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# *Brucella abortus* Δ*rpoE1* confers protective immunity against wild type challenge in a mouse model of brucellosis

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## Abstract

The *Brucella abortus* general stress response (GSR) system regulates activity of the alternative sigma factor, $\sigma^{E1}$, which controls transcription of approximately 100 genes and is required for persistence in a BALB/c mouse chronic infection model. We evaluated the host response to infection by a *B. abortus* strain lacking $\sigma^{E1}$ (Δ*rpoE1*), and identified pathological and immunological features that distinguish Δ*rpoE1*-infected mice from wild-type (WT), and that correspond with clearance of Δ*rpoE1* from the host. Δ*rpoE1* infection was indistinguishable from WT in terms of splenic bacterial burden, inflammation and histopathology up to 6 weeks post-infection. However, *Brucella*-specific serum IgG levels in Δ*rpoE1*-infected mice were 5 times higher than WT by 4 weeks post-infection, and remained significantly higher throughout the course of a 12-week infection. Total IgG and *Brucella*-specific IgG levels peaked strongly in Δ*rpoE1*-infected mice at 6 weeks, which correlated with reduced splenomegaly and bacterial burden relative to WT-infected mice. Given the difference in immune response to infection with wild-type and Δ*rpoE1*, we tested whether Δ*rpoE1* confers protective immunity to wild-type challenge. Mice immunized with Δ*rpoE1* completely resisted WT infection and had significantly higher serum titers of *Brucella*-specific IgG, IgG2a and IFN-γ after WT challenge relative to age-matched naïve mice. We conclude that immunization of BALB/c mice with the *B. abortus* GSR pathway mutant, Δ*rpoE1,* elicits an adaptive immune response that confers significant protective immunity against WT infection.

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**Keywords**

antibody; ecfG; phyR; brucellosis; general stress response; rpoE1; vaccine

## Introduction

*Brucella* spp. are Gram-negative intracellular pathogens that inflict a significant burden on agricultural production and human health on a global scale (1). During the course of intracellular infection, *Brucella* must adapt to changes in the host environment including oxidative burst, low pH, and nutrient limitation. *Brucella* and other species in the class Alphaproteobacteria employ a conserved regulatory system, known as the general stress response (GSR), to adapt to such non-optimal growth conditions (2, 3). The GSR has been functionally characterized in Alphaproteobacteria that inhabit a range of ecological niches including freshwater (2, 4-7), plant roots and soil (8-10), plant leaf surfaces (8, 11-16), the interior of mammalian cells (17, 18), arthropod vectors and mammalian reservoir hosts (19, 20). In the case of animal- and plant-associated Alphaproteobacteria, the GSR has been implicated in establishment or maintenance of host-microbe interactions (8, 17, 19).

In *B. abortus*, the GSR is directly regulated by the LovhK-PhyR two-component signaling system. LovhK is a soluble histidine kinase that responds to variable environmental cues: it binds a flavin mononucleotide (FMN) cofactor, autophosphorylates in response to blue light *in vitro* (21), and controls transcription and cellular adaptation to a range of acute stress conditions (18, 22). Phospho-LovhK (LovhK~P) transfers its phosphoryl group to the anti-anti-σ factor, PhyR, which increases PhyR affinity for the anti-$\sigma^{E1}$ factor NepR (17). Binding of PhyR~P to NepR is proposed to release the GSR σ-factor, $\sigma^{E1}$, allowing its association with RNA polymerase (RNAP) to regulate transcription of genes required for acute stress survival and infection (17, 18, 22) (Figure 1). We have previously reported that *B. abortus* mutants missing the GSR regulatory genes encoding $\sigma^{E1}$ or PhyR are attenuated specifically during the chronic phase of infection in a BALB/c mouse model (17, 18). However, these studies only measured bacterial burden in the spleen over an 8-12 week period; the corresponding effects of infection on host pathophysiology and immune response remain uncharacterized. This study focuses on defining features of murine host pathology and immune response associated with infection by a *B. abortus* strain missing the core GSR regulator, $\sigma^{E1}$ (Δ*rpoE1*). We have further assessed the efficacy of the *B. abortus ΔrpoE1* mutant as a live attenuated vaccine.

Mice infected with Δ*rpoE1* had less spleen inflammation in the chronic phase (>6 weeks post-infection) than mice infected with WT *B. abortus.* This reduced splenomegaly correlated with a ≈3 log decrease in the number of splenic bacteria and decreased immunohistochemical staining of *Brucella* antigen in fixed spleens. Host clearance of *B. abortus ΔrpoE1* strongly correlated with increased *Brucella*-specific IgG production relative to WT-infected mice, providing evidence that the Δ*rpoE1* strain elicits a more robust humoral response than WT. Mice immunized with a single dose of $5\times10^4$ **c**olony **f**orming **u**nits (CFU) of Δ*rpoE1* were completely protected from a WT challenge at 9 weeks post-immunization, demonstrating that Δ*rpoE1* is a live attenuated vaccine. Vaccinated mice had

higher total IgG, IgG2a, *Brucella*-specific IgG, and IFN-y levels than aged-matched naïve mice after WT challenge.

Our data provide evidence that *B. abortus* Δ*rpoE1* elicits a robust immune response that corresponds with reduced splenic inflammation and clearance of Δ*rpoE1* from the host. We further demonstrate that the *B. abortus* Δ*rpoE1* strain is an effective live attenuated vaccine in a mouse model of infection. There remains a need for more safe and effective brucellosis vaccines (23, 24), and the data presented here illustrate the potential of Δ*rpoE1* for development as a new brucellosis vaccine.

## Materials and Methods

### Bacterial Strains and Culture Conditions

Experiments conducted with live *B. abortus* strain 2308 were performed at the University of Chicago, Howard Taylor Ricketts Laboratory on the campus of Argonne National Laboratory. Experiments were performed at **B**io**s**afety **L**evel 3 (BSL3) conditions as required by the Centers of Disease Control rules and regulations governing the use of Select Agents. Growth and maintenance of strains is as previously described (25).

### Animal Infection

All animal experiments followed protocols approved by the University of Chicago Institutional Animal Care and Use Committee and the Institutional Biosafety Committee. For infections, we used the wild-type *B. abortus* strain 2308 and the *B. abortus ΔrpoE1* strain (17). Bacterial strains were grown for 2 days on **S**chaedler **B**lood **A**gar (SBA) at 37°C before being suspended in sterile **p**hosphate-**b**uffered **s**aline (PBS; pH 7.2, GE Healthcare), to a concentration of $5 \times 10^5$ CFU/mL. Bacterial titers were first estimated spectrophotometrically at 600 nm ($OD_{600}$); the CFU per inoculum was confirmed post-infection by serial dilution plating on **t**ryptic **s**oy **a**gar (TSA). Eight week old female BALB/c mice (Harlan Laboratories, Inc.) were infected by **i**ntra**p**eritoneal (IP) injection with 100 μL of bacteria at a concentration of $5 \times 10^5$ CFU/mL (for a total infectious dose of $5 \times 10^4$ CFU per mouse). At 1, 2, 4, 6, 9, 12 weeks post-infection, animals (n = 8 per time point) were asphyxiated with $CO_2$ prior to collection of blood via cardiac-puncture and aseptic removal of spleens. Serum from 8 mice for each strain and time point was separated from blood using a serum separation tube (Sarstedt). After serum was collected, samples were subsequently stored at −20°C until the end of the experiments. For each sample, five mouse spleens were weighed and splenic bacterial burden (CFU/spleen) was measured by homogenizing the spleens in 5 mL PBS with 0.1% Triton X-100, before serial dilution and plating on TSA. The three remaining mouse spleens were fixed and prepared for histology (described below).

