* an obligate intracellular microorganism that inhabits monocytes/macrophages [1] and that has been recognized as a potential biological weapon [2]. After primary infection, only one-half of patients will experience acute Q fever, mainly as a mild, self-limited, flu-like syndrome or, more rarely, as pneumonia, hepatitis, myocarditis, pericarditis, meningitis, or encephalitis [3]. Men are symptomatic more often than women, with a man:woman ratio of 2.5, yet the seroprevalence is not higher among men [3]. Some middle-aged men exhibit hyperinflammatory forms of Q fever, with granulomatous hepatitis and autoantibodies that poorly respond to antimicrobial agents [1]. In contrast, children are symptomatic less often than adults, even if they are exposed as often as adults; boys and girls are similarly affected by Q fever [3, 4]. Taken together, the results of these epidemiological studies suggest that sex hormones may be involved in the control of clinical expression of Q fever.

Progesterone and estradiol, the main female sex hormones, are involved in the control of infections caused by intracellular microorganisms [5]. A progressive and parallel increase in estrogens and progesterone is observed during pregnancy, in which the alteration of cell-mediated immunity is associated with increased susceptibility to intracellular organisms [6]. Hence, repeated pregnancies in BALB/c mice infected with *C. burnetii* result in disseminated infection [7], and acute Q fever during pregnancy results in chronic infection [8]. Progesterone increases the susceptibility of ovarectomized rats to intrauterine chlamydial infection [9]. Treatment with estrogen enhances the resistance to certain pathogens, such as *Streptococcus pneumoniae, Mycoplasma* species, and *Paracoccidiodes brasiliensis* [10, 11], and suppresses the protective effect against *Listeria monocytogenes* [12], *Chlamydia trachomatis* [9], *Salmonella* species [13], and *Mycobacterium avium* [14]. This heterogeneity of estrogen's effects on host resistance likely results from variations in estrogen levels and in responsiveness of immune effectors to estrogens [15].

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The purpose of the present study was to assess the effect of sex on *C. burnetii* infection by use of a murine model that recreates sex-dependent differences observed in humans. We show here that female mice have lower *C. burnetii* loads in tissue and lower granuloma numbers than male mice. Ovarectomy of female mice led to increased *C. burnetii* loads and granuloma numbers, both of which were corrected by administration of 17β-estradiol at doses corresponding to levels in plasma during the late phase of pregnancy. Hence, estrogens control *C. burnetii* infection and host response to infection and may account for host-dependent variations in clinical presentation of Q fever in humans.

MATERIALS AND METHODS

***Preparation of* C. burnetii.** *C. burnetii* bacteria (Nine Mile strain) were cultivated as described elsewhere [16]. BALB/c mice were injected intraperitoneally (ip) with 10^8 *C. burnetii* organisms. Ten days later, mice were killed, and their spleens were homogenized. Spleen homogenates were added to L929 cells, and cultures were maintained in antibiotic-free Eagle MEM supplemented with 4% fetal bovine serum and 2 mmol/L L-glutamine (Invitrogen) for 2 passages. Infected cells were sonicated, and the sonicates were centrifuged at 8000 *g* for 10 min. Bacteria were layered on 25%–45% linear Renografin gradient, and the gradients were centrifuged. Purified bacteria were then collected, washed, and suspended in serum-free Hanks' balanced salt solution (Invitrogen) before being stored at −80°C. The concentration of organisms was determined by Gimenez staining [17], and the bacterial viability was assessed by use of the LIVE/DEAD *Bac*Light bacterial viability kit (Molecular Probes), as described elsewhere [18]. Only *C. burnetii* preparations containing >90% viable organisms were used.

