Extended Safety and Efficacy Studies of the Attenuated *Brucella* Vaccine Candidates 16M$\Delta vjbR$ and S19$\Delta vjbR$ in the Immunocompromised IRF-1$^{-/-}$ Mouse Model

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The global distribution of brucellosis and high incidence in certain areas of the world warrant the development of a safer and efficacious vaccine. For the past 10 years, we have focused our attention on the development of a safer, but still highly protective, live attenuated vaccine for human and animal use. We have demonstrated the safety and protective efficacy of the vaccine candidates 16M$\Delta vjbR$ and S19$\Delta vjbR$ against homologous and heterologous challenge in multiple immunocompetent animal models, including mice and deer. In the present study, we conducted a series of experiments to determine the safety of the vaccine candidates in interferon regulatory factor-1-knockout (IRF-1$^{-/-}$) mice. IRF-1$^{-/-}$ mice infected with either wild-type *Brucella melitensis* 16M or the vaccine strain *Brucella abortus* S19 succumb to the disease within the first 3 weeks of infection, which is characterized by a marked granulomatous and neutrophilic inflammatory response that principally targets the spleen and liver. In contrast, IRF-1$^{-/-}$ mice inoculated with either the 16M$\Delta vjbR$ or S19$\Delta vjbR$ vaccine do not show any clinical or major pathological changes associated with vaccination. Additionally, when 16M$\Delta vjbR$- or S19$\Delta vjbR$-vaccinated mice are challenged with wild-type *Brucella melitensis* 16M, the degree of colonization in multiple organs, along with associated pathological changes, is significantly reduced. These findings not only demonstrate the safety and protective efficacy of the *vjbR* mutant in an immunocompromised mouse model but also suggest the participation of lesser-known mechanisms in protective immunity against brucellosis.

Brucellosis, a bacterial disease caused by members of the genus *Brucella*, is an important zoonosis of nearly worldwide distribution (22). In humans, the disease is characterized by episodes of fever, arthritis, endocarditis, and osteomyelitis (22). In animals, the most common manifestations are abortions and infertility, which result in extensive economic losses (28). To date, five out of the nine known *Brucella* species are capable of infecting humans, with the most pathogenic being *Brucella melitensis*, followed by *Brucella suis* and *Brucella abortus*. These three species have been classified as biological warfare agents by the Centers for Disease and Control and Prevention (CDC) due to their low infectivity dose when aerosolized and substantial capacity to cause a chronic debilitating illness (9). Vaccination against brucellosis has been integral for disease prevention in animals; however, the currently licensed vaccine strains for veterinary usage, such as S19, RB51, or Rev 1, are unacceptable for human use due to residual virulence that can result in the development of the disease (24, 27). To date, a safe and protective vaccine for use against human brucellosis is still needed.

Although many novel DNA and subunit vaccines against brucellosis are being developed, the live attenuated vaccines confer the greatest level of protection due to their ability to generate more persistent memory responses than nonliving preparations (7, 24). This protection is observed not only for *Brucella* vaccines but also for other infectious pathogens as well (18, 21, 30). At least 10 live attenuated vaccines are currently licensed in the United States (USDA website).

In the past, classic attenuation processes were somewhat empirical and unpredictable, which raised multiple concerns regarding safety, including reversion to wild-type virulence and vaccine shedding. Both of these adverse events pose a risk to vaccinated or even unvaccinated individuals with impaired immunity. Advances in molecular biology have led to the identification of genes that are essential for bacterial replication and persistence *in vivo* and *in vitro*. On the basis of this knowledge, our laboratory has designed a rational live attenuated vaccine for brucellosis via deletion of the *vjbR* gene (BMEII1116), which encodes the *luxR*-like quorum-sensing-related transcriptional regulator. VjbR is required for adequate VirB expression, which is essential for survival in phagocytes and virulence in mice (32). The *vjbR* mutants of *B. melitensis* and *B. abortus* are highly attenuated in mice and macrophages while conferring high levels of protective immunity, making such mutants ideal vaccine candidates (2, 3).

In the present study, we conducted a series of experiments designed to provide additional evidence of the safety and efficacy of the vaccine candidates 16M$\Delta vjbR$ and S19$\Delta vjbR$ by demonstrating their performance in the interferon regulatory factor-1-knockout (IRF-1$^{-/-}$) mouse model, in which mice are known to succumb to wild-type infection (16).

MATERIALS AND METHODS

Mice. Six- to 8-week old, female B6.129s2-*Irf1^{tm1Mak}*/J mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed un-
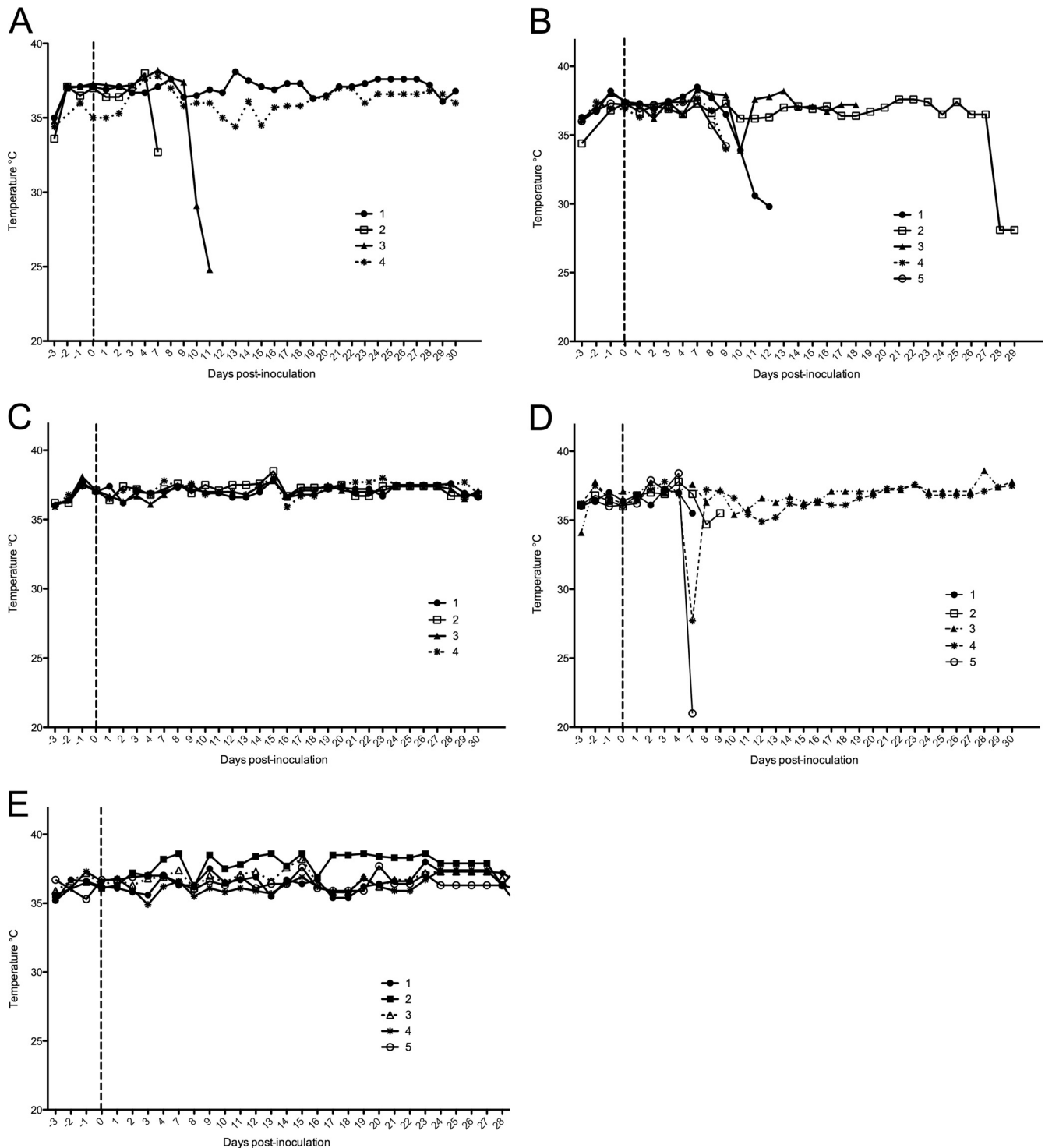
FIG 1 Temperature profiles from infected immune-incompetent mice. IRF-1$^{-/-}$ mice were implanted with transponders and monitored daily over a 33-day period following inoculation with 1×10^6 CFU/mouse of either 2308 (A), S19 (B), S19$\Delta vjbR$ (C), 16M (D), or 16M$\Delta vjbR$ (E). The dotted lines denote the day of inoculation; temperature profiles to the left of the dotted lines denote basal body temperatures prior to inoculation. Each symbol represents an individual mouse tracked for 30 days postinoculation.