### Histology

Spleens prepared for histology (n = 3) were first fixed with formalin for 7 days. Formalin was removed and replaced with fresh formalin and fixed for another 7 days before washing and subsequently transferring spleens to 70% ethanol. Whole spleens were submitted for tissue embedding, **h**ematoxylin and **e**osin (H & E) staining, and immunohistochemistry to

Nationwide Histology (Veradale, Washington). For immunohistochemistry, goat anti-*Brucella* IgG was used (Tetracore, Inc). We subsequently analyzed and scored slides at the University of Chicago. Pictures of fixed mouse spleens were taken at the University of Chicago Integrated Light Microscopy Facility on a Cambridge Research and Instrumentation whole slide scanner fitted with an Allied Vision Technologies Stingray F146C color camera.

### Determination of Antibody and Cytokine Responses

Mouse serum **i**mmuno**g**lobulin **G** (IgG) and **i**nter**f**ero**n** gamma (IFN-γ) titers were measured using an **e**nzyme-**l**inked **i**mmuno**s**orbent **a**ssay (ELISA) in 96-well ELISA plates (Nunc-immuno MaxiSorb, Sigma). Total mouse serum IgG, IgG1, and IgG2a titers were measured using mouse-specific ELISA kits by following manufacturer's instructions (eBioscience). To determine *Brucella*-specific IgG titers, ELISA plates were coated with 100 μL of heat-killed bacterial suspensions prepared in PBS ($OD_{600}$=2.0) from *B. abortus* grown for 2 days on SBA. Cultures were heat-killed at 65°C for 1 hour, cooled to room temperature, and treated with kanamycin (50 μg/mL) and gentamycin (50 μg/mL) to prevent bacterial growth. Serum IFN-γ titers were measured using the OptiEIA mouse IFN-γ ELISA kit (BD Biosciences) following the manufacturer's instructions. The signal from all ELISA plates were measured at 450 nm with a 570 nm background correction using a Tecan Infinite M200 PRO fluorescence plate reader. All values presented are the mean ± the **s**tandard **e**rror of the **m**ean (SEM) from at least 5 mice for each time-point and condition.

### Vaccination Studies

For vaccine studies, eight-week-old female BALB/c mice were vaccinated by IP injection (100 μL) with $5 \times 10^5$ CFU/mL of the *B. abortus ΔrpoE1* strain in PBS. After 9 weeks, naïve age-matched mice and Δ*rpoE1*-vaccinated mice were challenged with 100 μL of $5 \times 10^5$ CFU/mL of WT *B. abortus*. Three weeks post-challenge, bacterial burden, spleen weight, and immune responses were measured as described above. Primers specific to the *rpoE1* locus were used to differentiate WT from Δ*rpoE1* bacteria isolated from the spleens of vaccinated mice. WT bacteria gave a PCR product of 1141 **b**ase **p**airs (bp) while the Δ*rpoE1* strain gave a PCR product of 568 bp, using the primers Δ*rpoE1*_confirm_up 5′-CCTTTCGAGTGCTGATGCAT-3′ and Δ*rpoE1*_confirm_dn 5′ –GCTGACAGCAAGACCAACC-3′.

## Results

### Mice infected with *Brucella abortus* Δ*rpoE1* have reduced chronic splenomegaly, which correlates with reduced splenic bacterial burden

Mice infected with WT *B. abortus* had pronounced spleen swelling (i.e. splenomegaly, assessed by measuring spleen weight), which peaked at 4 **w**eeks **p**ost-**i**nfection (wpi) and slowly decreased during the chronic phase of infection (approximately 4 – 12 wpi). Peak spleen weight at this time point was 0.70 ± 0.09 grams; peak bacterial burden was ≈$10^7$ **c**olony **f**orming **u**nits per spleen (CFU/spleen). As previously described, mouse splenomegaly correlates with an increase in splenic bacterial burden (26).

Through the first four weeks of infection, WT- and Δ*rpoE1*-infected mice were statistically indistinguishable in terms of splenomegaly and bacterial burden (Figure 2). Beginning 6 wpi, mice infected with Δ*rpoE1* exhibited reduced splenomegaly and bacterial burden compared to WT-infected mice, though mean values were not statistically distinct at this time point (Figure 2). By 9 wpi we observed statistically significant reductions in both spleen weight ($P<0.0001$) and bacterial burden ($P<0.001$). Δ*rpoE1*-infected mice had a 2.5-fold reduction in spleen weight and a 3.0 log reduction in splenic bacterial burden relative to WT-infected mice (Figure 2). Both spleen weight and bacterial burden remained constant between 9 and 12 wpi in Δ*rpoE1*-infected mice, but decreased slightly in this period in WT-infected mice (Figure 2). These results demonstrate that host splenomegaly is reduced in Δ*rpoE1*-infected mice during the chronic phase relative to WT-infected mice. Reduced splenomegaly in the chronic phase is correlated with clearance of *B. abortus ΔrpoE1* from the murine host.

## Spleens of Δ*rpoE1*-infected mice have reduced pathologic features and contain less *Brucella* antigen

To assess differences in pathology of mice infected with WT and Δ*rpoE1* strains in the early and chronic phase, we harvested spleens at 1 and 9 wpi. Spleens were fixed, mounted, and subjected to **h**ematoxylin and **e**osin (H&E) staining. We scored spleens for white to red pulp ratio, average number of lymphoid follicles, germinal center size, marginal zone thickness, extramedullary hematopoiesis, histiocytic proliferation and presence of granulomas. Our results are summarized in Table 1 (data are scored on a scale of 0-3, with 0 being no apparent pathology). Naïve (i.e. uninfected) spleens scored 0 in all categories. Spleens of Δ*rpoE1*- and WT-infected mice were inflamed and contained *Brucella* granulomas at 1 wpi, consistent with what has been previously reported for BALB/c infection with WT *B. abortus* (27). However, there were modest differences in scored histopathology between strains at this early time point. Though spleen weight and total CFU per spleen were statistically equivalent at 1 wpi (Figure 2), Δ*rpoE1*-infected mice had lower extramedullary hematopoiesis, histiocytic proliferation and fewer granulomas than WT (Table 1, Figure S1). These data provide evidence of differences in interaction of Δ*rpoE1* and WT *Brucella* strains with the murine host in the acute phase, though infections look similar with respect to spleen colonization and bulk inflammation.

By 9 wpi, spleens of Δ*rpoE1*-infected mice had reduced pathologic features in all scored categories relative to 1 wpi (Table 1, Figure 3). In contrast, spleens of mice infected with WT had significantly reduced white to red pulp ratio due to disruption of lymphoid follicles (follicular lysis) and red pulp expansion while also displaying significantly increased marginal zones. In addition, spleens of WT-infected mice had higher extramedullary hematopoiesis, histiocytic proliferation, granulomas and presence of *Brucella* immunoreactivities than Δ*rpoE1* at 9 wpi (Table1, Figure 3). Images of naïve, WT- and Δ*rpoE1*-infected mouse spleens at 9 wpi are presented with histological features labeled in Figure 3.