Mice. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Université de la Méditerranée. C57BL/6 mice (25 males and 100 females) were obtained from Charles River Laboratories. Females were divided into 4 groups: 25 control mice, 25 mice that were sham ovarectomized, 25 mice that were ovarectomized at 6 weeks of age, and 25 ovarectomized mice treated with 17β-estradiol pellets 2 weeks after ovarectomy. Ovaries were removed through bilateral incisions. For the sham ovarectomy, the ovaries were identified, and the incision was then closed. For the estrogen-restoration experiments, mice were treated with 0.1-mg 17β-estradiol pellets (SE121; Innovative Research of America), leading to a constant 17β-estradiol concentration of 60–100 pg/mL/day over a period of 60 days [19]. Two weeks after implantation of the pellet, all mice were injected ip with 5×10^5 *C. burnetii* organisms. All the infections were made at the same time, and the clinical status of mice was recorded daily. They were killed serially before infection (day 0) and 4, 7, 14, and 21 days after infection. Blood was collected by retro-orbital puncture at the time of death. Organs were aseptically excised, and tissue samples were fixed with 10% formalin and embedded in paraffin.

***Histologic analysis and immunohistologic detection of* C. burnetii.** All the slides were coded and read in a blinded fashion. The 5-μm sections of paraffin-embedded tissues were stained with hematoxylin-eosin-saffron, to assess the presence of granulomas. Granulomas were detected by optical examination and quantified by use of the image analyzer SAMBA 2005 (SAMBA Technologies; Alcatel TITN) [20]. The results are expressed as the number of granulomas per surface unit (millimeters squared). Formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated in graded alcohol for 2 min each, and rinsed in PBS for 5 min. Each tissue section was incubated with rabbit antibodies (Abs) to *C. burnetii* (diluted 1:50) for 60 min, as described elsewhere [21]. Bacteria were revealed by use of the Immunostain-*Plus* kit (Zymed; CliniSciences), according to the manufacturer's instructions. In brief, slides were incubated with biotin-conjugated Abs directed against rabbit immunoglobulins, followed by peroxidase-labeled streptavidin with amino-ethylcarbazole as substrate. Slides were counterstained with Mayer hematoxylin for 10 min. Negative controls were performed with normal rabbit serum. The bacteria were numbered by optical examination, and the tissue area was determined as described above. The results are expressed as the number of bacteria per millimeter squared of tissue.

Ab determination. Blood was allowed to clot at room temperature and was centrifuged at 700 *g* for 10 min. The resulting serum was stored at −20°C until it was analyzed for the presence of anti–*C. burnetii* Abs. In brief, slides with smears of formaldehyde-inactivated organisms were incubated with serial dilutions of serum for 30 min. After being washed in PBS, the bacteria were labeled with fluorescein-conjugated goat Abs directed against mouse IgG (Beckman Coulter) at a 1:50 dilution for 30 min. The slides were then washed in PBS and examined by fluorescence microscopy. The starting dilution for the serum sample was 1:25, and samples were titered to end point, as described elsewhere [7].

Statistical analysis. Statistical calculations were performed with SAS software (version 5; SAS Institute) [22]. Data were compared by use of Wilcoxon's signed rank test. Results are expressed as mean ± SD. Differences were considered to be significant when $P < .05$.

RESULTS

Effect of sex on* C. burnetii *infection. To assess the effect of sex on *C. burnetii* infection, male and female C57BL/6 mice were injected ip with *C. burnetii* bacteria, and the number of

bacteria in organs was determined by immunohistochemical analysis. Bacteria were not found in the lungs, heart, or mesenteric lymph nodes but were detected as red rods in granulomatous infiltrates in the spleen and the liver (figure 1*A* and 1*B,* respectively). However, the bacteria load was different in males and females. In the spleen, bacteria were detectable after 4 days and peaked after 7 days. At this time, the number of bacteria was significantly higher in male than in female mice (4.3 ± 0.7 vs. 1.6 ± 0.3 bacteria/mm^2; $P < .001$). Thereafter, bacteria load decreased steadily, to undetectable levels after 21 days (figure 1*C*). In the liver, the number of bacteria was low, compared with that in the spleen: after 4 days of infection, there were 0.2 ± 0.04 bacteria/mm^2 in the livers of male mice and 0.1 ± 0.02 bacteria/mm^2 in livers of female mice ($P = .10$). The bacteria load steadily decreased thereafter, to undetectable levels after 14 days (figure 1*D*). Taken together, these results indicate that splenic load of *C. burnetii* was lower in female mice than in male mice.

