Attenuated *Coxiella burnetii* Phase II Causes a Febrile Response in Gamma Interferon Knockout and Toll-Like Receptor 2 Knockout Mice and Protects against Reinfection

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*Coxiella burnetii* is a highly infectious obligate intracellular bacterium. The phase I form is responsible for Q fever, a febrile illness with flu-like symptoms that often goes undiagnosed. The attenuated *C. burnetii* phase II (having a truncated “O” chain of its lipopolysaccharide) does not cause disease in immunocompetent animals; however, phase II organisms remain infectious, and we questioned whether disease could be produced in immunodeficient mice. To study *C. burnetii* phase II infections, febrile responses in gamma interferon knockout (IFN-γ−/−), BALB/c, Toll-like receptor 2 knockout (TLR2−/−), and C57BL/6 mice were measured using the Nine Mile phase II (NMII) strain of *C. burnetii*. Immunocompetent mice showed minimal febrile responses, unlike those obtained with IFN-γ−/− and TLR2−/− mice, which showed elevated rectal temperatures that were sustained for ~15 days with transient increases in splenic weights. Reinfection of IFN-γ−/− and TLR2−/− mice with *C. burnetii* NMII 30 days after primary infection protected mice as evident by reduced febrile responses and a lack of splenic inflammation. Although minimal detection of *Coxiella* in TLR2−/− mouse spleens was observed, greater colonization was evident in the IFN-γ−/− mice. Cytokine analysis was performed on infected peritoneal macrophages isolated from these mice, and immunocompetent macrophages showed robust tumor necrosis factor alpha, IFN-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF) but no interleukin-12 (IL-12) responses. IFN-γ−/− macrophages produced elevated levels of IL-6, IL-10, and IL-12, while TLR2−/− macrophages produced GM-CSF, IL-12, and minimal IL-10. To distinguish immunity conferred by innate or adaptive systems, adoptive transfer studies were performed and showed that immune lymphocytes obtained from immunocompetent mice protected against a subsequent challenge with NMII, indicating that adaptive immunity mediates the observed protection. Thus, our data show that NMII is capable of eliciting disease in immunocompromised mice, which may help in evaluation of vaccine candidates as well as the study of host-pathogen interactions.

*Coxiella burnetii*, the etiological agent of Q fever, a worldwide zoonotic disease, is an obligate intracellular gram-negative bacterium that inhabits replication vacuoles of monocytes/macrophages (30). Cattle, sheep, goats, farm-raised cats, and some wild animals are considered the primary reservoirs of this pathogen. These animals shed bacteria into the environment through birthing fluids, parturition products, milk, and feces, and due to high resistance to desiccation and extreme temperatures, *Coxiella* can persist in the environment for several weeks. *C. burnetii* is highly infectious (1 to 10 organisms) in humans. Inhalation of aerosolized bacteria is the most common mode of transmission, although the ingestion of contaminated food, such as unpasteurized dairy products, has also been suggested as an infectious route (33). Because of its resistance in the environment and the low airborne infection dose required, *C. burnetii* is catalogued as a class B select agent

Q fever is a febrile illness with a flu-like presentation. Acute infection lasts 1 to 3 weeks after exposure, and the first symptoms include fever, chills, headache, weakness, and night sweats. Approximately one-third of patients develop acute respiratory disease, and abortions in pregnant women can occur (20, 32). If the infection becomes chronic, possible multiorgan complications result, such as pneumonia, hepatitis, and rarely pericarditis or endocarditis. The control of the *Coxiella* infection is characterized by the formation of granulomas in peripheral organs, which depends on the interaction with Toll-like receptor 4 (TLR4) (17), provoking the recruitment of inflammatory cells. A role of TLR2 in the protection against *C. burnetii* has also been reported (51).

*Coxiella* poses a public health problem in European countries, Canada, Japan, the Middle East, and Australia (4, 8, 24). In the United States, sero-epidemiologic studies suggest that *C. burnetii* infections have increased in cattle in the last decades; however, the number of reported human infections remains low (21). From 1948 through 1999, 436 cases of Q fever were reported in the United States, with an average of 20 cases annually. Q fever became a nationally reportable disease in 1999, and from 2000 through 2004, 255 cases were reported (51 annual cases) (25). Despite this linear increase in the docu-
mented cases since 1999, a recent national survey indicated that as many as 75% of diagnosed Q fever cases are not reported, and in many cases, Q fever is likely undiagnosed (14). It is also believed that armed forces’ personnel in Iraq are at an increased risk for Q fever (1, 13, 23).

Vaccination against Q fever remains problematic. Currently, there is no vaccine licensed in the United States. The formalin-killed phase I cellular vaccine Q-Vax is available only in Australia and is protective, but preexisting immunity can cause severe adverse reactions (45). The irradiated *C. burnetii* and its chloroform-methanol extracts are also immunogenic and protective, although severe adverse reactions have been reported. Subunit vaccines show minimal side effects; however, few data support the use of these preparations in humans (45). Thus, the reactogenicity associated with current vaccines warrants the development of protective and safer new vaccines (30).

The measurement of febrile responses has been previously employed as a parameter to follow disease after challenge (5, 36, 39, 42, 44, 47). Fever has been associated with improved survival during acute infections. It is known to be induced by a variety of different mediators (endogenous pyrogens) in response to exogenous microbial compounds that interact with T cells and monocytes/macrophages. These mediators include proinflammatory cytokines (interferons [IFNs], tumor necrosis factor alpha [TNF-α], interleukin-1α [IL-1α], IL-1β, and IL-6), as well as lymphotxin, macrophage-inflammatory protein-1β, granulocyte-macrophage colony-stimulating factor (GM-CSF), or complement components C3a and C5a. These endogenous pyrogens are able to induce the synthesis of central mediators in the central nervous system, such as prostaglandins, causing the increase in body temperatures.

