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Chlamydia-Specific IgA Secretion in the Female Reproductive Tract Induced via Per-Oral Immunization Confers Protection against Primary *Chlamydia* Challenge

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ABSTRACT *Chlamydia trachomatis* is an obligate intracellular pathogen that causes sexually transmitted disease. In women, chlamydial infections may cause pelvic inflammatory disease (PID), ectopic pregnancy, and infertility. The role of antibodies in protection against a primary *Chlamydia* infection is unclear and was a focus of this work. Using the *C. muridarum* mouse infection model, we show that intestinal mucosa is infected via intranasal (i.n.) or per-oral (p.o.) *Chlamydia* inoculation and that unlike the female reproductive tract (FRT) mucosa, it halts systemic *Chlamydia* dissemination. Moreover, p.o. immunization or infection with *Chlamydia* confers protection against per-vaginal (p.v.) challenge, resulting in significantly decreased bacterial burden in the FRT, accelerated *Chlamydia* clearance, and reduced hydrosalpinx pathology. In contrast, subcutaneous (s.c.) immunization conferred no protection against the p.v. challenge. Both p.o. and s.c. immunizations induced *Chlamydia*-specific serum IgA. However, IgA was found only in the vaginal washes and fecal extracts of p.o.-immunized animals. Following a p.v. challenge, unimmunized control and s.c.-s.c.-immunized animals developed *Chlamydia*-specific intestinal IgA yet failed to develop IgA in the FRT, indicating that IgA response in the FRT relies on the FRT to gastrointestinal tract (GIT) antigen transport. Vaginal secretions of p.o.-immunized animals neutralize *Chlamydia in vivo*, resulting in significantly lower *Chlamydia* burden in the FRT and *Chlamydia* transport to the GIT. We also show that infection of the GIT is not necessary for induction of protective immunity in the FRT, a finding that is important for the development of p.o. subunit vaccines to target *Chlamydia* and possibly other sexually transmitted pathogens.

KEYWORDS *Chlamydia*, vaccine, mucosa, vaccination, IgA, antibodies, female reproductive tract, mucosal vaccines, neutralizing antibodies

The occurrence of new sexually transmitted infections (STIs) worldwide has been on the rise, with over 1 million new cases reported daily (1). In the United States, the situation is just as concerning, with significant increases in new cases of chlamydia, gonorrhea, and syphilis (2). Young adults (aged 15 to 24) account for around half of the new STIs. Between 2014 and 2018, chlamydial infections in the United States increased by 19%, with infection rates two times higher in women than in men (2). In women, about 80% of chlamydial infections are asymptomatic and may lead to the development of pelvic inflammatory disease (PID), chronic pelvic pain, hydrosalpinx, and tubal infertility (3–5). In addition, women infected with *Chlamydia* are at a higher risk of acquiring HIV infections and developing human papillomavirus (HPV)-associated cervical cancer (6, 7). Despite extensive efforts, the development of effective mucosal vaccines against STIs, including *Chlamydia*, has been for the most part unsuccessful, with the exception of the vaccine against HPV, which is administered parenterally (8).

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Parenteral immunization, however, induces mainly systemic immunity and protection against some mucosally acquired pathogens, such as influenza virus, HPV, and poliovirus. In contrast, mucosal immunization induces systemic and local mucosal immunity, which is essential for protection against most mucosal pathogens, including *Chlamydia*.