Effect of sex on formation of granulomas. Because the formation of granulomas is associated with protective cell-mediated immune response [23], we examined the expression of granulomas in tissues from male and female mice. Granulomas consisted of mononuclear aggregates, with few polymorphonuclear leukocytes, and were scattered throughout liver lobules, portal spaces, and splenic red pulp (figure 2*A* and 2*B*). Granulomas were present in the spleen by day 4, and their numbers peaked at day 7, with significantly more granulomas in male mice than in female mice (2.9 ± 0.3 vs. 1.1 ± 0.2 granulomas/mm^2; $P < .001$). Thereafter, the numbers of granuloma decreased steadily, and differences between male and female mice were not significant at days 14 and 21 (figure 2*C*). In the liver, the number of granulomas peaked at day 4, but there was no difference between male and female mice at this time. The number of granulomas decreased thereafter, to undetectable levels after 21 days (figure 2*D*). Taken together, these results show that there were fewer splenic granulomas in *C. burnetii*–infected female mice than in *C. burnetii*–infected male mice.

Effect of ovarectomy on* C. burnetii *infection. To understand why female mice had lower bacterial loads than male mice, female mice were ovarectomized, and *C. burnetii* infection was assessed. First, the titer of Abs to *C. burnetii* was determined. In control and ovarectomized mice, no Abs were detected before *C. burnetii* infection, but Abs were present 7 days after infection and achieved highest titers at day 21 (figure 3*A*). The titer of specific Abs was significantly higher in ovarectomized mice than in sham-ovarectomized mice, at day 21 (3000 ± 180 vs. 800 ± 80; $P < .001$). Second, *C. burnetii* load was markedly affected by ovarectomy. Ovarectomy significantly

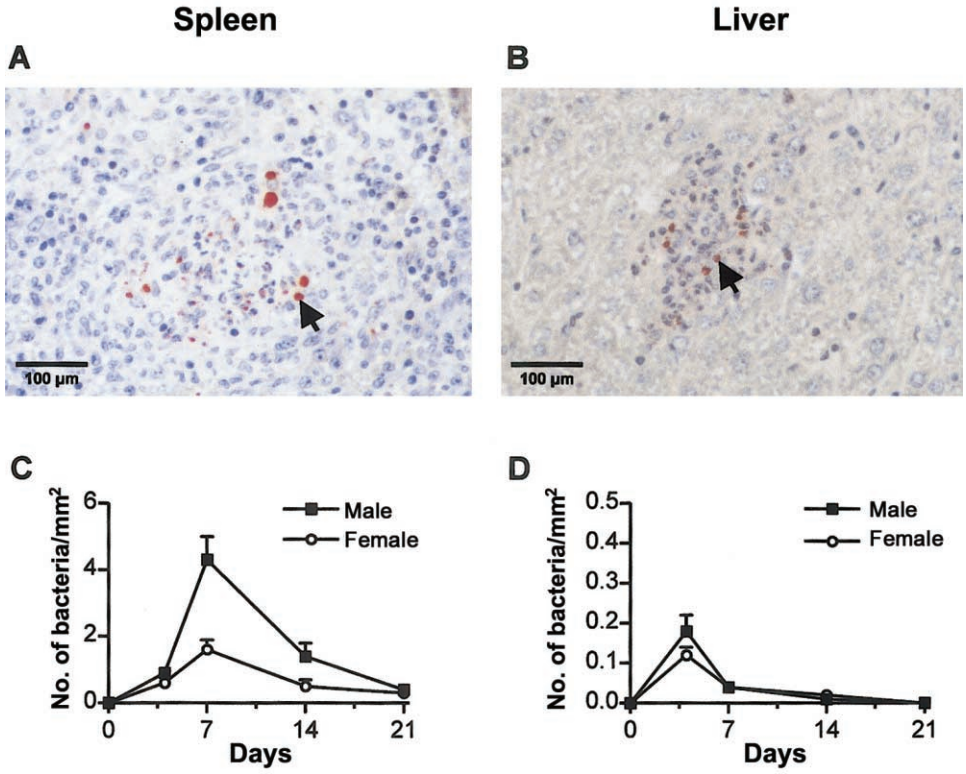

Figure 1. *Coxiella burnetii* load in mouse tissues. Male and female mice were infected with *C. burnetii* and killed at different times. Bacteria *(arrows)* were revealed by immunostaining in the spleen *(left)* and the liver *(right). A* and *B,* Representative micrographs of tissues from female mice at day 7 after infection (original magnification, ×400). *C* and *D,* Mean ± SD no. of bacteria/mm^2; data are the average of 5 mice/time point.