der specific-pathogen-free conditions and acclimated for 2 weeks prior to infection. All experimental procedures and animal care were performed in compliance with the institutional animal care guidelines.

Bacterial strains. Strains used in these experiments include *B. melitensis* 16M$\Delta vjbR$ (engineered for a previous study and used as a vaccine candidate) (35), S19$\Delta vjbR$ (engineered for a previous study and used as a vaccine candidate) (2), *B. abortus* S19 (NVSL, Ames, IA), the virulent strain *Brucella melitensis* 16M biovar 1 (originally obtained from ATCC and reisolated by this lab from an aborted goat fetus) (2, 12), and the wild-type strain *Brucella abortus* 2308 obtained from an aborted fetus by

A   B
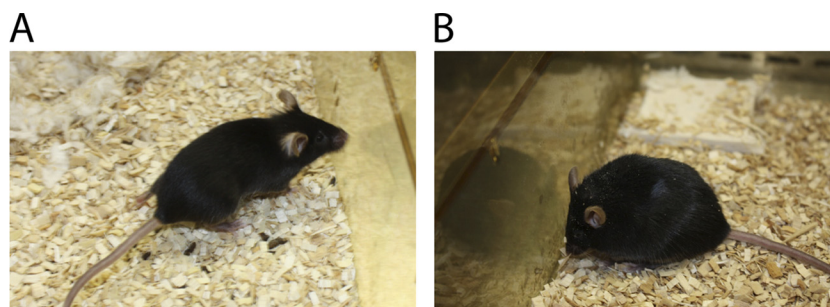
FIG 2 Health checks of immune-incompetent mice. Differences in behavior of IRF-1$^{-/-}$ mice infected with 1×10^6 CFU/mouse of either 16MΔ*vjbR* (A) or 16M (B) were noticeable by day 10 postinoculation. Note the abnormal posture and ruffled coat in the mouse inoculated with the wild-type strain.

B. Deyoe (NADC). Bacteria were grown on tryptic soy agar (TSA; Difco, Becton Dickinson) or Farrell's medium (TSA supplemented with *Brucella* selective supplement [Oxoid Limited] at 37°C with 5% [vol/vol] CO_2).

To prepare organisms for animal infections, the *Brucella* strains were harvested from the surface of the plates after 3 days of incubation using phosphate-buffered saline (PBS), pH 7.2 (Gibco). The bacteria were resuspended to a final concentration of 1×10^6 CFU/mouse on the basis of optical density readings using a Klett meter and a standardized light-scattering curve. Actual viable counts were confirmed retrospectively by serial dilution, plating, and enumeration.

Measurement of body temperature. Implantable temperature transponders (IPTT-300) and a handheld reader (DAS-7007; Bio Medic Data Systems, DE) were used according to the manufacturer's instructions. Briefly, the mouse-specific transponders were preprogrammed with individual number identifiers before they were implanted into each individual mouse. One week prior to inoculation with the *Brucella* strains, the transponders were injected into the subcutaneous tissue along the left lateral aspect of the flank, using the insertion device included with the microchips. Prior to inoculation, the basal temperature was monitored for 3 days for each individual mouse. Once mice were inoculated, the temperature was monitored for the first 30 days and for 1 week following challenge in those individuals that survived past the initial 30 days. Measurement of the temperature was achieved by holding the handheld detector approximately 5 to 10 cm from the implanted chip. Temperature was monitored once or twice daily, always at the same time of the day. A temperature drop below 32°C was chosen as an endpoint indicating bacterial sepsis.

Evaluation of virulence of *Brucella* Δ*vjbR* mutant in IRF-1$^{-/-}$ mice. Six groups of five mice each were injected intraperitoneally with 1×10^6 CFU/mouse with either (i) *B. melitensis* 16M, (ii) *B. melitensis* 16MΔ*vjbR*, (iii) *B. abortus* 2308, (iv) *B. abortus* S19, (v) *B. abortus* S19Δ*vjbR*, or (vi) PBS. Mice were monitored daily for clinical evidence of disease. Any infected animal with substantial reduced mobility, abnormal postural changes, ruffled coat, and a temperature below 32°C was considered a mortality and immediately euthanized via carbon dioxide asphyxiation. At 30 days postinoculation, a selection of animals that survived the infection for the first month were also euthanized to determine the bacterial colonization and associated pathological findings. At each time point, spleens and livers were collected, weighed, and homogenized in 1 ml of peptone saline. Serial dilutions were prepared, and 100-μl aliquots of the different dilutions were plated in duplicate onto TSA or Farrell's medium. Levels of infection were expressed as the mean ± standard error (SE) of the individual log number of CFU/organ (see Fig. 8 and 9).

Evaluation of protective efficacy of *vjbR* mutants in IRF-1$^{-/-}$ mice. Groups of four female IRF-1$^{-/-}$ mice were vaccinated intraperitoneally with 1×10^6 CFU of either *B. melitensis* 16MΔ*vjbR* or *B. abortus* S19Δ*vjbR*. These mice were monitored for any sign of clinical disease, including body temperature changes as described above. At 8 weeks postvaccination, mice were challenged intraperitoneally with 5×10^6 CFU of *B. melitensis* wild-type 16M/mouse. At 1 week postchallenge, animals were euthanized by CO_2 asphyxiation, and the spleens and livers were removed, weighed, and homogenized in 1 ml of peptone saline. Serial dilutions were prepared, and 100-μl portions were plated onto TSA plates. Levels of infection were expressed as the mean log number of CFU/organ ± SE recovered from mice.

Evaluation of histological changes in IRF-1$^{-/-}$ mice inoculated with the different *Brucella* strains. Multiple organs, including spleen, liver, heart, kidney, lung, intestine, lymph nodes, and brain, from *Brucella*-infected mice were collected at death, 30 days postinoculation, or 1 week postchallenge, placed in 10% buffered formalin, paraffin embedded, and stained with hematoxylin and eosin. Histological changes between groups were assessed by a board-certified veterinary pathologist.

Statistical analysis. Bacterial burdens from mutant clearance as well as efficacy studies were expressed as the mean number of CFU ± SE and presented graphically as the $\log_{10}$ number of *Brucella* CFU recovered per organ. Culture-negative organs were assigned a value of 4 CFU, which is below the limit of detection of 5 CFU/organ. Spleen weight data from challenge were plotted as the mean spleen weight (in mg) ± SE. A log-rank analysis (Mantel-Cox test) using GraphPad Prism software was used to determine the significance of survival curves among mice.