Animal studies have been instrumental in identifying protective immune responses to chlamydial infections and have guided vaccine development. Overall, for resolution of an infection and the development of immunity to reinfection, $CD4^+$ T cells and cytokines, such as gamma interferon (IFN-γ) and interleukin 12 (IL-12), have been shown to be essential (9–12), while $CD8^+$ T cells do not appear to be important (10–13). Although antibodies induced by most vaccines are key to their effectiveness, whether and how *Chlamydia*-specific antibodies confer protection against a primary infection is unclear (14, 15). Chlamydial infections induce *Chlamydia*-specific antibodies (16, 17), which could aid in protective immunity by neutralizing *Chlamydia* elementary bodies (EBs) in the female reproductive tract (FRT) mucosa, by activating the complement, as well as other cellular effector functions. *Chlamydia* exists in two different forms, namely, as infectious EBs and as noninfectious, intracellular reticulate bodies (RBs). Therefore, antibodies specific for antigens of the extracellular EB stage could serve to neutralize EBs and activate a number of other antibody-mediated effector functions. In guinea pigs, resistance to a secondary infection is decreased when humoral immunity is suppressed (18) and the transfer of convalescent-phase serum to naive animals significantly reduces bacterial shedding from the FRT (19). This indicates that antibodies play a role in controlling *Chlamydia* infections. Studies done with mice that lack B cells or IgA specifically have revealed no clear role for B cells or IgA in the resolution of a primary genital *Chlamydia* infection, as mice lacking B cells or IgA resolve infections at a rate similar to that of controls (15, 20, 21). Adoptive transfer of convalescent-phase sera does not protect mice against a primary infection; however, it does protect against a secondary infection (14). In women, secretory IgA (sIgA) in the FRT was shown to be inversely correlated with *Chlamydia* burden (22), indicating that IgA plays an important role in protection against this pathogen.

The mouse-specific pathogen *Chlamydia muridarum* is widely used for studying pathogenesis and immune responses because it induces a long-term FRT pathology similar to the one caused by *Chlamydia trachomatis* in women (23, 24). In both animals and humans, *Chlamydia* establishes long-term infections in the gastrointestinal tract (GIT), which present with no obvious pathology (25–27). In mice, infection of the GIT by *C. muridarum* induces immunity to a *C. muridarum* per-vaginal (p.v.) challenge (27). However, a deliberate infection with a nonattenuated pathogen poses inherent risks and would be perceived unsafe by the public and therefore would be an unacceptable form of immunization. This opens up the possibility that per-oral (p.o.) immunization with noninfectious chlamydial antigens may be an effective approach for the development of vaccines to target *Chlamydia* and possibly other STIs. We hypothesized that immune responses in the FRT may be induced in the gut-associated lymphoid tissue (GALT). Based on previous reports that mucosal priming followed by subcutaneous (s.c.) boosting induces long-lasting mucosal and systemic immunity (28–30), we hypothesized that p.o. priming with inactivated (killed) *Chlamydia* EBs followed by s.c. boosting would induce immunity to *Chlamydia* challenge in the FRT. Here, we address these hypotheses and show that inactivated *Chlamydia* EBs administered to the GIT are just as effective as live *Chlamydia* in inducing protection against *Chlamydia* challenge in the FRT. Moreover, we show that IgA secretion in the FRT is induced following immunization or infection of the GIT and not the FRT. FRT IgA effectively neutralizes *Chlamydia* and impedes its invasion in the FRT lamina propria and systemic dissemination.

RESULTS

***C. muridarum* infects the GIT following p.o. (intragastric) or i.n. administration.** Although known for its ability to cause STIs and long-term pathology in humans, *C. trachomatis* also infects and persists in the GIT (31, 32). In mice, the nonpathogenic infection of the GIT by *C. muridarum* can occur following a p.o. exposure (e.g., via

