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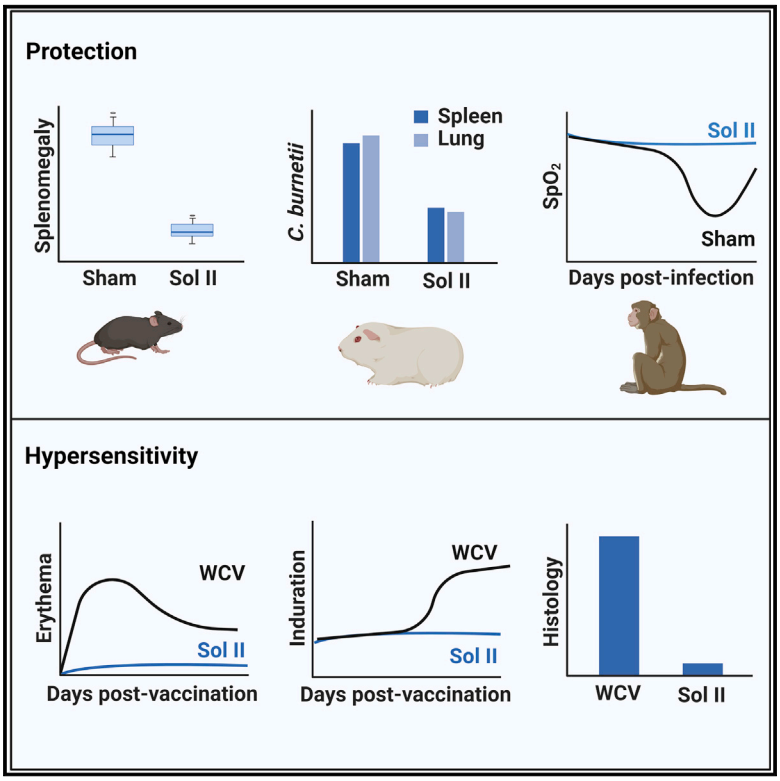
Soluble antigens derived from *Coxiella burnetii* elicit protective immunity in three animal models without inducing hypersensitivity

Graphical abstract



Highlights

- Solubilized fraction is purified from *C. burnetii* phase II cell lysate (Sol II)
- Sol II elicits protection in mice, guinea pigs, and macaques against Q fever
- No evidence of vaccine-mediated hypersensitivity in pre-sensitized guinea pigs
- Q fever protection improved further by Sol I immunization

Authors

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In brief

Q fever is a zoonotic disease with no widely approved vaccine that poses a serious threat to public health and national security. In this study, Gregory et al. show that a soluble bacterial extract is both safe and provides significant protection against Q fever in three animal models of infection.

Gregory et al., 2021, Cell Reports Medicine 2, 100461
December 21, 2021
https://doi.org/10.1016/j.xcrm.2021.100461

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Soluble antigens derived from *Coxiella burnetii* elicit protective immunity in three animal models without inducing hypersensitivity

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https://doi.org/10.1016/j.xcrm.2021.100461

SUMMARY

Q fever is caused by the intracellular bacterium *Coxiella burnetii*, for which there is no approved vaccine in the United States. A formalin-inactivated whole-cell vaccine (WCV) from virulent *C. burnetii* NMI provides single-dose long-lived protection, but concerns remain over vaccine reactogenicity. We therefore sought an alternate approach by purifying native *C. burnetii* antigens from the clonally derived avirulent NMII strain. A soluble bacterial extract, termed Sol II, elicits high-titer, high-avidity antibodies and induces a CD4 T cell response that confers protection in naive mice. In addition, Sol II protects against pulmonary *C. burnetii* challenge in three animal models without inducing hypersensitivity. An NMI-derived extract, Sol I, enhances protection further and outperforms the WCV gold standard. Collectively, these data represent a promising approach to design highly effective, non-reactogenic Q fever vaccines.

INTRODUCTION

Coxiella burnetii is a Gram-negative, obligate intracellular pathogen that causes the zoonotic disease Q fever. Ruminants, mostly sheep and goats, are considered the main natural reservoir for *C. burnetii*, and transmission to humans occurs most commonly via the inhalation of contaminated aerosols.[1,2] Acute Q fever accounts for approximately half of all cases and results in general influenza-like symptoms, as well as retro-orbital pain, atypical pneumonia, and hepatitis. The disease is self-limiting and can be treated effectively with antibiotics, particularly doxycycline.[3–5] In ~5% of cases, Q fever manifests into a chronic form that is far more severe and is less responsive to antibiotic treatment. Chronic Q fever is characterized by chronic fatigue and a potentially fatal endocarditis.[2,6] Asymptomatic Q fever accounts for the remainder of individuals infected with the pathogen.

Several attempts have been made over the years to generate Q fever vaccines. The most successful to date is a killed whole-cell vaccine (WCV) made from formalin-inactivated *C. burnetii* phase I Henzerling strain (Q-Vax; Commonwealth Serum Laboratories, Australia), which is able to confer lifelong protective immunity in humans after a single dose.[7] Encouraged by these data, the Australian government implemented a nationally funded vaccine program with Q-Vax to protect those deemed to be at high risk of infection, such as abattoir workers. This resulted in a 50% decline in Q fever notification rates during the 4 years the program was active.[8] However, safety concerns surrounding the use of Q-Vax, particularly in individuals who have been previously exposed to *C. burnetii*, has limited the widespread use of this vaccine outside Australia.[9]

Severe local and systemic adverse reactions have been well documented with Q-Vax, and therefore vaccination is restricted to serologically and skin test-negative individuals, assessed pre-immunization. In addition, *C. burnetii* phase I is classified as a tier 2 Biological Select Agent and Toxin (BSAT). Purifying vaccine material from this virulent strain of *C. burnetii* presents significant challenges both in terms of increased biosafety and biosecurity requirements for propagating the bacterium as well as manufacturing costs for large-scale production.

Upon serial passage *in vitro*, *C. burnetii* transitions from a smooth lipopolysaccharide (LPS) phase I variant to an energetically favorable rough LPS phase II variant.[10,11] *C. burnetii* phase II derivatives produce a truncated LPS containing lipid A as well as inner and outer core sugars but lack O-antigen altogether.[12] A major consequence for phase II bacteria is that without full-length LPS masking surface pathogen-associated molecular patterns (PAMPs), these antigenic variants induce a more robust pro-inflammatory response, compared with *C. burnetii* phase I. *In vitro* infection of primary cell lines with *C. burnetii* Nine Mile phase II (NMII) results in elevated levels of tumor necrosis factor (TNF) and interleukin-12 (IL-12), compared with cells infected

with *C. burnetii* NMI.[13,14] Without the shielding properties of full-length LPS, *C. burnetii* NMII is readily detected *in vivo* and cleared, rendering the bacterium avirulent in an immunocompetent host and therefore BSAT exempt.

While *C. burnetii* NMI LPS likely contributes to the protection afforded by inactivated *C. burnetii* NMI vaccines, it is doubtful that it is the sole antigen responsible. The authors of an LPS protection study concede that contaminating antigens present with LPS, due to the difficulties in purification, may contribute to the level of protection that they saw with their model.[15] In addition, LPS alone would not constitute a good vaccine candidate since glycans are T cell-independent antigens that fail to induce sustained T cell responses critical not only for clearance of *C. burnetii* but also for robust T cell memory responses.[16–18] Several studies have previously demonstrated durable antibody titers elicited by vaccination with purified *C. burnetii* proteins.[19,20]

Aside from disruptions to LPS biosynthesis, *C. burnetii* NMII shares an almost identical genome to *C. burnetii* NMI.[21,22]. Included is a functional T4BSS that secretes many immunodominant antigens identified in convalescent serum and polyclonal serum from various animal models immunized with killed *C. burnetii*.[23–26]. Therefore, it is reasonable to assume that many of the antigens driving protection in *C. burnetii* NMI-killed WCVs are also present in *C. burnetii* NMII. Furthermore, the ability to cultivate *C. burnetii* NMII at Biosafety Level 2 (BSL2) makes it far more attractive for use in large-scale vaccine manufacturing than is virulent *C. burnetii* NMI.

Currently, *C. burnetii* M44 (RSA461) is the only phase II strain to demonstrate any vaccine-mediated efficacy against Q fever and was used extensively in Russia during the 1960s.[27] Isolated following repeated passage through a guinea pig, *C. burnetii* M44 was administered orally as a live attenuated vaccine that elicited an 80% seroconversion rate in vaccinated humans.[27] Minimal side effects were initially reported following immunization; however, further evaluation of animal models inoculated with *C. burnetii* M44 revealed significant safety concerns, including Q fever-related lesions, myocarditis, and long-term persistence.[28,29]

Other attempts to make vaccine material from *C. burnetii* NMII have thus far proved unsuccessful.[30–32] Despite a similar breadth of immunoreactive antigens, mice immunized with a formalin-inactivated virulent strain of *C. burnetii* NMI produce significantly higher immunoglobulin G (IgG) titers than those immunized with a formalin-inactivated avirulent strain of *C. burnetii* NMII.[33] In addition, a higher frequency of antigen-specific $CD4^+$ T cells was detected in mice immunized with *C. burnetii* NMI than in those immunized with NMII. Although fixed *C. burnetii* NMI antigens have been well documented in their capacity to induce effective immunity, it remains unclear whether the inclusion of adjuvants would enhance the host response to weakly immunogenic NMII antigens. In a recent study comparing the reactogenicity of formalin-fixed NMI with NMII, a severe local response was observed with both vaccine formulations in guinea pigs.[34] Thus, there remains a considerable need to develop a Q fever vaccine that can provide effective immunity without inducing hypersensitivity.

In this study, we created a Q fever vaccine from native antigens found in the Select Agent-exempt *C. burnetii* NMII strain Sol II, with an appropriate adjuvant to enhance a protective response. We found that mice vaccinated with Sol II developed high-avidity antibodies and robust cellular responses that could transfer protective immunity into naive mice. Furthermore, Sol II conferred protection in guinea pigs and a non-human primate (NHP) model of pulmonary Q fever. A hypersensitivity model using previously sensitized guinea pigs revealed there were also no adverse local reactions associated with Sol II immunization. Lastly, we show that a derivative of NMI solubilized antigens (Sol I) can be used to provide additional protection against aerosol Q fever that is not entirely attributable to NMI LPS.