RESULTS

Temperature changes in IRF-1$^{-/-}$ mice infected with the different *Brucella* strains. To determine possible temperature fluctuations due to the inoculation with the vaccine candidates, mice were monitored daily pre- and postinoculation for up to 30 days using implantable temperature transponders. Basal preinoculation temperatures were in the range of 35°C to 37.8°C. Mice inoculated with either the 16MΔ*vjbR* or S19Δ*vjbR* mutant did not
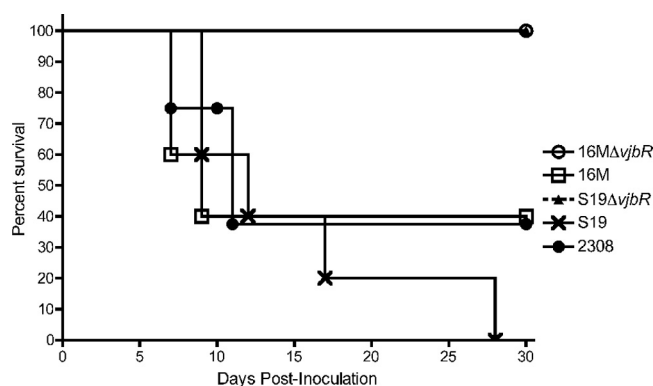
FIG 3 Survival of IRF-1$^{-/-}$ mice infected with 1×10^6 CFU/mouse of either 2308, S19, S19Δ*vjbR*, 16M, or 16MΔ*vjbR*. Mice inoculated with Δ*vjbR* vaccine candidates survived longer than mice inoculated with either 16M ($P < 0.005$), 2308 ($P < 0.005$), or S19 ($P < 0.005$).
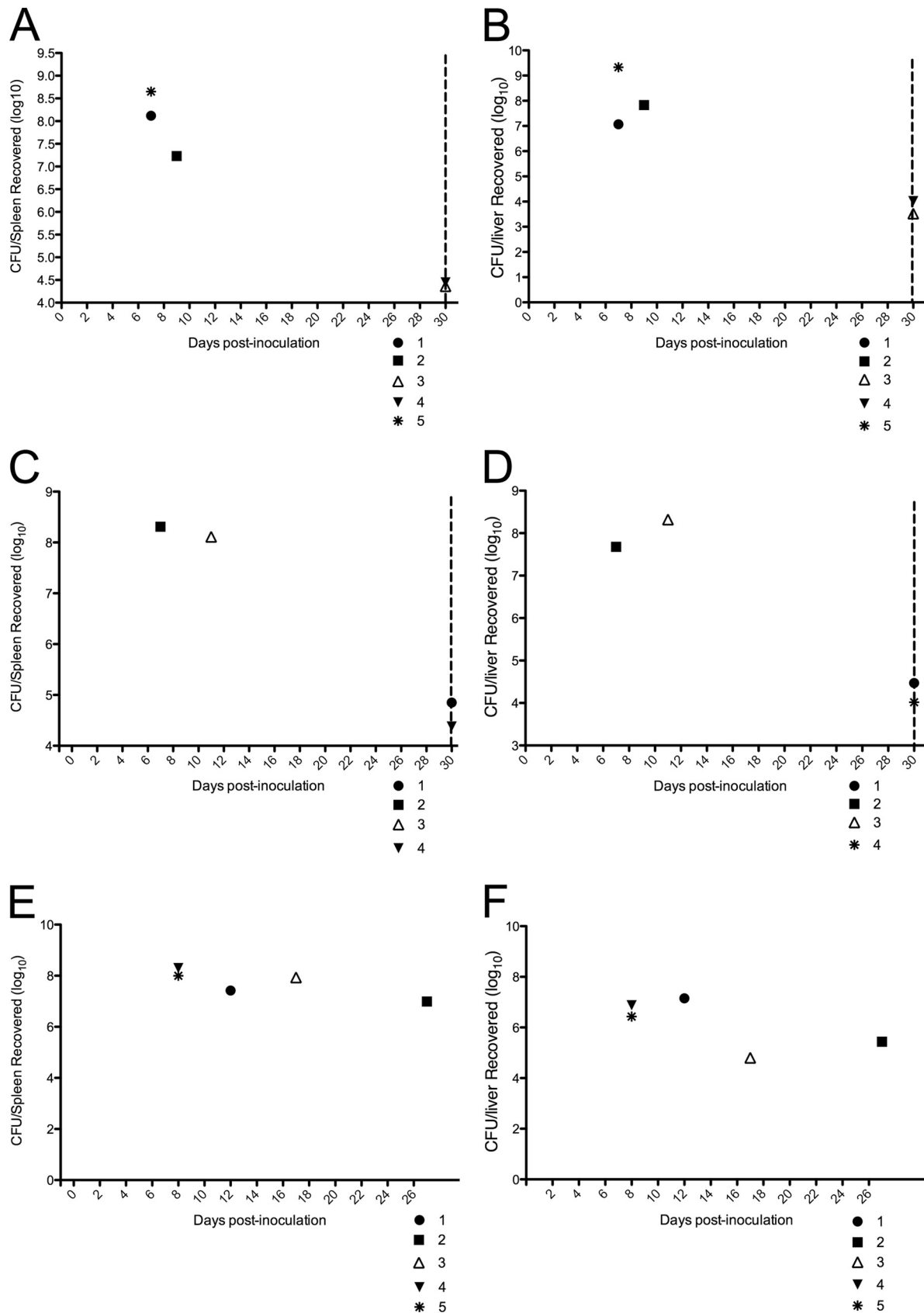
FIG 4 Bacterial colonization in the spleens and livers in terminally ill IRF-1$^{-/-}$ mice inoculated with 1×10^6 CFU/mouse of either 16M (A and B), 2308 (C and D), or S19 (E and F). The dotted lines denote 30 days postinfection, and animals that did not succumb to the infection prior to this time point were euthanized to determine the extent of bacterial colonization. Data are expressed as log$_{10}$ number of CFU/organ.
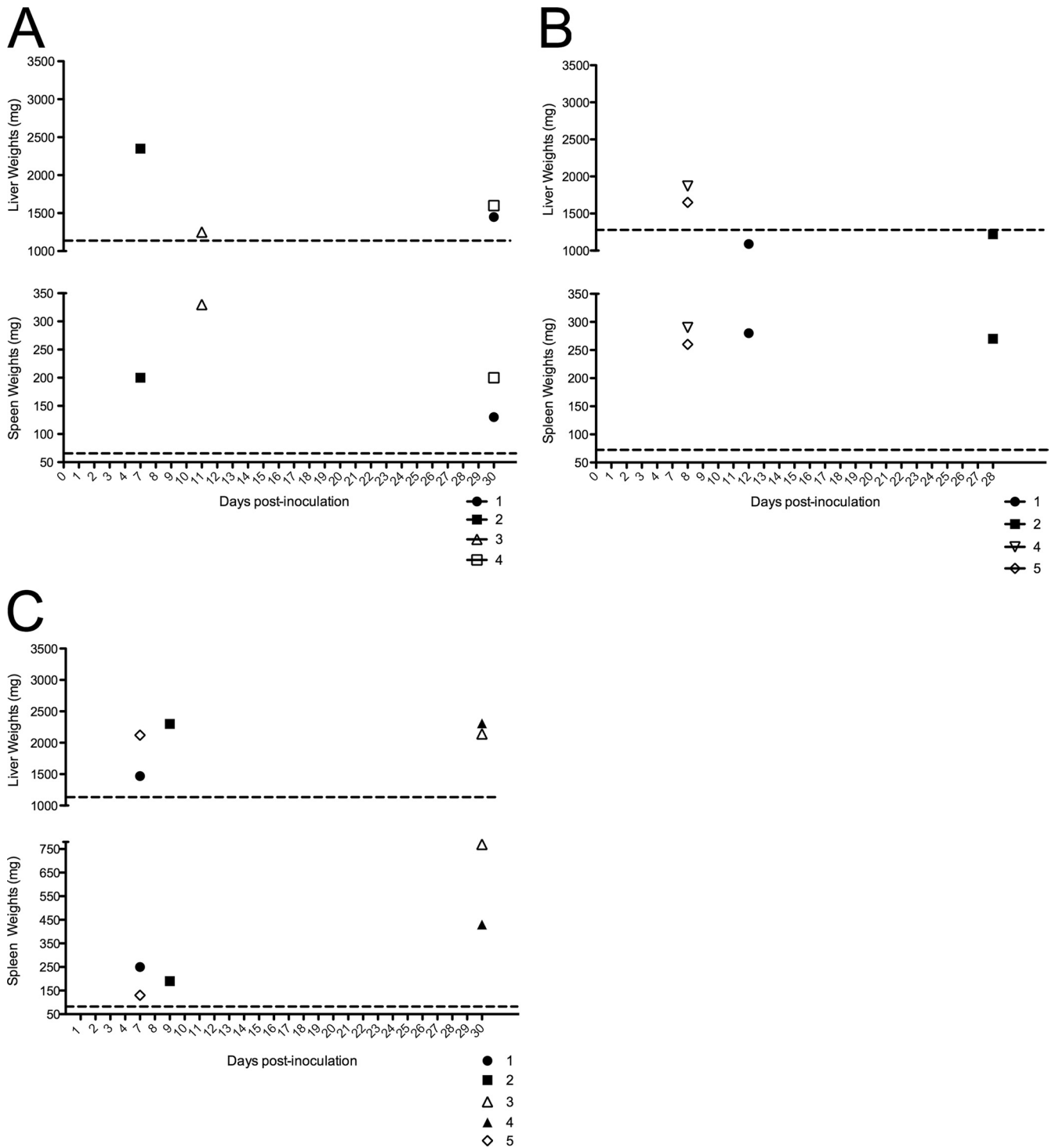
FIG 5 Spleen and liver weights in terminally ill IRF-1$^{-/-}$ mice inoculated with 1×10^6 CFU/mouse of either 2308 (A), S19 (B), or 16M (C). Each point represents an individual mouse and the spleen or liver weight at the time of death. The dotted lines represent the average normal weight of each organ for naïve animals.