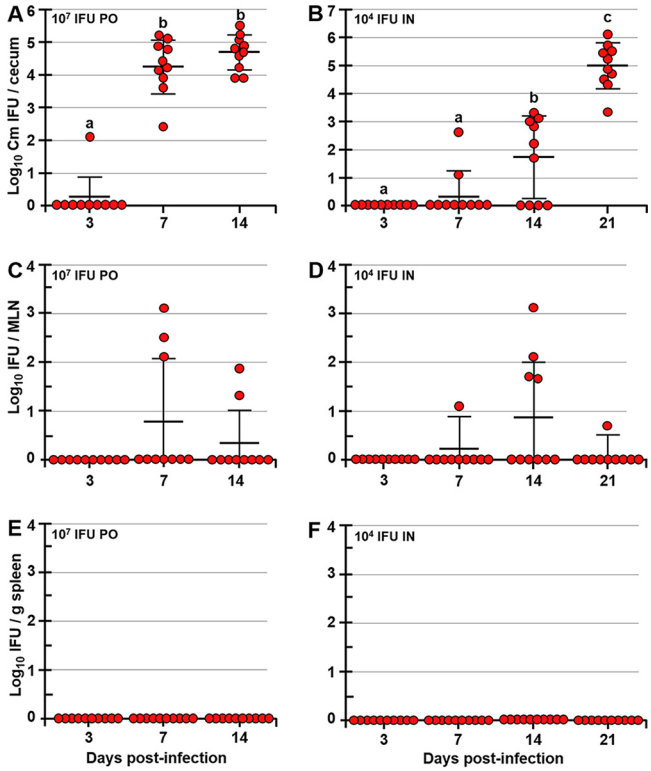

FIG 1 Following p.o. or i.n. administration, *Chlamydia* infects the GIT of mice but does not disseminate to the spleen. Shown are *C. muridarum* titers in ceca (A and B), MLNs (C and D), and spleens (E and F) of p.o.- or i.n.-infected mice. Mice were infected p.o. via gastric gavage with 10^7 or i.n. with 10^4 IFU of *Chlamydia* and at 3, 7, 14, and 21 dpi, and *Chlamydia* titers in tissues were determined. *Chlamydia* titers are expressed as $\log_{10}$ number of IFU per cecum, per MLN, or per gram of spleen. Data are representative of those from two separate experiments and are expressed as means ± SDs. Group means were separated using Tukey's multiple-comparison test and were declared significantly different at a *P* value of <0.05 ($n = 10$ mice per time point). Group means that do not share a lowercase letter are significantly different.

coprophagy or grooming). However, host cells can also spread *Chlamydia* from the FRT to the GIT internally (33), and this systemic spread of *Chlamydia* and infection of the spleen specifically were proposed to be critical for the long-term hydrosalpinx pathology in the FRT (33). On the other hand, p.o. and intranasal (i.n.) infections with *Chlamydia* induce protective immunity to a primary *Chlamydia* challenge in the FRT (27, 34). In light of these observations, we hypothesized that like p.o. inoculation, i.n. inoculation with live *Chlamydia* EBs leads to the colonization of the GIT and that GALT represents a firewall that prevents *Chlamydia* systemic dissemination. To address these hypotheses, mice were infected i.n. or p.o. (intragastrically) with live *C. muridarum* EBs and *Chlamydia* titers were examined in ceca, mesenteric lymph nodes (MLNs), and the spleen for up to 21 days postinfection (dpi). Following p.o. or i.n. inoculation, *Chlamydia* infected the GIT, with ceca of all inoculated animals becoming positive by 7 or 21 dpi, respectively (Fig. 1A and B). The delayed *Chlamydia* spread to the GIT after the i.n. inoculation was likely due to the lower *Chlamydia* dose used for i.n. than for p.o. inoculation (10^4 versus 10^7 inclusion-forming units [IFU], respectively). While at 7, 14, and 21 dpi p.o. or i.n., a small proportion of MLNs were positive for live *Chlamydia* (Fig. 1C and D), no *Chlamydia* was detected in spleens of p.o.- or i.n.-infected mice at any time point examined (Fig. 1E and F). Within ceca, chlamydial inclusions were observed in the epithelium, as well as in the subepithelial compartment (Fig. 2A to C). These findings indicate that unlike the FRT (33, 35), GIT mucosa effectively contains *Chlamydia* and halts its spread to the deeper tissues, such as the spleen. To examine whether p.o. immunization with killed *Chlamydia* EBs induces protection in the FRT, two studies were