In this current work, we showed the induction of disease in immunodeficient mice after infection with the clonal Nine Mile phase II strain (NMII) of *C. burnetii*. We measured febrile responses to quantify clinical symptoms in immunocompromised mice and to evaluate the effect of the infection and reinfection with NMII. Our results show that NMII is capable of eliciting disease in IFN-γ−/− and TLR2−/− mice. These findings suggest that the use of NMII in immunocompromised mice might provide an additional screening tool for new vaccines against Q fever.

**MATERIALS AND METHODS**

*C. burnetii* preparation and mouse infections. *C. burnetii* phase II organisms of the NMII (RSA443, clone 4) strain were cultured in African green monkey kidney (Vero) fibroblasts (CCL-81; American Type Culture Collection), as previously described (9).

Breeder pairs of TLR2−/− mice on a C57BL/6 background were obtained from the Jackson Laboratory. IFN-γ−/− mice on a BALB/c background were obtained as previously described (46). Pathogen-free female BALB/c and C57BL/6N mice were obtained from the National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD. All mice were maintained at the Montana State University Animal Resources Center under pathogen-free conditions in individual ventilated cages under HEPA-filtered barrier conditions and were fed sterile food and water ad libitum.

All animal care and procedures were in accordance with institutional policies for animal health and well-being. Seven-week-old female TLR2−/−, C57BL/6, IFN-γ−/−, and BALB/c mice were infected intraperitoneally (i.p.) (five mice/group) with 1 × 108 NMII or left uninfected, and rectal temperatures were measured daily. For the reexposure experiments, mice were i.p. infected with 1 × 108 CFU 30 days after the primary infection. Rectal temperatures were measured daily for another 10 days, after which lymphoid tissues were harvested for splenic weights and enumeration of genomic DNA copies.

Rectal temperatures. Rectal temperatures of *C. burnetii* NMII-infected and uninfected mice were measured using an electronic thermometer (Physitemp-Model BAT-12, Clifton, NJ) provided with a rectal probe (Physitemp-Model RET-3). The probe was washed with 70% ethanol and sterile phosphate-buffered saline (PBS) between interanimal measurements. Normal body temperatures of 35.5°C to 36.5°C were measured and were consistent with previously reported normal temperatures (47).

Antibody ELISA. Antibody titers in serum samples were determined by an enzyme-linked immunosorbent assay (ELISA) adapted from previously described methods (31). Briefly, heat-killed *Coxiella* (5 × 109 bacteria/ml) in sterile PBS was used to coat Maxisorp Immunoplate II microtiter plates (Nunc, Roskilde, Denmark) at 100 μl/well, and the plates were incubated overnight at room temperature. Various dilutions of serum samples were prepared in ELISA buffer (PBS, 0.5% bovine serum albumin, 0.05% Tween 20) and incubated overnight at 4°C. Specific reactivities against *Coxiella* were determined with horseradish peroxidase conjugates of the following detecting antibodies (1.0 μg/ml): goat anti-mouse immunoglobulin (IgG), IgG1, IgG2a, and IgG2b antibodies (Southern Biotechnology Associates, Birmingham, AL). Following 90 min of incubation at 37°C and a washing step, the specific reactivity was determined by the addition of the ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) enzyme substrate (Moss, Inc., Pasadena, CA)] at 100 μl/well, and the absorbance was measured at 415 nm on a Bio-Tek Instruments ELX808 microtiter plate reader (Winooski, VT). End point titers were expressed at the reciprocal log2 of the last sample dilution giving an absorbance of ≥0.1 optical density unit above negative control values after 1 h of incubation, similar to that previously described (49).

**Peritonal macrophage isolation.** Peritoneal macrophages from C57BL/6, TLR2−/−, BALB/c, and IFN-γ−/− mice were induced with a single i.p. injection of 1.0 ml of thioglycolate medium (Difco, Detroit, MI) as previously described (31), and 3 days later the peritoneal exudate of each mouse was washed with sterile RPMI 1640 medium (Gibco BRL-Life Technologies, Grand Island, NY) containing 2% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA) without antibiotics. Peritoneal cells were washed twice in the same medium without antibiotics.

Infection of macrophages with *C. burnetii* NMII. RAW264.7 (American Type Culture Collection, Manassas, VA) and peritoneal macrophages from C57BL/6, TLR2−/−, BALB/c, and IFN-γ−/− mice were evaluated for cytokine production after infection with *C. burnetii* NMII. Macrophages were added at 1.25 × 106 cells/well in RPMI 1640 medium containing 2% fetal calf serum (31) without antibiotics and were allowed to adhere to plastic in 24-well microtiter dishes (BD Labware, Franklin Lakes, NJ) for 3 h at 37°C. The wells were washed to remove nonadherent cells. The nonadherent cells were collected and counted to determine the number of cells that remained adherent to the plastic. After overnight culture at 37°C in complete medium without antibiotics, cells were infected at various *C. burnetii* NMII-to-macrophage ratios (1:1 to 100:1) for 8 h or 24 h at 37°C. Supernatants were collected and frozen until analysis by cytokine ELISA.

**Cytokine ELISAs.** Cytokine secretion of infected macrophages was detected using cytokine-specific ELISAs for IFN-γ, IL-1α, IL-1β, TNF-α, IL-6, IL-10, IL-12p40, IL-12p70, GM-CSF, and transforming growth factor β (TGF-β). The ELISA protocols used were identical to those assays previously described for each cytokine (29, 31). For development, horseradish peroxidase-conjugated antibodies were used (29, 31) and developed with ABTS peroxidase substrate (Moss, Inc., Pasadena, CA) at 100 μl/ml and a 1:1 optical density unit above negative control values after 1 h of incubation. The absorbance was read at 415 nm on a Bio-Tek Instruments ELX808 microtiter plate reader. To determine the amount of cytokine present in the test samples, various dilutions of recombinant murine cytokines (R&D Systems, Minneapolis, MN) were used to establish standard curves from which values for the test samples could be extrapolated.