show a statistically significant fluctuation in the temperature at any time point (Fig. 1E and C). Temperatures postinoculation were in the range of 35.4°C to 37.8°C (Fig. 1). In contrast, temperature changes were evident in several mice infected with either *B. melitensis* 16M, *B. abortus* 2308, or *B. abortus* S19 and were characterized by a marked episode of hypothermia prior to death (Fig.

1A, B, and D). There was no detectable evidence of fever at any of the time points evaluated.

Survival of *Brucella* Δ*vjbR* mutants in IRF-1$^{-/-}$ mice. To determine the effect of the *vjbR* gene deletion in an immunocompromised mouse model, IRF-1$^{-/-}$ mice were infected intraperitoneally with 1×10^6 CFU/mouse of either *B. melitensis*
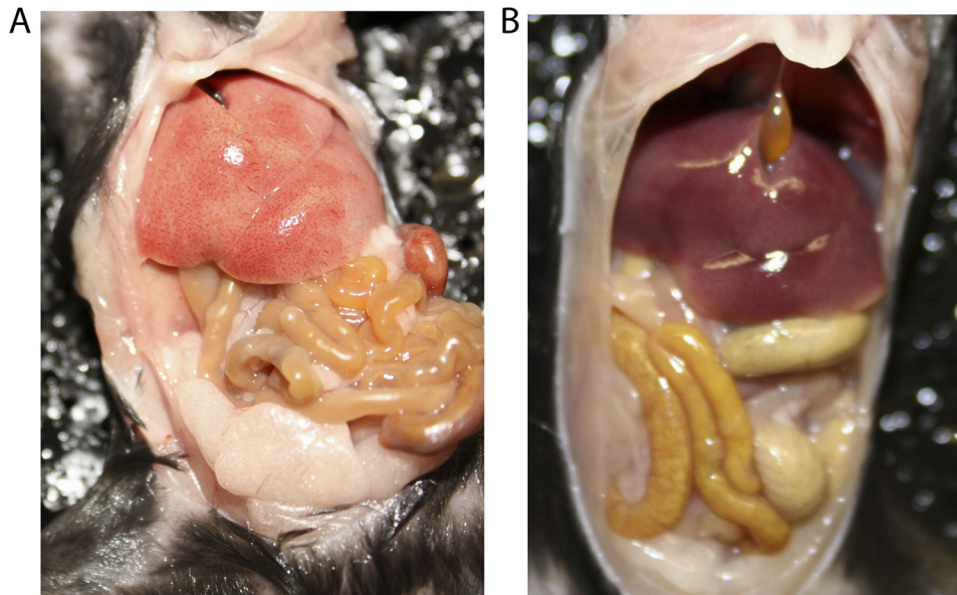
FIG 6 Gross pathological changes observed at day 30 in mice infected with 1×10^6 CFU/mouse of either 16M (A) or 16M$\Delta vjbR$ (B). Note the markedly enlarged and pale liver and spleen in the mouse inoculated with 16M. The abdominal cavity in the mouse inoculated with the 16M$\Delta vjbR$ was unremarkable.

16M$\Delta vjbR$, *B. abortus* S19$\Delta vjbR$, *B. melitensis* 16M, or *B. abortus* 2308 or S19. At 5 days postinoculation, mice that received the *B. melitensis* 16M strain started showing clinical evidence of disease, characterized by lethargy, ruffled coat, hunched posture, hypothermia, and anorexia (Fig. 2). By 10 days postinoculation, 60% of mice had succumbed to the infection (Fig. 3). Similarly, mice inoculated with *B. abortus* 2308 exhibited signs of illness by day 7, and 50% of mice were euthanized by day 11 postinoculation due to imminent deterioration (Fig. 3). Interestingly, mice vaccinated with the vaccine strain S19 also elicited clinical signs of illness, but at a time point later than that observed with the virulent strains, i.e., at day 10 postinoculation, with a 100% mortality rate by day 28 (Fig. 3). In contrast, 100% of mice vaccinated with either the 16M$\Delta vjbR$ or S19$\Delta vjbR$ mutant exhibited no signs of disease ($P < 0.0001$), and 100% survived beyond day 30 postinoculation (Fig. 3). Two animals from the groups inoculated with *B. melitensis* 16M and *B. abortus* 2308 exhibited no signs of disease beyond day 30 postinoculation. These data clearly demonstrate that *vjbR* mutants are less virulent in immunocompromised mice.

