



Eosinophils Affect Antibody Isotype Switching and May Partially Contribute to Early Vaccine-Induced Immunity against *Coxiella burnetii*

Lindsey Ledbetter,^a Rama Cherla,^a Catherine Chambers,^a Yan Zhang,^a Guoquan Zhang^a

^aDepartment of Veterinary Pathobiology, University of Missouri, Columbia, Missouri, USA

ABSTRACT *Coxiella burnetii* is an obligate intracellular Gram-negative bacterium which causes human Q fever. An acidified citrate cysteine medium (ACCM-2) has been developed which mimics the intracellular replicative niche of *C. burnetii* and allows axenic growth of the bacteria. To determine if *C. burnetii* cultured in ACCM-2 retains immunogenicity, we compared the protective efficacies of formalin-inactivated *C. burnetii* Nine Mile phase I (PIV) and phase II (PIIV) vaccines derived from axenic culture 7, 14, and 28 days postvaccination. PIV conferred significant protection against virulent *C. burnetii* as early as 7 days postvaccination, which suggests that ACCM-2-derived PIV retains immunogenicity and protectivity. We analyzed the cellular immune response in spleens from PIV- and PIIV-vaccinated mice by flow cytometry at 7 and 14 days postvaccination and found significantly more granulocytes in PIV-vaccinated mice than in PIIV-vaccinated mice. Interestingly, we found these infiltrating granulocytes to be SSC^{high} CD11b⁺ CD125⁺ Siglec-F⁺ (where SSC^{high} indicates a high side scatter phenotype) eosinophils. There was no change in the number of eosinophils in PIV-vaccinated CD4-deficient mice compared to the level in controls, which suggests that eosinophil accumulation is CD4⁺ T cell dependent. To evaluate the importance of eosinophils in PIV-mediated protection, we vaccinated and challenged eosinophil-deficient Δ dblGATA mice. Δ dblGATA mice had significantly worse disease than their wild-type counterparts when challenged 7 days postvaccination, while no significant difference was seen at 28 days postvaccination. Nevertheless, Δ dblGATA mice had elevated serum IgM with decreased IgG1 and IgG2a whether mice were challenged at 7 or 28 days postvaccination. These results suggest that eosinophils may play a role in early vaccine protection against *C. burnetii* and contribute to antibody isotype switching.

KEYWORDS ACCM-derived phase I vaccine, CD4⁺ T cells, *Coxiella burnetii*, antibody isotype switching, eosinophils, granulocytes

Coxiella burnetii is an obligate intracellular Gram-negative bacterial pathogen and the causative agent of human Q fever. This disease manifests acutely as a flu-like illness although it can escalate to a chronic and often fatal disease. Chronic Q fever commonly presents as endocarditis (1–4) and occurs in <5% of acutely infected patients. Among those who develop chronic disease, fatality is observed in 25 to 60% of patients when the disease is left untreated (5). Long-term (\geq 18 months) administration of doxycycline and hydroxychloroquine is the preferred treatment (2, 6, 7). However, even with the recommended antibiotic regimen, one in three Q fever patients continues to experience diminished health 2 years postdiagnosis (4, 8, 9). This globally distributed pathogen is spread to humans via aerosols from infected ruminants (1, 2) and therefore serves as an occupational hazard for individuals working closely with livestock (10–12). The highly infectious nature of *C. burnetii* (13–15), coupled with its

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Address correspondence to Guoquan Zhang, zhangguo@missouri.edu.

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prolonged environmental stability (14) and ease of dissemination (16, 17), makes it an important zoonotic pathogen. *C. burnetii* is an NIH category B priority pathogen because it serves as a threat to our national security, with potential uses in bioterrorism (18). A recent outbreak in the Netherlands highlights the relevance of this disease to human health, with more than 4,000 human cases reported (19, 20). Considering the threat of chronic manifestations and the failure of antibiotic therapies, creation of a safe and effective vaccine remains an important public health and national biosecurity goal.

C. burnetii undergoes antigenic phase variation upon serial passage in eggs, tissue culture, or synthetic medium. During this process, virulent phase I organisms lose the O antigen and outer core regions of their lipopolysaccharide (LPS) and become avirulent phase II organisms (1, 21, 22). Phase I organisms are able to replicate in immunocompetent animals and cause disease, while phase II organisms are rapidly cleared and do not cause disease (13).

A formalin-inactivated whole-cell vaccine produced from the Henzerling strain in phase I (Q-VAX) has been shown to elicit long-lasting protection in animal models and human vaccinees (10, 23–25). Despite its high protective efficacy, Q-VAX is not approved for use in the United States due to adverse reactions, especially in previously sensitized individuals (10, 23, 26–29). Safe use of this vaccine requires multiple screening procedures, which precludes a mass vaccination program. Understanding what is needed to confer protection with minimal side effects is essential to developing an intervention that is both safe and effective.

Previous work suggests that both humoral and cell-mediated immunity are involved in vaccine protection against *C. burnetii* (25, 30–33); however, the contribution of innate immunity remains unknown. The innate immune response stimulates adaptive immunity and tailors adaptive responses to different types of microbes. As such, the innate immune system is a useful tool which can be manipulated to enhance vaccine protection. In fact, the use of Toll-like receptor agonists as immunoadjuvants has proved effective when they are incorporated into vaccines against multiple pathogens (34–39).

Here, we use the recently developed host cell-free culture system (40) and assess the immunogenicity of formalin-inactivated *C. burnetii* Nine Mile phase I (PIV) and phase II (PIIV) vaccines. The data indicate that PIV elicits protection superior to that of PIIV at all time points examined. Furthermore, using flow cytometry to examine the cellular immune response, we find that PIV and PIIV differ in the accumulation of eosinophils in the spleen. This accumulation appears to be CD4⁺ T cell dependent as PIV-vaccinated CD4-deficient mice do not have elevated eosinophils in their spleens. Increased splenomegaly and bacterial burden in PIV-vaccinated eosinophil-deficient Δ dblGATA mice compared to levels in wild-type (WT) mice challenged at 7 days postvaccination (dpv) suggests a partial role for eosinophils in early vaccine protection. Additionally, elevated serum IgM coupled with decreased IgG1 and IgG2a subclass antibodies in Δ dblGATA mice suggests a role for these cells in antibody isotype switching. These findings highlight the utility of the axenic culture system for the manufacture of *C. burnetii* vaccines and provide new insights into the contribution of innate immunity to vaccine protection against *C. burnetii*, which can be applied to Q fever vaccine development.

RESULTS

Host cell-free *C. burnetii* retains immunogenicity and protectivity. Q-VAX is produced in embryonated eggs (41), and while this allows for the generation of large quantities of material at a relatively low cost, residual egg proteins often remain in the final vaccine preparation and contribute to the high incidence of vaccine reactions (42). An acidified citrate cysteine medium (ACCM-2) has been developed which mimics the intracellular replicative niche of *C. burnetii* and allows axenic growth of the bacteria. This medium removes the potential for host contaminants, while still allowing the generation of large quantities of material in a short time (~3 logs of growth in 6 days) (43). Previous reports have shown that *C. burnetii* strains grown in ACCM-2 as well as a

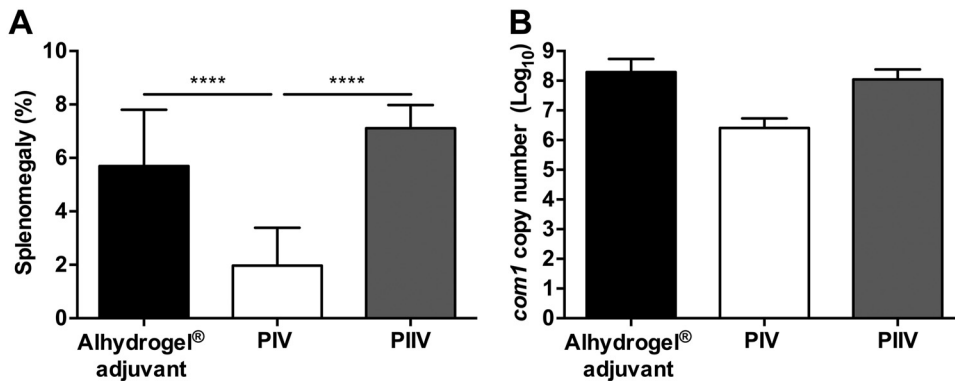


FIG 1 Host cell-free *C. burnetii* retains immunogenicity and protectivity. BALB/c mice were vaccinated subcutaneously (s.c.) with 10 μ g of formalin-inactivated *C. burnetii* Nine Mile phase I (PIV) or phase II (PIIV) vaccine and challenged via intraperitoneal (i.p.) route with 5×10^7 *C. burnetii* NMI bacteria at 28 days postvaccination (dpv). Mice receiving Alhydrogel adjuvant alone served as unvaccinated controls. Splenomegaly (A) and bacterial burden in the spleen (B) were evaluated at 14 days postinfection (dpi) to compare protective efficacies. Results are expressed as percent splenomegaly [(spleen weight/body weight) \times 100]. Bacterial burden was determined by real-time quantitative PCR (qPCR) and is expressed as log₁₀ *C. burnetii* com1 gene copy numbers. Each experimental group includes four mice, with error bars representing the standard deviations from the means. ****, $P < 0.0001$, as determined by one-way ANOVA with Tukey's multiple-comparison test.