RESULTS

Soluble extracts from *C. burnetii* phase II retain antigen diversity

We used a combination of mild detergents to solubilize integral membrane proteins and insoluble antigens from *C. burnetii* phase II cell lysate. Detergents were selected based on previously published data highlighting their use in purifying proteins for application in vaccine studies.[35–37] Detergents with increasing polarity and strength were selected to maximize protein diversity and capture as many antigens as possible in their native structure (Table S1). A mild, non-denaturing detergent, *n*-octylglucoside (OG), was used initially to isolate membrane proteins through the disruption of lipid-lipid and lipid-protein interactions.[38] Treatment of *C. burnetii* lysate with OG yielded a variety of soluble proteins, broadly between 12 and 50 kDa (Figure 1A).

Next, we used Anzergent 3-14 (Anz) to purify any additional membrane proteins we were not able to capture with OG solubilization. Like OG, Anz has a net neutral charge but is capable of breaking protein-protein interactions due to its opposing polar regions.[39] However, the zwitterionic polarity of Anz means that many of these proteins are likely to maintain their native conformation and are therefore used in many structural studies of integral membrane proteins.[40,41] Solubilization with Anz resulted in the capture of additional proteins >50 kDa.

Finally, we used sodium lauroyl sarcosinate (sarkosyl) to capture any remaining insoluble antigens from *C. burnetii* lysate. Sarkosyl has previously been demonstrated to be highly effective at solubilizing proteins from inclusion bodies and allows refolding without aggregation.[42–44] In addition, sarkosyl is effective at extracting insoluble antigens that may contribute toward immunogenicity, including glycoproteins and carbohydrates.[45,46] Sarkosyl treatment resulted in the extraction of additional proteins between 10 and 60 kDa (Figure 1A).

The solubilized fractions were pooled (Sol II) and run on a western blot against convalescent serum from mice, guinea pigs, and NHPs. The immunoblot demonstrates that Sol II is cross-reactive with a broad range of *C. burnetii*-specific antibodies present in previously infected animals (Figure 1B). The data also suggest that there are some significant differences between animal models in antigen recognition. In addition, we confirmed the presence of endogenous proteins by running a western blot against an immunodominant *C. burnetii* protein, cbu_1910, which has been previously identified as a potential vaccine candidate[26,47–49] (Figure 1C). Antigens purified from *C. burnetii* NMII were also analyzed by mass spectrometry to determine some of the most abundant antigens present (Table S2).

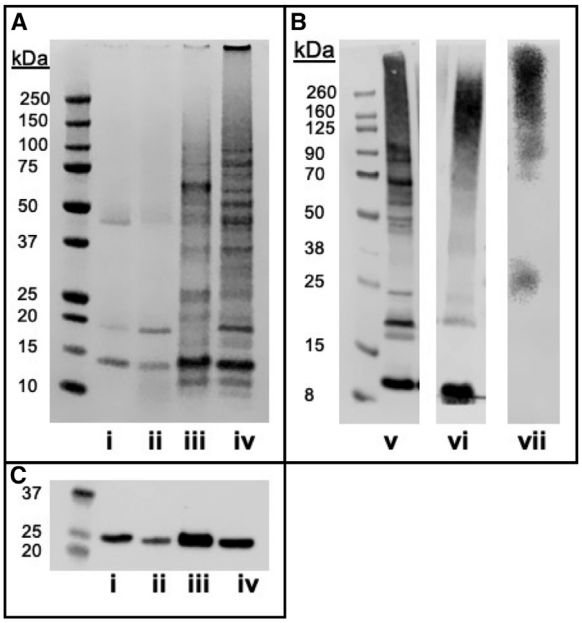

Figure 1. Soluble extracts from *C. burnetii* phase II contain diverse antigens

(A) Single detergent extracts of 10 μg protein from *n*-octylglucoside (i), Anzegent 3-14 (ii), and Sarkosyl (iii) were run on an SDS-PAGE gel alongside a pooled sample of all of the antigens that make up Sol II material (iv).

(B) Sol II material was run on a western blot and immunoblotted against convalescent serum isolated from a mouse (v), guinea pig (vi), and non-human primate (vii).

(C) Sol II material was also blotted against a cbu_1910 monoclonal antibody to confirm the presence of this immunodominant *C. burnetii* antigen.

Sol II immunization produces high-avidity antibodies

We immunized mice with Sol II in combination with different adjuvants to evaluate the elicited humoral immune response from Sol II vaccination and the contributions of adjuvant choice toward IgG isotype switching. C57BL/6 (BL6) mice were vaccinated subcutaneously (s.c.) in a prime-boost manner with 10 μg Sol II plus an adjuvant at a dose recommended by the manufacturers (see Method details). Serum was collected at days 7 (post prime) and 21 (post-boost) to evaluate IgM and IgG response via ELISA. At day 7, QuilA and monophosphoryl lipid A (MPLA) were the only adjuvants to increase detectable IgM and IgG antibody responses to Sol II above the unadjuvanted control (Figure 2A). However, by day 21, each of the groups receiving an adjuvanted vaccine had higher titers of both IgM and IgG compared with the unadjuvanted control.

Each of the adjuvants tested has a propensity to drive the immune response toward either a Th1 (CpG and MPLA), T helper 2 (Th2) (alum, TiterMax Gold, and incomplete Freund's adjuvant [IFA]) or a balanced Th1/Th2 (QuilA). Adjuvant bias toward Th2 appeared to correlate with elevated antibody responses, as expected. To explore this further, we examined IgG subclasses IgG1 (indicating Th2 bias) and IgG2c (Th1 bias) of vaccinated mice. IgG1 responses were significantly higher in Sol II-vaccinated mice with Th2 agonists (alum, TiterMax Gold, and IFA) as well as MPLA, whereas IgG2c responses were significantly higher in Sol II-vaccinated mice with Th1 agonists CpG and QuilA (Figure 2B). From these data, we calculated the ratio of IgG2c to IgG1 to profile the type of immune response favored by the selected adjuvants. Only CpG and QuilA had an IgG2c/IgG1 ratio above 1, with CpG resulting in an IgG2c ratio of >6 (Figure 2C). Thus, adjuvant choice plays a significant role in engaging Th1 and Th2 responses in mice immunized with Sol II. Previous studies have indicated Th1 immune responses are an important correlate of protective immunity against Q fever.[15,50,51] For this reason, CpG was selected as the adjuvant of choice for all future Sol II immunizations.

We evaluated the impact of vaccine regime and antigen dose on the immune response to Sol II vaccination by immunizing mice with a low dose (10 μg) of Sol II + CpG in either a prime, prime-boost, or prime-boost-boost manner. In addition, mice were immunized with a high dose (40 μg) of Sol II + CpG or WCV, both in a prime-boost manner. Sera were collected from mice 35 days after initial immunization and assayed via ELISA. There was a negligible difference in IgM responses between a single dose and prime-boost immunization, but a significant increase following a second booster immunization. Similarly, IgG responses to Sol II were elevated after each additional immunization. Equivalent IgG titers were measured from mice vaccinated with a high dose and those receiving a prime-boost-boost vaccine, although IgM titers were significantly reduced in the former. The lowest antibody response was seen in mice immunized with WCV, with IgM titers higher than IgG (Figure 2D).

IgG1 and IgG2c subclasses from dose-variable Sol II immunized mice were also calculated. The ratio of IgG2c to IgG1 increased with an increasing number of doses. However, mice immunized prime-boost with a higher dose of Sol II exhibited the largest ratio of IgG2c to IgG1, 3.2. The ratio of IgG2c to IgG1 antigen-specific antibodies contrasted further in mice immunized with WCV (Figure 2E).

An avidity assay was designed to determine the impact variable dosing regimens have on the quality of antibodies elicited and calculate an avidity index (AI).[52] The avidity of polyclonal serum from mice immunized with Sol II in a prime-boost dosing was significantly higher than that from mice immunized with a single prime dose (Figure 2F). Similarly, AI remained high in mice immunized with Sol II in a prime-boost-boost or the higher dose. High-avidity antibodies were also detected in WCV serum, suggesting a possible correlation between avidity and protection. It is interesting to note that despite WCV immunization not resulting in a high titer of antibodies, these antibodies are of high avidity and indicate Th1 immune skewing.

Mice and guinea pigs demonstrate protection against *C. burnetii* infection following immunization with Sol II

Next, we evaluated the protective immunity of Sol II in mice and guinea pigs against an aerosol challenge of *C. burnetii* NMI. Sol II- and WCV-immunized mice did not demonstrate any weight loss during the 2-week infection, whereas sham-vaccinated mice demonstrated significant weight loss (10%) on days 4 ($p = 0.0001$) and 9 ($p = 0.0038$) post-infection, compared with Sol II mice (Figure 3A). In addition, sham-immunized mice exhibited splenomegaly (spleen weight as a percentage of body weight),

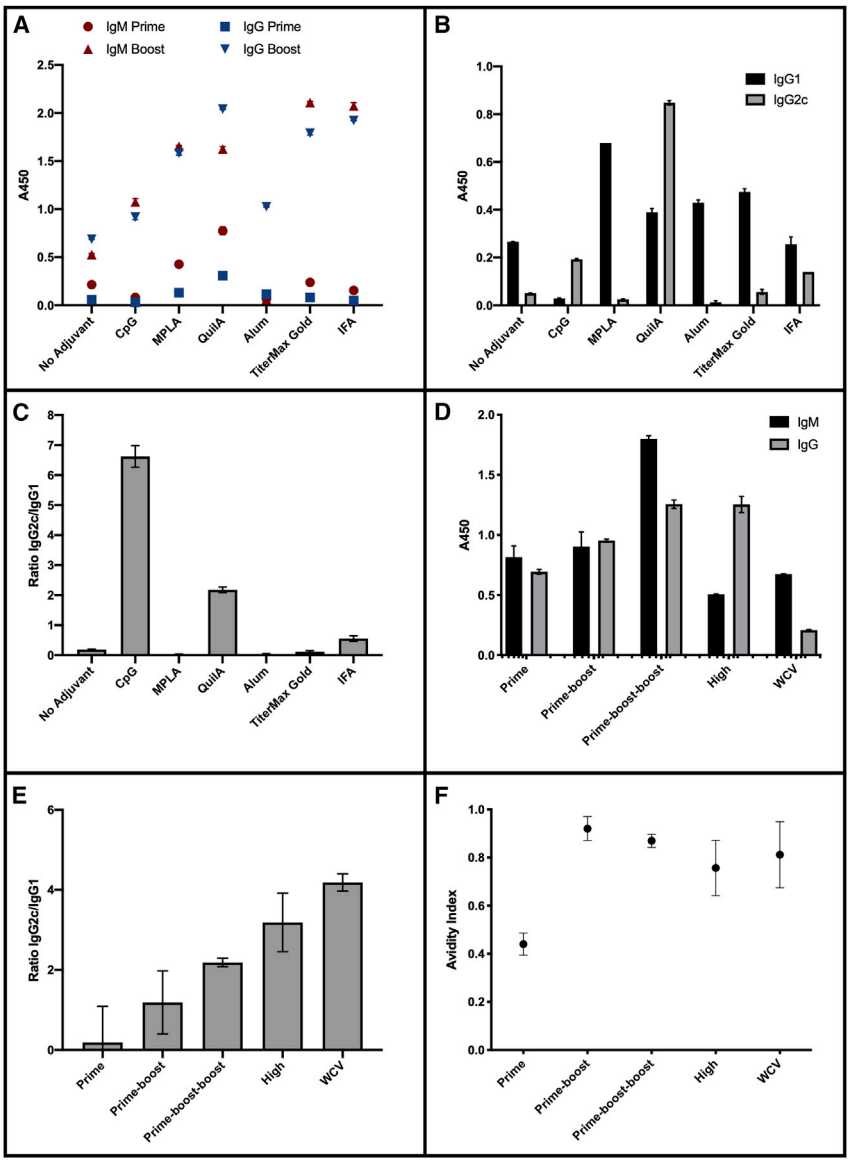

Figure 2. Sol II immunization produces high-avidity antibodies

(A) IgM and IgG responses were elevated in all adjuvant groups, compared with Sol II alone, and increased following boost immunization.