To assess T-cell-derived cytokines, spleens were aseptically removed 10 days after infection of mice i.p. with 1 × 108 *C. burnetii* NMII organisms. Lymphocytes were prepared as previously described (29) and resuspended in a complete medium consisting of RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 1 mM nonessential amino acids, penicillin-streptomycin (10 U/ml), and 10% fetal bovine serum (Atlanta Biologicals). Lymphocytes were cultured in 24-well tissue plates at 5 × 106 cells/ml in complete medium alone or in the presence of heat-killed *Coxiella* NMII at various *C. burnetii*-to-lymphocytes ratios (1:1 to 100:1) in a total volume of 1 ml for 72 h at 37°C. The supernatants were collected by centrifugation and stored at −80°C. Capture ELISA was employed to quantify, on duplicate sets of samples, the levels of IFN-γ, TNF-α, IL-6, IL-10, IL-4, IL-13, and TGF-β produced by lymphocytes, as previously described (29, 31).

Adoptive transfer studies. Six weeks after i.p. infection of BALB/c, IFN-γ−/−, C57BL/6, and TLR2−/− mice with *C. burnetii* NMII, T cells (T-cell isolation kit; Miltenyi Biotec, Auburn, CA) and B cells (mouse B-cell negative isolation kit; Dynal Biotec ASA, Oslo, Norway) were obtained and combined at ratios of 1:1.

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The purity of lymphocyte populations was ≥98% for B cells and 96% for T cells as determined by fluorescence-activated cell sorter analysis. Immune or naïve T and B cells (3 × 10⁶ cells) were intravenously (i.v.) injected into naïve recipient BALB/c, IFN-γ−/−, C57BL/6, and TLR2−/− mice. One day later, separate groups of mice were i.p. infected with 1 × 10⁸ Coxiella burnetii NMII, and rectal temperatures were monitored daily. At 3 and 10 days postchallenge, spleens were harvested, weighed, and evaluated for the number of C. burnetii genomic copies.

**Tissue detection of C. burnetii by quantitative PCR.** C. burnetii replication in spleens of mice during infection and reinfection was quantified by using TaqMan quantitative PCR of genome equivalents. Briefly, spleens were aseptically removed, and DNA was extracted by using a DNeasy tissue kit (QIAGEN, Hilden, Germany). The tissues were weighed and homogenized in detergent lysis buffer and proteinase K for 3 h at 55°C according to the manufacturer’s instructions.

The primers (Orps6-F [5′-CGCGTTCGTCAAATCCAAATA-3′] and Orps6-R [5′-GACGCTTCCATTTCCAAA-3′]) for the stationary-phase sigma factor (ppsS) were used, and the probe (5′-ACGCTTGCAGCAATTACGCACA-3′) sequences were designed with PrimerExpress software (Applied Biosystems, Foster City, CA). Purified C. burnetii (NMII strain) DNA was used as the template to generate standard curves, as previously described (9). The concentration and purity were determined by measuring the A₂₆₀ and A₆₀₀ (9). Quantitative PCR was performed in 96-well plates with a final volume of 25 μl consisting of 12.5 μl of TaqMan universal master mix (PE Applied Biosystems, Foster City, CA), 1 μl of ppsS primer (100 nM) (0.5 μl of forward primer and 0.5 μl of reverse primer), 1 μl of DNA template, and 10.5 μl of distilled water per well, using an ABI 7500 real-time PCR system (Applied Biosystems), with one cycle of 95°C for 5 min to activate AmpliTaq Gold DNA polymerase (Applied Biosystems) and then 40 cycles of 95°C for 15 s and 60°C for 1 min to amplify the target DNA. The threshold cycle values obtained were extrapolated into the standard curves, and the DNA copies per ml of sample and then per spleen were determined.

**Statistical analysis.** Analysis of variance followed by a post hoc Tukey test was applied to show differences in body temperatures between immunocompetent and immunodeficient mice after infection with C. burnetii NMII. The Student t test was used to evaluate differences between variations in the averages of spleen weights, differences in cytokine production levels, and differences in the DNA copies of C. burnetii NMII in the quantitative PCR analysis.

**RESULTS**

**Infection with C. burnetii NMII induces fever in immunodeficient mice.** Previous studies have shown that control of C. burnetii infections requires IFN-γ (10, 11, 27, 41) and recognition by the innate immune system via TLR2 (28, 51). In addition, the role of TLR4 in the recognition of C. burnetii was also described (17). Thus, we questioned whether disease could be induced in mice deficient in IFN-γ or TLR2 using the avirulent NMII. To test this possibility, daily rectal temperatures were measured in infected and uninfected mice. Measurement of febrile responses has been previously employed to follow disease after infection with various pathogens (5, 36, 39, 42, 44, 47). Infected BALB/c mice showed no significant changes in their rectal temperatures after infection with C. burnetii NMII compared to uninfected mice. In contrast, IFN-γ−/− mice showed significant increases in their rectal temperatures (P < 0.001) compared to BALB/c mice (Fig. 1A and 2A and B). Likewise, TLR2−/− mice showed immediate increases in their body temperatures following infection (Fig. 1C and 3A and B). Immunocompetent C57BL/6 mice also showed significant increases in their temperatures (P < 0.001) after infection with NMII compared to uninfected mice (Fig. 3B), although these increases were significantly less than in TLR2−/− mice (Fig. 1C) (P < 0.001). These results show that the infection with C. burnetii NMII can cause a fever response in immunodeficient mice but not in immunocompetent BALB/c mice and only a mild fever for a few days in C57BL/6 mice.