### Δ*rpoE1* infection elicits an increased IgG antibody response relative to WT infection

To test whether there are differences in antibody or cytokine responses in mice infected with Δ*rpoE1* and WT, we harvested serum from uninfected (naïve) control mice and infected mice at 1, 2, 4, 6, 9 and 12 wpi. We measured serum levels of total IgG, *Brucella*-specific IgG, IgG1, IgG2a, and IFN-γ by **e**nzyme-**l**inked **i**mmuno**s**orbent **a**ssays (ELISA). Total serum IgG was higher across the 12-week experiment in both WT- and Δ*rpoE1*-infected mice relative to uninfected control (Figure 4A). WT- and Δ*rpoE1*-infected mice had similar total IgG levels with the exception of 6 wpi when total IgG serum levels were ≈2 times higher in Δ*rpoE1* (9.4 mg/mL versus 5.5 mg/mL; Figure 4A). We next measured IgG specific to *B. abortus*. Serum harvested from WT- and Δ*rpoE1*-infected mice was incubated in ELISA plates coated with a suspension of heat-killed *B. abortus*; measurements were normalized to uninfected control serum. The level of *Brucella*-specific IgG was ≈6 times higher in Δ*rpoE1*-infected mice by 4 wpi (Figure 4B). Levels peaked at 6 wpi when antibodies specific to *B. abortus* antigen(s) were 7 times higher in Δ*rpoE1*-infected compared to WT-infected mice (one-way ANOVA with Bonferroni correction; $P<0.001$) (Figure 4B). Thus the difference in total IgG levels we observe between strains is largely attributable to a response to *B. abortus*-specific antigen(s).

WT *B. abortus* infection is reported to stimulate a robust CD4+ T helper 1 (Th1) response (28, 29) without a significant Th2 response (30). Though BALB/c is regarded as a Th2-dominant mouse strain (31), BALB/c can mount a Th1 response against bacterial and parasitic infections (32, 33); there is evidence that BALB/c do not actually present a Th2 bias following WT *Brucella* infection (34). Given the specific defect of the Δ*rpoE1* strain in the chronic phase of infection, and the known role of the Th1 cytokine IFN-γ in control of chronic infection (35), we sought to test whether BALB/c infected with WT and Δ*rpoE1* may show a differential skew in Th1/Th2 responses. To this end, we profiled IgG sub-classes IgG1 and IgG2a and the cytokine IFN-γ in harvested sera. The ratio of IgG1 to IgG2a can serve as an indicator of Th1 versus Th2 responses in the host: high IgG2a/IgG1 ratios indicate a Th1 response, and a low ratio indicates a Th2 response (36, 37). IFN-γ is an established Th1 cytokine, and a marker for Th1 response (38).

Both WT- and Δ*rpoE1*-infected mice displayed similar increases in IgG1 and IgG2a across the 12-week experiment with levels of IgG1 peaking at approximately 9 wpi and IgG2a peaking at 6 wpi (Figure 4C,D). The IgG2a/IgG1 ratio suggests a mixed Th1/Th2 response, though relative increase in IgG2a was much larger than IgG1 after infection. IFN-γ levels increased by a factor of ≈2 over the first four weeks of infection, and decreased gradually back to levels measured at 1 wpi by the end of the 12-week experiment (Figure 4E). We observed no significant differences between WT- and Δ*rpoE1*-infected mice for IgG1, IgG2a, or IFN-γ at any time point. Together, these data provide evidence that Δ*rpoE1* elicits a stronger antibody response than WT *B. abortus* in BALB/c mice (Figure 4A-B), while the Th1/Th2 profile of Δ*rpoE1*-infected mice does not appear to differ from WT infection (Figure 4C-E).

### Vaccination with Δ*rpoE1* protects mice against WT *B. abortus* challenge

*Brucella* infection in humans is correlated with the incidence of infection of their ungulate livestock, which can range above 70% at the herd level in certain regions of the world (39-42). Current efforts to control disease in livestock, and by extension, humans, are limited by the availability of broadly effective and safe animal vaccines (24). The observation that *B. abortus* Δ*rpoE1* elicits a strong humoral response (Figure 4B) that corresponds with clearance of infection from the host (Figure 2) suggested that Δ*rpoE1* may be an effective live-attenuated vaccine. To test this hypothesis, we vaccinated mice with the Δ*rpoE1* strain via IP injection ($5 \times 10^5$ CFU/mL). At 9 weeks post-vaccination, we challenged Δ*rpoE1* vaccinated and naïve age-matched mice with $5 \times 10^4$ CFU of WT *B. abortus*. We measured spleen weights and bacterial burden in the spleen at 3 **w**eeks **p**ost-**c**hallenge (wpc) (Figure 5A).

Naïve mice exhibited pronounced splenomegaly with a mean spleen weight of 0.68 ± 0.14 grams at 3 wpc (Figure 5B). Δ*rpoE1*-vaccinated mice had significantly lower spleen weight of 0.23 ± 0.08 grams (t-test; P<0.01). Reduced splenomegaly corresponded with reduced bacterial burden: Δ*rpoE1* vaccinated mice had ≈3 logs less splenic bacteria than naïve mice at 3 wpc (t-test; <0.001) (Figure 5C). As presented above, the Δ*rpoE1* strain persists at low levels in the spleen at 12 wpi (Figure 2B). Thus, it was necessary to determine whether *B. abortus* isolated from the spleens of vaccinated mice at 3 wpc was Δ*rpoE1*, WT, or a mixture of both strains. To this end, we PCR amplified the *rpoE1* locus of all bacterial colonies isolated from a spleen section of vaccinated mice. All bacteria isolated from plated spleen homogenates of vaccinated mice (n ≥ 48) were confirmed to be the Δ*rpoE1* genotype. Thus, vaccination with Δ*rpoE1* provided complete protection against WT *B. abortus* challenge in the context of this assay.

### Mice vaccinated with Δ*rpoE1* have higher antibody and IFN-γ responses than naïve mice upon WT challenge

We next assessed whether naïve and Δ*rpoE1*-vaccinated mice exhibited differences in antibody or IFN-γ production upon WT challenge. Total IgG serum levels were significantly higher in Δ*rpoE1*-vaccinated mice at 3 wpc (t-test; P<0.0001) (Figure 5E). *Brucella*-specific IgG levels, measured as described above, were ≈5 times higher in Δ*rpoE1*-vaccinated mice than naïve control mice (Figure 5F). There was no difference in total IgG1 levels between naïve and Δ*rpoE1*-vaccinated mice at 3 wpc (Figure 5G). Total IgG2a serum levels were ≈2 times higher in Δ*rpoE1*-vaccinated mice (t-test; $P$=0.020), which is consistent with a stronger Th1 response (Figure 5H). Finally, IFN-γ response was significantly higher in Δ*rpoE1*-vaccinated mice than unvaccinated naïve mice (t-test; $P$=0.019) (Figure 5I). We conclude that vaccination with Δ*rpoE1* results in significantly increased antibody production and IFN-γ production relative to naïve mice upon WT challenge.