newer formulation, ACCM-D, retain *in vivo* infectivity in mouse and guinea pig models (43, 44). To determine if ACCM-2-derived *C. burnetii* retains immunogenicity, we vaccinated BALB/c mice subcutaneously (s.c.) with 10 μ g of PIV or PIIV with aluminum hydroxide adjuvant (Alhydrogel) and challenged mice intraperitoneally (i.p.) with 5×10^7 *C. burnetii* Nine Mile phase I (NMI) bacteria 28 days later. An aluminum hydroxide adjuvant was selected for these vaccination studies due to its broadly accepted use in FDA-approved vaccines for humans (45). To assess disease severity, we evaluated splenomegaly and bacterial burden in the spleen at 14 days postinfection (dpi). Similar to previous reports of vaccines generated from chicken eggs or cell culture (25), we found that PIV grown in ACCM-2 significantly reduces splenomegaly compared to results with PIIV and adjuvant controls (Fig. 1A). Additionally, we found that bacterial burden in the spleens of PIV-vaccinated animals was reduced compared to that with PIIV and adjuvant controls (Fig. 1B). These results indicate that axenically generated PIV retains immunogenicity and protectivity.

PIV protects BALB/c mice as early as 1 week postvaccination. While it has been established that PIV is protective at 28 dpv, it remains unknown how early mice are protected from developing clinical disease following vaccination. To test this, we vaccinated BALB/c mice as described previously and then challenged mice at 7 or 14 dpv with 1×10^7 *C. burnetii* NMI bacteria by i.p. injection. Due to the high degree of splenomegaly (~8%) observed with a challenge dose of 5×10^7 bacteria (Fig. 1A), we lowered the challenge dose to 1×10^7 bacteria for all subsequent infections. PIV had significantly reduced splenomegaly compared to that with PIIV and adjuvant controls when challenged at 7 or 14 dpv (Fig. 2A). Additionally, mice vaccinated with PIV had a reduction in splenic bacterial burden at all time points examined (Fig. 2B). Taken together, these data suggest that immunity develops as early as 1 week postvaccination.

PIV significantly increases SSC^{high} granulocytes in the spleen. PIV significantly reduces splenomegaly and bacterial burden in BALB/c mice challenged with virulent *C. burnetii* NMI. However, PIIV fails to protect mice from clinical disease. The immunological differences between PIV and PIIV that lead to such disparate outcomes remain unknown. Considering the early time point at which this difference becomes apparent (~7 dpv), we hypothesized that innate cells may be differentially recruited to the spleen following vaccination. To test this hypothesis, we used flow cytometry to analyze cellular recruitment to the spleens of adjuvant control and PIV- or PIIV-

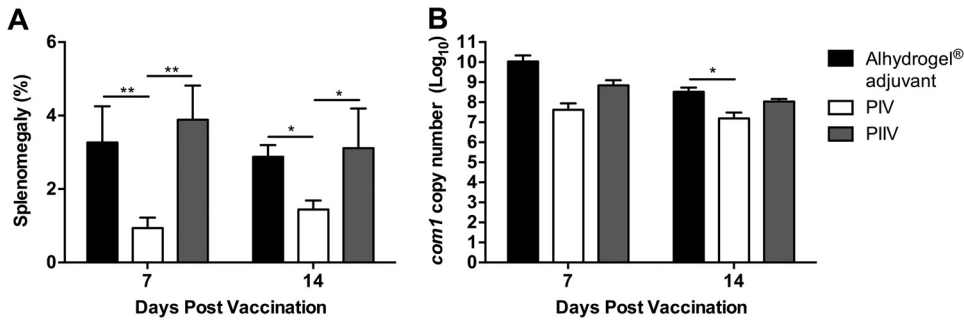


FIG 2 PIV protects BALB/c mice as early as 1 week postvaccination. BALB/c mice were vaccinated s.c. with 10 μ g of PIV or PIIV and challenged via the i.p. route with 1×10^7 *C. burnetii* NMI bacteria at 7 or 14 dpv. Mice receiving Alhydrogel adjuvant alone served as unvaccinated controls. Splenomegaly (A) and bacterial burden in the spleen (B) were evaluated at 14 dpi to compare protective efficacies. Results are expressed as percent splenomegaly [(spleen weight/body weight) \times 100]. Bacterial burden was determined by real-time quantitative PCR (qPCR) and is expressed as log₁₀ *C. burnetii* com1 gene copy numbers. Each experimental group includes four mice, with error bars representing the standard deviations from the means. *, $P < 0.05$; **, $P < 0.01$, as determined by one-way ANOVA with Tukey’s multiple-comparison test.

vaccinated BALB/c mice at 7 and 14 dpv. Naïve animals were used to establish baseline values for all populations examined. Figure 3A details the gating strategy used for the initial analysis. Cellular debris and doublets were removed before gating on lymphocytes, monocytes, and granulocytes based on their forward (FSC) and side scatter (SSC)

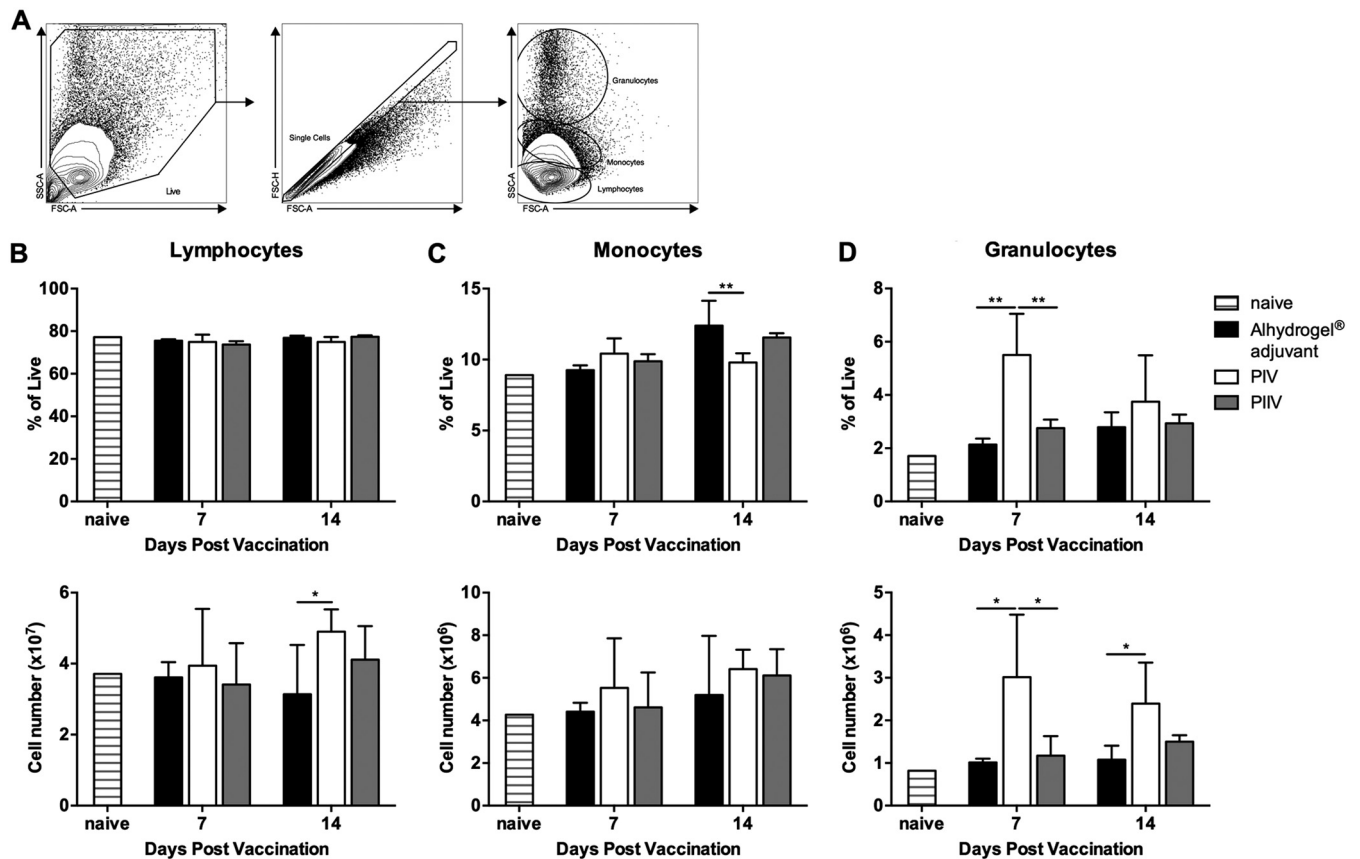


FIG 3 PIV significantly increases high side scatter granulocytes in the spleen. BALB/c mice were vaccinated s.c. with 10 μ g of PIV or PIIV, and spleens were processed for flow cytometry as described in Materials and Methods. Mice receiving Alhydrogel adjuvant alone served as unvaccinated controls. Naïve mice were used to establish baseline values. (A) Gating strategy. Forward (FSC) and side scatter (SSC) were used to identify lymphocytes, monocytes, and granulocytes as well as to remove cell debris from the analysis. (B to D) Population frequencies and absolute cell counts were determined at 7 and 14 dpv. Each experimental group includes 4 to 6 mice, with error bars representing the standard deviations from the means. *, $P < 0.05$; **, $P < 0.01$, as determined by one-way ANOVA with Tukey’s multiple-comparison test.