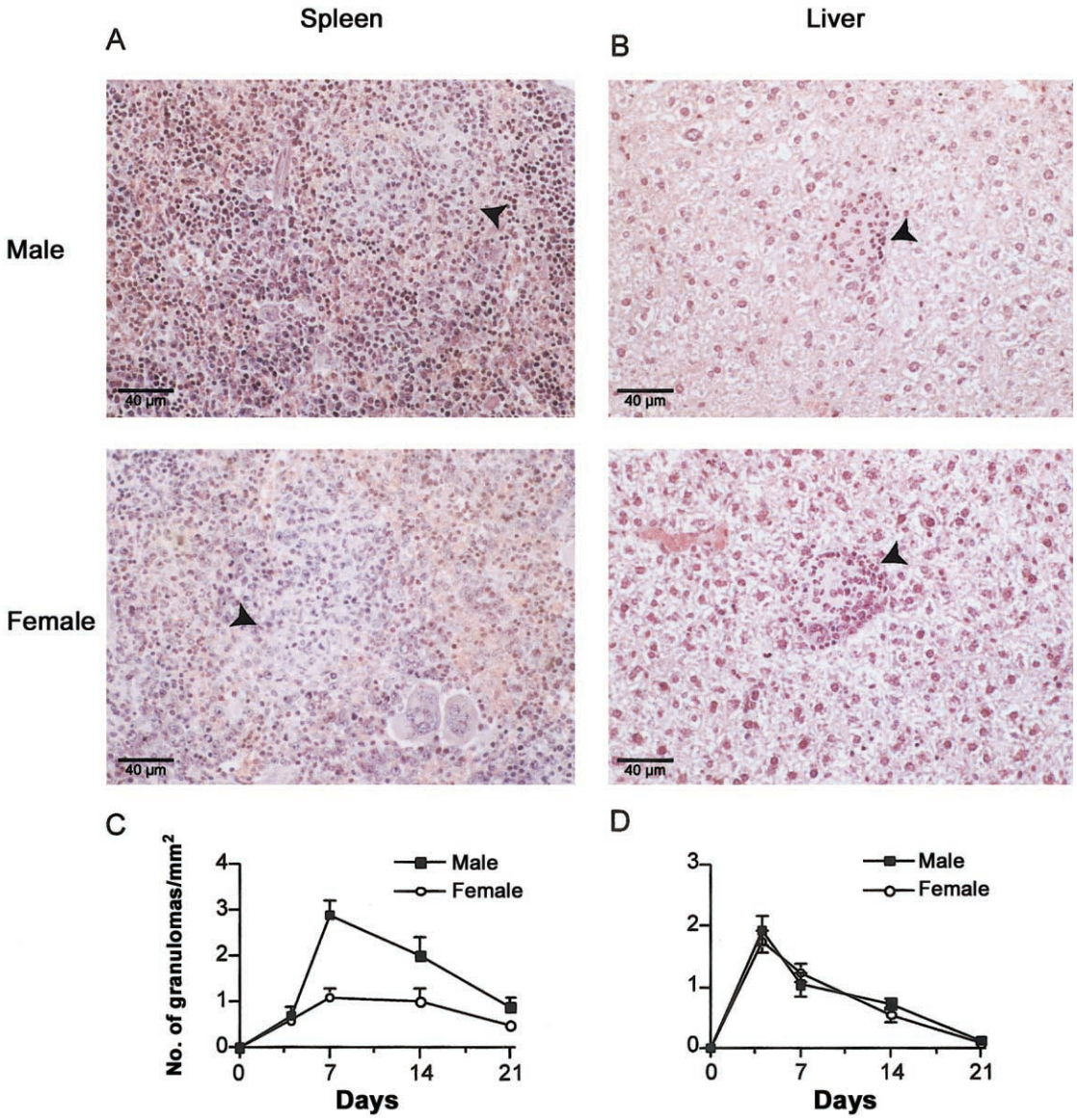

Figure 2. Granuloma expression in mouse tissues. Male and female mice were infected with *Coxiella burnetii* and killed at different times. Granulomas *(arrowheads)* were revealed by histochemical analysis in the spleen *(left)* and the liver *(right). A* and *B,* Representative micrographs of tissues (original magnification, ×250). *C* and *D,* Mean ± SD no. of granulomas/mm^2; data are the average of 5 mice/time point.