**Infection with C. burnetii NMII provokes splenomegaly in immunodeficient mice.** Splens of infected immunocompetent
and immunodeficient mice were harvested and weighed at weekly intervals for up to 3 weeks postinfection (Fig. 1B and D). Infection of IFN-γ−/− mice showed significant increases in the splenic weights at 7 days after infection (Fig. 1B) \((P < 0.001)\). At 2 weeks after the infection, the IFN-γ−/− splenic weights were reduced, but they were still significantly greater than splenic weights of infected BALB/c mice \((P < 0.001)\).

FIG. 2. Reinfection with \(C. burnetii\) NMII provokes less severe fever in IFN-γ−/− mice and no increases in their splenic weights. (A and B) IFN-γ−/− (A) and BALB/c (B) mice were infected i.p. with \(1 \times 10^8\) \(C. burnetii\) NMII organisms (5/group) or PBS (10/group). Rectal temperatures were measured daily. Thirty days after primary infection, mice were rechallenged with same dose of \(C. burnetii\) NMII or PBS, and rectal temperatures were measured. Half of the PBS groups were infected with \(C. burnetii\) NMII; the other half were left uninfected. The normal body temperature used as a control to compare febrile responses was between 35.5 and 36.5°C. *, \(P < 0.001\) for Coxiella/Coxiella-reinfected versus PBS/PBS mice; †, \(P < 0.001\) for PBS/Coxiella-infected versus PBS/PBS mice. (C) Mice were sacrificed at 10 days after the second challenge and splenic weights measured. Reinfection of IFN-γ−/− mice provoked increases in rectal temperatures, but these were less severe than after the primary infection. There were no increases in splenic weights of IFN-γ−/− mice after the second infection. *, \(P < 0.001\) for PBS/Coxiella mice versus PBS/PBS mice and Coxiella/Coxiella-reinfected mice. Data represent the means for individual mice from two experiments ± standard deviations of the means. n.s., not significant.

FIG. 3. Reinfection with \(Coxiella burnetii\) NMII provokes less severe fever in TLR2−/− mice and no increases in splenic weights. (A and B) TLR2−/− (A) and C57BL/6 (B) mice were infected i.p. with \(1 \times 10^8\) \(C. burnetii\) NMII organisms (5/group) or PBS (10/group). Rectal temperatures were measured daily. Thirty days after primary infection, mice were rechallenged with same dose of \(C. burnetii\) NMII or PBS, and rectal temperatures were measured. Half of the PBS group were infected with \(C. burnetii\) NMII, and the other half were left uninfected. The normal body temperature used as a control to compare febrile responses was between 35.5 and 36.5°C. *, \(P < 0.001\) for Coxiella/Coxiella-reinfected versus PBS/PBS mice and for PBS/Coxiella-infected versus PBS/PBS mice; †, \(P < 0.001\) for PBS/Coxiella versus Coxiella/Coxiella-reinfected mice. (C) Mice were sacrificed 10 days after the second infection, and splenic weights were measured. As observed with IFN-γ−/− mice, reinfection of TLR2−/− mice provoked increases in rectal temperatures, but they were less severe than after the primary infection. No increases in the splenic weights of TLR2−/− mice were observed after the second challenge. *, \(P < 0.001\) for PBS/Coxiella mice versus PBS/PBS mice and Coxiella/Coxiella-reinfected mice. Data represent the means for individual mice from two experiments ± standard deviations of the means. n.s., not significant.
observed between TLR2−/− and C57BL/6 mice. Slight increases in splenic weights of BALB/c mice were observed at 1 week postinfection, but this was not evident with the C57BL/6 mice.

**Previous exposure to C. burnetii NMII reduces fever in immunodeficient mice after a reinfection.** To assess whether prior exposure to *C. burnetii* NMII could protect immunodeficient mice from fever, IFN-γ−/− and TLR2−/− mice, as well as their respective immunocompetent controls, were infected i.p., as before, monitored for primary fever responses, and then 30 days later reinfeected with the same dose of *C. burnetii* NMII. For each group, a PBS-dosed group was used as a primary fever response control for the twice-infected mice, or some mice were left uninfected to serve as a normal temperature control group. Primary infection of IFN-γ−/− mice provoked a fever response that persisted for 2 weeks (Fig. 2A), and beyond 2 weeks, the IFN-γ−/− mice recovered with no subsequent fever episodes until the second infection. Infected BALB/c mice showed no significant incidence of a fever response (Fig. 2B). When infected IFN-γ−/− mice were reinfeected with *C. burnetii* NMII, the magnitude of the induced febrile response was as potent as after the primary infection, but it was of shorter duration, lasting only 3 to 4 days (Fig. 2A). IFN-γ−/− mice given a primary infection on day 30 of this experiment reproduced the long-term febrile response. Twice-infected BALB/c mice again showed no febrile responses (Fig. 2B).

Infection of TLR2−/− mice induced a rapid febrile response that lasted 1 week, followed by a persistently low-grade febrile episode on days 8 to 16 and another one on days 27 to 30 (Fig. 3A). When these mice were twice infected with the same dose of *C. burnetii* NMII, a delay of 4 days in the febrile response was observed, and the magnitude and duration of this response were less than those after the primary infection. TLR2−/− mice given a primary infection on day 30 of this experiment also reproduced the long-term febrile responses. C57BL/6 mice developed febrile responses soon after primary infection and recovered by day 8, with no subsequent episodes (Fig. 3B). The magnitude of these responses was also less than that observed for the TLR2−/− mice. When these C57BL/6 mice were reinfeected with *C. burnetii* NMII, the magnitude and duration of the second febrile responses were similar to those obtained following the primary infection (Fig. 3B), but these febrile responses were similar in magnitude to those obtained in the twice-infected TLR2−/− mice (Fig. 3A). These results show that IFN-γ−/− mice and TLR2−/− mice are susceptible to infections with *C. burnetii* NMII resulting in disease.