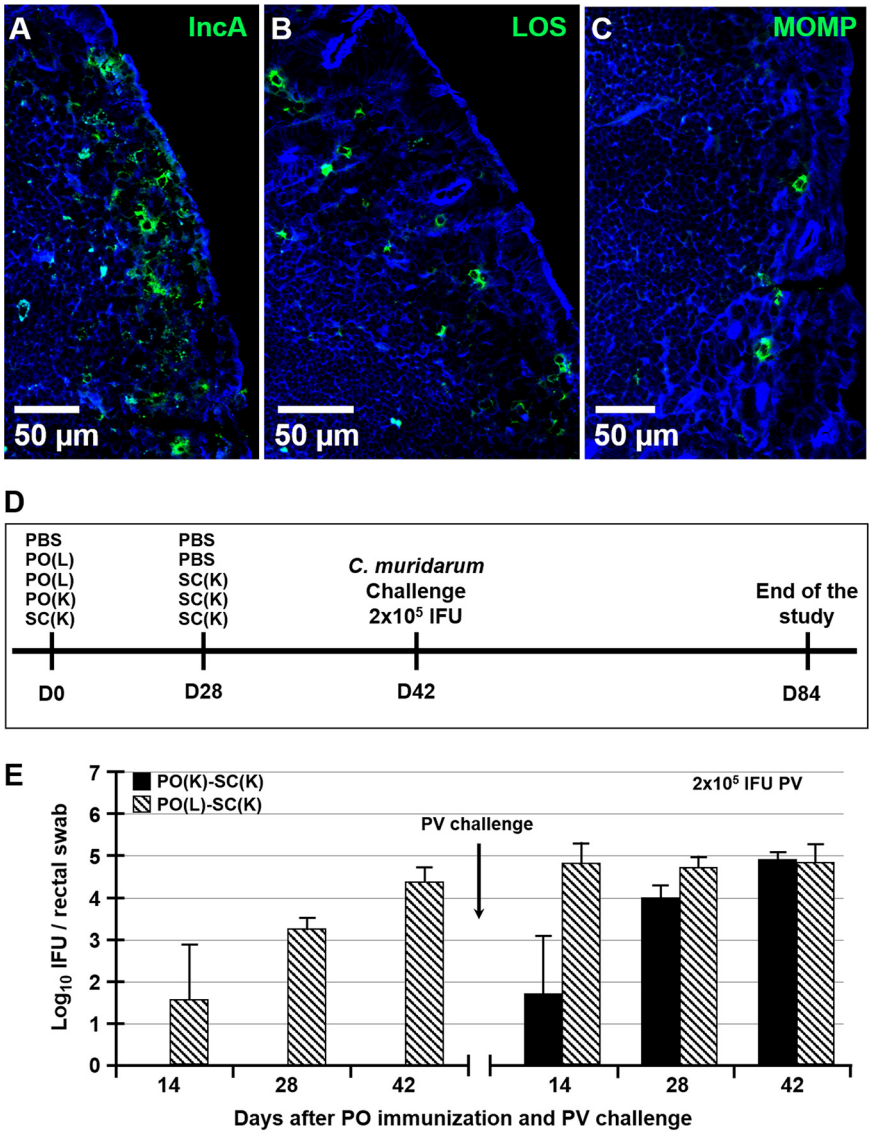

FIG 2 *Chlamydia* inclusions in ceca of p.o.-infected mice and immunization and challenge timeline. Shown are chlamydial inclusions in ceca of mice p.o. infected with 10^7 IFU of *C. muridarum* at 7 dpi. Tissues were stained with antibodies specific for *Chlamydia* antigens IncA (A), LOS (B), or MOMP (C) (green) and actin-binding phalloidin-Alexa Fluor 350 (blue). (D) Timeline of prime-boost immunizations and p.v. challenge with *C. muridarum*. Mice were primed p.o. or s.c. with live or killed *Chlamydia* EBs at day 0 and then s.c. boosted at day 28 with killed EBs. Two weeks later (day 42), mice were p.v. challenged with 2×10^5 IFU of *C. muridarum*. Six weeks after p.v. challenge (day 84), the studies were terminated and FRT pathology and *Chlamydia* burdens were determined. (E) Rectal swab titers of PO(K)-SC(K) and PO(L)-SC(K) mice collected at days 14, 28, and 42 prior to and 14, 28, and 42 after p.v. challenge with 2×10^5 IFU of *Chlamydia*.