## Discussion

Genes encoding the GSR system (Figure 1) are determinants of chronic brucellosis in a BALB/c mouse model of disease (17). A goal of this study was to define features of the host response that distinguish wild-type (WT) *B. abortus* infection from infection with a strain

harboring a null allele of the GSR sigma factor, *rpoE1* (Δ*rpoE1*). Deletion of *rpoE1* had no effect on splenic bacterial burden or spleen inflammation in the acute phase (up to 6 wpi). However, histological analysis of Δ*rpoE1* and WT infected spleens at 1 wpi revealed pathologic features that differ at this time point including lower histiocytic proliferation and extramedullary hematopoiesis, and fewer granulomas in Δ*rpoE1* at this early time point (Table 1). As previously reported (17, 18), deletion of *rpoE1* resulted in significant reduction of splenic bacteria and decreased splenomegaly in the chronic phase (> 6 wpi) of infection relative to WT (Figure 2).

Reduced splenic bacterial burden of Δ*rpoE1*-infected mice during the chronic phase of infection suggested that this strain may elicit a stronger host immune response than WT *B. abortus*. To test this hypothesis, we measured serum levels of specific cytokines and antibodies known to be important in host-mediated clearance of *Brucella* infection (26, 35, 43, 44). We observed no significant difference in IFN-γ levels between WT- and Δ*rpoE1*-infected mice over a 12-week experiment. We did observe a peak in total IgG and *Brucella*-specific IgG in the serum of Δ*rpoE1*-infected mice at 6 wpi, which corresponds with clearance of Δ*rpoE1* from the spleen. This IgG peak was significantly reduced in WT-infected mice. These data provide evidence that Δ*rpoE1* elicits a more robust humoral response than WT *B. abortus*. At this time, we cannot discern whether clearance of the Δ*rpoE1* strain is mediated or enhanced by these antibodies, or if antibody production is simply a consequence of antigen release triggered by host clearance of Δ*rpoE1* by innate or cell-mediated mechanisms known to control *Brucella* spp. infection in mice (43, 45-48).

While a number of *Brucella* live vaccines have been developed (26, 49-52), there is a need for more effective vaccines (23, 24). Vaccines in current use are documented to cause spontaneous abortion and arthritis in livestock and have varying levels of protective efficacy (23, 50, 53). Accordingly, we tested the efficacy of *B. abortus* Δ*rpoE1* as a live attenuated vaccine. Though a low number of Δ*rpoE1* brucellae remained in the spleen at the time of vaccination, Δ*rpoE1*-vaccinated mice showed no signs of spleen inflammation and completely resisted splenic colonization by WT brucellae. This stands in contrast to BALB/c mice vaccinated with some commercially available vaccines; vaccinated mice are not significantly protected against splenic colonization upon WT challenge (54). Serum from vaccinated mice and naïve mice after WT challenge revealed significantly higher levels of total IgG, *Brucella*-specific IgG, IgG2a, and IFN-γ in Δ*rpoE1*-vaccinated mice. The increase in IgG2a, but not IgG1, and the significantly higher IFN-γ levels in vaccinated mice post-challenge (Figure 5) suggests that Δ*rpoE1* elicits Th1-mediated protection (30, 55).

We do not currently understand the mechanism by which the *B. abortus* GSR regulatory system mediates bacterial survival in the host during the chronic phase of infection. Transcriptional profiling approaches have defined the GSR regulon in *B. abortus* (17, 18), and these studies have demonstrated that a variety of outer membrane and other cell envelope proteins, stress response proteins, and genes of unknown function are under direct or indirect transcriptional control of RpoE1 ($\sigma^{E1}$). The roles of these GSR-regulated genes in maintenance of chronic infection remain largely undefined, though several including *rpoH1* (56), *ba14K* (18, 57), *pgm* (58, 59), *wrpA* (60), *cydAB* (61), *cydX* (62), the urease gene cluster (63), and flagellar genes (64) have been linked to *B. abortus* virulence or host-

*Brucella* interaction. Alterations in the *B. abortus* envelope due to dysregulation of the GSR in the chronic phase of infection may reveal *B. abortus* antigens that are otherwise repressed. This could explain the increased antibody response elicited by Δ*rpoE1*, and subsequent clearance from the host (Figure 2; Figure 4 A-B). Alternatively, it is possible that *B. abortus* encounters particular physical or chemical stressors during the chronic phase of infection that result in death of Δ*rpoE1*, which has known acute stress survival defects *in vitro* (17). The exact nature of such a chronic phase stress in this scenario is not obvious given that Δ*rpoE1* has no deficiency in entry or replication in activated macrophages *in vitro*, or in infection and colonization of mouse spleens over the first 6 weeks of infection (Figure 2) (17). Future studies will elucidate the specific links between Δ*rpoE1* clearance from the host, activation of host immunity, and Δ*rpoE1*-mediated protection against wild-type *B. abortus* infection.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Works Cited

1. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human brucellosis. Lancet Infect Dis. 2006; 6:91–99. [PubMed: 16439329]
2. Fiebig A, Herrou J, Willett J, Crosson S. General Stress Signaling in the Alphaproteobacteria. Annu Rev Genet. 2015; 49:603–625. [PubMed: 26442844]
3. Francez-Charlot A, Kaczmarczyk A, Fischer HM, Vorholt JA. The general stress response in Alphaproteobacteria. Trends Microbiol. 2015; 23:164–171. [PubMed: 25582885]
4. Foreman R, Fiebig A, Crosson S. The LovK-LovR two-component system is a regulator of the general stress pathway in Caulobacter crescentus. J Bacteriol. 2012; 194:3038–3049. [PubMed: 22408156]
5. Herrou J, Foreman R, Fiebig A, Crosson S. A structural model of anti-anti-sigma inhibition by a two-component receiver domain: the PhyR stress response regulator. Mol Microbiol. 2010; 78:290–304. [PubMed: 20735776]
6. Herrou J, Rotskoff G, Luo Y, Roux B, Crosson S. Structural basis of a protein partner switch that regulates the general stress response of alpha-proteobacteria. Proc Natl Acad Sci U S A. 2012; 109:E1415–E1423. [PubMed: 22550172]
7. Herrou J, Willett JW, Crosson S. Structured and Dynamic Disordered Domains Regulate the Activity of a Multifunctional Anti-sigma Factor. MBio. 2015; 6:e00910. [PubMed: 26220965]
8. Gourion B, Sulser S, Frunzke J, Francez-Charlot A, Stiefel P, Pessi G, Vorholt JA, Fischer HM. The PhyR-sigma(EcfG) signalling cascade is involved in stress response and symbiotic efficiency in Bradyrhizobium japonicum. Mol Microbiol. 2009; 73:291–305. [PubMed: 19555458]