**Prior exposure to C. burnetii NMII protects against subsequent colonization in immunodeficient mice.** To ascertain whether the initial exposure to *C. burnetii* could protect immunodeficient mice against colonization by a second infection, mice were evaluated at 10 days after secondary infection for their splenic weights and *C. burnetii* genomic copies. Once- or twice-infected BALB/c mice showed no measurable inflammation of their spleens compared to age-matched spleens from uninfected mice (Fig. 2C). On the other hand, C57BL/6 mice did show increased splenic weights after a single infection or two infections (Fig. 3C). However, infection of IFN-γ−/− mice and TLR2−/− mice protected against subsequent inflammation following the second infection (Fig. 2C and 3C). The splenic weights in twice-infected IFN-γ−/− mice and TLR2−/− mice were not significantly different from those in PBS control groups, suggesting that the primary infection protected these mice against subsequent infection. However, during a primary infection, the splenic weights in IFN-γ−/− mice and TLR2−/− mice increased 1.5-fold and 3-fold, respectively, compared to splenic weights in twice-infected mice (P < 0.001) or in the PBS control groups (P < 0.001).

Quantitative PCR to determine genomic DNA copy number showed a significant reduction (P < 0.001) in genomic DNA copies of *Coxiella* present in the spleens of twice-infected IFN-γ−/− mice compared to the spleens of these mice 10 days after a single infection with *C. burnetii* (Fig. 4A). No significant differences were observed between *Coxiella* genomic DNA copies detected in BALB/c spleens from once- or twice-infected mice (Fig. 4A). There were significant differences between once- or twice-infected BALB/c and IFN-γ−/− mice (Fig. 4A). There was a difference in *Coxiella* genomic DNA copies in spleens from once- or twice-infected C57BL/6 mice, but this difference was greater in TLR2−/− mice, in which spleens from twice-infected mice showed a significant reduction (P < 0.001) in *Coxiella* genomic DNA copies (Fig. 4B).
Moreover, this level of detection for twice-infected TLR2−/− mice was at the threshold of sensitivity for this assay (Fig. 4B). Singly infected TLR2−/− mice showed a significant enhancement (P < 0.001) of Coxiella genomic DNA copies in their spleens compared to the spleens from similarly NMII-infected C57BL/6 mice, but spleens from twice-infected TLR2−/− mice showed significantly fewer Coxiella genomic DNA copies than those from twice-infected C57BL/6 mice (Fig. 4B).

**Subdued inflammatory responses by C. burnetii NMII-infected macrophages from IFN-γ−/− and TLR2−/− mice.** To evaluate what proinflammatory cytokines may be induced after infection with *C. burnetii* NMII, in vitro infections of RAW264.7 and peritoneal macrophages were performed at various bacterium-to-macrophage ratios. Cytokines were measured from the collected supernatants at 8 h and 24 h postinfection. Infection of RAW264.7 cells for 8 h showed increased production of TNF-α and IL-6 and, to a lesser extent, IFN-γ and GM-CSF. A slight increase in IL-10 was measured, and no production of IL-1α, IL-1β, IL-12p40, IL-12p70, or TGF-β was observed. Samples from 24 h of infection showed a similar trend. Depicted are the means from two experiments ± standard deviations of the means.

![Graphs showing cytokine production](http://iai.asm.org/)

**FIG. 5.** RAW264.7 cells produce proinflammatory cytokines in response to NMII. RAW 264.7 macrophages were infected with *C. burnetii* NMII at various bacterium-to-macrophage ratios of between 1:1 and 100:1. Macrophages were allowed to adhere overnight, infected, and cultured for 8 h, and supernatants were harvested for cytokine ELISAs. RAW264.7 cells produced increased levels of TNF-α and IL-6 and, to a lesser extent, IFN-γ and GM-CSF. A slight increase in IL-10 was measured, and no production of IL-1α, IL-1β, IL-12p40, IL-12p70, or TGF-β was observed. Samples from 24 h of infection showed a similar trend. Depicted are the means from two experiments ± standard deviations of the means.

In twice-infected C57BL/6 mice, serum IgG, IgG2a, and IgG2b antibody responses were significantly more elevated than those in singly infected mice (Fig. 7B) (P < 0.001). In TLR2−/− mice, significant increases (P < 0.001) in IgG, IgG2a, and IgG2b levels were observed after two NMII infections than after one infection. IgG antibody responses were significantly lower in TLR2−/− mice infected once with NMII than in C57BL/6 mice, but no differences were observed after...
two infections. IgG2a production was enhanced in TLR2−/− mice infected twice compared to twice-infected C57BL/6 mice. By contrast, IgG2b levels in twice-infected TLR2−/− mice infected twice compared to twice-infected C57BL/6 mice.

**Th cell cytokine responses following antigen restimulation.**
The lack of IgG1 antibodies in serum and the high production of IgG2a antibodies regardless of the mouse strain tested suggest that Th1-type immune responses are induced. Thus, antigen restimulation assays were conducted to ascertain the cytokine profiles in response to *Coxiella* antigens. BALB/c, IFN-γ−/−, C57BL/6, and TLR2−/− mice were infected i.p. with *C. burnetii* NMII, and at 10 days postinfection splenic lymphocytes were isolated and restimulated with heat-killed *Coxiella* (Table 1). Elevations in IFN-γ were detected in BALB/c and C57BL/6 lymphocyte cultures but to a lesser extent in TLR2−/− lymphocyte cultures. Interestingly, IFN-γ−/− lymphocytes produced significantly higher levels of IL-10 (P < 0.001) in the absence of IFN-γ pressure than lymphocytes from BALB/c or C57BL/6 mice (Table 1). There were no differences detected in the production of IL-6 in BALB/c and IFN-γ−/− mice, but it was elevated in C57BL/6 mice. No TNF-α, IL-4, IL-13, or TGF-β were detected in any of the restimulation lymphocyte cultures from any of the infected mice. These results might offer a possible explanation for the reduction of fever and inflammation observed in immunodeficient mice after rechallenge with NMII. The protection observed in IFN-γ−/− mice after a subsequent rechallenge may be IL-10 dependent.