increased the number of splenic bacteria at days 4 and 7, compared with numbers of bacteria in spleens of sham-ovarectomized mice (figure 3*B*). A maximum increase of 4-fold was observed at day 7 in ovarectomized mice (4.5 ± 0.7 vs. 1.2 ± 0.1 bacteria/mm^2; $P < .002$) and was equivalent to the bacterial load found in infected male mice (4.3 ± 0.7 bacteria/mm^2). In the livers of ovarectomized mice, the bacteria load was significantly increased at day 4 (0.6 ± 0.08 vs. 0.1 ± 0.03; $P < .001$) and day 7 (0.3 ± 0.06 vs. 0.04 ± 0.006; $P < .01$), compared with sham-ovarectomized mice (figure 3*C*). Taken together, these results show that ovarectomy increased bacteria load in the spleens and livers of mice.

Effect of ovarectomy on formation of granulomas. Because ovarectomy affected *C. burnetii* infection, we wondered if it also affected the formation of granulomas. The ovarectomy did not modify the time course of granuloma formation in the spleen and the liver (figure 4) or the structure of granulomas (data not shown). After 7 days of infection, the number of granulomas in the spleen was significantly higher in ovarectomized mice than in sham-ovarectomized mice (3.4 ± 0.6 vs. 1.0 ± 0.2 granulomas/mm^2; $P < .001$; figure 4*A*). The number of granulomas was similar in ovarectomized mice and in male mice (compare with figure 2*C*). After 4 days of infection, the number of granulomas in the liver was significantly higher in

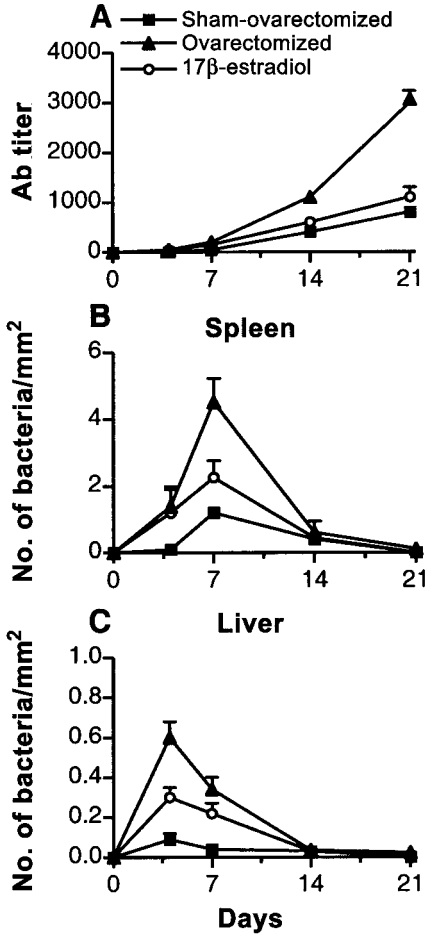

Figure 3. *Coxiella burnetii* load in ovarectomized female mice. Mice were or were not ovarectomized, and 17β-estradiol was administered to ovarectomized mice. Then they were infected with *C. burnetii* and killed at different times. *A,* Titer of circulating antibodies (Abs) to *C. burnetii;* data are the mean ± SD of 5 mice/time point. *B* and *C,* Bacteria were revealed by immunostaining in the spleen *(B)* and the liver *(C).* Data are the mean ± SD no. of bacteria/mm^2 and are the average of 5 mice/time point.

ovarectomized mice than in sham-ovarectomized mice (4.2 ± 0.7 vs. 2.0 ± 0.2; $P < .005$; figure 4*B*). Taken together, these results show that the ovarectomy markedly increased the number of granulomas in the spleen and the liver of mice.