Evaluation of bacterial colonization in IRF-1$^{-/-}$ mice. In addition to the signs of illness described above, bacterial colonization in selected tissues was determined in an effort to identify any correlation between death and the degree of colonization. Spleens and livers from animals that succumbed to the infection at the different time points were harvested, weighed, and homogenized in PBS, and the bacterial burden was determined by growth on solid medium. All mice inoculated with *B. melitensis* 16M that died within the first 2 weeks of infection (Fig. 3) had an overwhelming bacterial burden in the spleens (7.07, 7.83, and 9.33 $\log_{10}$ CFU/spleen) and livers (8.12, 8.65, and 7.23 $\log_{10}$ CFU/liver) (Fig. 4A and B). Animals that survived the initial 30 days without signs of disease demonstrated a reduced but still high level of bacterial colonization in the spleens (4.36 and 4.45 $\log_{10}$ CFU/spleen) and livers (3.52 and 4.02 log CFU/liver). Similar degrees of colonization were evident in mice that were inoculated with *B. abortus*

2308. Mice that died within the first 2 weeks of infection had an extremely elevated bacterial burden in the spleens (8.11 and 8.31 log CFU/spleen) and livers (7.68 and 8.32 $\log_{10}$ CFU/liver) (Fig. 4C and D). Animals that survived the initial 30 days of infection had a reduced but still significant bacterial burden in both organs (Fig. 4). Interestingly, S19-inoculated animals also demonstrated an overwhelming infection, with approximately 7 log units present in the spleens and livers, even at 28 days postinoculation (Fig. 4E and F). Reduced splenic colonization by the 16M$\Delta vjbR$ or S19$\Delta vjbR$ strain was evident by 30 days postinoculation, and only 20 CFU was obtained from the spleen from each mouse (Fig. 4). Increased splenic colonization by the 16M, 2308, or S19 strain correlated with increased splenic and hepatic weights (Fig. 5), suggesting an increased inflammatory response to the infection that could be confirmed only by histopathology. The overwhelming amount of bacteria in the tissues from animals inoculated with 16M, 2308, or S19 along with the clinical signs indicates a severe septicemic process as the cause of death in these animals.

Evaluation of gross and histopathologic changes in IRF-1$^{-/-}$ mice inoculated with different *Brucella* strains. To determine whether gross and histologic changes were observed in IRF-1$^{-/-}$ inoculated with the different *Brucella* strains, a full necropsy as well as histopathologic examination was performed in all animals that succumbed to the infection and at 30 days postinfection. Gross changes were most prominent in the liver and spleen of *B. melitensis* 16M-inoculated mice, followed by *B. abortus* 2308- and S19-inoculated mice. All the livers from 16M-, 2308-, and, to a lesser degree, S19-infected mice were pale and enlarged (Fig. 6). The spleens from all mice inoculated with 16M and, to a lesser degree, 2308 and S19 mice were pale, markedly enlarged, and friable. Approximately 20% of mice inoculated with 16M or 2308 had a moderate amount of yellow to clear, gelatinous exudate within the abdominal cavity, consistent with ascites. The 16M- and 2308-inoculated animals that survived the initial 30 days postinfection had similar lesions but to a lesser extent. None of the
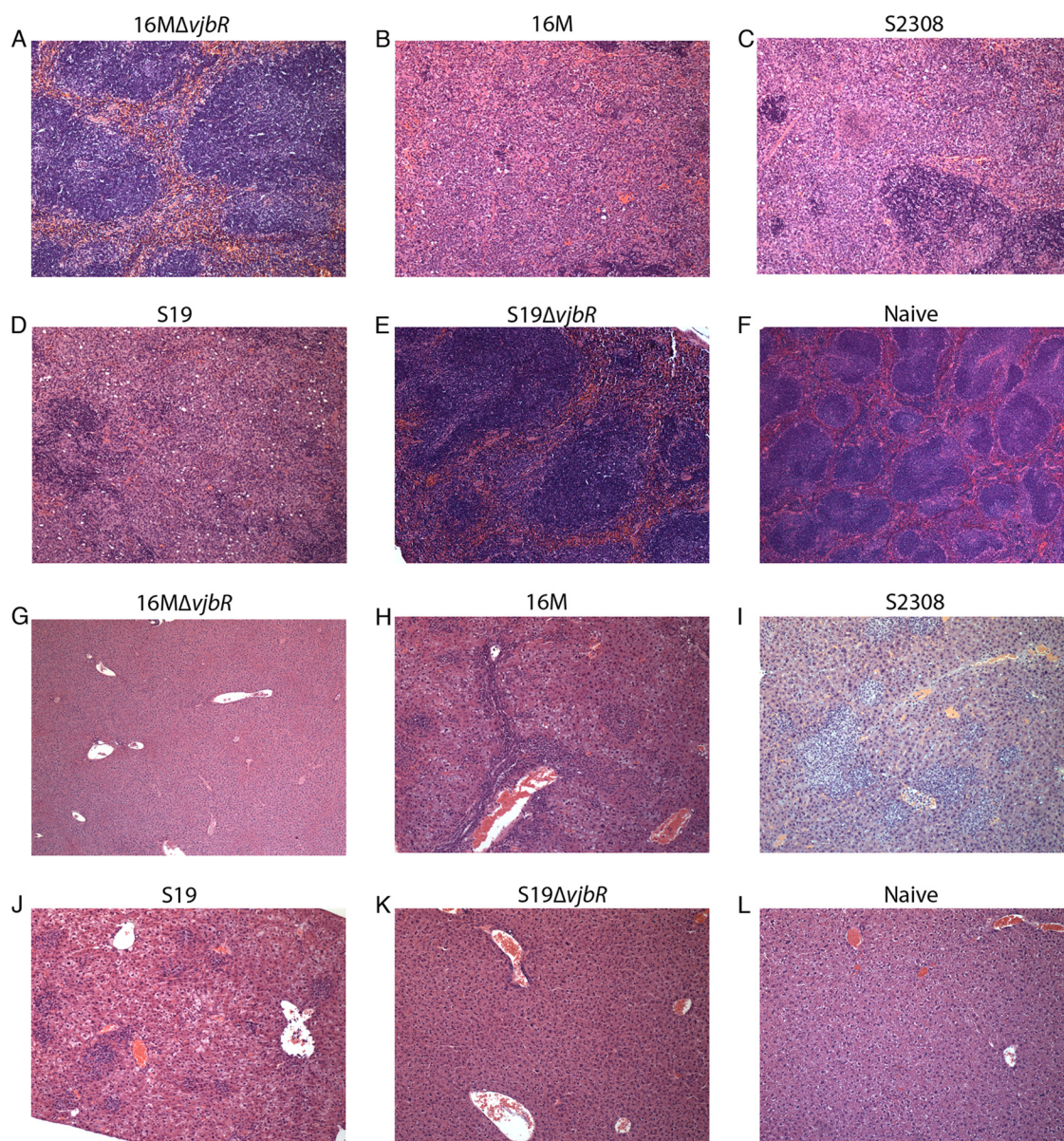
FIG 7 Microscopic changes observed in the spleens (A to F) and livers (G to L) of IRF-1$^{-/-}$ mice infected with either 16MΔ*vjbR* (A and G), 16M (B and H), 2308 (C and I), S19 (D and J), or S19Δ*vjbR* (E and K) or naïve mice (F and L). Note the complete obliteration of the normal splenic parenchyma and replacement with a histiocytic infiltrate in 16M-, 2308-, and S19-inoculated mice (B, C, and D). None of the mice infected with the *vjbR* mutants exhibited significant changes in the spleen. All livers inoculated with 16M, 2308, or S19 (H, I, and J) exhibited multifocal, random neutrophilic and histiocytic infiltrates. The livers from Δ*vjbR* mutant-infected mice are unremarkable.

animals that were inoculated with either the 16MΔ*vjbR* or S19Δ*vjbR* strain exhibited any gross pathological changes (Fig. 6).

Histopathologic evaluation of the spleens from mice inoculated with either *B. melitensis* 16M, *B. abortus* 2308, or *B. abortus* S19 exhibited similar changes, which consisted of a marked histiocytic to granulomatous splenitis with multiple foci of splenic necrosis (Fig. 7) that in some instances completely obliterated the normal tissue architecture. These changes were more prominent in mice that died more acutely. By 30 days, the degree of inflammatory response was mildly reduced but was still very pronounced. Changes in the liver in 16M-, 2308-, and S19-inoculated mice consisted of a multifocal, random neutrophilic hepatitis at

the earlier time points that progressed to a histiocytic infiltrate with epithelioid cells and microgranulomas at the later stages of the infection (Fig. 7). Other changes were evident in multiple organs and included a neutrophilic to lymphohistiocytic infiltrate in multiple lymph nodes, lung, brain, kidney, intestine, and heart (in 2 mice, 1 inoculated with 2308 and 1 inoculated with 16M), indicating that these mice died from a severe septicemic process. None of the animals that received the vaccine candidates and examined at 30 days postinoculation had any evidence of the *Brucella*-associated pathological findings mentioned above (Fig. 7).