characteristics. The frequency of lymphocytes was not significantly altered at either time point though there was a significant increase in the number of lymphocytes in PIV-vaccinated mice compared to levels in the adjuvant controls at 14 dpv (Fig. 3B). While there were significant changes in the frequency of monocytes in PIV-vaccinated mice compared to levels in the PIIV-vaccinated and adjuvant control mice at 14 dpv, there were no significant differences in the numbers of monocytes at either time point (Fig. 3C). However, there was a significant increase in the frequency of granulocytes with a high SSC (SSC^{high}) phenotype in PIV-vaccinated mice compared to the levels in PIIV-vaccinated and adjuvant control mice at 7 dpv. Additionally, the total number of granulocytes in the spleen was significantly higher in PIV-vaccinated mice than in the PIIV-vaccinated and adjuvant control mice (Fig. 3D). Overall, these data demonstrate that PIV elicits granulocyte accumulation in the spleen early in the vaccine response.

SSC^{high} granulocytes express CD11b and the interleukin-5 receptor. Following the observation that PIV elicits significant accumulation of granulocytes in the spleen, we further analyzed this population to determine which granulocytic cells were responding. Figure 4A details the gating strategy used for the analysis. Cellular debris and doublets were removed as previously described. PIV-vaccinated animals had a significantly higher frequency of SSC^{high} cells that expressed CD11b than PIIV-vaccinated or adjuvant control mice. Furthermore, the total number of SSC^{high} CD11b⁺ cells in the spleens of PIV-vaccinated animals was significantly higher than that in PIIV-vaccinated animals or adjuvant controls (Fig. 4B).

Granulocytic cells expressing CD11b include neutrophils and eosinophils. To distinguish these two populations, we next looked at interleukin-5 receptor alpha subunit (IL-5R α , CD125) expression. CD125 is expressed on eosinophils and absent from neutrophils (46). Interestingly, PIV-vaccinated mice had significantly more CD125⁺ cells than PIIV-vaccinated animals or adjuvant controls (Fig. 4C). These results suggest that the granulocyte population recruited to the spleens of PIV-vaccinated mice may be eosinophils.

SSC^{high} CD11b⁺ CD125⁺ cells are Siglec-F⁺ eosinophils. While CD125 is most commonly expressed on eosinophils, there are subsets of B1 cells which also express CD125 (47). To determine the phenotype of these cells, we evaluated their expression of Siglec-F, an eosinophil-specific marker, as well as CD19/B220 (Fig. 5A). PIV-vaccinated mice had significantly more Siglec-F⁺ positive cells at 7 dpv than PIIV-vaccinated animals and adjuvant controls (Fig. 5B). Less than 0.1% of the SSC^{high} CD11b⁺ CD125⁺ cells were CD19/B220⁺ (Fig. 5C). Taken together, these data indicate that PIV and PIIV differ in the early recruitment of eosinophils to the spleen.

Eosinophil accumulation in PIV-vaccinated mice is CD4⁺ T cell dependent. CD4⁺ T cells are potent producers of IL-5, which promotes eosinophil differentiation, maturation, migration, and activation (48, 49). Therefore, to determine if eosinophil accumulation in PIV-vaccinated spleens is dependent on signals from CD4⁺ T cells, we vaccinated CD4-deficient mice with 10 μ g of PIV and assessed eosinophil numbers 7 days later. Interestingly, we observed a defect in eosinophil accumulation in PIV-vaccinated CD4-deficient mice compared to the level in PIV-vaccinated WT mice (Fig. 6A). There was no difference in the numbers of eosinophils between naive WT and CD4-deficient mice, which indicates that this phenomenon was not due to an inherent defect in CD4-deficient mice. Overall, these data suggest that eosinophil accumulation in PIV-vaccinated mice is CD4⁺ T cell dependent.

Next, we assessed whether the loss of eosinophils in CD4-deficient mice had any effect on the production of gamma interferon (IFN- γ), an essential cytokine for *C. burnetii* clearance (50). To test this, we isolated total splenocytes from naive, adjuvant-treated, and PIV-vaccinated WT and CD4-deficient mice for *ex vivo* restimulation as described previously (51). IFN- γ levels were measured by enzyme-linked immunosorbent assay (ELISA) 3 and 6 days postrestimulation with either medium alone or 1×10^7 *C. burnetii* NMI bacteria. Splenocytes from PIV-vaccinated WT mice produced significant levels of IFN- γ in response to *C. burnetii* NMI at all time points (Fig. 6B). However, while