Effect of 17β-estradiol on* C. burnetii *infection and formation of granulomas. Ovarectomized mice were treated with 17β-estradiol, and *C. burnetii* infection and formation of granulomas were assessed. First, in ovarectomized mice treated with 17β-estradiol, circulating Abs to *C. burnetii* achieved titers of 1100 ± 200 after 21 days, levels significantly ($P < .002$) lower than those in untreated ovarectomized mice but not different from those in sham-ovarectomized mice (figure 3*A*). Second, the treatment of ovarectomized mice with 17β-estradiol significantly reduced the bacteria load in the spleen (2.3 ± 0.5 bacteria/mm^2 after 7 days of infection; $P < .01$) and the liver (0.3 ± 0.05 bacteria/mm^2 after 4 days of infection; $P < .02$), compared with untreated ovarectomized mice (figure 3*B* and 3*C*). However, the bacteria load remained significantly higher ($P < .02$ for splenic infection and $P < .04$ for liver infection) than in sham-ovarectomized mice. Third, the numbers of granulomas in the spleen (1.5 ± 0.2 granulomas/mm^2) and in the liver (2.3 ± 0.3 granulomas/mm^2) were significantly ($P < .005$) lower in ovarectomized mice treated with 17β-estradiol than in untreated ovarectomized mice, at days 7 and 4 (figure 4*A* and 4*B,* respectively), similar to numbers found in sham-ovarectomized mice. Thereafter, the number of granulomas found in the spleen and the liver of mice ovarectomized and treated with 17β-estradiol decreased to the levels of those found in sham-ovarectomized and ovarectomized mice. Taken together, these results show that the administration of 17β-estradiol to ovarectomized mice reduced *C. burnetii* load and prevented up-regulated formation of granulomas.

DISCUSSION

In the present report, we have shown that sex affects *C. burnetii* infection in mice, thus emphasizing the results of epidemiological studies showing that men are infected more frequently and exhibit more-severe clinical presentations of Q fever than women [3]. First, the comparison of *C. burnetii* infection in

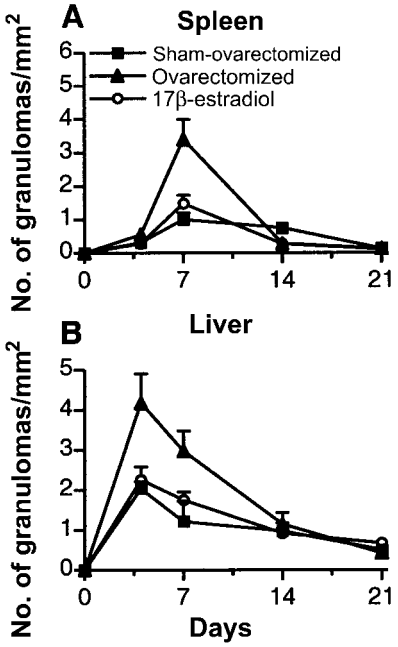

Figure 4. Granuloma counts in ovarectomized female mice. Mice were or were not ovarectomized, and 17β-estradiol was administered to ovarectomized mice. Then they were infected with *Coxiella burnetii* and killed at different times. Granulomas were revealed by histochemical analysis in the spleen *(A)* and the liver *(B).* Data are the mean ± SD no. of granulomas/mm^2 and are the average of 5 mice/time point.