9. Bastiat B, Sauviac L, Bruand C. Dual control of Sinorhizobium meliloti RpoE2 sigma factor activity by two PhyR-type two-component response regulators. J Bacteriol. 2010; 192:2255–2265. [PubMed: 20154128]
10. Bastiat B, Sauviac L, Picheraux C, Rossignol M, Bruand C. Sinorhizobium meliloti sigma factors RpoE1 and RpoE4 are activated in stationary phase in response to sulfite. PLoS One. 2012; 7:e50768. [PubMed: 23226379]
11. Kaczmarczyk A, Campagne S, Danza F, Metzger LC, Vorholt JA, Francez-Charlot A. Role of Sphingomonas sp. strain Fr1 PhyR-NepR-sigmaEcfG cascade in general stress response and identification of a negative regulator of PhyR. J Bacteriol. 2011; 193:6629–6638. [PubMed: 21949070]
12. Kaczmarczyk A, Hochstrasser R, Vorholt JA, Francez-Charlot A. Complex two-component signaling regulates the general stress response in Alphaproteobacteria. Proc Natl Acad Sci U S A. 2014; 111:E5196–5204. [PubMed: 25404331]
13. Kaczmarczyk A, Hochstrasser R, Vorholt JA, Francez-Charlot A. Two tiered histidine kinase pathway involved in heat shock and salt sensing in the general stress response of Sphingomonas melonis Fr1. J Bacteriol. 2015; 197:1466–1477. [PubMed: 25666137]
14. Francez-Charlot A, Frunzke J, Reichen C, Ebneter JZ, Gourion B, Vorholt JA. Sigma factor mimicry involved in regulation of general stress response. Proc Natl Acad Sci U S A. 2009; 106:3467–3472. [PubMed: 19218445]
15. Gourion B, Francez-Charlot A, Vorholt JA. PhyR is involved in the general stress response of Methylobacterium extorquens AM1. J Bacteriol. 2008; 190:1027–1035. [PubMed: 18024517]
16. Metzger LC, Francez-Charlot A, Vorholt JA. Single-domain response regulator involved in the general stress response of Methylobacterium extorquens. Microbiology. 2013; 159:1067–1076. [PubMed: 23596318]
17. Kim HS, Caswell CC, Foreman R, Roop RM, Crosson S. The Brucella abortus General Stress Response System Regulates Chronic Mammalian Infection and Is Controlled by Phosphorylation and Proteolysis. J Biol Chem. 2013; 288:13906–13916. [PubMed: 23546883]
18. Kim HS, Willett JW, Jain-Gupta N, Fiebig A, Crosson S. The Brucella abortus virulence regulator, LovhK, is a sensor kinase in the general stress response signalling pathway. Mol Microbiol. 2014; 94:913–925. [PubMed: 25257300]
19. Abromaitis S, Koehler JE. The Bartonella quintana extracytoplasmic function sigma factor RpoE has a role in bacterial adaptation to the arthropod vector environment. J Bacteriol. 2013; 195:2662–2674. [PubMed: 23564167]
20. Tu N, Lima A, Bandeali Z, Anderson B. Characterization of the general stress response in Bartonella henselae. Microb Pathog. 2015; 92:1–10. [PubMed: 26724735]
21. Swartz TE, Tseng TS, Frederickson MA, Paris G, Comerci DJ, Rajashekara G, Kim JG, Mudgett MB, Splitter GA, Ugalde RA, Goldbaum FA, Briggs WR, Bogomolni RA. Blue-light-activated histidine kinases: two-component sensors in bacteria. Science. 2007; 317:1090–1093. [PubMed: 17717187]
22. Sycz G, Carrica MC, Tseng TS, Bogomolni RA, Briggs WR, Goldbaum FA, Paris G. LOV Histidine Kinase Modulates the General Stress Response System and Affects the virB Operon Expression in Brucella abortus. PLoS One. 2015; 10:e0124058. [PubMed: 25993430]
23. Olsen SC, Stoffregen WS. Essential role of vaccines in brucellosis control and eradication programs for livestock. Expert Rev Vaccines. 2005; 4:915–928. [PubMed: 16372886]
24. Wang Z, Wu Q. Research progress in live attenuated Brucella vaccine development. Curr Pharm Biotechnol. 2013; 14:887–896. [PubMed: 24372253]
25. Willett JW, Herrou J, Briegel A, Rotskoff G, Crosson S. Structural asymmetry in a conserved signaling system that regulates division, replication, and virulence of an intracellular pathogen. Proc Natl Acad Sci U S A. 2015; 112:E3709–3718. [PubMed: 26124143]
26. Grillo MJ, Blasco JM, Gorvel JP, Moriyon I, Moreno E. What have we learned from brucellosis in the mouse model? Veterinary Research. 2012; 43
27. Enright FM, Araya LN, Elzer PH, Rowe GE, Winter AJ. Comparative histopathology in BALB/c mice infected with virulent and attenuated strains of Brucella abortus. Vet Immunol Immunopathol. 1990; 26:171–182. [PubMed: 2124401]