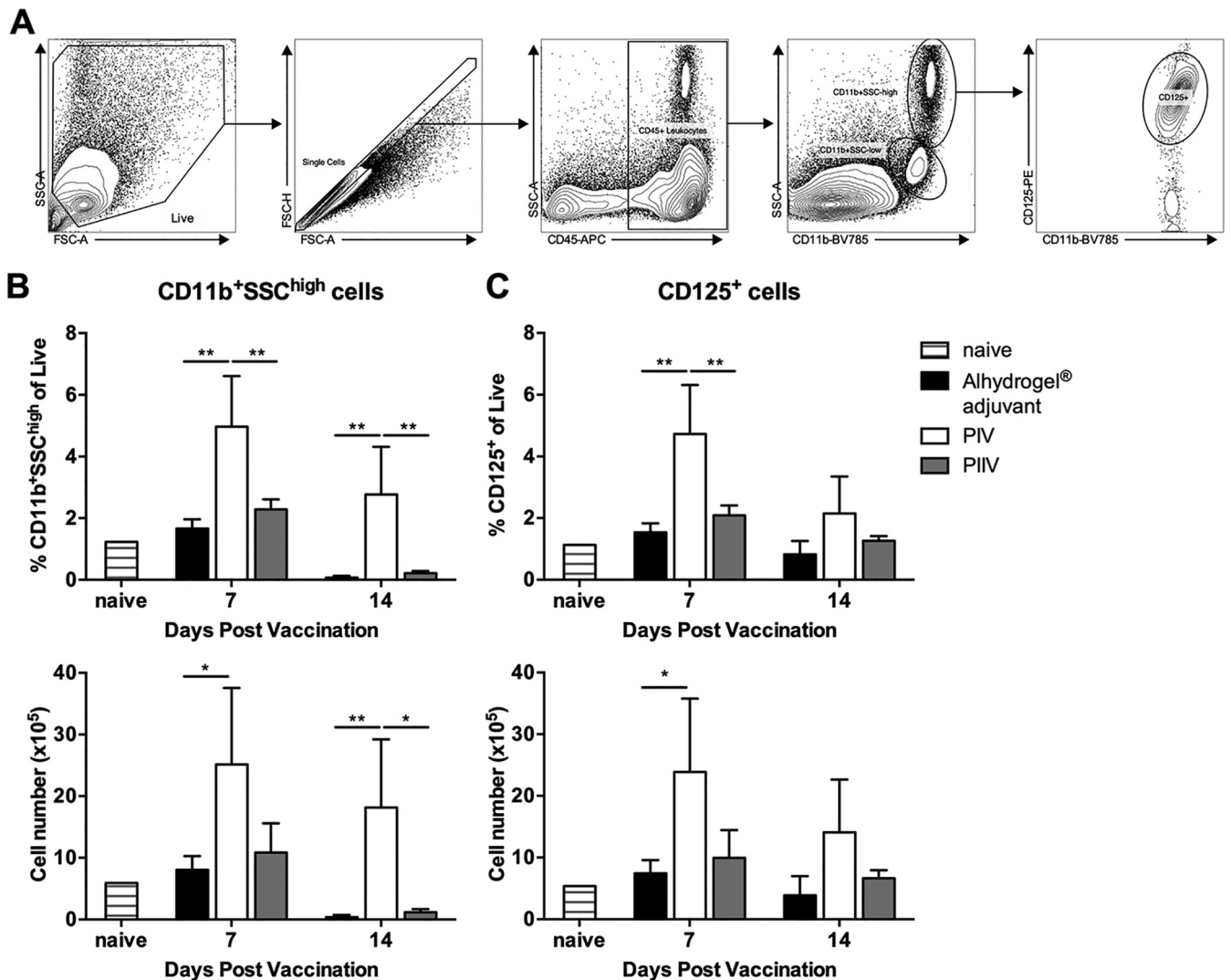


FIG 4 SSC^{high} granulocytes express CD11b and the interleukin-5 receptor. BALB/c mice were vaccinated s.c. with 10 μ g of PIV or PIIV, and spleens were processed for flow cytometry as previously described. Mice receiving Alhydrogel[®] adjuvant alone served as unvaccinated controls. Naïve mice were used to establish baseline values. (A) Gating strategy. FSC and SSC were used to remove cell debris from the analysis. CD45⁺ leukocytes were then identified and further subgated based on SSC and expression of CD11b. CD11b⁺ SSC^{high} granulocytes were then further subgated based on expression of the interleukin-5 receptor alpha subunit (IL-5R α , CD125). (B and C) Population frequencies and absolute cell counts were determined at 7 and 14 dpv. Each experimental group includes 4 to 6 mice, with error bars representing the standard deviation from the mean. *, $P < 0.05$; **, $P < 0.01$, as determined by one-way ANOVA with Tukey's multiple-comparison test.

PIV-vaccinated CD4-deficient mice were still able to respond to *C. burnetii* NMI, there was a significant defect in their production of IFN- γ at both 3 and 6 days poststimulation compared to the level in PIV-vaccinated WT mice. These data suggest a CD4-dependent mechanism of IFN- γ production which could be partially related to the absence of significant eosinophil accumulation.

Eosinophils may partially contribute to early PIV protection. Following the observation that eosinophils are recruited to the spleens of PIV-vaccinated mice, we evaluated whether these cells were important for vaccine-mediated protection. To test this, we vaccinated eosinophil-deficient Δ dblGATA mice with 10 μ g of PIV and challenged them i.p. with 1×10^7 *C. burnetii* NMI bacteria 7 days later. Disease severity was evaluated by comparing body weight loss, splenomegaly, and bacterial burden at 14 dpi. Compared to WT PIV-vaccinated mice, Δ dblGATA mice displayed no significant loss in body weight throughout infection (Fig. 7A). However, Δ dblGATA mice had significantly higher splenomegaly and bacterial burden than their WT PIV-vaccinated coun-

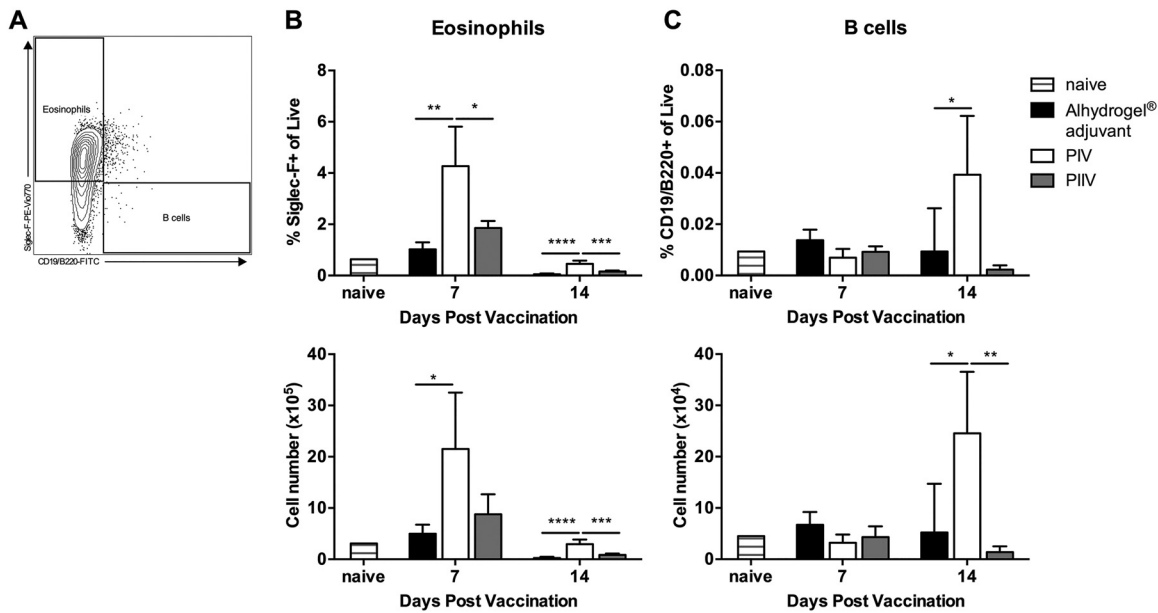


FIG 5 $SSC^{\text{high}} CD11b^+ CD125^+$ cells are Siglec-F⁺ eosinophils. BALB/c mice were vaccinated s.c. with 10 μg of PIV or PIIV, and spleens were processed for flow cytometry. Mice receiving Alhydrogel adjuvant alone served as unvaccinated controls. Naive mice were used to establish baseline values. (A) Gating strategy. $SSC^{\text{high}} CD11b^+ CD125^+$ cells were examined for expression of Siglec-F (eosinophils) and CD19/B220 (B cells). (B and C) Population frequencies and absolute cell counts were determined at 7 and 14 dpv. Each experimental group includes 4 to 6 mice, with error bars representing the standard deviations from the means. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, as determined by one-way ANOVA with Tukey's multiple-comparison test.

terparts (Fig. 7B and C). These data indicate that eosinophils may partially contribute to the early vaccine response against *C. burnetii* infection.

Eosinophils are known to play a role in priming the early IgM response as well as eliciting Th2 cells through the secretion of cytokines (49). In order to evaluate the effect of eosinophil deficiency on antibody-mediated immunity, we measured *C. burnetii* NMI-specific IgM and IgG by ELISA. At 7 dpv, there was significantly more NMI-specific IgM in serum from $\Delta\text{dblGATA}$ mice than in that from their WT counterparts (Fig. 7D). At this time point, however, there was no significant difference in IgG levels.

To evaluate the secondary response, we challenged $\Delta\text{dblGATA}$ mice at 7 dpv and again measured NMI-specific IgM and IgG by ELISA. At 14 dpi, there was a significant increase in the serum concentration of NMI-specific IgM in $\Delta\text{dblGATA}$ mice (Fig. 7E) compared to that in the WT. Again, no significant difference in IgG levels was measured between PIV-vaccinated cohorts. However, while there was not a significant difference in total IgG, there were significant decreases in IgG subclasses associated with eosinophil deficiency. Specifically, there was significantly less NMI-specific IgG1 and IgG2a in $\Delta\text{dblGATA}$ mice than in their WT counterparts (Fig. 7E). Collectively, these data suggest that eosinophils may partially contribute to the early vaccine response as well as antibody isotype switching.

Eosinophils contribute to antibody isotype switching in late PIV responses.

Next, we examined whether eosinophils also contribute to the late PIV response. WT and $\Delta\text{dblGATA}$ mice were vaccinated as previously described and challenged i.p. with 1×10^7 *C. burnetii* NMI bacteria 28 days later. Body weight loss, splenomegaly, and bacterial burden were examined to assess disease severity at 14 dpi. No body weight loss was observed in the $\Delta\text{dblGATA}$ cohort compared to WT (Fig. 8A). Additionally, there was no significant difference in levels of splenomegaly or bacterial burdens between PIV-vaccinated WT and $\Delta\text{dblGATA}$ mice (Fig. 8B and C). These results suggest that eosinophils may not play a role in later protection.