**Immune T and B cells confer long-term protection against fever.** In our previous experiments, the results showed that a prior exposure to *C. burnetii* NMII in immunodeficient mice induced protection against a subsequent challenge 4 weeks later. In order to ascertain whether protection was due to the activation of the innate immunity or to the development of acquired immunity, adoptive transfer studies using immune or naive T and B cells were performed. Immunocompetent and immunodeficient mice were infected i.p. with *C. burnetii* NMII, and 6 weeks later T and B cells were purified, combined at 1:1 ratios, and adoptively transferred (3 × 10⁶ cells/mouse) into naive recipient mice. One day after the adoptive transfer, mice were challenged with NMII, and rectal temperatures were monitored daily. Spleens were harvested at 3 or 10 days after infection and weighed, and quantitative PCR analysis was performed to detect *C. burnetii* genomic copies (see Fig. 10). Naïve immunocompromised or immunocompetent mice adoptively transferred with immune T and B cells from immunocompetent mice exposed to NMII 6 weeks earlier were protected against fever after subsequent challenge with NMII.

When immune T and B cells from BALB/c mice were transferred into naive IFN-γ−/− recipients, no fever episodes were observed in these mice following challenge with NMII, as opposed to the case for IFN-γ−/− mice treated with PBS, which suffered significant increases (P < 0.001) in their body temperatures (Fig. 8C). When immune T and B cells obtained from infected IFN-γ−/− mice were transferred into naive IFN-γ−/− recipients, a significant reduction of fever was observed for only 2 days (days 3 and 4 postchallenge), as occurred with naive IFN-γ−/− mice that received naive BALB/c T and B cells. Splenic weight measurements at 3 and 10 days postchallenge showed a significant reduction (P < 0.001) in inflammation after infection in IFN-γ−/− recipients that received infected IFN-γ−/− or infected BALB/c T and B cells compared to IFN-γ−/− recipients receiving PBS or naive BALB/c mice.
cells (Fig. 8D). However, when naive BALB/c mice received naive IFN-γ−/− or infected IFN-γ−/− T and B cells, significant increases in their body temperatures (P < 0.001) were observed for 2 days following challenge with NMII (Fig. 8A) compared to PBS-treated BALB/c mice. In agreement with the data in Fig. 1 and 2A, no significant increases in the temperatures were observed when naive PBS-treated BALB/c mice were infected with NMII. Interestingly, when infected BALB/c cells were transferred into BALB/c mice, slight increases (not significant) in the temperatures were observed compared to the PBS group. Splenic weights of BALB/c recipients given immune T and B cells from infected BALB/c mice showed

TABLE 1. Cytokine production by splenic lymphocyte cells from BALB/c, IFN-γ−/−, C57BL/6, and TLR2−/− mice at 10 days postinfection with C. burnetii NMII

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>BALB/c</th>
<th>IFN-γ−/−</th>
<th>C57BL/6</th>
<th>TLR2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ag</td>
<td>Medium</td>
<td>Ag</td>
<td>Medium</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5.7 ± 2.1*</td>
<td>0.3 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.8 ± 2.1</td>
<td>0.2 ± 0.1</td>
<td>2.2 ± 0.2</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>IL-10</td>
<td>4.0 ± 1.0</td>
<td>0.1 ± 0.1</td>
<td>6.2 ± 0.1*</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>TGF-β</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

* T cells were antigen (Ag) restimulated with heat-killed Coxiella (C. burnetii-to-lymphocytes ratio, 100:1) or medium and cultured in complete medium for 72 h at 37°C (5% CO2). †, P < 0.001 for Ag-pulsed cells from BALB/c mice versus IFN-γ−/− mice. †, P < 0.001 for Ag-pulsed cells from C57BL/6 mice versus TLR2−/− mice.
significant reductions ($P < 0.001$) at 3 and 10 days after challenge with NMII (Fig. 8B). BALB/c recipients given immune lymphocytes from infected IFN-γ−/− mice showed a reduction in splenic weights only on day 3 postchallenge (Fig. 8B).

Infection of C57BL/6 mice with NMII showed the expected slight increases in the body temperatures (Fig. 9A), as previously observed (Fig. 1C and 3B). When immune T and B cells obtained from infected C57BL/6, infected TLR2−/−, or naive TLR2−/− mice were adoptively transferred into naive C57BL/6 recipients, no significant reductions in fever were observed following infection with NMII (Fig. 9A). Moreover, no significant differences in the splenic weights of these mice were observed (Fig. 9B). However, very remarkable differences were observed in the body temperatures of TLR2−/− recipients given immune T and B cells and subsequently challenged with NMII (Fig. 9C). When immune T and B cells from infected C57BL/6 donors were transferred into naive TLR2−/− recipients, basically no fever was observed ($P < 0.001$) compared to PBS-treated TLR2−/− mice infected with NMII, which had elevated body temperatures that lasted for more than 1 week. The reduction of fever was less apparent, although statistically significant ($P < 0.001$), in TLR2−/− recipients that received immune T and B cells from infected TLR2−/− mice compared to the PBS-treated TLR2−/− mice. When cells from naive C57BL/6 mice were transferred into TLR2−/− recipients, no significant differences in the temperatures were observed compared to PBS-treated mice, but significant increases (Fig. 9C) ($P < 0.001$) were observed compared to the body temperatures of naive TLR2−/− recipients that received immune T and B cells from infected C57BL/6 cells. Splenic weights of naive TLR2−/− recipients that received immune T and B cells from infected TLR2−/− or C57BL/6 mice were significantly lower ($P < 0.001$) than the splenic weights from PBS-treated TLR2−/− mice or those given naive C57BL/6 cells at 3 days after challenge (Fig. 9D). At 10 days postchallenge, a significant reduction ($P < 0.001$) in splenic weights of TLR2−/− recipients given immune T and B cells from infected C57BL/6 mice was observed compared to TLR2−/− recipients given PBS or T and B cells from naive C57BL/6 mice or from infected TLR2−/− mice.