Evaluation of immune protection provided by 16MΔ*vjbR* and S19Δ*vjbR* in IRF-1$^{-/-}$ mice against 16M challenge. In order
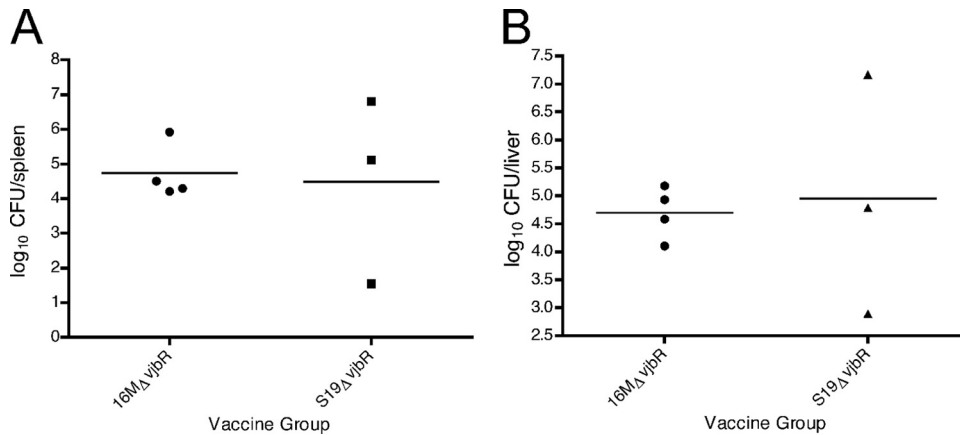
FIG 8 16M recovery from spleens and livers 1 week postchallenge in IRF-1$^{-/-}$ mice. Groups of four mice were vaccinated with 1×10^6 of either 16MΔ*vjbR* or S19Δ*vjbR* for 8 weeks prior to challenge. Data are expressed as log$_{10}$ number of CFU/organ in the spleens (A) or livers (B).

to determine the vaccination efficacy elicited by either the 16MΔ*vjbR* or S19Δ*vjbR* mutant, the level of protection provided by the vaccine candidate was evaluated against intraperitoneal *B. melitensis* 16M wild-type challenge at 8 weeks postinoculation. The 8-week time point was chosen because by 4 weeks postvaccination both vaccine candidates were already cleared from the host (data not shown). At 1 week postchallenge (9 weeks postvaccination), there was a marked decrease in the splenic and hepatic bacterial loads (4-log-unit reduction) from the mice vaccinated with the 16MΔ*vjbR* mutant as well as from the animals that received the S19Δ*vjbR* mutant (Fig. 8). This is only an observation, and it is solely based on the previous finding (Fig. 3A and B) that at 1 week postinfection the 16M infection was overwhelming (9 log units). Reduced bacterial loads correlated with decreased splenic and hepatic weights, which indicated a reduced inflammatory response (Fig. 9). This indicates that the vaccine candidates were able to induce partial protective immunity in IRF-1$^{-/-}$ mice.

**Evaluation of temperature changes in mice vaccinated with the *vjbR* mutants and challenged intraperitoneally.** To deter-mine temperature fluctuations due to the challenge dose, mice were monitored daily for 1 week postchallenge using transplantable temperature transponders. None of the mice showed a statistically significant fluctuation in temperature at any time point (Fig. 10).

Evaluation of gross and histologic findings in mice vaccinated with the *vjbR* mutants and challenged with wild-type strain 16M. In order to determine the gross and microscopic changes associated with wild-type challenge, a full necropsy and microscopic evaluation of the spleen, liver, kidneys, heart, lung, intestine, and brain were performed. Gross findings were most obvious in naïve but challenged mice and consisted of pale, enlarged livers and spleens. In *vjbR*-vaccinated mice, the only consistent finding was a mild to moderate increase in the spleen size (Fig. 9). Histopathologic evaluation of the spleens revealed a markedly reduced to absent inflammatory response compared to nonvaccinated but challenged animals and mainly consisted of a mild and multifocal neutrophilic to granulomatous hepatitis. In contrast, the nonvaccinated but challenge mice had a marked neutrophilic to granulomatous inflammatory response with areas of hepatocellular degeneration and necrosis (Fig. 11). There were no
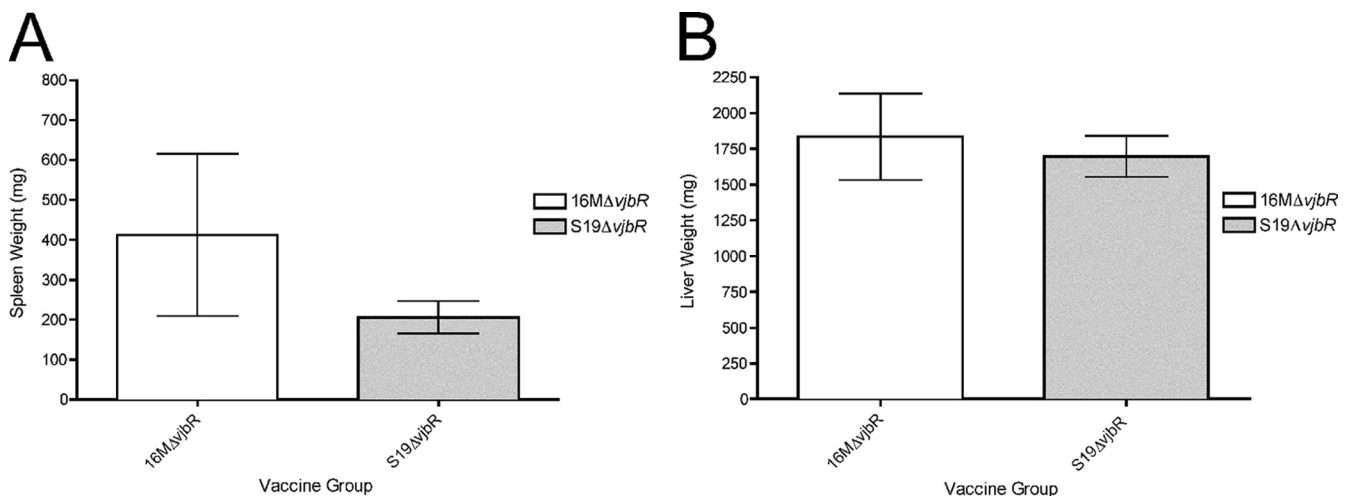
FIG 9 Spleen (A) and liver (B) weights of IRF-1$^{-/-}$ mice vaccinated with either 16MΔ*vjbR* or S19Δ*vjbR* and challenged intraperitoneally with 16M. Data represent the average of 4 mice per group.
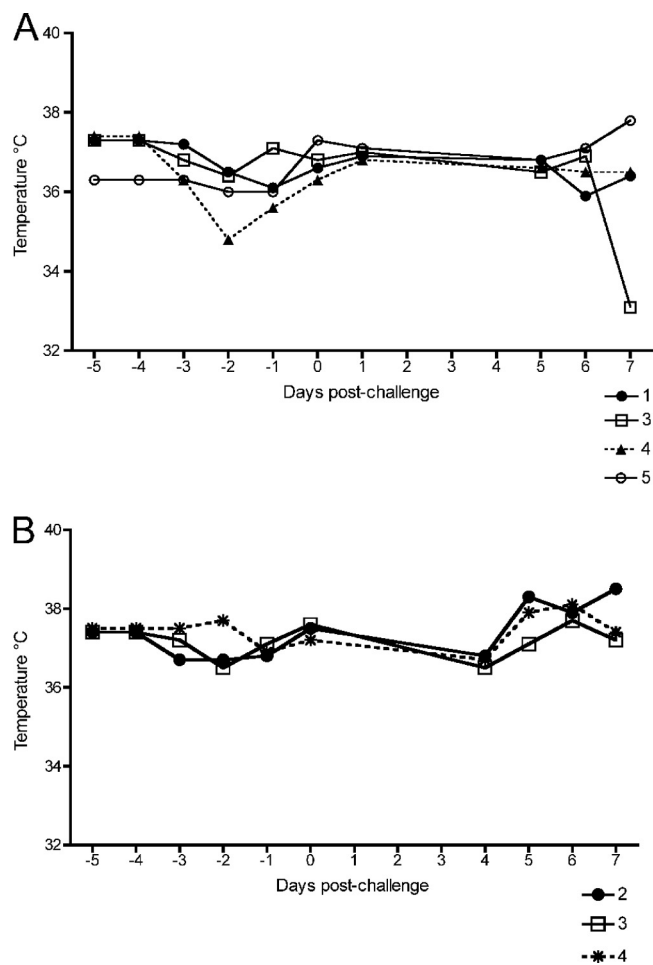
FIG 10 Temperature profiles collected over a 1-week period from IRF-1⁻/⁻ mice vaccinated with 1 × 10⁶ CFU/mouse of either S19Δ*vjbR* or 16MΔ*vjbR* and challenged at 8 weeks postvaccination with 1 × 10⁶ CFU/mouse of wild-type *B. melitensis* 16M.