To determine if eosinophils play a role in the IFN- γ response, we measured IFN- γ in serum from PIV-vaccinated WT and $\Delta\text{dblGATA}$ mice by ELISA. Interestingly, there was significantly less IFN- γ present in $\Delta\text{dblGATA}$ mice at 14 dpi than in their WT counter-

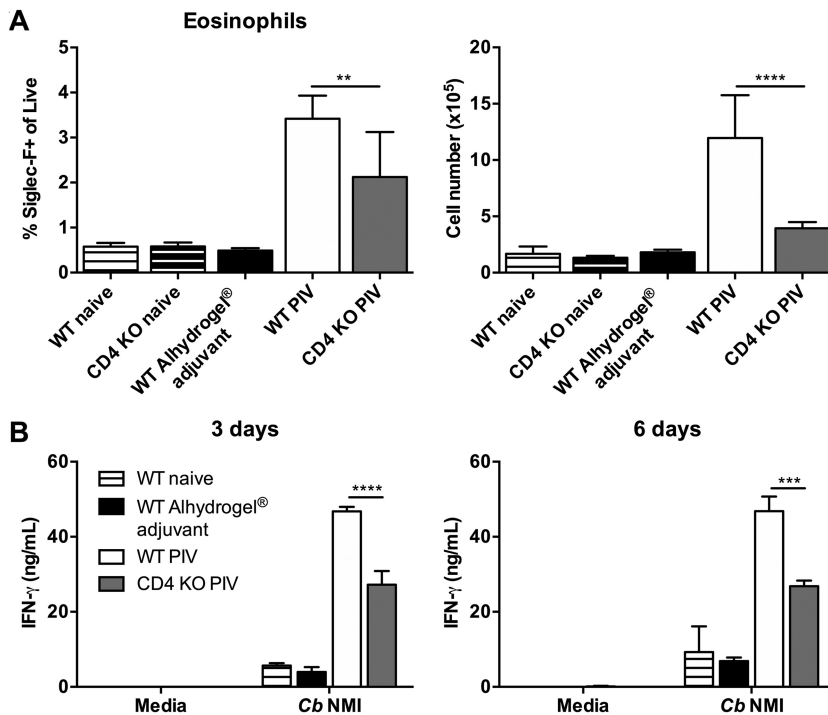


FIG 6 Eosinophil accumulation in PIV-vaccinated mice is CD4⁺ T cell dependent. WT C57BL/6 and CD4-deficient mice were vaccinated s.c. with 10 μ g of PIV, and 7 days later spleens were harvested for flow cytometry or *ex vivo* restimulation studies. Mice receiving Alhydrogel adjuvant alone served as unvaccinated controls. Naïve mice were used to establish baseline values. (A) SSC^{high} CD11b⁺ CD125⁺ Siglec-F⁺ eosinophils were enumerated using the gating strategy described in the legends for Fig. 4 and 5. (B) Total splenocytes were isolated and restimulated with medium or with 1×10^7 *C. burnetii* NMI bacteria for 3 and 6 days, and cell culture supernatants were evaluated for IFN- γ production by cytokine-specific ELISA. Each experimental group includes 5 mice, with error bars representing the standard deviations from the means. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, as determined by one-way ANOVA with Tukey's multiple-comparison test. Cb, *C. burnetii*; KO, knockout.

parts (Fig. 8D). This suggests that eosinophils may contribute to the cytokine response following *C. burnetii* infection.

When evaluating the primary antibody response to vaccination, we found that at the time of challenge there was no significant difference in total serum IgM (Fig. 8E). Interestingly, we did find a significant decrease in total serum IgG in Δ dblGATA mice compared to the level in their WT counterparts. At 2 weeks postchallenge we detected elevated serum IgM (Fig. 8F) in Δ dblGATA mice and no difference in serum IgG, similar to the phenotype seen in Δ dblGATA mice challenged at 7 dpv. In line with previous results, we also found Δ dblGATA mice to have significantly reduced IgG1 and IgG2a subclass antibodies (Fig. 8F). Taken together, these data indicate a role for eosinophils in isotype switching for the generation of *Coxiella*-specific antibodies.

DISCUSSION

There is currently no FDA-approved vaccine available against *C. burnetii* infection due to a high incidence of adverse reactions in vaccinated populations. It remains unclear what vaccine components elicit these delayed-type hypersensitivity reactions. This, coupled with a lack of knowledge regarding the immunological mechanisms of vaccine protection, has prevented the rational design of a safe and effective new-generation vaccine against Q fever.

In this study, we sought to test the protective efficacies of PIV and PIIV generated by axenic culture methods. Reactogenicity has been attributed, in part, to residual egg proteins from the vaccine manufacturing process (41). By utilizing the axenic culture system, we removed the possibility of contamination by host-derived components. In line with previous results utilizing formalin-inactivated whole-cell vaccines derived from

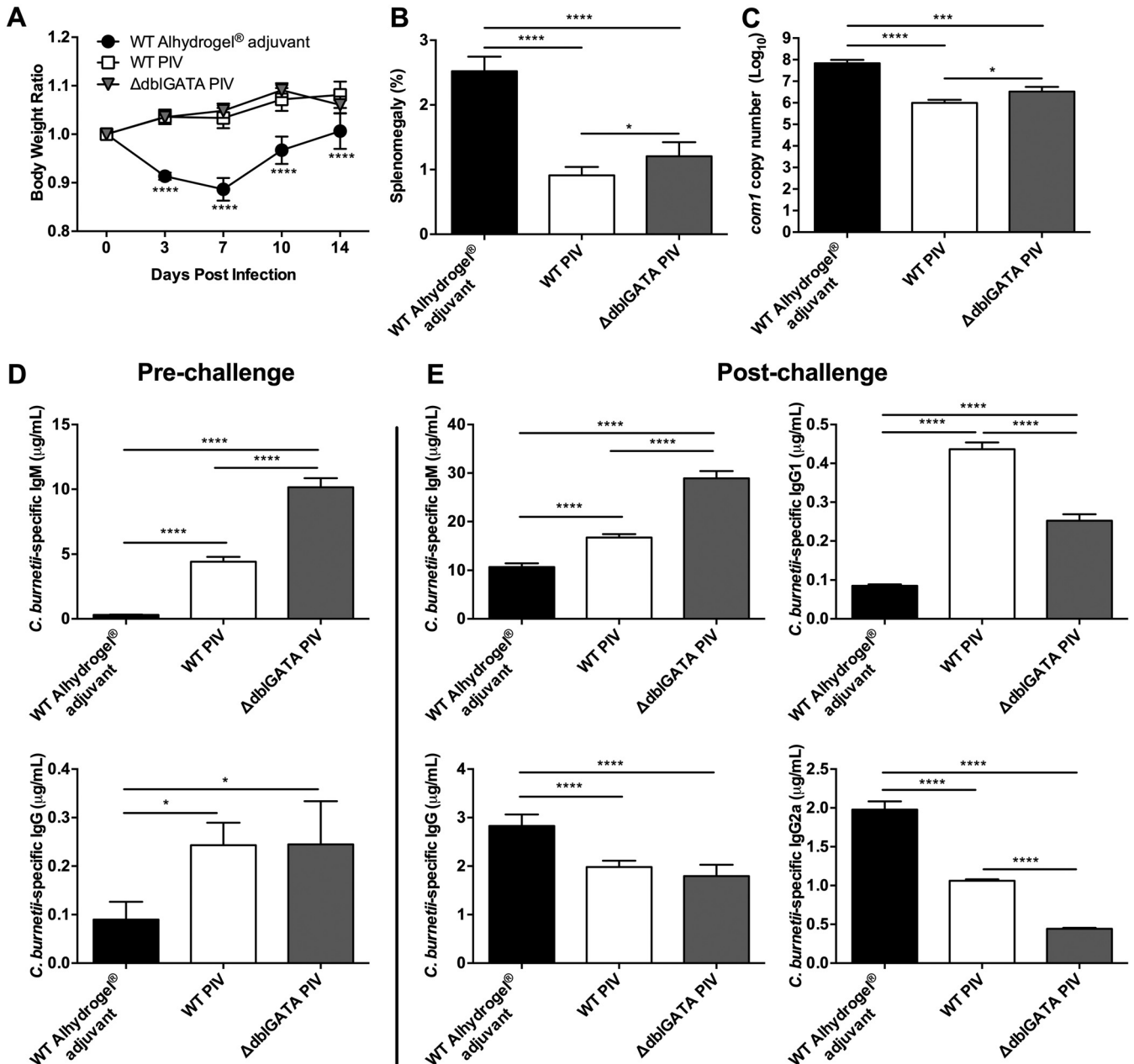


FIG 7 Eosinophils play a role in early PIV protection. WT and eosinophil-deficient Δ dblGATA mice were vaccinated s.c. with 10 μ g of PIV 7 days prior to i.p. challenge with 1×10^7 *C. burnetii* NMI bacteria. (A) Relative body weight was measured throughout infection. Splenomegaly (B) and bacterial burden in the spleen (C) were evaluated at 14 dpi to assess disease severity. *C. burnetii* NMI-specific serum IgM and IgG levels were evaluated at 7 dpv (D) or 14 dpi (E). Results are expressed as percent splenomegaly [(spleen weight/body weight) \times 100]. Bacterial burden was determined by qPCR and is expressed as log₁₀ *C. burnetii* com1 gene copy numbers. Each experimental group includes 5 mice, with error bars representing the standard deviations from the means. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$, as determined by two-way ANOVA with Dunnett's multiple-comparison test (for panel A) or by one-way ANOVA with Tukey's multiple-comparison test (for panels B to E).

chicken eggs or tissue culture (25, 32, 52, 53), axenically generated PIV was able to prevent clinical disease in BALB/c mice. There was a significant reduction in splenomegaly and an approximately 2-log decrease in *C. burnetii* genomic equivalents. Further investigation is required to determine if there is a decrease in adverse reactions with the use of this vaccine. Baeten et al. recently published a standardized guinea pig model for Q fever vaccine reactogenicity testing that could prove useful for these studies (54).