(B and C) Adjuvant selection was found to influence either IgG1 or IgG2c subclass production (B), with CpG resulting in the biggest IgG2c:IgG1 differential (C).

(D) Mice were immunized with an increasing number of doses of Sol II and CpG, resulting in an increased IgG response.

(E) Similarly, increases in immunization frequency or dose resulted in a greater shift in IgG2c responses, relative to IgG1.

(F) The avidity of polyclonal serum from immunized mice demonstrated a higher affinity for Sol II following boost immunizations or a higher dose.

Data are represented as means ± SEMs.

2.6%, 2 weeks post-infection that was significantly reduced in both Sol II- (0.86%, p = 0.0001) and WCV- (0.47%, p = 0.0001 immunized mice (Figure 3B). Bacterial burdens in the spleen and lungs were significantly reduced in the Sol II- (p = 0.0001 and 0.0012, respectively) and WCV- (p = 0.0001 and 0.0018, respectively) immunized mice, compared with sham (Figure 3C).

Eight days post-infection, sham-immunized guinea pigs lost ~5% of their starting body weight, whereas the Sol II group had gained an average of 3% (Figure 3D). Sham-immunized guinea pigs exhibited splenomegaly (0.4%) 2 weeks post-infection that was significantly reduced in both Sol II- (0.16%, p = 0.0175) and WCV- (0.14%, p = 0.0138) immunized guinea pigs (Figure 3E). Bacterial burdens in spleen and lungs were significantly reduced in the Sol II- (p = 0.0335 and 0.0003, respectively) and WCV- (p = 0.0280 and 0.0003, respectively) immunized guinea pigs, compared with sham (Figure 3F).

CD4$^+$ T cells from Sol II-immunized mice confer protection when transferred into a naive mouse

Polyclonal serum, lymphocytes, or CD4$^+$ T cells were transferred from sham, Sol II-, or WCV-immunized mice into naive mice to evaluate the contributions of cellular and humoral immunity in Sol II-mediated protection against Q fever. Adoptively transferred mice were subsequently challenged with *C. burnetii* NMI by large-particle aerosol (LPA) and evaluated for 2 weeks. Vaccinated mice were included as a comparison to adoptive transfer recipients.

Mice that received serum from Sol II- or WCV-immunized mice did not show any difference in splenomegaly, compared with sham (Figure 4A). However, partial mitigation of splenomegaly was observed when either lymphocytes or CD4$^+$ T cells from Sol II- or WCV-immunized mice were transferred into naive mice. Passive transfer of serum was also ineffective at reducing the amount of detectable *C. burnetii* in the lungs of infected mice (Figure 4B). CD4$^+$ T cells from Sol II- and WCV-immunized mice were able to confer significant levels of protection in recipient mice, similar to the levels observed with those immunized. Adoptively transferred CD4$^+$ T cells were more effective at reducing bacterial burden than lymphocytes, which only demonstrated a partial rescue of the phenotype observed with vaccinated mice. In addition, the bacterial burden was significantly reduced in the lungs following infection of adoptively transferred mice.

NHPs immunized with Sol II demonstrate protection against pulmonary Q fever

NHPs were immunized by prime-boost with sham, Sol II, or Q-Vax and rested for 8 weeks before aerosol challenge with *C. burnetii* NMI. There were no observable or significant

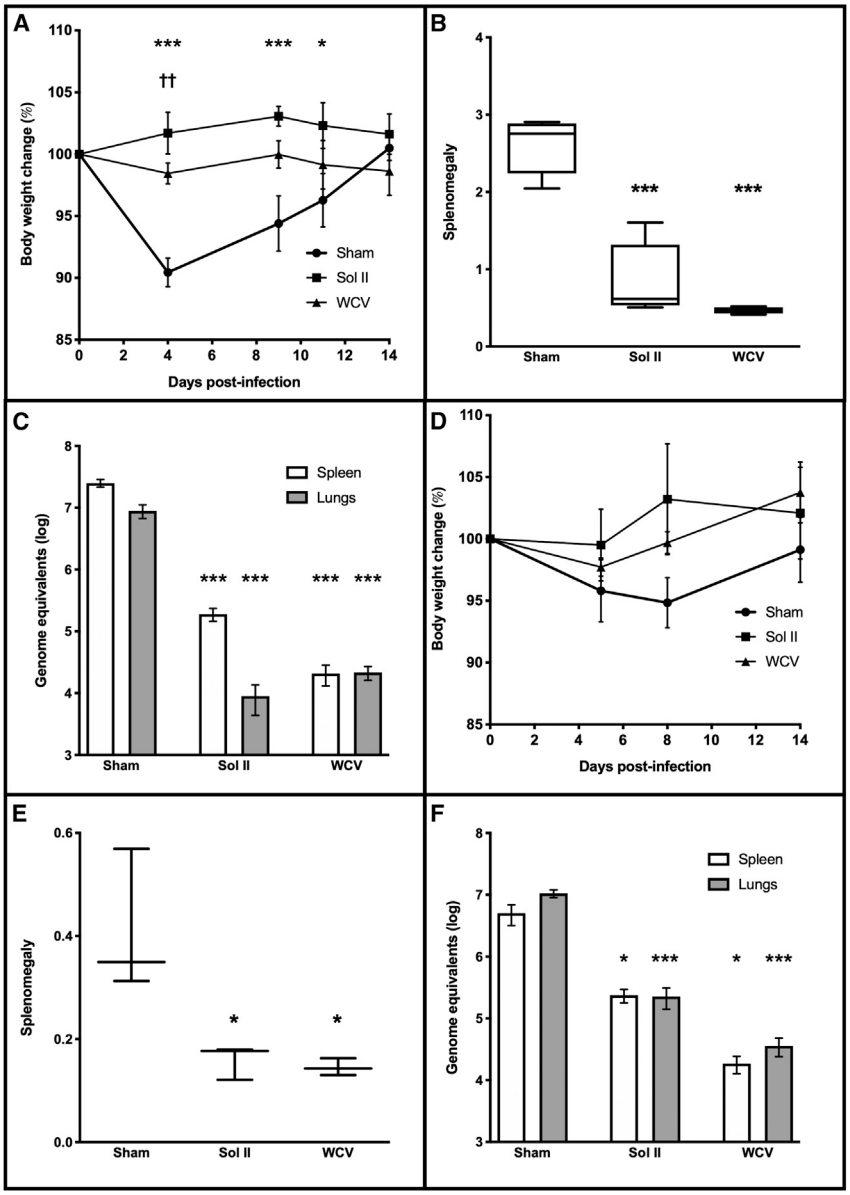

Figure 3. Mice and guinea pigs infected with *C. burnetii* have reduced bacterial numbers in primary and secondary tissues following vaccination with Sol II

Sham vaccinated mice demonstrated significant weight loss 4 days post-infection before recovering to initial weight.

(A) Sol II- and WCV-immunized mice did not demonstrate any weight loss.

(B) Splenomegaly was significantly reduced in both Sol II- and WCV-immunized mice.

(C) Bacterial burden in spleen and lungs of Sol II- and WCV-immunized mice were significantly reduced, compared with sham.

(D) Sham vaccinated guinea pigs demonstrated significant weight loss 8 days post-infection before recovering to initial weight. Sol II- and WCV-immunized guinea pigs did not demonstrate any weight loss.

(E) Splenomegaly was significantly reduced in both Sol II- and WCV-immunized guinea pigs.

(F) Bacterial burden in spleen and lungs of Sol II- and WCV-immunized guinea pigs were significantly reduced, compared with sham .

Data are represented as means ± SEMs. Data for (A) were analyzed using 2-way ANOVA with Sidak's correction for multiple comparisons; all other data were analyzed using 1-way ANOVA with Dunnett's correction for multiple comparisons. *Significance of statistical difference between Sol II and sham, †significance of statistical difference between WCV and sham (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

differences in weight between the three immunized groups over the course of infection (Figure 5A). Sham immunized NHPs developed a measurable fever response (40.2°C highest recorded) ~7 days post-infection that was not detectable in either Sol II- or Q-Vax-immunized NHPs (Figure 5B). Cumulative body temperature readings for sham immunized NHPs were significantly higher over the 2-week infection period than those immunized with Sol II ($p = 0.0114$).

Heart rate readings for sham and Q-Vax-immunized NHPs recorded elevated heart rates by ~30 bpm between days 2 and 10 (33 and 27 bpm, respectively), whereas Sol II-immunized NHPs increased by only 12 bpm (Figure 5C). In addition, Sol II immunized NHPs had returned to their resting heart rate (132 bpm) by day 14, whereas heart rates recorded from sham and Q-Vax-immunized NHPs were still above baseline (136 and 147 bpm, respectively). Respiratory rate readings were taken to determine the impact of a pulmonary infection on respiration. The respiratory rate of sham immunized NHPs was significantly higher than Sol II NHPs on days 7 ($p = 0.0328$), 10 ($p = 0.0261$), and 14 ($p = 0.0359$; Figure 5D). Sham immunized NHPs had an increased respiratory rate of 33 breaths/min between days 3 and 7, which remained significantly higher than basal respiratory rate for the duration of the two-week infection. Conversely, Sol II and Q-Vax immunized NHPs did not show any significant changes in respiratory rate (>4.5 bpm).