changes observed in the lung, brain, heart, kidneys, or intestine in *vjbR*-vaccinated mice (data not shown).

DISCUSSION

One of the biggest challenges for producing an effective *Brucella* vaccine is to develop an immunization strategy that can induce a robust and prolonged memory response while still being safe. In the case of brucellosis, live attenuated vaccines are considered to be the vaccine of choice due to their overall ability to induce the most potent and effective memory response (7, 24). Two clear examples of the success of the live attenuated vaccines are S19 and Rev 1, both of which induce a protective memory response in animals (15, 20, 27). Other clear examples of the importance of live attenuated vaccines in controlling diseases throughout the world include *Mycobacterium bovis* BCG against tuberculosis and the smallpox vaccine. While S19 and Rev 1 have been extremely valuable for the past 50 years, one of the main safety concerns is their virulence in humans. These vaccines were obtained empirically by serial passage or repeated passage in culture of an active organism, without identification of the molecular basis of attenuation (27). Due to the more stringent regulatory environment, it is

unlikely that a live attenuated vaccine generated empirically would be approved for use, based on safety concerns that include the possibility of reversion to wild-type virulence. Therefore, the molecular basis for attenuation of a live vaccine needs to be both stable and well-defined, as is the case for multiple live attenuated *Brucella* vaccines under investigation (1, 2, 8, 13, 17, 23, 34, 36).

Our pursuit of the identity of genes essential for bacterial replication and persistence *in vivo* and *in vitro* led us to the generation of rationally designed, live attenuated vaccine candidates 16MΔ*vjbR* and s19Δ*vjbR*. The LuxR-like transcriptional regulator VjbR has been shown to positively influence the expression of the type IV secretion system and flagellar genes, both of which are necessary for bacterial virulence and survival (2, 3, 6). Our previous results have demonstrated that the *B. melitensis* and *B. abortus* Δ*vjbR* mutant strains conferred, with a single dose, a strong protective immune response against wild-type challenge in the mouse model (2, 3). The protective efficacy was demonstrated by different parameters, such as reduced bacterial burden in the spleen, decreased pathology in the spleen and liver, and an overall reduction in the inflammatory response, evidenced by a reduction in splenomegaly. Additionally, Δ*vjbR* mutants exhibit decreased survival in macrophages compared with the vaccine strain S19 (2).

As mentioned above, safety is one of the main concerns and a major challenge in the development of live vaccines. In this work, we extended the safety studies of the *vjbR* vaccine candidate strains in order to provide enough assurance for its potential future use in humans. The first step was the development of an unmarked deletion mutant to prevent future rearrangements in the *vjbR*::Tn5 or *vjbR*::Km^r mutation since loss of either element could potentially restore the mutant to the virulent form during subcultivation. Additionally, removal of the antibiotic marker eliminates any potential risk associated with its use in humans. Another important concern regarding the safety of live vaccines is that primary or secondary exposure, i.e., exposure to bacteria shed by vaccine recipients, could potentially pose a risk to immunocompromised individuals. To address this question, we utilized the IRF-1⁻/⁻ mouse model to determine the effect of the vaccine candidates in these mice. IRF-1⁻/⁻ mice are a valuable means to test these candidates, since it has been shown over the last 10 years that a single intraperitoneal inoculation of wild-type *Brucella melitensis* or *Brucella abortus* at 1 × 10⁶ CFU/mouse causes significant illness and death in these mice (16).

IRF-1 is a member of a multigene family of transcription factors that regulates an array of genes involved in cell differentiation and growth (19). Additionally, the transcription factor IRF-1 is involved in many aspects of innate and adaptive immune responses, including the differentiation of naïve CD4⁺ T cells into effector helper T cells (10, 11, 33). The T helper type 1 (T_H1)-, T helper type 2 (T_H2)-, and interleukin 17 (IL-17)-producing T helper (T_H17) populations play a critical role in immunity and are characterized by their distinct patterns of cytokine production. T_H1 cells mainly produce gamma interferon (IFN-γ) and mediate cellular immune responses to intracellular pathogens (26). T_H2 cells typically produce IL-4 and regulate humoral immune responses to extracellular pathogens (26). It is known that IRF-1⁻/⁻ mice have impaired T_H1 responses to *Brucella abortus* 2308 and *Brucella melitensis* 16M (16). Although impairment of IL-12 production in IRF-1⁻/⁻ macrophages has been considered the main cause of the imbalance in T_H1 versus T_H2 differentiation, the exact mechanism was unknown until recently, when it was demon-