28. Oliveira SC, Zhu Y, Splitter GA. Recombinant L7/L12 ribosomal protein and gamma-irradiated Brucella abortus induce a T-helper 1 subset response from murine CD4+ T cells. Immunology. 1994; 83:659–664. [PubMed: 7875746]
29. Street NE, Schumacher JH, Fong TA, Bass H, Fiorentino DF, Leverah JA, Mosmann TR. Heterogeneity of mouse helper T cells. Evidence from bulk cultures and limiting dilution cloning for precursors of Th1 and Th2 cells. J Immunol. 1990; 144:1629–1639. [PubMed: 1968485]
30. Agranovich I, Scott DE, Terle D, Lee K, Golding B. Down-regulation of Th2 responses by Brucella abortus, a strong Th1 stimulus, correlates with alterations in the B7.2-CD28 pathway. Infect Immun. 1999; 67:4418–4426. [PubMed: 10456882]
31. Watanabe H, Numata K, Ito T, Takagi K, Matsukawa A. Innate immune response in Th1- and Th2-dominant mouse strains. Shock. 2004; 22:460–466. [PubMed: 15489639]
32. Barbi J, Brombacher F, Satoskar AR. T cells from Leishmania major-susceptible BALB/c mice have a defect in efficiently up-regulating CXCR3 upon activation. J Immunol. 2008; 181:4613–4620. [PubMed: 18802063]
33. Garcia-Pelayo MC, Bachy VS, Kaveh DA, Hogarth PJ. BALB/c mice display more enhanced BCG vaccine induced Th1 and Th17 response than C57BL/6 mice but have equivalent protection. Tuberculosis (Edinb). 2015; 95:48–53. [PubMed: 25467292]
34. Vitry MA, De Trez C, Goriely S, Dumoutier L, Akira S, Ryffel B, Carlier Y, Letesson JJ, Muraille E. Crucial role of gamma interferon-producing CD4+ Th1 cells but dispensable function of CD8+ T cell, B cell, Th2, and Th17 responses in the control of Brucella melitensis infection in mice. Infect Immun. 2012; 80:4271–4280. [PubMed: 23006848]
35. Murphy EA, Sathiyaseelan J, Parent MA, Zou BX, Baldwin CL. Interferon-gamma is crucial for surviving a Brucella abortus infection in both resistant C57BL/6 and susceptible BALB/c mice. Immunology. 2001; 103:511–518. [PubMed: 11529943]
36. Brazolot Millan CL, Weeratna R, Krieg AM, Siegrist CA, Davis HL. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. Proc Natl Acad Sci U S A. 1998; 95:15553–15558. [PubMed: 9861007]
37. Snapper CM, Paul WE. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science. 1987; 236:944–947. [PubMed: 3107127]
38. Romagnani S. The Th1/Th2 paradigm. Immunol Today. 1997; 18:263–266. [PubMed: 9190109]
39. Ahmed MO, Elmeshri SE, Abuzweda AR, Blauo M, Abouzeed YM, Ibrahim A, Salem H, Alzwam F, Abid S, Elfahem A, Elrais A. Seroprevalence of brucellosis in animals and human populations in the western mountains region in Libya, December 2006-January 2008. Euro Surveill. 2010; 15
40. Gwida M, Al Dahouk S, Melzer F, Rosler U, Neubauer H, Tomaso H. Brucellosis - regionally emerging zoonotic disease? Croat Med J. 2010; 51:289–295. [PubMed: 20718081]
41. McDermott JJ, Arimi SM. Brucellosis in sub-Saharan Africa: epidemiology, control and impact. Vet Microbiol. 2002; 90:111–134. [PubMed: 12414138]
42. Osoro EM, Munyua P, Omulo S, Ogola E, Ade F, Mbatha P, Mbabu M, Ng'ang'a Z, Kairu S, Maritim M, Thumbi SM, Bitek A, Gaichugi S, Rubin C, Njenga K, Guerra M. Strong Association Between Human and Animal Brucella Seropositivity in a Linked Study in Kenya, 2012-2013. Am J Trop Med Hyg. 2015; 93:224–231. [PubMed: 26101275]
43. Baldwin CL, Parent M. Fundamentals of host immune response against Brucella abortus: what the mouse model has revealed about control of infection. Vet Microbiol. 2002; 90:367–382. [PubMed: 12414157]
44. Vitry MA, Mambres DH, De Trez C, Akira S, Ryffel B, Letesson JJ, Muraille E. Humoral Immunity and CD4(+) Th1 Cells Are Both Necessary for a Fully Protective Immune Response upon Secondary Infection with Brucella melitensis. Journal of Immunology. 2014; 192:3740–3752.
45. Oliveira SC, de Almeida LA, Carvalho NB, Oliveira FS, Lacerda TL. Update on the role of innate immune receptors during Brucella abortus infection. Vet Immunol Immunopathol. 2012; 148:129–135. [PubMed: 21700343]
46. Pei J, Ding X, Fan Y, Rice-Ficht A, Ficht TA. Toll-like receptors are critical for clearance of Brucella and play different roles in development of adaptive immunity following aerosol challenge in mice. Front Cell Infect Microbiol. 2012; 2:115. [PubMed: 22973560]

47. Weiss DS, Takeda K, Akira S, Zychlinsky A, Moreno E. MyD88, but not toll-like receptors 4 and 2, is required for efficient clearance of Brucella abortus. Infect Immun. 2005; 73:5137–5143. [PubMed: 16041030]
48. Byndloss MX, Tsolis RM. Brucella spp. Virulence Factors and Immunity. Annu Rev Anim Biosci. 2016; 4:111–127.
49. Arenas-Gamboa AM, Rice-Ficht AC, Fan Y, Kahl-McDonagh MM, Ficht TA. Extended safety and efficacy studies of the attenuated Brucella vaccine candidates 16 M(Delta)vjbR and S19(Delta)vjbR in the immunocompromised IRF-1−/− mouse model. Clin Vaccine Immunol. 2012; 19:249–260. [PubMed: 22169089]
50. Avila-Calderon ED, Lopez-Merino A, Sriranganathan N, Boyle SM, Contreras-Rodriguez A. A history of the development of Brucella vaccines. Biomed Res Int. 2013; 2013:743509. [PubMed: 23862154]
51. Conde-Alvarez R, Arce-Gorvel V, Gil-Ramirez Y, Iriarte M, Grillo MJ, Gorvel JP, Moriyon I. Lipopolysaccharide as a target for brucellosis vaccine design. Microb Pathog. 2013; 58:29–34. [PubMed: 23219811]
52. Godfroid J, Scholz HC, Barbier T, Nicolas C, Wattiau P, Fretin D, Whatmore AM, Cloeckaert A, Blasco JM, Moriyon I, Saegerman C, Muma JB, Al Dahouk S, Neubauer H, Letesson JJ. Brucellosis at the animal/ecosystem/human interface at the beginning of the 21st century. Prev Vet Med. 2011; 102:118–131. [PubMed: 21571380]
53. Moriyon I, Grillo MJ, Monreal D, Gonzalez D, Marin C, Lopez-Goni I, Mainar-Jaime RC, Moreno E, Blasco JM. Rough vaccines in animal brucellosis: structural and genetic basis and present status. Vet Res. 2004; 35:1–38. [PubMed: 15099501]
54. Jimenez de Bagues MP, Elzer PH, Jones SM, Blasco JM, Enright FM, Schurig GG, Winter AJ. Vaccination with Brucella abortus rough mutant RB51 protects BALB/c mice against virulent strains of Brucella abortus, Brucella melitensis, and Brucella ovis. Infect Immun. 1994; 62:4990–4996. [PubMed: 7927779]
55. Clapp B, Skyberg JA, Yang X, Thornburg T, Walters N, Pascual DW. Protective live oral brucellosis vaccines stimulate Th1 and th17 cell responses. Infect Immun. 2011; 79:4165–4174. [PubMed: 21768283]
56. Delory M, Hallez R, Letesson JJ, De Bolle X. An RpoH-like heat shock sigma factor is involved in stress response and virulence in Brucella melitensis 16M. J Bacteriol. 2006; 188:7707–7710. [PubMed: 16936018]
57. Vemulapalli TH, Vemulapalli R, Schurig GG, Boyle SM, Sriranganathan N. Role in virulence of a Brucella abortus protein exhibiting lectin-like activity. Infect Immun. 2006; 74:183–191. [PubMed: 16368972]
58. Ugalde JE, Comerci DJ, Leguizamon MS, Ugalde RA. Evaluation of Brucella abortus phosphoglucomutase (pgm) mutant as a new live rough-phenotype vaccine. Infect Immun. 2003; 71:6264–6269. [PubMed: 14573645]
59. Ugalde JE, Czibener C, Feldman MF, Ugalde RA. Identification and characterization of the Brucella abortus phosphoglucomutase gene: role of lipopolysaccharide in virulence and intracellular multiplication. Infect Immun. 2000; 68:5716–5723. [PubMed: 10992476]
60. Herrou J, Czyz DM, Willett JW, Kim HS, Chhor G, Babnigg G, Kim Y, Crosson S. WrpA is an atypical flavodoxin-family protein under regulatory control of the Brucella abortus general stress response system. J Bacteriol. 2016; 198:1281–1293. [PubMed: 26858101]
61. Endley S, McMurray D, Ficht TA. Interruption of the cydB locus in Brucella abortus attenuates intracellular survival and virulence in the mouse model of infection. J Bacteriol. 2001; 183:2454–2462. [PubMed: 11274104]
62. Sun YH, de Jong MF, den Hartigh AB, Roux CM, Rolan HG, Tsolis RM. The small protein CydX is required for function of cytochrome bd oxidase in Brucella abortus. Front Cell Infect Microbiol. 2012; 2:47. [PubMed: 22919638]
63. Sangari FJ, Seoane A, Rodriguez MC, Aguero J, Garcia Lobo JM. Characterization of the urease operon of Brucella abortus and assessment of its role in virulence of the bacterium. Infect Immun. 2007; 75:774–780. [PubMed: 17101645]