Blood oxygen saturation (SpO_2) was also measured as an additional indicator of impaired respiration. Levels of SpO_2 did not deviate from a normal range of >96% in either Sol II- or Q-Vax-immunized NHPs over 2 weeks (Figure 5E). However, in sham immunized NHPs, levels dropped to 95% by day 7 and by day 10 there was a further, significant decrease to an average of 92.5%. The cumulative SpO_2 readings for sham immunized NHPs were significantly lower over the 2-week infection period than those immunized with Sol II ($p = 0.0202$). NHPs were euthanized 2 weeks post-infection and spleens were collected to determine the severity of splenomegaly (Figure 5F). There was a statistically significant reduction in splenomegaly in three of four spleens collected from Sol II-immunized NHPs, compared

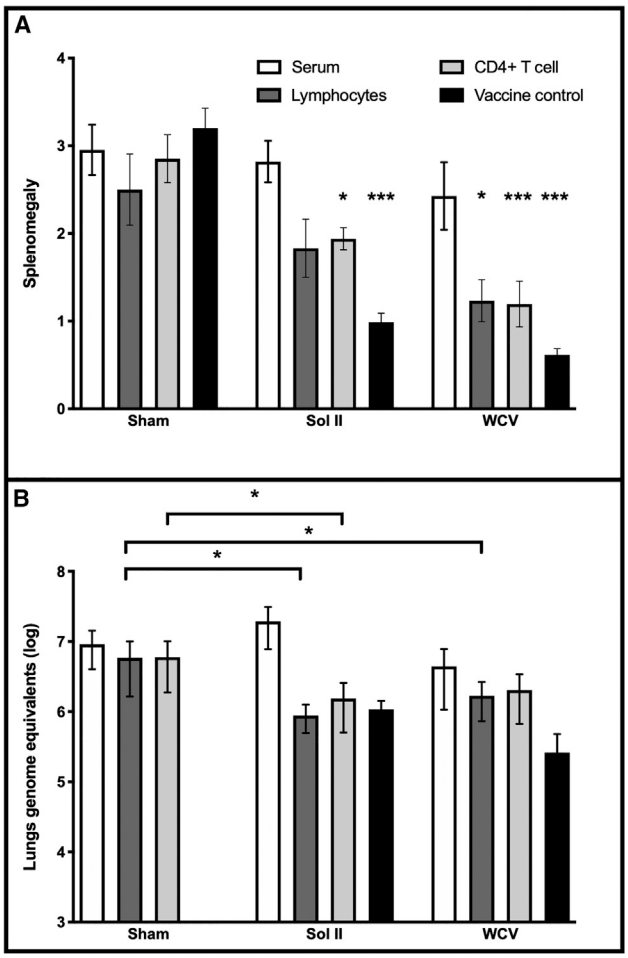

Figure 4. CD4$^+$ T cells from Sol II-vaccinated mice confer protection when transferred into a naive mouse

(A) Splenomegaly was significantly reduced in mice that received either lymphocytes or CD4$^+$ T cells from Sol II or WCV donor mice.

(B) Similarly, the bacterial burden enumerated from the lungs of mice that received either lymphocytes or CD4$^+$ T cells from Sol II or WCV donor mice was significantly reduced.

Data are represented as means ± SEMs and analyzed using 1-way ANOVA with Dunnett's correction for multiple comparisons. *Significance of statistical difference relative to sham (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

with sham. There were no measurable differences between Q-Vax- and sham immunized NHPs. Blood collected at time points for blood chemistry analysis throughout the 2-week infection did not reveal any significant findings (Table S3).

Sol II does not induce hypersensitivity in a previously sensitized guinea pig model

We also aimed to determine whether the vaccine material produced a hypersensitivity response in previously sensitized individuals, similar to Q-Vax. Hairless guinea pigs were sensitized by infection with a sublethal dose of *C. burnetii* NMI via LPA and subsequently rested for 7 weeks to resolve infection. Re-sensitization was achieved with a single subcutaneous immunization of either WCV, Sol II, or sham (S-WCV, S-Sol II, and S-Sham). Naive guinea pigs immunized with WCV were included as controls (N-WCV).

Previously sensitized guinea pigs immunized with WCV developed significant erythema soon after vaccination, with the largest area measured on day 2 (4.25 mm) (Figure 6A). The area of erythema reduced over the remaining days of the experiment but remained significantly larger than S-Sol II as late as 9 days post-vaccination ($p = 0.0251$). S-Sol II did not produce any significant erythema above S-Sham or N-WCV guinea pigs. As erythema reduced at the vaccine site of S-WCV guinea pigs, induration increased (Figure 6B). Significant induration was measured 10 days post-vaccination in S-WCV guinea pigs ($p = 0.0190$, compared with S-Sol II) and lasted the duration of the experiment (D11 $p = 0.0096$, D12 $p = 0.0179$, D13 $p = 0.0007$, D14 $p = 0.0003$). Induration at the vaccine site increased by 4 mm in S-WCV guinea pigs, whereas there was no measurable difference in any other group, including S-Sol II. By day 14, an eschar was visible in S-WCV guinea pigs at the site of vaccination (Figure S1).

Skin and s.c. tissue at the site of vaccination were excised during necropsy and evaluated for immunopathology. The severity of inflammation at the vaccine site for each guinea pig in their respective group was scored 0–5 in a blind fashion. Minimal or mild multifocal inflammation was scored 1 and severe inflammation, hemorrhages, and giant cell formation were scored 5 (Figure 6C). Skin sections in S-WCV guinea pigs were scored significantly higher for severity of inflammation than S-Sol II ($p = 0.0312$), S-Sham ($p = 0.0020$), or N-WCV ($p = 0.0485$). Marked inflammation was attributed to N-WCV, suggesting that inflammation is due to the recruitment of antigen-presenting cells, rather than a hypersensitivity response. No inflammation was found in S-Sham guinea pigs.

All guinea pigs in the S-WCV group were found to have moderate to large numbers of epithelioid macrophages, plasma cells, lymphocytes, and rare Langhans giant cells within the dermis and subcutis (Figure 6D). Multifocal aggregates of viable and degenerate heterophils within the dermis forming abscesses were found with small amounts of hemorrhage and edema within the area of inflammation. In addition, myofibers of the panniculus muscle in the area of inflammation were either lost or hypereosinophilic and shrunken, indicating degeneration. Vaccine sites from S-Sol II guinea pigs were found to contain small to moderate numbers of lymphocytes and plasma cells scattered throughout the dermis and subcutis. Few myofibers in the affected area were moderately shrunken, indicating degeneration, and moderate numbers of heterophils with lymphocytes and plasma cells extended to the deep dermis. N-WCV guinea pigs exhibited small numbers of lymphocytes, macrophages, and plasma cells within the subcutis and multifocally in the dermis. No significant immunopathologic observations were found in S-Sham guinea pigs.

Lung tissue from all infected guinea pigs was evaluated and demonstrated the presence of mild bronchus-associated lymphoid tissue (BALT) hyperplasia, indicating an immune response to successful pulmonary infection. Heart tissue was also collected from all of the guinea pigs in the study. While S-Sol II, S-Sham, and N-WCV groups did not show any significant findings, sections of the heart from S-WCV guinea pigs had small

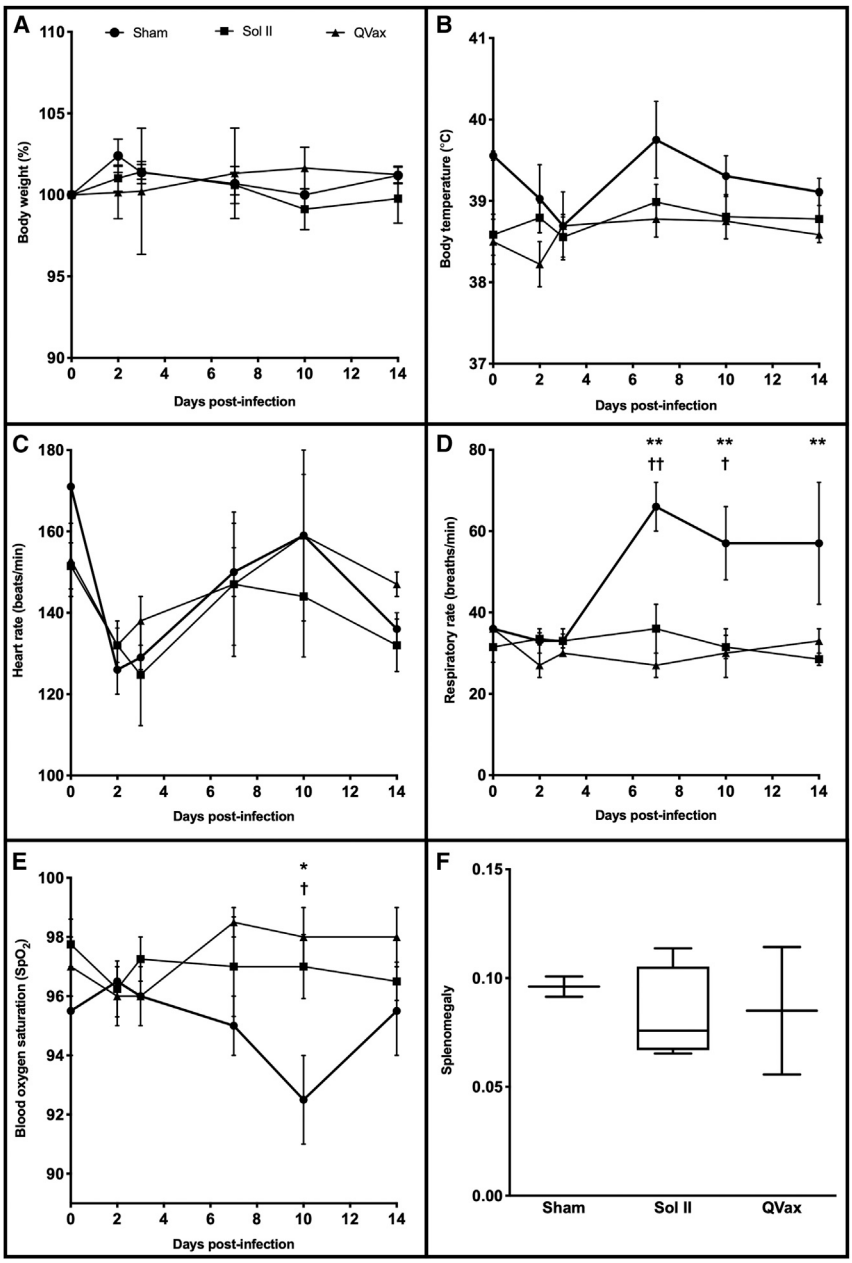

Figure 5. NHPs immunized with Sol II demonstrate protection against *C. burnetii* aerosol challenge

(A) No measurable changes in weight loss were recorded in any of the groups post-infection.