Naive

Naive + 16M Challenge

S19Δ*vjbR*

16MΔ*vjbR*
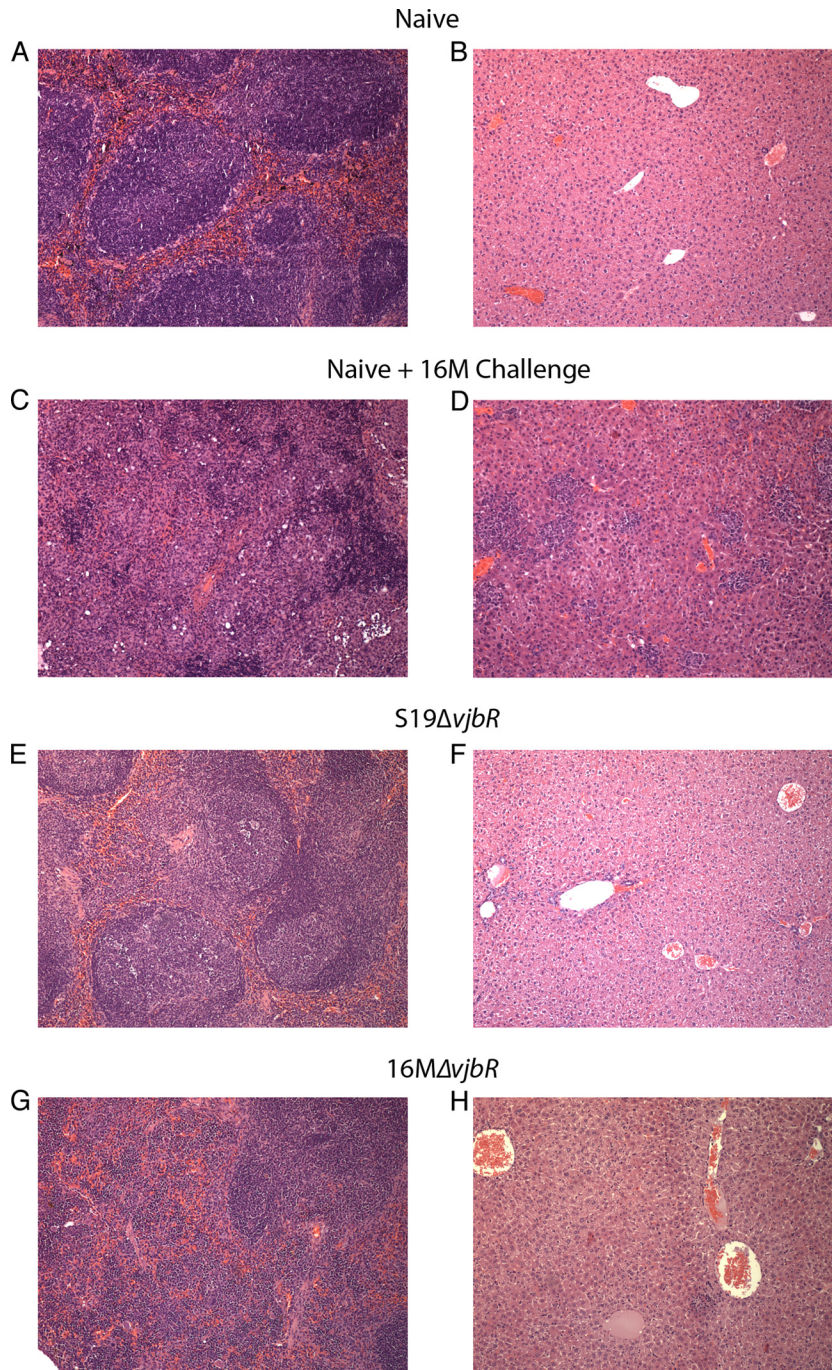
FIG 11 Microscopic changes observed in the spleens (A, C, E, and G) or livers (B, D, F, and H) of IRF-1$^{-/-}$ mice vaccinated with either S19Δ*vjbR* (E and F) or 16MΔ*vjbR* (G and H) and challenged at 8 weeks postvaccination with 1 × 10^6 CFU/mouse of wild-type *B. melitensis* 16M. Results for naïve but challenged mice (C and D) and naïve mice (A and B) are presented for comparison. Note the marked reduction in the inflammatory response in both the spleen and liver in animals that received the Δ*vjbR* mutants.

strated that the IRF-1 gene is essential for T$_H$1 differentiation acting on the IL12rb1 promoter in CD4$^+$ T cells (14). As a result, IRF-1$^{-/-}$ mice infected with the most virulent *Brucella* strains have an impaired IL-12 production and succumb to the infection within the first month of inoculation, favoring a T$_H$1 over a T$_H$2 response (16). Despite this, attenuated strains such as the vaccine strain RB51 are cleared from these mice without causing a marked

inflammatory response (16). In these experiments, none of the Δ*vjbR* mutants, regardless of the background strain, caused death or any significant *Brucella*-associated pathological changes, confirming the reduced virulence of these strains. This is further supported by the lack of clinical signs of disease such as lethargy, anorexia, weight loss, abnormal posture, or temperature changes. To the authors' knowledge, this is the first time that telemetry

monitoring of the body temperature in mice infected with *Brucella* spp. has been performed. Although it has been maintained for years that mice infected with *Brucella* spp. do not develop fever, temperature has never been monitored to detect possible fluctuations that can indicate disease. Previous investigations in rodents have demonstrated that mice are unlikely to recover from an infection by fungus or bacteria if there is a marked temperature drop (usually below 32 to 33°C) (29, 31). Interestingly, we were able to observe a similar drop in body temperature in animals that were obviously sick, and most importantly, once the temperature dropped below 32 to 33°C, the mice became terminally ill. Temperature measurement provides a useful and objective parameter of health, while interpretation of other clinical signs such as depression, piloerection, badly groomed coat, decreased locomotion, and abnormal posture may be relative and potentially subjective. Additionally, the severity of the symptoms may differ among individuals. It has long been recognized that septic patients can have variable thermoregulatory responses (5). Mice, rats, and guinea pigs routinely exhibit hypothermia in response to endotoxin challenge under normal laboratory conditions (4). The potential mechanisms of hypothermia related to septicemia are somewhat speculative, but probably, as tissue oxygen delivery decreases, maximal tissue oxygen extraction capability is exceeded, oxygen consumption declines, and heat production decreases. Other factors that might lower body temperature include malnutrition, cell senescence, and venous congestion causing decreased liver and or gastrointestinal metabolism.

Bacterial sepsis is defined as a clinical syndrome characterized by the active multiplication of bacteria that results in an overwhelming infection and a systemic inflammatory response. The pathophysiology of sepsis begins with the entry of the organism into the bloodstream, and it is characterized by simultaneous activation of inflammation and coagulation in response to the microbial insult (25). This inflammatory process is normally accompanied by activation of circulating and fixed phagocytic cells and by generation of proinflammatory and anti-inflammatory mediators. As the bacteria multiply, the response becomes uncontrolled and unregulated, exerting harmful systemic effects resulting in death. This is the case for IRF-1^{−/−} mice inoculated with either *Brucella melitensis* 16M, *Brucella abortus* 2308, or *Brucella abortus* S19, but it was not the case for animals vaccinated with the Δ*vjbR* mutants. At different time points, terminally ill mice inoculated with the *Brucella* strains were euthanized to determine the extent of bacterial colonization prior to death. All moribund mice had an extremely high bacterial burden in the spleens and livers as well as a marked inflammatory response, evidenced by histopathology. An extremely high bacterial burden associated with a severe neutrophilic and granulomatous inflammatory response in multiple organs clearly indicates that these mice were unable to control the infection, leading to a systemic spread and death. In contrast, none of the animals inoculated with the vaccine candidates had evidence of an inflammatory response by 30 days postinfection, and 20 CFU was recovered from the spleens of only two of five animals at 30 days postinfection (data not shown), indicating that the Δ*vjbR* mutants are unable to colonize IRF-1^{−/−} mice to the same extent as the wild-type organism or the vaccine strain S19. This provides further evidence of the increased safety of the Δ*vjbR* mutants.

The protective immunity induced in immunocompetent mice by vaccination with the Δ*vjbR* mutant strains has been previously evaluated (2, 3). The use of immunocompromised mouse models to determine induction of protective efficacy can be an important tool to elucidate other possible immune mechanisms that may play a role in adaptive immunity. IRF-1-knockout mice vaccinated with the vaccine candidates and challenged with the wild-type strain 16M survived, while they exhibited a significant reduction in bacterial colonization, reduced pathology, and no detectable evidence of clinical disease, including no temperature fluctuations after 1 week postinfection. Evidence of protection in the absence of IFN-γ signaling raises questions regarding the components of the innate and adaptive immunity that play a role in the control of the *Brucella* infection. Investigations are in progress to obtain an understanding between synergy and mutual interdependence between the different cellular immune responses as well as humoral immunity.

Our results extend our previous findings by demonstrating that considerable bacterial attenuation in immunocompromised individuals can be achieved if the *vjbR* gene is deleted. Future studies to determine the correlates of immune protection in immunocompetent and immunocompromised mice as well as toxicity studies are under way.

ACKNOWLEDGMENT

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