A recent improvement on ACCM-2, ACCM-D, allows for the generation of 5- to

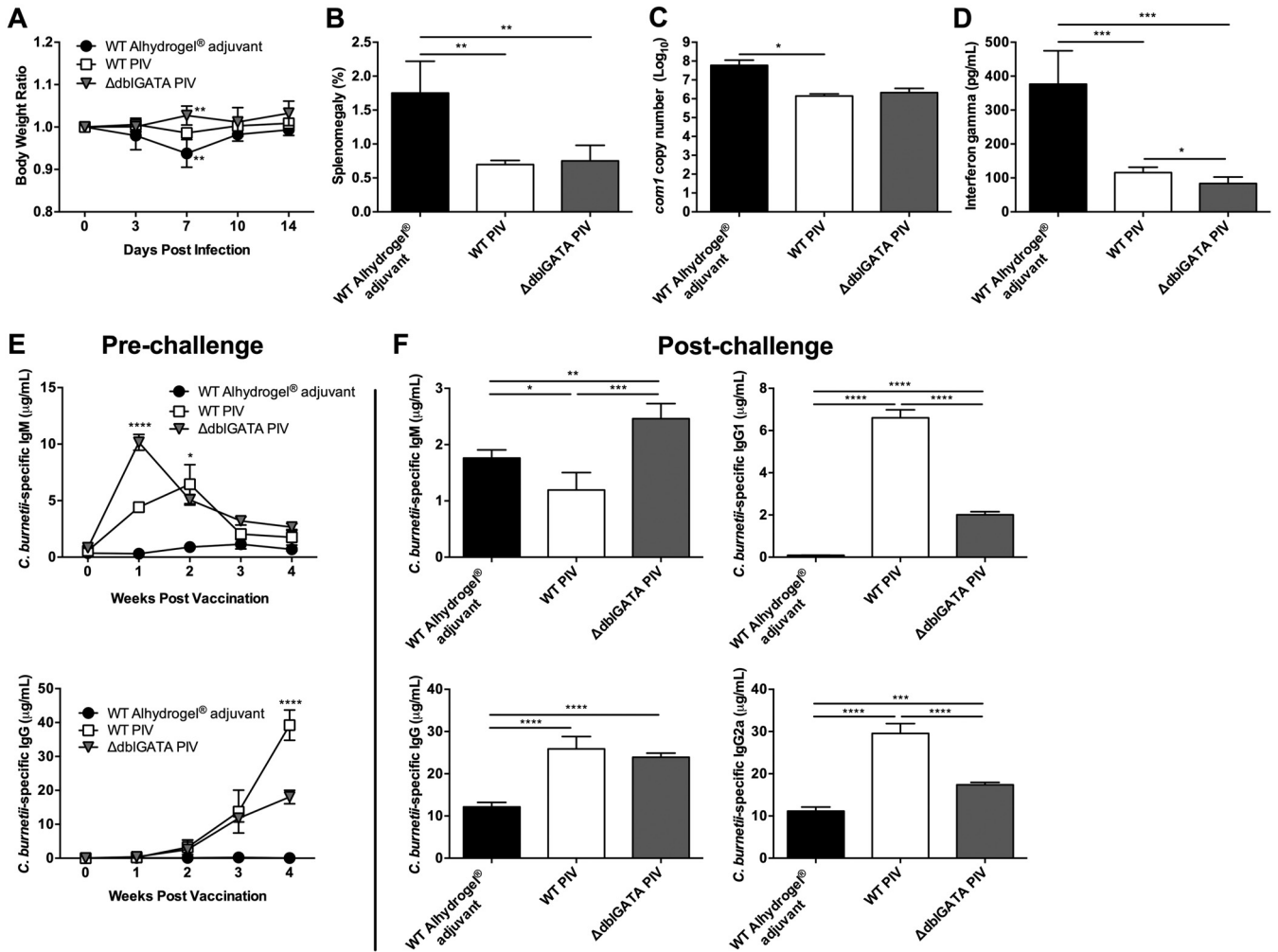


FIG 8 Eosinophils contribute to antibody isotype switching in late PIV responses. WT and ΔdblGATA mice were vaccinated s.c. with 10 μg of PIV 4 weeks prior to i.p. challenge with 1 × 10⁷ *C. burnetii* NMI bacteria. (A) Relative body weight was measured throughout infection. Splenomegaly (B) and bacterial burden in the spleen (C) were evaluated at 14 dpi to assess disease severity. (D) Serum IFN-γ levels were quantified at 14 dpi by cytokine-specific ELISA. *C. burnetii* NMI-specific serum IgM and IgG levels were measured weekly until 28 dpv (E) as well as at 14 dpi (F). Results are expressed as percent splenomegaly [(spleen weight/body weight) × 100]. Bacterial burden was determined by qPCR and is expressed as log₁₀ *C. burnetii* com1 gene copy numbers. Each experimental group includes 4 mice, with error bars representing the standard deviations from the means. * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001; **** *P* < 0.0001, as determined by two-way ANOVA with Dunnett’s multiple-comparison test (panels A and D) or by one-way ANOVA with Tukey’s multiple-comparison test (panels B, C, and E).

10-fold more bacteria with increased viability (55). Future studies examining the immunogenicity/protectivity of vaccines propagated in this defined axenic medium would be interesting, considering these changes in *C. burnetii* replication dynamics. Furthermore, the higher bacterial yield would improve the feasibility of this propagation method for future vaccine manufacturing.

While whole-cell inactivated *C. burnetii* vaccines have been demonstrated to be highly efficacious in lieu of adjuvant (10, 23, 24, 56, 57), these studies were carried out in healthy adults. It has recently been reported that children’s risk of exposure to *C. burnetii* in South West Queensland is similar to the high risk of abattoir workers (58); however, Q-VAX is not currently recommended for children (59). Vaccines which are successfully tested in healthy adults often fail in hypo/nonresponders such as young children and the elderly (60). Immunopotential in these risk groups should be the focus in designing a new-generation Q fever vaccine, and adjuvantation is one approach to this aim. Additionally, the use of adjuvant for immunopotential may allow a reduction in the amount of antigen required to generate protection. This “dose-

sparing" approach may, in turn, reduce adverse reactions as they appear to occur in a dose-dependent manner (57).

Previous reports in human cohorts suggested that it took at least 2 to 3 weeks for protection to develop (10, 23, 24). However, this was based on the incidence of clinical Q fever cases in vaccinated groups. Considering an incubation period of 2 to 3 weeks, it is likely that these patients were exposed prior to vaccination. Using our previously established mouse splenomegaly model, we determined that axenically generated PIV is able to generate a protective response as early as 7 dpv. At this time point, there was a significant reduction in splenomegaly as well as a roughly 4-log decrease in *C. burnetii* genomic equivalents. This finding has important implications in the context of *C. burnetii*'s use as a biological weapon. A vaccine which generates protection in a short time frame is not only a great prophylactic candidate but also a potential therapeutic.

The early time point at which PIV was able to significantly reduce splenomegaly suggested that innately driven mechanisms were involved in early vaccine protection. Indeed, we found significantly more SSC^{high} granulocytes in the spleens of PIV-vaccinated mice at all time points. There also appeared to be an increase in the number of lymphocytes at 14 dpv. Considering the kinetics of the anti-*Coxiella* antibody response, it is likely that this initial increase is due to the expansion of B cells in response to antigen stimulus. We saw peak serum IgM titers at 1 week postvaccination and began to detect measurable changes to serum IgG as early as 2 weeks postvaccination.

Further characterization of the SSC^{high} granulocyte population indicated that the cells were CD11b⁺ CD125⁺ Siglec-F⁺ eosinophils. Eosinophils are typically associated with clearance of helminth infections and allergic diseases (61, 62); however, they are also capable of bridging innate and adaptive immunity (49). These granulocytic cells contain preformed cytokines, chemokines, and growth factors that are able to be released quickly following activation (63). These mediators can activate neutrophils, immature dendritic cells, B cells, and T cells (64).

Major basic protein (MBP) has been shown to increase superoxide production in neutrophils (65). Elliott et al. have shown that neutrophils are important for the secondary response against *C. burnetii* (66). It is possible that eosinophil activation aids in the bactericidal activity of neutrophils, which could be important for protection. Alternatively, eosinophil-derived neurotoxin (EDN) released from eosinophils stimulated with CpG DNA can elicit the maturation of immature dendritic cells (67, 68). Dendritic cells are known to play a pivotal role in generating protective adaptive responses following vaccination against many intracellular pathogens, including *C. burnetii* (69). Therefore, it is possible that eosinophils are the first cells to respond in a cascade involving other innate populations, which then prime naive B and T cells. However, eosinophils can also express major histocompatibility complex class II (MHC-II) and costimulatory molecules and serve as antigen-presenting cells to directly activate B and T cells (64, 70). In fact, previous studies have found that alum adjuvant induces eosinophil recruitment and activation of B cells to produce antigen-specific IgM (71).