(B) Sham immunized NHPs developed a fever following infection, which was absent in both Sol II- and Q-Vax-immunized groups.

(C) There was a measurable increase in the heart rate of all NHPs following infection.

(D and E) The respiratory rate (D) and blood oxygen saturation (E) of NHPs were monitored as indicators of pulmonary function. In both cases, sham immunized NHPs had a faster respiratory rate and lower blood oxygen saturation than those immunized with Sol II or Q-Vax.

(F) Spleens were collected during necropsy and no significant difference between sham and Sol II or Q-Vax groups was recorded.

Data are represented as means ± SEMs and analyzed using 2-way ANOVA with Sidak's correction for multiple comparisons. *Significance of statistical difference between Sol II and sham, †significance of statistical difference between WCV and sham (*$p < 0.05$, **$p < 0.01$).

foci of lymphocytes within the epicardial fat and mild degeneration of adipocytes (data not shown).

Sol I immunization provides additional protection over Sol II + NMI LPS and WCV against aerosol *C. burnetii* infection in mice

To determine the contribution of NMI LPS to vaccine-mediated protection against *C. burnetii*, we purified a second vaccine formulation from *C. burnetii* NMI (Sol I) using the same method previously described for Sol II. In addition, we purified NMI LPS and combined it with the Sol II formulation. Mice were immunized with either sham, WCV, Sol II + NMI LPS, or Sol I and challenged via intratracheal LPA, 7 weeks following boost. Sol II + NMI LPS and Sol I were both administered with CpG. After 6 days post-infection, mice immunized with either sham, WCV, or Sol II + NMI LPS demonstrated a weight loss of ~5%–7% (Figure 7A). However, Sol I-immunized mice did not demonstrate any weight loss (<1%) and had a significantly higher body mass index compared with sham ($p = 0.0041$).

Pathology of infected mice revealed splenomegaly in sham vaccinated mice that was significantly reduced in WCV-, Sol II + NMI LPS-, and Sol I-immunized groups ($p < 0.0001$ for each group) (Figure 7B). Furthermore, lung weight relative to body weight, as an indicator of consolidation, was significantly reduced in WCV-, Sol II + NMI LPS-, and Sol I-immunized groups ($p = 0.0118$, 0.0118, and 0.0007, respectively) (Figure 7C). Bacterial burdens in the spleen and lungs were enumerated for genome equivalents (GEs) by qPCR and were found to be significantly reduced in WCV- ($p = 0.0117$ and 0.0095, respectively) Sol II + NMI LPS- ($p = 0.0109$ and 0.0174, respectively), and Sol I-immunized mice ($p = 0.0099$ and 0.0006, respectively), compared with sham (Figure 7D).

Lung tissue from all of the mice was excised during necropsy and evaluated for histopathology. Sham immunized mice had only a small number of lymphocytes multifocally surrounding bronchioles and blood vessels. However, the presence of lymphocytes in these areas was much more frequent in WCV-, Sol II + NMI LPS-, or Sol I-immunized mice, indicating BALT hyperplasia. In most sections of sham immunized lung tissue, there were large areas of alveolar septae, especially adjacent to bronchioles,

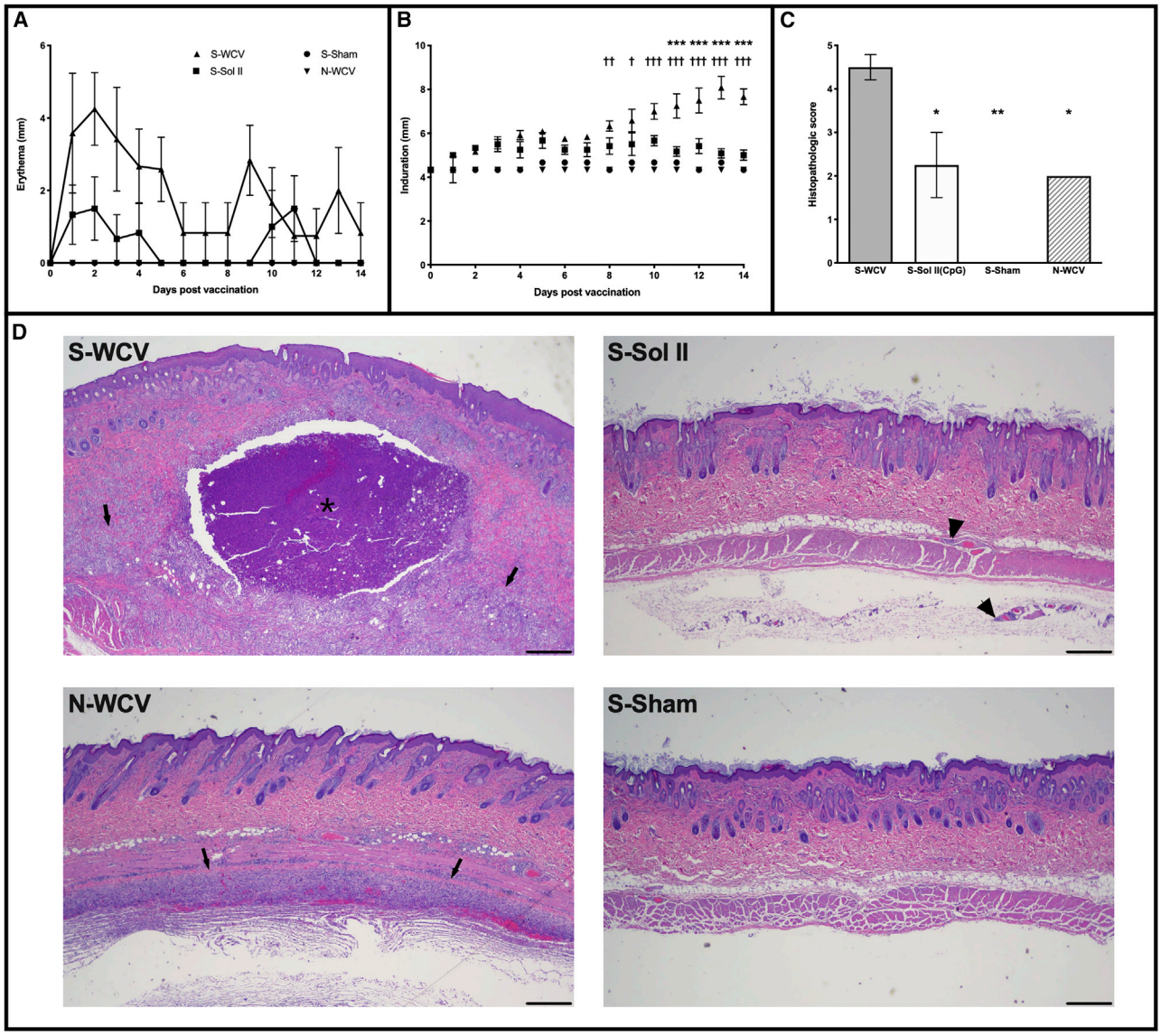

Figure 6. Sol II does not induce hypersensitivity in a previously sensitized guinea pig model

(A) Erythema at the site of vaccine was measured daily, with the largest measurements associated with the S-WCV vaccine site within the first 3 days post immunization.

(B) S-WCV guinea pigs also exhibited significant induration at the vaccine site from day 9 until the end of the study.

(C) Skin tissue at the vaccine site was excised during necropsy and the histopathology evaluated by a veterinary pathologist. The data show marked, diffuse inflammation in S-WCV and a significantly reduced grade for S-Sol II and the controls.

(D) Representative micrographs for all skin sites are presented, showing granulomatous inflammation and Langhans-type giant cells. Arrows indicate granulomatous inflammation with fibrosis (S-WCV, N-WCV), asterisk indicates abscess (S-WCV), arrowheads indicate perivascular inflammation (S-Sol II). Magnification 2×; scale bar, 500 μm.

Data are represented as means ± SEMs. Data for (A) and (B) were analyzed using 2-way ANOVA with Sidak's correction for multiple comparisons. Data for (C) were analyzed using 1-way ANOVA with Dunnett's correction for multiple comparisons. *Significance of statistical difference between S-Sol II and S-WCV, †significance of statistical difference between S-Sham and S-WCV (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

which were moderately expanded by macrophages, lymphocytes, and neutrophils and resulted in emphysema. In Sol II + NMI LPS-immunized mice, septae were only occasionally infiltrated, resulting in moderate emphysema, whereas in WCV- and Sol I-immunized mice, only mild peripheral emphysema was observed. In addition, cellular infiltration resulted in the loss of alveolar spaces and severe or moderate consolidation for sham or Sol II + NMI LPS-immunized mice, respectively. Consolidation in WCV- and Sol I-immunized mice was determined to be mild or not present, respectively (Figure 7E).