Quantitative PCR analysis was performed on spleens at 3 and 10 days after challenge with C. burnetii NMII, and the data revealed that the adoptive transfer of immune T and B cells obtained from BALB/c and C57BL/6 mice prevented the colonization by Coxiella NMII (Fig. 10). The genomic DNA copy numbers for NMII in spleens of IFN-γ−/− mice treated with...
immune T and B cells from infected BALB/c or IFN-γ−/− mice were significantly reduced (P < 0.001) compared to those in PBS-treated or naive BALB/c mice (Fig. 10C) at 3 and 10 days after challenge. BALB/c mice treated with lymphocytes from immunized BALB/c or IFN-γ−/− mice showed a significant reduction in the genomic copies at 10 days postchallenge (Fig. 10A). No reductions in the NMII genomic copies were observed in C57BL/6 recipient mice (Fig. 10B). By contrast, TLR2−/− mice that received immune T and B cells from infected C57BL/6 or TLR2−/− mice showed a significant reduction (P < 0.001) in NMII colonization at 3 and 10 days after challenge. These results demonstrate that the immune response induced in immunocompetent mice after a prior exposure with NMII possesses an acquired component that lasts for at least 6 weeks and is able to protect against a subsequent challenge with C. burnetii NMII.

**DISCUSSION**

For these studies, the avirulent C. burnetii NMII strain, which contains a chromosomal mutation affecting the biosynthesis of its LPS (18), was used to investigate potential disease mechanisms in immunocompromised mice. The NMII LPS lacks the O-polysaccharide chain of virulent organisms in phase I (38). Repeated passage of the virulent NMII through embryonated eggs caused a spontaneous mutation in its genome (12), resulting in a chromosomal deletion that caused the lack of the O chain of its LPS, the only known virulence factor of Coxiella (51), and consequently resulted in the pathogen's attenuation. C. burnetii's LPS is considered less endotoxic than the LPS of Enterobacteriaceae (48), but it has been shown to induce proinflammatory cytokine production in mice (40). The phenotypic differences of the two phases influence the interactions with the host cells. C. burnetii is an obligate intracellular bacterium that resides in monocytes/macrophages. The CR3 receptor has been shown to be involved in the entry of C. burnetii phase II organisms into monocytes, via the phagolysosomal pathway, whereas the complex of leukocyte integrin and the integrin-associated protein of human monocytes appears to bind to phase I (26). Recently, it was shown that the impaired fusion of bacterial phagosomes with lysosomes enhances the survival of phase I in monocytes (15). Intracellular stages of C. burnetii include a metabolically active large-cell variant that is...
different from the metabolically inactive small-cell variant observed extracellularly. Recently, the large-cell variant has been described as the replicative form of *C. burnetii* (9). Here we describe infection of immunodeficient mice with *C. burnetii* that results in a febrile response and protects against a subsequent febrile response, splenomegaly, and colonization. Phase II attenuated strains have been employed in animal models (3, 7, 37) and in vitro studies (50–52). The use of immunocompromised animals in infection studies with *C. burnetii* was previously described for SCID (lacking T and B cells) (2) and SCIDbg (lacking T, B, and NK cells) mice (3). In the latter study, the innate immune responses induced against the infection with the virulent phase I were not able to control the replication of the microorganisms in the spleens of the infected animals, and severe splenomegaly, loss of body weight, and systemic histopathological damage were observed. When immunodeficient mice were infected with NMII, a transient splenomegaly, but no loss of body weight or other clinical symptoms, was observed. The use of immunodeficient mice and attenuated bacteria might serve to evaluate vaccine candidates against *C. burnetii*, as well as to study host-pathogen interactions that otherwise remain obscured in immunocompetent animals.

One of the major immune responses against acute infections is fever, and febrile responses have been previously quantified as a parameter of disease after challenge of animals with different pathogens or in animals subjected to stress (5, 36, 39, 42, 44, 47). Various bacterial components, including LPS, can stimulate a spectrum of host endogenous pyrogens to potentiate a fever response. These include the proinflammatory cytokines, IFNs, TNF-α, IL-1α, IL-1β, and IL-6, as well as other soluble molecules such as lymphotixin, GM-CSF, and the complement factors C3a and C5a. In the present work, daily rectal temperatures were monitored as clinical symptoms of disease following i.p. infection with *C. burnetii* NMII. Our results show that infection of IFN-γ−/− mice and TLR2−/−...
mice with C. burnetii NMII induced febrile responses that lasted for at least 2 weeks. Infection of IFN-γ−/− mice showed febrile responses of ≥1°C above normal temperatures that persisted for 2 weeks. In TLR2−/− mice, the initial febrile responses were of shorter duration than in IFN-γ−/− mice, which lasted several days, but were also ≥1.5°C above normal temperatures; they then declined to ≤0.6°C above normal temperature, which persisted for 9 to 10 days. A second bout of low-grade febrile responses was observed a short time later. Nonetheless, the primary infection with C. burnetii NMII protected against subsequent febrile responses when the mice were challenged again with NMII. In the IFN-γ−/− mice, the febrile responses attributed to secondary infection were of shorter duration, lasting only 4 or 5 days, and did not have the intensity of the primary infection. Likewise, in TLR2−/− mice, the febrile responses were also of shorter duration, lasting only 1 week, and were without the intensity of the primary infection. In the IFN-γ−/− mice and TLR2−/− mice, increased splenic weights were observed only 7 to 10 days after primary infection and not by 10 days after reinfection. Quantitative PCR analysis of these spleens revealed that reinfection of immunodeficient mice with NMII provoked a reduction in genomic DNA copies of C. burnetii, suggesting immune clearance from these mice following a second exposure to NMII. Surprisingly, no detectable levels of C. burnetii (below threshold of detection) were observed in the spleens of TLR2−/− mice 10 days after reinfection. Based on these results, a primary infection of immunodeficient mice with C. burnetii NMII can cause disease, as evidenced by the produced febrile responses, and this preexposure to C. burnetii NMII is protective against subsequent infection with NMII.