Evaluating the role of eosinophils in the host defense against *C. burnetii* using a more clinically relevant aerosol model of infection is an exciting area for future research. While eosinophils are canonically known for their role in clearance of parasitic infections, these cells are frequently involved in allergic airway inflammation (61, 62). With this in mind, it is possible that eosinophils could be involved in *C. burnetii* lung pathology. Previous reports have shown that MBP can dampen superoxide anion production by guinea pig alveolar macrophages (72). Considering that *C. burnetii* possesses superoxide dismutase abilities (73–76), which promote its survival within the intracellular compartment, it is conceivable that this function of MBP would aid in bacterial survival. However, recent reports have shown that eosinophils also aid in clearance of prominent pulmonary pathogens like respiratory syncytial virus (77–82) and *Mycobacterium tuberculosis* (83, 84). Indeed, Shi et al. (85) have previously demonstrated that airway eosinophils, or those isolated from the peritoneal cavity of

hypereosinophilic IL-5 transgenic mice, migrate to paratracheal lymph nodes following instillation into the trachea and act as antigen-presenting cells to stimulate antigen-specific CD4⁺ T cell responses.

In order to understand the mechanism of eosinophil accumulation, we studied potential upstream signaling events that would elicit eosinophil accumulation in PIV-vaccinated spleens. CD4⁺ T cells can recruit eosinophils through their secretion of chemoattractants like IL-5 and eotaxin-1. Therefore, to test whether CD4⁺ T cells were required for eosinophil accumulation, we vaccinated CD4-deficient mice and compared their splenic eosinophil numbers to those of WT mice. Interestingly, we found that CD4-deficient mice had a defect in tissue eosinophilia compared to that of WT mice, which suggests a CD4⁺ T cell-dependent role for this phenomenon. Additionally, we found that PIV-vaccinated CD4-deficient mice had a defect in IFN- γ production upon *ex vivo* restimulation with *C. burnetii* NMI. This could be due to the absence of CD4⁺ T cells; however, it is also possible that the lack of significant eosinophil accumulation contributes to this phenotype. While eosinophils are best known for their release of type 2 cytokines (IL-4, IL-5, and IL-13), it has become recently appreciated that these cells can also secrete many proinflammatory cytokines, including tumor necrosis factor alpha (TNF- α) and IFN- γ (86). Indeed, PIV-vaccinated eosinophil-deficient Δ dblGATA mice had significantly lower serum IFN- γ than PIV-vaccinated WT mice at 14 dpi, which suggests that eosinophils contribute to the IFN- γ response following *C. burnetii* infection. Further studies are required to determine which cell population(s) serves as an early IFN- γ producer in our vaccine model.

To evaluate the contribution of eosinophils to PIV protection, we vaccinated eosinophil-deficient Δ dblGATA mice and challenged them at either 7 or 28 dpv. Δ dblGATA mice have a deletion in the GATA1 promoter, which is important for eosinophil development (87). We found that Δ dblGATA mice had a defect in protection when challenged at 7 dpv. This suggests that eosinophils may play a partial role in the early protective response against *C. burnetii*. Considering that the changes in splenomegaly and bacterial burden were relatively small, albeit statistically significant, it is possible that eosinophils do not make a biologically significant contribution to vaccine immunity. However, it would be interesting to evaluate vaccine protection using a hypereosinophilic IL-5 transgenic mouse model. Eosinophil recruitment to the spleen, while not sufficient for vaccine protection, could enhance the overall vaccine response. In this case, adjuvant selection targeting eosinophils would be an interesting approach to consider.

At 2 weeks postchallenge, Δ dblGATA mice vaccinated 7 days prior displayed elevated serum IgM with no difference in total IgG compared to the level of WT. However, there was a significant decrease in both IgG1 and IgG2a subclass antibodies. While there was no difference in the total IgG concentration, the fact that we saw decreases in certain IgG subclasses indicates that the absence of eosinophils likely affects the ratio of each subclass (IgG1, IgG2a, IgG2b, and IgG3), which shapes the overall antibody response. Indeed, eosinophils have been shown to promote B cell responsiveness to both T-dependent and T-independent antigens in the periphery (88). Furthermore, previous work by Chu et al. has shown that eosinophils are required for the maintenance of long-lived plasma cells in the bone marrow (89, 90). Δ dblGATA mice have normal migration of plasmablasts to the bone marrow; however, they fail to develop into mature plasma cells (91). In line with these results, we found Δ dblGATA mice challenged at 28 dpv to have elevated serum IgM with decreased IgG1 and IgG2a subclass antibodies. These data indicate that eosinophils contribute to isotype switching and help shape the humoral response.

Cumulatively, these data have led us to propose a working model (Fig. 9) to describe the role of eosinophils in PIV-mediated protection. In our proposed model, antigen-presenting cells, such as dendritic cells (92), process and present vaccine antigens to naive CD4⁺ T cells. These activated helper cells secrete IL-5, which is a potent chemoattractant and activator of eosinophils. Eosinophils then accumulate in the spleens of vaccinated animals, where they are poised to respond to subsequent infection. Upon

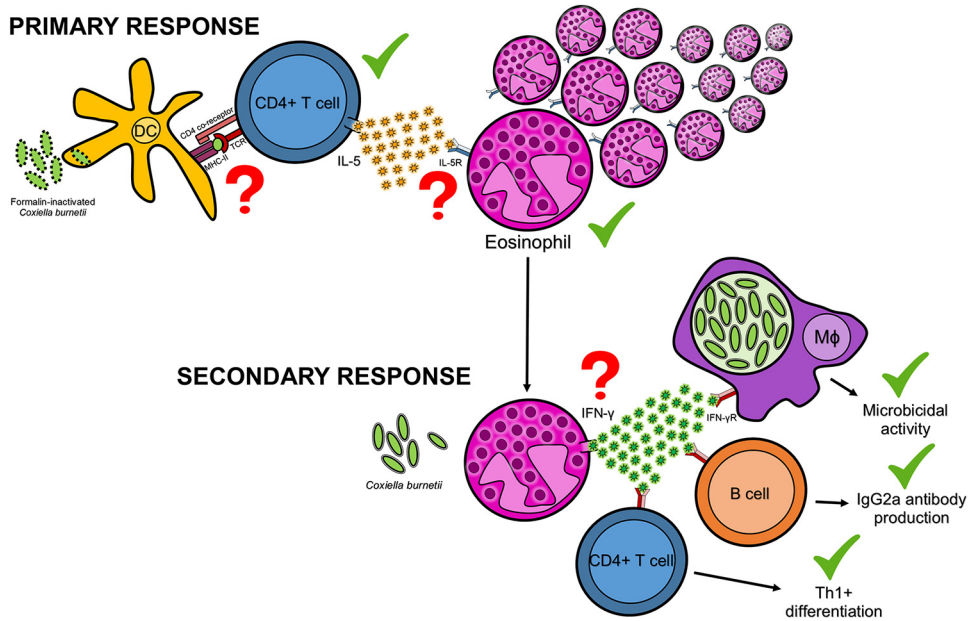


FIG 9 A working model for eosinophils in PIV-mediated protection. During the primary response, vaccine antigens are taken up by antigen-presenting cells, such as dendritic cells, processed, and presented to naive CD4⁺ T cells. These naive cells become activated T helper cells and can secrete IL-5, a potent chemoattractant and activator of eosinophils. Eosinophils then accumulate in the spleens of vaccinated animals, where they are poised to respond to subsequent infection. Upon secondary challenge, eosinophils can release preformed IFN- γ from their granules. IFN- γ contributes to host immunity against *C. burnetii* through activation of macrophages and enhancement of their microbicidal activity, promotion of IgG2a secretion from B cells, and expansion of Th1⁺ helper cells. Steps denoted with a green check mark (✓) have been demonstrated either experimentally in the manuscript or previously in the literature. Red question marks (?) denote steps which require further experimentation.

secondary challenge, eosinophils can release preformed IFN- γ from their granules. IFN- γ aids in bacterial clearance through activation of macrophages and enhancement of their microbicidal activity (93), promotion of IgG2a secretion from B cells (94, 95), and expansion of Th1⁺ CD4⁺ T cells (96). While it still remains to be demonstrated that eosinophils secrete IFN- γ in response to *C. burnetii*, it is known that IFN- γ promotes the previously mentioned host defense mechanisms against *C. burnetii* (25, 31, 50, 97–101).

In summary, this study provides novel information regarding the utility of axenic culture for the generation of highly efficacious vaccines against *C. burnetii*. Additionally, our results shed light on a previously uncharacterized mechanism of vaccine protection involving CD11b⁺ CD125⁺ Siglec-F⁺ eosinophils. Further understanding of the mechanisms of vaccine protection will guide future rational vaccine design and adjuvant selection.