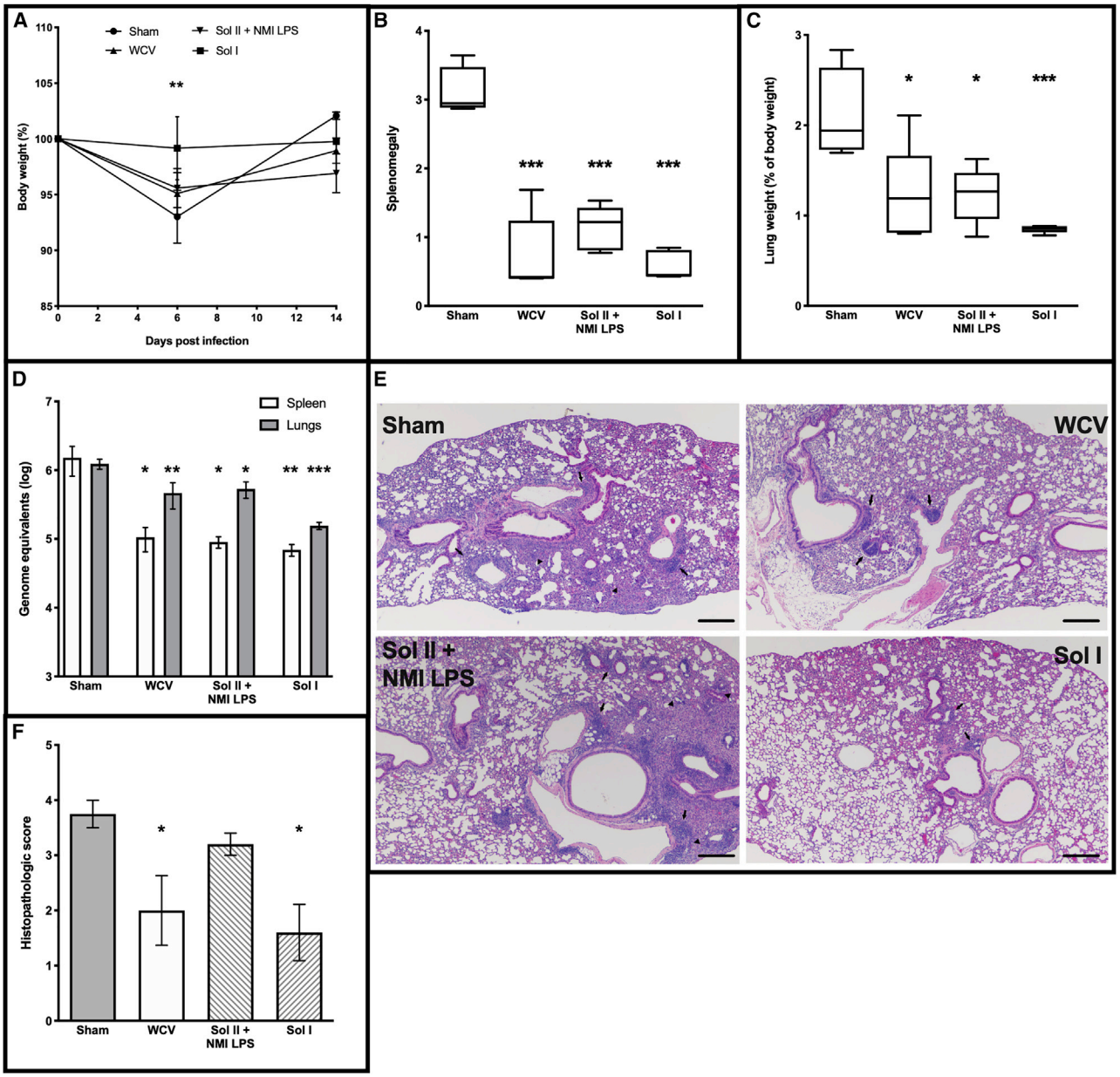

Figure 7. Sol I immunization provides additional protection over Sol II + NMI LPS and WCV against aerosol *C. burnetii* infection in mice

Sham, Sol II + NMI LPS-, and WCV-vaccinated mice demonstrated measurable weight loss 6 days post-infection (dpi) before recovering to initial weight.

(A) Sol I-immunized mice did not demonstrate any weight loss and had a significantly higher body index 6 dpi compared with sham.

(B) Splenomegaly was significantly reduced in all vaccinated groups, compared with sham.

(C) The lungs of sham immunized mice were significantly more enlarged than those observed in the three vaccine groups.

(D) GEs enumerated from infected lungs and spleen were reduced most significantly in mice immunized with Sol I.

(E) Representative micrographs of lungs from each group show varying degrees of moderate interstitial inflammation, multifocal areas of consolidation (arrowheads), and mild lymphoid hyperplasia (arrows). Images were scored for severity of inflammation, which was significantly reduced in mice immunized with WCV or Sol I. H&E stained; magnification 4×; scale bar, 250 μm. Data are represented as means ± SEMs.

Data for (A) were analyzed using 2-way ANOVA with Sidak's correction for multiple comparisons. All other data were analyzed using 1-way ANOVA with Dunnett's correction for multiple comparisons. *Significance of statistical difference relative to sham (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

The severity of pulmonary inflammation was graded for each mouse, with minimal interstitial inflammation scored 1 and severe diffuse inflammation with consolidation scored 5 (Figure 7F). Lung sections in sham mice were scored significantly higher for severity of inflammation than WCV ($p = 0.0467$), or Sol I ($p = 0.0144$). There was no significant difference in the severity of inflammation between sham and Sol II + NMI LPS.

DISCUSSION

Despite international efforts spanning >60 years, a safe and effective vaccine against Q fever for human use remains elusive. In that time, the only licensed vaccine is a formalin-inactivated whole-cell derivative of virulent *C. burnetii* Henzerling, Q-Vax. Early studies into the efficacy of Q-Vax in protecting individuals against Q fever proved hugely successful, with several reports of 100% protection lasting for up to 5 years post-immunization.[53,54] However, mounting concerns over vaccine reactogenicity and high manufacturing costs has limited the widespread use of Q-Vax. In this study, we have developed a Q fever vaccine that does not require BSAT-restricted BSL3 manufacturing, is non-reactogenic, and is comparable to Q-Vax, or a WCV surrogate, in its capacity to elicit protective immunity against an aerosol challenge of *C. burnetii* in three animal models.

Previous studies have shown that several purified proteins from *C. burnetii* can provide significant levels of protection in guinea pigs and mice.[19,20] However, recombinant *C. burnetii* proteins appear to be less effective at protecting against infection, despite inducing measurable antibody responses.[55] This may suggest that key, highly antigenic proteins undergo post-translational modifications upon *C. burnetii* expression that are necessary for eliciting optimal protection. Mice immunized with Sol II demonstrated a strong serological response to antigen, which significantly improved when co-administered with chosen adjuvants. Both CpG and QuilA were found to be strong Th1 polarizing adjuvants, as indicated by the IgG2c skewed response, which has been suggested to be an important correlate of protective immunity against Q fever.[15,50,56]

Further analysis of serological responses to Sol II revealed a dose-dependent correlation with an increasing IgG2c ratio. Increasing the number of doses or administering a higher dose also resulted in the development of high avidity polyclonal serum, indicating affinity maturation. Some studies have shown that vaccines capable of eliciting high-avidity antibody responses have improved functional activity against respiratory pathogens than those inducing low-avidity antibodies.[57,58] Correlative improvements between high-avidity antibodies and vaccine performance may be due to increased levels of opsonophagocytosis or a more direct bactericidal activity.[59–61] However, the role of opsonization in antibody-mediated immunity (AMI) to *C. burnetii* remains unclear, as increased uptake into primary dendritic cells does not appear to have any impact on bacterial growth rate.[62] High-avidity antibodies may also contribute toward neutralization, a mechanism that may have greater significance in protection against *C. burnetii* than is currently appreciated and warrants further investigation.[32,63,64]

One of the most striking clinical features of infection in our NHP model was evidence of acute respiratory distress. Sham immunized NHPs were tachypneic with hypoxemia ~10 days post-infection. Hypoxemia is similarly presented in human clinical cases of Q fever.[65,66] Saturated blood oxygen returned to normal levels by day 14, but the respiratory rate in sham immunized NHPs remained high. A previous study comparing Q fever models of rhesus and cynomolgus macaque described significant liver pathology following infection using serological biomarkers.[67] Blood chemistry was performed for all NHPs at time points throughout the study, but no significant findings between the groups were made. Serum AST levels increased significantly in all infected NHPs 3 days post-infection but returned to within normal levels by day 7. Interestingly, blood urea nitrogen/creatinine (BUN/Cr) levels were elevated in sham and Q-Vax NHPs between days 10 and 14 but remained low in the Sol II group, which can be an early indicator of kidney damage.

Despite Sol II generating a humoral response consisting of high-avidity and high-titer antibodies, we were unable to transfer measurable protection from Sol II- or WCV-derived serum into a naive mouse. This was unexpected given previous studies suggesting a role for AMI to *C. burnetii* and demonstrating passive protection of mice with *C. burnetii* antiserum.[15,62] One possible reason for this discrepancy may be due to technical differences in preparing immune serum. In this study, serum was depleted of complement before passive transfer, whereas in previous studies, this step was not performed, suggesting that complement may play a role in protection. However, in a follow-up study by Zhang et al.,[32] cobra venom factor was used to deplete endogenous C3 before passive transfer of immune serum to demonstrate that the complement pathways are dispensable for *C. burnetii* immunity. We were, however, able to generate measurable protection by adoptively transferring cellular immunity from Sol II- and WCV-immunized mice into a naive recipient, thus highlighting the significance of $CD4^+$ T cell-mediated immunity in controlling *C. burnetii* infection.[68]

One of the major limitations for the widespread use of Q-Vax are the serious adverse events following immunization. Adverse local and systemic events are often reported in individuals, including erythema and tenderness at the injection site as well as headaches, lethargy, and fever.[9] A recent study looking at the frequency of the adverse effects of Q-Vax in young adults reported 98% of participants complaining of local injection site reactions, 30% of which were severe. Adverse systemic events occurred in 60% of Q-Vax recipients, 3.8% of whom required medical assistance.[69] Furthermore, reactogenicity associated with Q-Vax immunization is exacerbated in individuals with pre-existing immunity.[9,70] To mitigate these hypersensitivity responses, individuals must be pre-screened and demonstrate that they are immunologically naive to *C. burnetii* by both skin test and serology. However, exclusionary factions can be extensive, with positivity rates for either skin test or serology anywhere between 5%–22%, varying significantly between urban and rural areas.[69,71] Seroprevalence among communities is a major contributory factor that has limited the widespread use of Q-Vax outside Australia.

The absence of hypersensitivity following Sol II immunization of previously sensitized guinea pigs suggests that the response is likely due to either formaldehyde or insoluble antigens not captured in Sol II but present in Q-Vax. Given the extensive cross-linking and formation of insoluble protein aggregates that arises from formaldehyde treatment, we suggest that this may contribute significantly to the persistence of antigen and the resulting reactogenic nature of Q-Vax.[72] In addition, trichloroacetic acid (TCA)-extracted soluble antigens from NMI have previously been shown to be less reactive than whole-cell preparations.[73] Further experiments are required to understand

the contributory factors of hypersensitivity and the immunological mechanisms that are involved.

A major antigen missing from Sol II formulation is NMI LPS, which plays an indispensable role in *C. burnetii* host immune evasion.[13,74,75] Furthermore, NMI LPS and analogs of the O-antigen sugars present have been shown in numerous publications to elicit protective immunity in murine models.[15,76] Despite this, NMI LPS alone has failed to become a viable *C. burnetii* vaccine due to a limited, and potentially inhibitory, effect on T cell activation.[77,78] Nevertheless, we were interested in the contribution NMI LPS may make to the protection we have already observed from the Sol II vaccine described here.