64. Fretin D, Fauconnier A, Kohler S, Halling S, Leonard S, Nijskens C, Ferooz J, Lestrate P, Delrue RM, Danese I, Vandenhaute J, Tibor A, DeBolle X, Letesson JJ. The sheathed flagellum of Brucella melitensis is involved in persistence in a murine model of infection. Cell Microbiol. 2005; 7:687–698. [PubMed: 15839898]

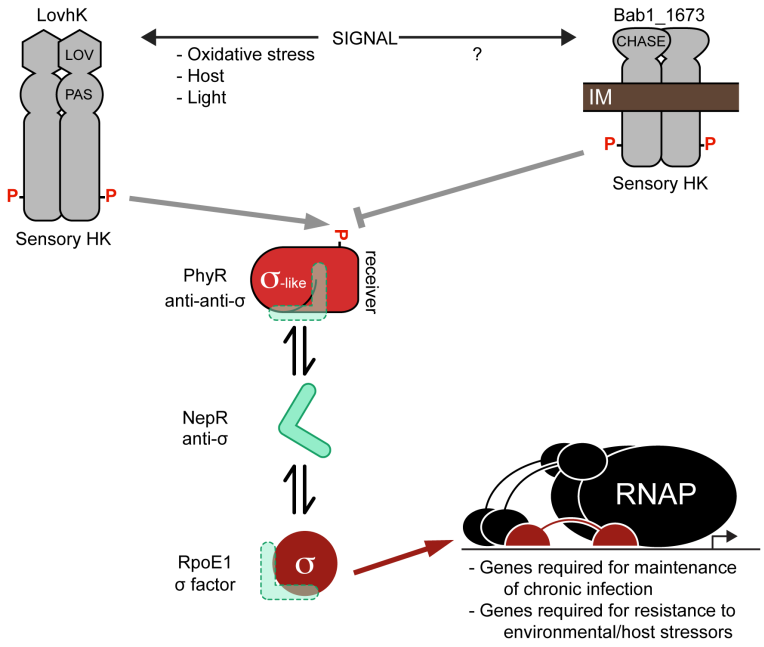


**Figure 1. Model of the *B. abortus* general stress response (GSR) regulatory system**

The stress-activated sensor histidine kinase (HK), LovhK, autophosphorylates and transmits a phosphoryl group to the anti-anti-σ factor, PhyR. The HK, Bab1_1673, represses PhyR activation. Phosphorylated PhyR (PhyR~P) binds the anti-σ factor, NepR. When released from NepR, the σ-factor $\sigma^{E1}$ associates with RNA polymerase (RNAP) to activate transcription of the GSR regulon.

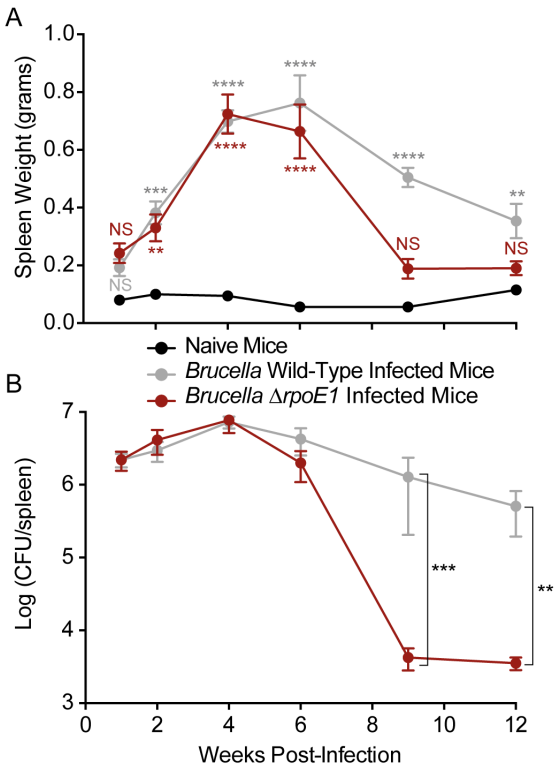


**Figure 2. Δ*rpoE1*-infected mice have reduced splenic bacterial burden and splenomegaly during the chronic phase of infection**

Female BALB/c mice were infected intraperitoneally with WT or Δ*rpoE1 B. abortus* and (A) spleen weights and (B) bacterial burden were measured at 1, 2, 4, 6, 9, and 12 weeks post-infection. Data presented are the mean ± the standard error of the mean (n = 5). Graphs represent data from naïve mice (black circle), WT-infected mice (grey circle), and Δ*rpoE1*-infected mice (red circle). One-way ANOVA was followed by a Bonferroni post-test to assess the significance in the mean differences between naïve mice and WT- or Δ*rpoE1*-infected mice (A) or between WT- and Δ*rpoE1*-infected mice (B). NS = not-significant, (*) = P<0.05, (**) = P<0.01, (***) = P<0.001, (****) = P < 0.0001.

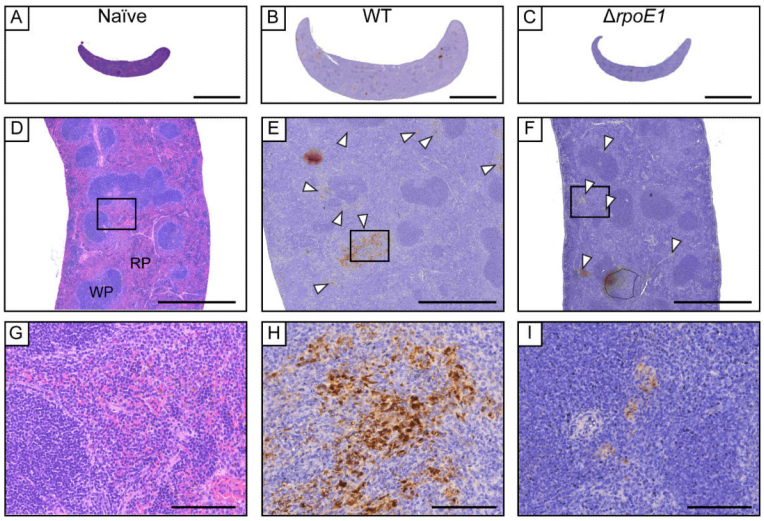


**Figure 3. Spleen histology of BALB/c mice infected with wild-type and Δ*rpoE1 B. abortus* strains**

At 9 weeks post-infection spleens were harvested, fixed, and slides were prepared. H&E staining was performed on all samples. Uninfected (A, D, G), WT-infected (B, E, H), or Δ*rpoE1*-infected (C,F,I) mouse spleen pictures were taken at 10X (A-C), 50X (D-F) or 400X magnification (G-I). *Brucella* antigen was visualized by immunohistochemistry with an anti-*Brucella* antibody (D-I). White arrowheads point to areas of extensive *Brucella* antigen staining. Scale bars are 5 mm (A), 1 mm (B), and 100 μm (C). WP = white pulp, RP = red pulp.