mice revealed higher bacteria load in male mice than in female mice. This result suggests that female sex hormones play a role in the control of *C. burnetii* infection. To assess the role of female hormones in the course of infections, ovarectomy of rodents is a suitable approach. Ovarectomized rats are susceptible to genital chlamydial infection [9], and ovarectomized mice have higher numbers of *M. avium* organisms in the lungs than do control mice [14]. Here, we have shown that ovarectomy increased the *C. burnetii* loads in mice, compared with untreated female mice. Although bacteria load was higher in male mice than in female mice only in the spleen, ovarectomy increased the bacterial load in both the spleen and the liver of female mice. This result suggests that the lack of female sex hormones caused by ovarectomy impairs the control of *C. burnetii* infection in both the spleen and the liver. The better control of *C. burnetii* infection in male mice than in ovarectomized female mice may be related to high amounts of androgens, which are able to modulate host response to infection [24]. The effect of ovarectomy on *C. burnetii* infection largely depends on estrogen, a major female sex hormone. In ovarectomized mice treated with exogenous 17β-estradiol, the bacteria load was decreased, but not to the level found in control mice. The partial effect of 17β-estradiol was not due to insufficient hormone supplementation, because the insertion of a 0.1-mg 17β-estradiol pellet provided continuous circulating levels equal to those found during the late phase of pregnancy [25]. The effect of estrogen on *C. burnetii* infection is reminiscent of results obtained in other infectious models. The administration of 17β-estradiol to *L. monocytogenes*–infected mice decreases the number of tissue bacteria [26]. Administration of estradiol to ovarectomized rats leads to complete protection from chlamydial infection [9]. Estradiol inhibits the morphological transition of *P. brasiliensis* and reduces the fungus load in the lungs of female mice [27]. The treatment of ovarectomized mice with exogenous estradiol restores *M. avium* load to the level found in sham-ovarectomized control mice [14]. This phenomenon differs from that seen in *Leishmania* species–infected hamsters, in which the presence of high levels of androgens, rather than low levels of estrogens, is responsible for the more-severe disease observed in males [28].

The second important feature of the present report is the effect of sex on the formation of granulomas, which reflects the development of cell-mediated immunity [29]. Indeed, the number of granulomas was higher in male mice than in female mice and was increased in ovarectomized mice. Increased formation of granulomas in ovarectomized mice was prevented by the administration of 17β-estradiol, thus demonstrating that formation of granulomas in *C. burnetii*–infected mice is regulated by estrogens. Such findings are reminiscent of results reported in other infectious models. In a murine model of vaginal candidosis, estrogens suppressed *Candida albicans*–specific, delayed-type hypersensitivity [29, 30]. In a murine model of burn injury in which delayed-type hypersensitivity was impaired, the ovarectomy restored cell-mediated immune response, and 17β-estradiol prevented this correction [31]. It is likely that estrogens affect mechanisms, including cytokines, that lead to the formation of granulomas. Hence, interferon (IFN)–γ, tumor necrosis factor (TNF), and interleukin (IL)–12/IL-18 have been shown to be involved in the formation of granulomas [32]. In some reports, estrogens up-regulated the production of IFN-γ and TNF [33, 34]. In other reports, estrogens decreased the release of IL-12, TNF, and IFN-γ and decreased cell-mediated immunity [26, 35]. In a model of immunization that used mice that were ovarectomized and treated with 0.1 mg of 17β-estradiol, the continuous estradiol exposure selectively enhanced Th1 cell priming, including the frequency of IFN-γ–producing T cells [36]. In mice ovarectomized and infected with *C. burnetii* (data not shown) or *M. avium* [14], the effect of estrogens is independent of modulation of IFN-γ expression. We cannot rule out the possibility that lower antigen loads in female mice result in low levels of granuloma formation. Indeed, estrogens partly decreased *C. burnetii* load and impaired the formation of granulomas. In the present report, we have suggested a model for the role of sex hormones in *C. burnetii* infection. In the absence of pregnancy, estrogens control the severity of infection and prevent the development of granulomatous hepatitis. This protective effect may be related to prevention of cell-mediated autoimmunity, as found in experimental autoimmune encephalomyelitis [35]. It is noteworthy that the occurrence of acute Q fever with hyperinflammatory response and autoantibodies is observed in males but not in females [1]. During pregnancy, high levels of female sex hormones may exert a suppressive effect on cell-mediated immune response and favor the production of immunoregulatory cytokines, such as IL-10 and IL-4 [6]. Hence, IL-10 and its receptor are up-regulated in women during the early phase of pregnancy, compared with women who are still menstruating [37]. Both cytokines have been reported to promote replication of *C. burnetii,* and IL-10 has been associated with chronic evolution of Q fever [38]. Sex hormone–mediated suppression of protective immune response may contribute to the chronic outcome of Q fever during pregnancy.

The present report has shown the role of sex and estrogen in *C. burnetii* infection and, more specifically, in the inflammatory response induced by bacterial infection. Estrogens limit both tissue infection and formation of granulomas. Such results may explain why men experience more-severe and more-inflammatory expression of Q fever than women. This finding is critical for preventing the risk of severe forms of Q fever in individuals exposed to natural or military sources of *C. burnetii.*

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