conducted (Fig. 2D). In the first study, groups of mice were p.o. fed live or killed EBs and then s.c. boosted with killed EBs [PO(L)-SC(K) and PO(K)-SC(K) mice]. Another group was s.c. primed and boosted with killed EBs [SC(K)-SC(K) mice], as this form of immunizations induces systemic but not mucosal immunity and allows for examination of the role of serum and mucosal antibodies in protection against a p.v. *Chlamydia* challenge. In the second study, a similar prime-boost immunization approach was used, with the difference that mice were intragastrically inoculated to ensure that antigen was administered to the GALT alone. Both p.o. and intragastric immunizations are referred to as p.o. immunizations. In the second study, we also added an immunization group in which mice were p.o. infected with live *Chlamydia*, but were s.c. boosted with

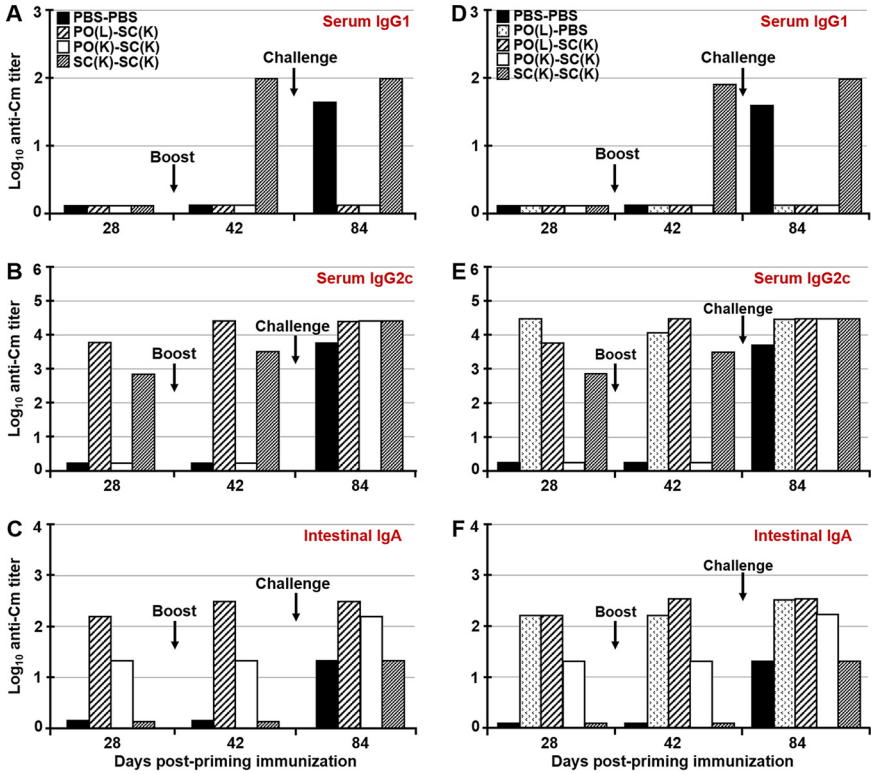

FIG 3 Serum and mucosal antibody titers following p.o. immunization or challenge with *C. muridarum*. Antibody titers in sera and intestinal extracts of mice 4 weeks after priming (day 28), 2 weeks after boosting (day 42), and 6 weeks after p.v. challenge (day 84). Two studies were conducted. In the first study, animals were fed live or killed *Chlamydia* EBs via a pipette, while in the second study (D to F), *Chlamydia* live or killed EBs were administered by gastric gavage, in order to preclude sublingual immunization. Samples from five animals from treatment group of each study were pooled and *Chlamydia*-specific IgG1, IgG2c, and intestinal IgA were analyzed by ELISA. Titers are expressed as $\log_{10}$ values of IgG1, IgG2c, or IgA antibodies detected in serum (A, B, D, and E) or fecal extracts (C and F) of mice following priming, boosting, and p.v. challenge with *C. muridarum*. Titers represent the highest dilutions of samples showing an absorbance value at 405 nm that is twice that of the negative control.