The increased protection afforded by Sol I over Sol II + NMI LPS and WCV could be due to subtle changes in NMI LPS present in these three vaccine groups. Alterations to the variable O-antigen region may arise from either hot phenol or formalin treatment that could negatively affect immunogenicity.[79] Alternatively, NMI LPS in the Sol I preparation may remain associated with adjacent outer membrane proteins that were otherwise digested by proteinase K in the NMI LPS purification. If so, then glycan-protein complexes are likely to modify the immune response mounted by NMI LPS, including T cell-mediated immunity. Glycoconjugate vaccines have previously been shown to facilitate $CD4^+$ T cell responses to LPS, due to protein carriers' chaperoning of glycans onto major histocompatibility complex (MHC) receptors for T cell presentation.[80] Given the lack of reactogenicity of Sol II and recent publications indicating reactogenicity in the absence of NMI LPS, Sol I is unlikely to be reactogenic.[81]

In summary, we have designed two Q fever vaccines using a detergent extraction method to isolate soluble antigens from virulent NMI and the Select Agent-exempt strain *C. burnetii* NMII. The vaccines described herein, Sol I and Sol II, contain a diverse variety of *C. burnetii* immunoreactive antigens that are expressed during infection in several animal models. Immunologic evaluation of Sol II, in combination with CpG, showed elicitation of high titer IgG and high-avidity polyclonal serum with a polarizing Th1 skew. Furthermore, Sol II induces a protective $CD4^+$ T cell response that can be transferred into a naive mouse. Sol II immunization provides measurable protection in three biologically relevant animal models, including NHPs, against an aerosol challenge of Q fever and obviates reactogenicity in a sensitized guinea pig model. Finally, we demonstrated additional protection afforded by NMI LPS by purifying soluble antigens from NMI. Despite the challenges of purifying Sol I in high containment, we believe that the significant levels of protection presented here warrant further study to elucidate the associated mechanisms of immunity and assessment of reactogenicity.

Limitations of the study

The aim of this study was to identify a Q fever vaccine that is both safe for use in previously sensitized individuals and capable of eliciting equal or greater protection than WCV/Q-Vax. A solubilized extract of the BSAT-exempt NMII strain of *C. burnetii* (Sol II) was shown to elicit robust protection in three animal models, comparable to WCV, and non-reactogenic in a previously sensitized guinea pig model. A secondary extract from virulent *C. burnetii* NMI (Sol I) increased protection further and outperformed WCV protection in a mouse challenge model. Mass spectrometry of Sol II identified an abundance of antigens that have previously been attributed to eliciting protective immunity against Q fever. Despite this, it remains unclear which of these antigens are immunodominant and therefore critical for inclusion in a vaccine against *C. burnetii*. Regarding the animal models we used to demonstrate efficacy, we opted for a sublethal aerosol challenge since it is the most common route of transmission, and ~95% of symptomatic human Q fever cases result in an acute febrile illness. However, chronic disease remains a significant manifestation of Q fever given the severity of prognosis. Our current animal models do not reflect this, nor do they address the protective efficacy of individuals with comorbidity (e.g., cardiac impairment) or alternate routes of transmission. Finally, waning immunity is a concern with any potential vaccine, and the long-term protection afforded by either Sol II or Sol I remains unknown.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2021.100461.

ACKNOWLEDGMENTS

This research was supported by the Defense Threat Reduction Agency, contract no. HDTRA1-14-C-0113. The views expressed in this article are those of the authors and do not reflect the official policy or position of the US Department of Defense or the US. Army. Additional support was provided by the National Institutes of Health Primate Research Center Grant NIH P51OD011104 and Institutional Training Grant T32 fellowship 5 OD 11083-11.

AUTHOR CONTRIBUTIONS

Conceptualization, A.E.G., E.J.V.S., and J.E.S.; methodology, A.E.G., E.J.V.S., and C.M.F.; investigation, A.E.G., E.J.V.S., A.P.F., and K.E.R.-L.; writing, A.E.G.; funding acquisition, J.E.S. and A.P.F.; resources and supervision, J.E.S.

DECLARATION OF INTERESTS

J.E.S., E.J.V.S., and A.E.G. are named inventors of patent US 2019/0083598 A1.

Received: May 5, 2021
Revised: September 27, 2021
Accepted: November 10, 2021
Published: December 6, 2021

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Cbu_1910 monoclonal	Laboratory collection	13C43
Goat anti-Mouse IgG (H+L) Secondary Antibody [HRP]	Novus Biologicals	RRID:AB_524788
Rabbit Anti-Guinea pig IgG H&L (HRP)	Abcam	RRID:AB_955426
Goat Anti-Monkey IgG H&L (HRP)	Abcam	RRID:AB_10866625
IRDye® 800CW Goat anti-Mouse IgG Secondary Antibody	LI-COR Biosciences	RRID:AB_2687825
Bacterial and virus strains		
Coxiella burnetii RSA439	Laboratory collection	N/A
Coxiella burnetii RSA493	Laboratory collection	N/A
Biological samples		
Q-Vax	Stephen Graves (Australian Rickettsial Reference Laboratory)	N/A
WCV	Laboratory collection	N/A
CpG ODN 1826	Invivogen	tlrl-1826-blk
Alhydrogel	Invivogen	vac-alu-250
QuilA	Invivogen	vac-quil
TiterMax Gold	MilliporeSigma	T2684-1ML
Monophosphoryl lipid A	Invivogen	vac-mpla
Incomplete Freund's Adjuvant	MilliporeSigma	F5506
CpG ODN 2007	Invivogen	tlrl-2007-blk
CpG ODN 2006	Invivogen	tlrl-2006-1
Chemicals, peptides, and recombinant proteins		
n-octylglucoside	MilliporeSigma	10634425001
Anzergent 3-14	Anatrace	AZ316 25 GM
sodium lauroyl sarcosinate	MilliporeSigma	1614374
ACCM-2 media	Sunrise Science	4700-300
Experimental models: organisms/strains		
C57BL/6NHsd mouse	Envigo	044
Hartley guinea pig	Charles River	051
Rhesus macaque	Tulane National Primate Research Center	N/A
Oligonucleotides		
C. burnetii qPCR com1 gDNA Forward and Reverse Primer	van Schaik et al., 2017[82]	5′-CGCGTTGTCTTCAAAGAACT-3′ and 5′-GCGTCGTGGAAAGCATAATA-3′
C. burnetii qPCR TaqMan probe	van Schaik et al., 2017[82]	5′FAM-CGGCCAATCGCAATACGCTG-3′TAMRA
Software and algorithms		
Prism 9.0	Graphpad Software Inc.	https://www.graphpad.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, James Samuel (jsamuel@tamu.edu).

Materials availability

This study did not generate new unique reagents or strains.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Female C57BL/6N mice (6-8 weeks old) and female Hartley guinea pigs (350-400 g) were purchased from Envigo and Charles River Laboratories, respectively. Male rhesus macaques (*Macaca mulatta*) of a similar age (> 3 years old) and weighing 3-5 kg were used. Animals were housed in isolator cages under pathogen-free conditions and provided with food and water *ad libitum*.

Animal research ethics statement

All procedures were performed under animal use protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Texas A&M University and Tulane National Primate Research Center to ensure compliance with PHS standards. Experiments were carried out in AAALAC-approved facilities in accordance with university and federal regulations.

METHOD DETAILS

C. burnetii inoculum and vaccine preparation

WCV material was prepared by propagating *C. burnetii* Nine Mile phase I (NMI) RSA493 in ACCM-2 media (Sunrise Science) at 37°C with 5% CO_2 and 2.5% O_2 for 7 days. Cultures were centrifuged at 15,000 x g for 20 min and resuspended in 0.9% NaCl (v/v) and 2% formalin (v/v) for 48 h. Fixed bacteria were washed with PBS three times before resuspending in PBS to an appropriate concentration. Q-Vax (Seqirus, Victoria, Australia) was kindly provided by Dr. Stephen Graves.

Sol II material was prepared by propagating *C. burnetii* Nine Mile phase II (NMII) RSA439 in ACCM-2 media for 14 days. Cultures were centrifuged at 15,000 x g for 20 min and resuspended in a lysis buffer of 25 mM Tris-HCl, 150mM NaCl pH 7.4 containing 1mM DTT, 1 mM EDTA-Na_2, 1 mg/mL lysozyme, 100 units benzonase nuclease (MilliporeSigma), 1 x protease inhibitor cocktail (Takara). Cell suspension was homogenized in an ice-cold sonication water bath for 30 min (30 s on/off). Cell lysate was centrifuged at 15,000 x g for 20 min and the supernatant retained at 4°C. The resulting pellet was resuspended in the lysis buffer to repeat the sonication and centrifugation steps. Next, the pellet was resuspended in a detergent buffer containing 25 mM Tris-HCl, 150mM NaCl pH 7.4, 50 mM n-octylglucoside (MilliporeSigma), 1 x protease inhibitor and rotated overnight at 4°C. Approx. 5 mL detergent buffer was used per gram of wet cell pellet. The lysate was centrifuged at 15,000 x g for 20 min and the supernatant retained at 4°C. Solubilization of the pellet was repeated by replacing the detergent in the detergent buffer with 0.4 mM Anzergent 3-14 (Anatrace) and rotated overnight. The lysate was centrifuged at 15,000 x g for 20 min and the supernatant retained at 4°C. Solubilization of the pellet was repeated by replacing the detergent in the detergent buffer with 30 mM sodium lauroyl sarcosinate (MilliporeSigma) and rotated overnight. The lysate was centrifuged at 15,000 x g for 20 min and the supernatant combined with all those previously collected. Excess detergent was removed by centrifuging solubilized proteins in a 3 kDA MWCO Amicon ultra-15 centrifugal filter (MilliporeSigma). Filter centrifugation was repeated three times before diluting the solubilized protein content to a concentration of 2 mg/mL. Immunoblotting to detect the presence of CBU_1910 (Com1) was determined with a purified monoclonal antibody (13C43). Protein content was calculated using a Detergent Compatible Bradford Assay Kit (Pierce) and the same batch of Sol II was used throughout the study.

Sol I material was prepared similarly to Sol II, using *C. burnetii* NMI. *C. burnetii* RSA493 was grown in 5 mL ACCM-2 media for 10 days before being used to inoculate 1 L ACCM-2 media. Cultures were grown for 14 days prior to cell lysis, which was performed using 0.1 mm glass disruptor beads. Soluble antigens were purified from the lysate by treating with detergents, as previously described. Protein content was determined using a Detergent Compatible Bradford Assay Kit (Pierce).