Control of wild-type Coxiella infections is characterized by the formation of granulomas in peripheral organs in a TLR4-dependent fashion (17), provoking the recruitment of inflammatory cells. Recently, it was shown that the impaired fusion of bacterial phagosomes with lysosomes enhances the survival of phase I in monocytes (15). Honstetter et al. reported that the recognition of LPS through TLR4 in macrophages induced phagocytosis and inflammatory responses against Coxiella (17), and they suggested that TLR2 was not involved in the phagocytosis of C. burnetii. However, a different study showed that TLR4-specific responses were not detected, and murine macrophages deficient for TLR2 were susceptible to infection with NMII. This work suggests that TLR4 is not involved in the recognition of C. burnetii and that TLR2 is necessary in the detection of Coxiella and the subsequent production of proinflammatory cytokines (51). It has also been described that murine macrophages defective in TLR2 may be dispensable for the clearance of C. burnetii NMII in vivo (28), as was observed for TLR4 (17). Our results showed that a deficiency in TLR2 provokes a febrile response and splenomegaly in mice, confirming that TLR2 is a necessary key in the regulation of immune responses against NMII. However, this deficiency is not dispensable, since the lack of TLR2 does not avoid the resistance and clearance of the bacteria after a second exposure. In vitro cytokine analysis revealed that TLR2−/− peritoneal macrophages did not produce IFN-γ, TNF-α, IL-1α, IL-1β, or IL-6 proinflammatory cytokines, which was consistent with previously reported data (51). However, significant febrile responses, which might be contributed by GM-CSF, IL-12, or other factors, were observed in these mice after primary infection. In vitro cytokine analysis revealed that TLR2−/− peritoneal macrophages produced elevated levels of GM-CSF and IL-12p70.

Previous reports have shown that activation by IFN-γ of guinea pigs monocytes, murine fibroblasts, and primary mouse macrophages controls the replication of C. burnetii (11, 15, 42, 53). IFN-γ can also regulate nitric oxide production by macrophages infected with C. burnetii in the replication of phase I (6) and the replication of NMII in fibroblasts activated with IFN-γ (19). Interestingly, infection of IFN-γ−/− peritoneal macrophages produced significantly greater amounts of IL-6, IL-12p40, and IL-12p70 than that of BALB/c macrophages. These increases in IL-6, and possibly IL-12, may account for the elevated body temperatures observed with these mice. Disease in IFN-γ−/− mice after infection with C. burnetii NMII was observed as enhanced febrile responses, transient splenic inflammation, and the lack of production of proinflammatory cytokines following in vitro infection of peritoneal macrophages. These results confirm the previously described role of IFN-γ in the protection of immunocompetent mice against phase II organisms of C. burnetii (6).

T and B cells obtained from immunocompetent BALB/c and C57BL/6 mice previously challenged with NMII protected against a subsequent challenge with NMII, showing that these cells remained immune for at least 6 weeks. This protection, observed after the adoptive transfer of T and B lymphocytes, resulted in a reduction in fever, splenomegaly, and Coxiella colonization. This result supports the idea of the induction of a long-term protection against C. burnetii NMII in immunocompetent mice. Cytokine analysis of antigen-restimulated lymphocytes showed that IFN-γ and IL-6 are involved in protection. The lack of Coxiella-specific IgG1 antibodies and the elevated production of IgG2a antibodies by BALB/c, IFN-γ−/−, and TLR2−/− mice after infection with NMII suggest that Th1-type immune responses are induced and contribute to the observed protection against a secondary challenge with NMII.

We have described in this work a valid model to study the disease caused by C. burnetii NMII in immunodeficient mice. This approach might serve to evaluate vaccine candidates, as well as to study host-pathogen interactions. We used the detection of febrile responses as a valid tool to quantify clinical symptoms in immunocompromised mice and evaluate the effect of the infection and reinfection with NMII. Phase II C. burnetii can also be used as an alternative in screening multiple vaccine candidates, as well as in optimizing their immunogenicity. Once identified, these vaccine candidates would need to be subjected to additional testing using a phase I C. burnetii challenge in normal mice to validate their efficacy. The use of NMII would further allow study of its pathogenicity outside biosafety level 3 (BSL-3) containment, and could allow for more focused studies in BSL-3 containment for infections with phase I C. burnetii. The approach used for our studies offers a simple method to follow disease, albeit in an immunocompromised mouse, but often these types of studies are important to learn about protective mechanisms, as with Pneumocystis carinii in SCID (16) or type I IFN receptor-deficient mice (35) or the use of attenuated Salmonella enterica Typhimurium vaccines in IFN-γ−/− mice, which become virulent in these mice.
(43). Our findings suggest that immunodeficient animals and attenuated bacteria might be a safe model of infection for screening new vaccines against Q fever under BSL-2 conditions.

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