phosphate-buffered saline (PBS) [PO(L)-PBS mice]. Control mice were administered sterile PBS p.o. and s.c. (PBS-PBS mice). In both studies, immunized and unimmunized controls were p.v. challenged with 2×10^5 live *Chlamydia* EBs at day 42 after priming (2 weeks after boosting immunization). Six weeks after the p.v. challenge (day 84), FRT pathology and *Chlamydia* clearance and burden in the FRT were evaluated (Fig. 2D). We confirmed that the stock of paraformaldehyde (PFA)-treated and sonicated EBs used for p.o. immunizations in both studies had no viable organisms by culturing the stock in HeLa cells and passaging the infection 6 times. In addition, we determined the titers of rectal swabs of PO(L)-SC(K) and PO(K)-SC(K) mice to ensure that PO(K)-SC(K) mice were not infected prior to p.v. challenge. Mice p.o. immunized with live EBs had positive rectal swabs at 14, 28, and 42 after p.o. priming, as well as 14, 28, and 42 dpi p.v. (Fig. 2E). In contrast, none of the mice p.o. immunized with killed EBs had positive rectal titers prior to p.v. challenge (Fig. 2E).

Serum and intestinal antibody responses following immunization and p.v. *Chlamydia* challenge. Prime-boost immunization influences the type, location, and the longevity of the immune response. Thus, the use of various prime-boost strategies allowed us to separately examine the role of systemic and mucosal antibodies in immunity to a *Chlamydia* challenge in the FRT. We hypothesized that the GALT, rather than the FRT, is the inductive site of IgA secretion not only in the GIT but also in the FRT. To address this hypothesis, serum IgG1 and IgG2c, as well as mucosal IgA responses in the FRT and the GIT, were examined. At day 28 (4 weeks after priming) none of the immunized mice from either study developed serum IgG1 titers (Fig. 3A

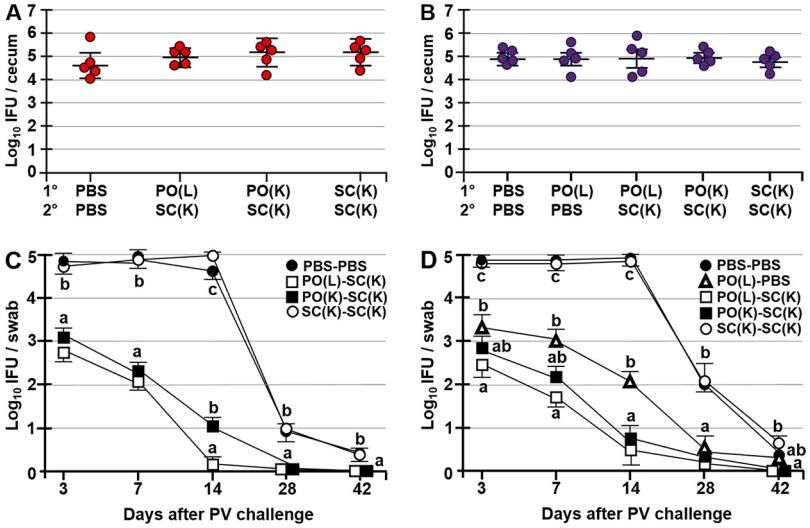

FIG 4 Immunization does not affect *Chlamydia* loads in ceca, but it enhances *Chlamydia* clearance from the FRT following a p.v. challenge. (A and B) *Chlamydia* titers in ceca of unimmunized and immunized mice at 6 weeks after p.v. challenge with 2×10^5 IFU of *C. muridarum*. (C and D) *Chlamydia* titers in vaginal swabs of mice at days 3, 7, 14, 21, 35, and 42 after p.v. challenge with 2×10^5 IFU of *C. muridarum*. *Chlamydia* titers are expressed as $\log_{10}$ number of IFU per cecum or per swab. Data are expressed as means ± SDs. Group means were separated using Tukey's multiple-comparison procedures and were declared significantly different at a *P* value of <0.05. Means that do not share a lowercase letter are significantly different from each other.