C. burnetii NMI clone 7 was grown in embryonated yolk sacs and purified by gradient centrifugation, as previously described.[83] Inoculum for animal infections were calculated based on genome equivalents (GE) determined by quantitative PCR (qPCR), as described below. Experiments involving *C. burnetii* NMI were performed in biosafety level 3 (BSL3) or animal biosafety level 3 (ABSL3) facilities at Texas A&M University Health Science Center and Tulane National Primate Research Center (TNPRC).

LPS

LPS was purified from *C. burnetii* NMI using a hot phenol extraction described elsewhere.[84] Briefly, *C. burnetii* NMI was propagated in ACCM-2 media for 10 days before 90% (w/v) aqueous phenol was added and heated to 70°C for 30 min with stirring. The resulting solution was dialyzed against ddH_2O for 5 days and the quality verified by silver stain and immunoblotting against *O*-antigen with a purified monoclonal antibody (H5A) (data not shown). The absence of contaminating proteins was confirmed by SDS-PAGE and immunoblotting against CBU_1910 (Com1) with a purified monoclonal antibody (13C43). A final dose of 5 μg NMI LPS was included in Sol II + NMI LPS formulations.

Immunization

Mice (n = 5/group) were immunized subcutaneously (SC) with 50 μL containing 10 μg (40 in high dose study) of Sol II, Sol I, or WCV in a prime, prime-boost, or prime-boost-boost schedule. Boost immunizations were administered after a two-week rest after the previous dose. Sol II and Sol I formulations were administered with 10 μg CpG ODN 1826 (Invivogen), unless stated otherwise. Adjuvants were administered in combination with Sol II vaccination at the following doses: 0.2% (w/v) Alhydrogel® (Invivogen), 50% (v/v) TiterMax Gold (MilliporeSigma), 10 μg QuilA (Invivogen), 2 μg MPLA (Invivogen), 50% (v/v) Incomplete Freund's Adjuvant (IFA; MilliporeSigma). Mice were rested for an additional seven weeks following final immunization. Blood was collected periodically via submandibular vein on days −3 (pre-prime), 7 (post-prime), 21 (post boost), and 56 (pre-challenge).

Guinea pigs (n = 5/group) were vaccinated SC with 100 μL containing 50 μg of Sol II with 20 μg CpG ODN 2007 (Invivogen) or WCV in a prime-boost schedule. Boost immunizations were administered after a two-week rest after the previous dose. Guinea pigs were rested for an additional seven weeks following final immunization. Blood was collected periodically via lateral saphenous vein on days −3 (pre-prime), 7 (post-prime), 21 (post boost), and 56 (pre-challenge).

Non-human primates (NHPs) were vaccinated SC with 500 μL containing 100 μg of Sol II with 50 μg CpG ODN 2006 (Invivogen) (n = 4/group) or 25 μg Q-Vax (n = 2/group) in a prime-boost schedule. Sham animals (n = 2/group) were also included as a negative control. Boost immunizations were administered after a two-week rest after the previous dose. NHPs were rested for an additional seven weeks following final immunization. Blood was collected periodically on days 0, 3, 7, 10, 14.

Adoptive and passive transfer

Immune sera were collected from vaccinated mice at 35 days post vaccination and pooled in equal amounts from each mouse. Serum was depleted of complement by heat inactivation at 55°C for 10 min. Each recipient naive mouse received 100 μL of pooled sera IP 24 h before challenge. The spleens and draining lymph nodes from the same donor mice were harvested and used for isolation of lymphocytes and $CD4^+$ T cells. Single cell suspensions were prepared by homogenization of spleens, passage through nylon mesh, and lysis of erythrocytes by RBC lysis buffer (BioLegend). $CD4^+$ T cells were purified by an exclusionary $CD4^+$ T cell isolation kit (Miltenyi). The viability of lymphocytes and $CD4^+$ T cells was determined by trypan blue staining and cells were diluted in PBS at a concentration of $2x10^7$ cells/mL. Adoptive transfer was performed by retro-orbital injection of $1x10^6$ lymphocytes or $CD4^+$ T cells in 50 μL to each naive recipient mouse 24 h before challenge.

Animal infection

Mice were anaesthetized by administering ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively) intraperitoneally (IP) prior to intratracheal infection with 1×10^6 GE *C. burnetii* NMI via a large-particle aerosol (LPA) device, as previously described.[85] Mice were monitored daily for clinical signs of disease and weighed every two days for fourteen days at which point they were euthanized. At necropsy, spleen and lungs were collected to determine splenomegaly and bacterial burden.

Guinea pigs were anesthetized with ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively) given IP prior to intratracheal infection with $1x10^4$ GE *C. burnetii* NMI via LPA, as previously described.[85] Guinea pigs were monitored daily for clinical signs of disease using a modified Karnofsky scale of disease severity (behavior/appearance, respiratory rate, weight loss) and rectal temperatures were measured.[85] At necropsy, spleen and lungs were collected for histological analysis and to determine splenomegaly and bacterial burden.

Rhesus macaques were implanted with biotelemetry devices and monitored for changes in respiratory rate, temperature, heart rate and capillary oxygen saturation for 1 week prior to inoculation with $1x10^5$ GE *C. burnetii* NMI via intratracheal LPA route. Sham-vaccinated animals were also included in each study. All animals were euthanized two weeks post infection, at which point spleens and lung tissues collected at necropsy for evaluation.

Reactogenicity

Hairless guinea pigs (n = 5/group) were challenged with $1x10^4$ GE *C. burnetii* NMI via intratracheal LPA and monitored daily for two weeks until the infection resolved. Guinea pigs were rested for an additional five weeks and then vaccinated SC with 50 μg WCV, 50 μg Sol II or PBS and monitored daily for induration and erythema at the injection site, axillary lymphadenopathy, body temperature and weight loss. Blood was collected via lateral saphenous vein on days 0, 7, and 14 post vaccination. The study was terminated 14 days post vaccination at which point skin sites, lungs, and heart were taken for histological analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

ELISA

96 well microtiter plates (Fisher Scientific; Pittsburgh, USA) were coated overnight at 4°C with 100 μl of 5 μg/ml WCV or Sol II. Plates were blocked with 200 μl of 3% (w/v) milk in PBS and incubated for 2 h at 37°C. Serum from individual animals was added in duplicates at a 1:100 dilution in 1% (w/v) skimmed milk with PBS, followed by a 2-fold serial dilution and incubated for 1 h at 37°C. Plates were washed with PBS plus 0.05% (v/v) Tween-20 (PBS-T) and sera was detected using a goat anti-mouse IgG (H+L)- HRP conjugate (Bio-rad) diluted 1:10,000 in 1% (w/v) skimmed milk at 37°C for 1 h. TMB substrate (EMD Millipore) was added, and the absorbance measured at 450 nm using a Spectra Max M2 plate reader (Molecular Devices). Serum endpoint titers were defined as the maximum

dilution to give an absorbance reading higher than the optical density at 450 nm of pre-immune/control serum plus three times the standard deviation.

Antibody avidity was measured using urea as the chaotropic agent in a modification of the ELISA-based method described elsewhere.[52] Briefly, 96 well microtiter plates (Fisher Scientific; Pittsburgh, USA) were coated overnight at 4°C with 100 μl of 5 μg/ml WCV or Sol II. Plates were blocked with 200 μl of 3% skimmed milk and incubated for 2 h at 37°C. Serum from individual animals was added in quadruplicate at a 1:100 dilution in 1% (w/v) skimmed milk, followed by a 3-fold serial dilution and incubated for 1 h at 37°C. After three washes with PBS-T, 100 μL 4 M urea were added to one-half of the serial diluted sera and PBS added to the other one-half plate. After a 15-min incubation at room temperature, plates were washed and sera was detected using a goat anti-mouse IgG (H+L)- HRP conjugate (Bio-rad) diluted 1:10,000 in 1% (w/v) skimmed milk at 37°C for 1 h. TMB substrate (EMD Millipore) was added, and the absorbance measured at 450 nm using a Spectra Max M2 plate reader (Molecular Devices). Avidity Index (AI) was determined by calculating the area under the antibody titration curve, defined by the following equation:

$$AI = \frac{A_U}{A_C}$$

Where A_U is the area under the urea-washed curve, and A_C is the area under the control curve.

Quantification of *C. burnetii* DNA

Spleens and lungs were collected from mice and guinea pigs fourteen days after challenge to measure *C. burnetii* gDNA using qPCR. Tissues were homogenized in PBS and then added to a tissue lysis buffer (Roche) containing proteinase K and incubated at 55°C overnight. The following day, 1% SDS (v/v) was added to all samples followed by one-hour incubation at room temperature. Samples were then processed using High Pure PCR Template Preparation Kit (Roche) according to the manufacture's recommendations. Purified genomic DNA was used in a TaqMan quantitative PCR with a standard curve generated from *C. burnetii* genomic DNA, com1 specific primers and probe (com1_L1: CGCGTTGTCTTCAAAGAACT, com1_R1: GCGTCGTGGAAAGCATAATA and 5′FAM-CGGCCAATCGCAATACGCTG-3′TAMRA).

Histopathology

Extracted tissues were fixed in 10% neutral buffered formalin for 72 h at room temperature and processed by AML Laboratories (Saint Augustine, USA). Fixed tissues were embedded in paraffin and 5 μm sections were taken prior to staining with hematoxylin and eosin. A board-certified pathologist evaluated samples in a blind fashion for severity of inflammation. Skin sites excised from guinea pigs for evidence of hypersensitivity in Figure 6 were graded as follows: 0, No significant lesions; 1, Minimal inflammation; 2, Mild multifocal inflammation; 3, Mild diffuse inflammation; 4, Moderate diffuse inflammation; 5, Marked diffuse inflammation. Lung tissue excised from mice for evidence of disease severity in Figure 7 were graded as follows: 0, No significant lesions; 1, Minimal interstitial inflammation; 2, Mild interstitial inflammation; 3, Moderate interstitial inflammation; 4, Severe multifocal inflammation with consolidation; 5, Severe diffuse inflammation with consolidation.

Statistics

Statistical analyses were performed using Prism 9.0 (Graphpad Software Inc.). Results were compared using one-way or two-way ANOVA with Dunnett's or Sidak's correction for multiple comparisons, respectively. Differences were considered significant if p value was ≤ 0.05, (*) ≤ 0.01 (**), or ≤ 0.001 (***).