MATERIALS AND METHODS

Animals. Four- to 12-week-old BALB/c and C57BL/6 mice were generated in our colony for use in these studies. Eosinophil-deficient Δ dblGATA [C.129S1(B6)-Gata1^{tm6Sho/J}] and CD4-deficient (B6.129S2-Cd4^{tm1Mak/J}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in sterile microisolator cages in a conventional animal facility or in an animal biosafety level 3 (ABSL3) facility at the University of Missouri Laboratory for Infectious Disease Research (MU-LIDR). Animals were provided food and water *ad libitum*. All research involving animals was conducted in accordance with the Animal Care and Use guidelines, and all animal use protocols were approved by the Animal Care and Use Committee at the University of Missouri. All infections were conducted in an ABSL3 facility at the MU-LIDR.

Bacterial strains. *C. burnetii* Nine Mile phase I (NMI) clone 7 (RSA 493) and Nine Mile phase II (NMII) clone 4 (RSA 439) were propagated in acidified citrate cysteine medium-2 (ACCM-2) as previously described (43). Bacteria were purified by centrifugation at 15,000 $\times g$ for 30 min, followed by two washes with sterile 1 \times phosphate-buffered saline (PBS). NMI was handled under biosafety level 3 (BSL3) conditions at the MU-LIDR.

Vaccination. Purified *C. burnetii* NMI and NMII were inactivated for 48 h in 10% formalin, followed by dialysis in deionized water. Antigen concentrations were then measured using a Micro BCA protein assay kit (Pierce, Rockford, IL) per the manufacturer's instructions. Mice were vaccinated subcutaneously

(s.c.) with 10 μg of formalin-inactivated phase I (PIV) or phase II (PIIV) whole-cell vaccine in 50 μl of sterile $1 \times \text{PBS}$ with 50 μl of aluminum hydroxide adjuvant (Alhydrogel adjuvant, 2%; InvivoGen, San Diego, CA) for a final delivered volume of 100 μl . Unvaccinated control mice were given 50 μl of PBS with 50 μl of adjuvant, for a final delivered volume of 100 μl . Vaccines were prepared with a 1:1 ratio of antigen/adjuvant per the manufacturer's instructions. Vaccinated and unvaccinated control mice were then either challenged to assess protectivity or sacrificed to assess cellular responses via flow cytometry or cytokine enzyme-linked immunosorbent assay (ELISA).

Infection. Vaccinated and unvaccinated control mice were challenged at 7, 14, or 28 days postvaccination (dpv) by intraperitoneal (i.p.) injection with a total of 1×10^7 or 5×10^7 *C. burnetii* NMI bacteria in 400 μl of sterile $1 \times \text{PBS}$. Mice were weighed throughout the course of infection. The protective efficacy of PIV and PIIV was evaluated 14 days postinfection (dpi) by comparing percent splenomegaly [(spleen weight/body weight) $\times 100$] against that of unvaccinated controls. Bacterial burden in the spleen was also measured using real-time quantitative PCR (qPCR).

qPCR. Spleen pieces were homogenized in 200 μl of lysis buffer (1 M Tris, 0.5 M EDTA, 7 mg/ml glucose, 28 mg/ml lysozyme) and filtered through a 100- μm -pore-size nylon mesh to remove any connective tissue. Ten microliters of proteinase K (20 mg/ml) was added to each sample prior to incubation at 60°C for 18 h. Next, 21 μl of 10% SDS was added to samples and incubated at room temperature for 1 h. Finally, DNA was extracted using a High Pure PCR Template Preparation kit (Roche, Indianapolis, IN) as directed by the manufacturer. Bacterial burden was determined by quantifying *com1* gene copy numbers using a standard curve with SYBR green (Applied Biosystems, Foster City, CA) on an Applied Biosystems StepOnePlus real-time PCR system. The standard curve was generated using recombinant plasmid DNA (*com1* gene ligated into pBluescript vector).

Spleen cell isolation. Spleen cells were harvested from BALB/c, C57BL/6, and CD4-deficient mice at 7 or 14 dpv or post-adjuvant treatment. Briefly, spleens were removed and homogenized. The cell suspension was then filtered through a 100- μm -pore-size nylon mesh to remove any connective tissue. Spleen cells were pelleted by centrifugation at $500 \times g$ for 8 min and resuspended in 5 ml of ammonium chloride-potassium (ACK) lysis buffer for 5 min at room temperature to lyse red blood cells. Remaining cells were then pelleted by centrifugation at $500 \times g$ for 8 min and resuspended in 2 ml of fluorescence-activated cell sorting (FACS) buffer (PBS supplemented with 0.5% bovine serum albumin [BSA], 2 mM EDTA, and 0.1% sodium azide) for counting.

Antibodies. Recombinant anti-mouse CD125 (IL-5Ra, clone REA343; conjugated to phycoerythrin [PE]), Siglec-F (clone REA798; PE-Vio770), and CD45R (B220, clone REA755; fluorescein isothiocyanate [FITC]) as well as rat anti-mouse CD19 (clone 6D5; FITC) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Rat anti-mouse CD45 (clone 30-F11; allophycocyanin [APC]) was purchased from ThermoFisher Scientific (Waltham, MA). Rat anti-mouse/human CD11b (clone M1/70; Brilliant Violet 785) was purchased from BioLegend (San Diego, CA).

Flow cytometry. Approximately 1×10^6 cells were added to each well of a 96-well plate for flow cytometry. Briefly, cells were blocked for 15 min at 4°C with Fc Block (BD Biosciences) in FACS buffer. Cells were then stained in FACS buffer with an antibody cocktail containing CD45, CD125, CD11b, Siglec-F, CD19, and CD45R for 30 min at 4°C. Cells were pelleted by centrifugation at $500 \times g$ for 8 min, followed by two washes with FACS buffer. A 2% paraformaldehyde solution was then used to fix cells for 15 min at 4°C. Finally, cells were pelleted by centrifugation at $500 \times g$ for 8 min and resuspended in FACS buffer for analysis. A total of 500,000 cellular events were collected for each sample using a Miltenyi MACSQuant Analyzer 10 or BD LSRFortessa. Compensation controls were utilized to account for spectral overlap before data analysis was performed using FlowJo, version 10.4.2. Absolute cell counts were determined based on live cell frequency and the total number of spleen cells recovered from each animal (see Table S1 in the supplemental material).

Cytokine ELISA. Splenocytes from naive, adjuvant-treated, or PIV-vaccinated C57BL/6 and CD4-deficient mice were restimulated *ex vivo* with 1×10^7 virulent *C. burnetii* NMI bacteria to evaluate gamma interferon (IFN- γ) levels in supernatants by ELISA as previously described (51). Briefly, 5×10^6 total splenocytes from each treatment group were added in duplicate to a 24-well plate in complete medium (RPMI 1640 medium supplemented with 10% fetal bovine serum [FBS], 10 mM HEPES buffer, 10 mM nonessential amino acids, and 10 mM sodium pyruvate). Cells were cultured alone or restimulated with *C. burnetii* NMI bacteria ($1 \times 10^7/\text{ml}$) for 3 or 6 days at 37°C. ELISAs were performed on supernatants for IFN- γ as directed by the manufacturer (Invitrogen-ThermoFisher Scientific).

Coxiella-specific ELISA. Sera from vaccinated and unvaccinated control mice were used for quantification of total IgM, IgG, IgG1, and IgG2a subclass antibodies. Microtiter plates (96-well) were coated with 100 μl of inactivated NMI antigen (0.5 $\mu\text{g}/\text{ml}$) or unlabeled anti-IgM or -IgG antibody (0.5 $\mu\text{g}/\text{ml}$, for the standard curve) (Southern Biotech, Birmingham, AL) in 0.05 M carbonate/bicarbonate coating buffer (pH 9.6) for 24 h at 4°C. Plates were blocked with 1% BSA in PBS-T buffer (0.05% Tween 20 in $1 \times \text{PBS}$) and then incubated for 2 h with 200 μl of diluted sample serum (1:200 to 1:1,200) or serially diluted pure IgM, IgG, IgG1, or IgG2a (Southern Biotech) at room temperature. Plates were washed four times with PBS-T buffer and then incubated with 100 μl of diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM, IgG, IgG1, or IgG2a (1:4,000 to 1:8,000) at room temperature for 1 h. Plates were washed again four times with PBS-T, followed by the addition of 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (ThermoFisher Scientific). Reactions were stopped using 1 M H_3PO_4 , and absorbance was measured at 450 nm using a SpectraMax (Molecular Devices, San Jose, CA) or Infinite F50 (Tecan, Switzerland) microplate reader.

Statistical analysis. Prism, version 5.0 (GraphPad Software, Inc., San Diego, CA), was used for all statistical analyses. Results represent means \pm standard deviations. Values were compared using

one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test or two-way ANOVA with Dunnett's multiple-comparison test. Differences were considered significant at a *P* value of <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00376-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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