and D). After boosting immunization (day 42), only SC(K)-SC(K) mice developed serum IgG1, while after p.v. challenge both unimmunized controls (PBS-PBS) and SC(K)-SC(K) mice developed serum IgG1 titers (Fig. 3A and D). In contrast, p.o.-immunized mice [PO(L)-SC(K), PO(K)-SC(K), and PO(L)-PBS mice] did not develop *Chlamydia*-specific serum IgG1 at any time point (Fig. 3A and D). Animals that were immunized p.o. with live *Chlamydia* or s.c. with inactivated *Chlamydia* developed serum IgG2c titers after priming immunization (days 28), while mice primed p.o. with inactivated *Chlamydia*, much like unimmunized controls, did not develop detectable serum IgG1 or IgG2c after priming or s.c. boosting (Fig. 3A, B, D, and E). After the p.v. challenge, all immunized and unimmunized animals developed serum IgG2c (Fig. 3B and E). As expected, only mice that were primed mucosally (p.o.) with live or inactivated *Chlamydia* developed intestinal IgA titers after priming and boosting (day 28 and 42), while all groups developed intestinal IgA following the p.v. challenge (Fig. 3C and F). Animals p.o. primed with inactivated *Chlamydia* developed measurable serum IgG2c antibodies only after the p.v. challenge (Fig. 3B and E), although they did develop *Chlamydia*-specific intestinal IgA after priming (Fig. 3C and F). IgA titers in fecal extracts of these mice were lower than in mice primed with live *Chlamydia* (Fig. 3C and F), likely indicating an inefficient uptake of larger noninfectious *Chlamydia* antigens, as opposed to the findings using antigen-carrying nanoparticles (28, 36). The presence of intestinal IgA in mice that were not inoculated p.o. with live *Chlamydia* [PBS-PBS, SC(K)-SC(K), and PO(K)-SC-(K) mice] at day 84 (6 weeks postchallenge) was likely due to *Chlamydia* dissemination to the GIT after p.v. challenge (33), or acquisition of infection via grooming or coprophagy (Fig. 3C and F).

p.o. immunization does not affect GIT colonization by *Chlamydia*, but it enhances *Chlamydia* clearance in the FRT. Although all p.o.-immunized mice developed *Chlamydia*-specific intestinal sIgA prior to p.v. challenge, the presence of these antibodies did not affect *Chlamydia* clearance from the GIT, as cecal *Chlamydia* titers were comparable among all groups in both studies (Fig. 4A and B). In contrast, mice p.o. immunized with live or inactivated EBs exhibited significantly lower *Chlamydia* titers in vaginal swabs starting at day 3 postchallenge and cleared the FRT *Chlamydia* weeks faster than SC(K)-SC(K) mice and unimmunized controls (Fig. 4C and D). In this regard,