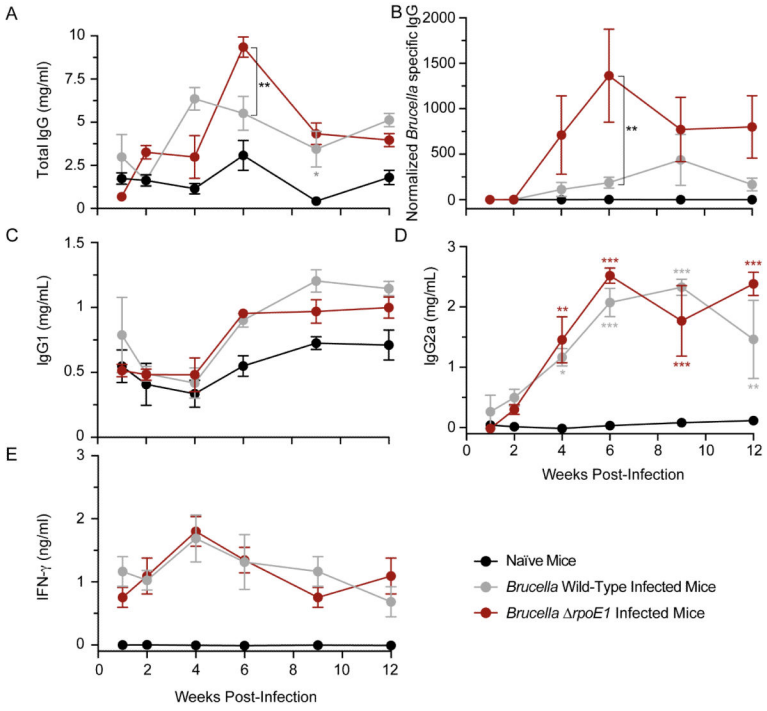


**Figure 4. Antibody and cytokine quantification in wild-type and Δ*rpoE1*-infected mouse sera**

Mouse serum was harvested at 1, 2, 4, 6, 9, and 12 weeks post-infection from naïve-control mice, WT-, or Δ*rpoE1*-infected mice. Amounts of total IgG (A) *Brucella*-specific IgG (B) IgG1 (C) IgG2a (D) and IFN-γ (E) were determined by ELISA (see Materials and Methods for details). Data presented are from naïve mice (black circle), WT-infected mice (grey circle), or Δ*rpoE1*-infected mice (red circle). Each data point (n = 5) is the mean ± the standard error of the mean. Unless otherwise indicated, one-way ANOVA was followed by a Bonferroni post test to assess the significance in the mean differences between naïve mice and WT- or Δ*rpoE1*-infected mice. NS = not significant, (*) = P<0.05, (**) = P<0.01, (***) = P<0.001, (****) = P < 0.0001.

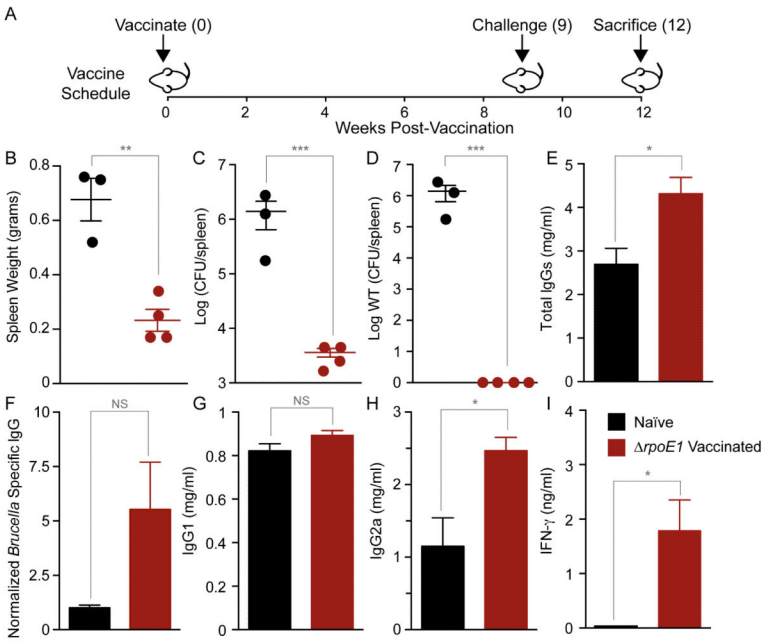


**Figure 5. Analysis of splenomegaly, bacterial burden, antibody, and cytokine profiles in Δ*rpoE1*-vaccinated mice**

Naïve age-matched control mice or Δ*rpoE1*-vaccinated mice were infected with WT *Brucella* 9 weeks post-vaccination (A). Spleen weights (B), total *Brucella* burden (C), and WT *Brucella* (D) were determined for each spleen. Quantitative ELISAs were performed to analyze serum levels of total IgG (E), *Brucella*-specific IgG (F), IgG1 (G) IgG2a (H), and IFN-γ (I). For vaccination studies, naïve mice (n = 3) are colored black and Δ*rpoE1*-vaccinated mice (n = 4) are colored red. Data presented are the mean ± SEM. T-tests were performed between naïve and Δ*rpoE1*-vaccinated mice. NS = not-significant, (*) = P<0.05, (**) = P<0.01, (***) = P<0.001, (****) = P < 0.0001.

## Table 1

Summary of histopathologic scoring of naïve mouse spleens, and spleens of mice infected with wild-type or Δ*rpoE1 B. abortus*.

| | Naïve | | *B. abortus* WT | | *B. abortus* Δ*rpoE1* | |
|---|---|---|---|---|---|---|
| **Week Post-infection (wpi)** | **1** | **9** | **1** | **9** | **1** | **9** |
| **white pulp to red pulp ratio** | Normal (≈ 1:1) | Normal (≈ 1:1) | Decrease Score 1 | Decrease Score 3 | Decrease Score 1 | Decrease Score 0.5 |
| **Average lymphoid follicles per field** | 8 to 12 | 8 to 12 | Decrease Score 1 | Decrease Score 2 | Decrease Score 1 | Decrease Score 0.5 |
| **Size of follicles** | normal | normal | Decrease Score 1 | Decrease Score 1 | Decrease Score 1 | Score 0 |
| **Marginal zone depletion** | normal | normal | Increase Score 1 | Increase Score 2 | Increase Score 1 | Increase Score 0-1 |
| **Extramedullary hematopoiesis** | minimal | minimal | Increase Score 2 | Increase Score 2 | Increase Score 1 | Increase Score 0-1 |
| **Histiocytic proliferation** | none | none | Increase Score 2 | Increase Score 3 | Increase Score 1 | Increase Score 0-1 |
| **Granulomas** | none | none | Increase Score 1 | Increase Score 3 | Increase Score 0-1 | Increase Score 0-1 |

Scores range from 0 to 3, and are based on masked evaluation of sectioned and stained spleen tissue. The presence of *Brucella* in tissue was confirmed by immunohistochemistry. 0 = normal pathology (all naïve spleens scored 0 in all categories); 1 = mild pathology, 2 = moderate pathology, 3 = severe pathology relative to uninfected (naïve) control.