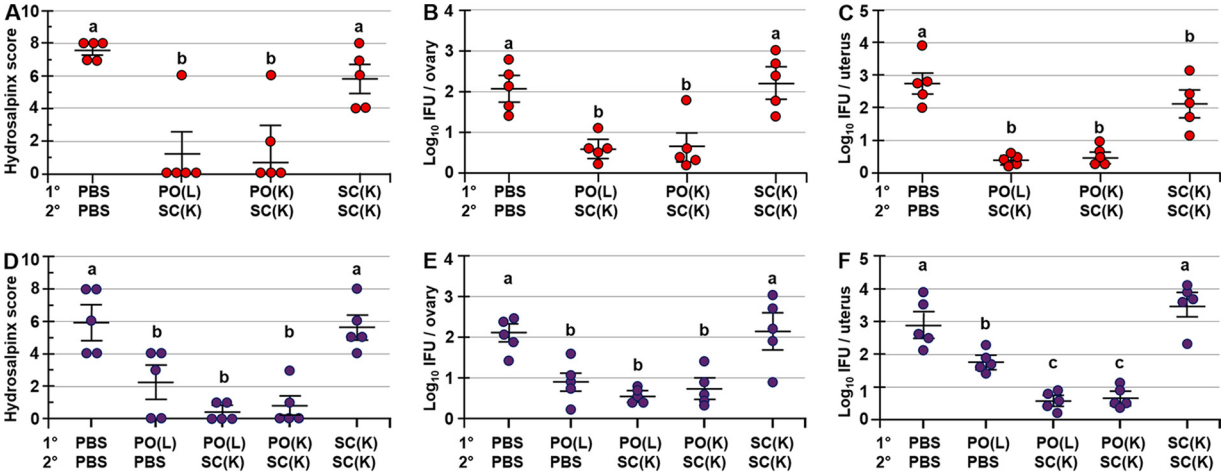

FIG 5 p.o. priming with live or killed *C. muridarum* significantly reduces FRT pathology and *Chlamydia* loads in ovaries/oviducts and uteri of mice following a p.v. challenge. (A and D) Hydrosalpinx scores and *Chlamydia* loads in ovaries/oviducts (B and E) and uteri (C and F) of C57BL/6J mice at 6 weeks after p.v. challenge with 2×10^5 IFU of *C. muridarum*. *Chlamydia* titers are expressed as $\log_{10}$ number of IFU per ovary/oviduct or uterus. Data are expressed as means ± SDs. Group means were separated using Tukey's multiple-comparison procedures and were declared significantly different at a *P* value of <0.05. Means that do not share a lowercase letter are significantly different from each other.

mice p.o. primed with live EBs and s.c. boosted with killed EBs [PO(L)-SC(K) mice] cleared the FRT *Chlamydia* faster than mice that were p.o. infected only and had significantly lower vaginal swab titers for up to day 28 postchallenge (Fig. 4C and D). *Chlamydia* titers in vaginal swabs of SC(K)-SC(K) mice were about 2 logs higher than for PO-SC-immunized mice at 3 dpi and were not different from the titers of unimmunized controls, indicating that serum antibodies provide no protection against primary infection. Across two studies, all SC(K)-SC(K) mice and unimmunized controls (PBS-PBS mice) (20/20) failed to completely clear FRT *Chlamydia*, even at day 42 post-p.v. challenge, and had comparable *Chlamydia* titers in vaginal swabs (Fig. 4C and D). Overall, mice primed p.o. with live or killed EBs and then boosted s.c. with killed EBs cleared *Chlamydia* from the FRT faster than mice from other immunization groups, including mice that were infected p.o. with live EBs alone (Fig. 4C and D).

p.o. infection or immunization with killed *Chlamydia* confers protection against p.v. *Chlamydia* challenge. To examine whether p.o. immunization confers protection against a primary p.v. *Chlamydia* challenge, we examined hydrosalpinx pathology and *Chlamydia* burdens in the uteri and combined ovary and oviduct tissues. In the first study, we found that p.o. priming with live or killed *Chlamydia*, followed by s.c. boosting [PO(L)-SC(K) and PO(K)-SC(K) mice], resulted in significantly reduced hydrosalpinx scores and *Chlamydia* burdens in the ovaries/oviducts and uteri compared to those of unimmunized controls and SC(K)-SC(K) animals (Fig. 5A to C). In the second study, p.o.-primed mice had significantly lower hydrosalpinx pathology than did unimmunized controls and SC(K)-SC(K)-immunized animals (Fig. 5). Although not statistically significant, p.o.-primed s.c.-boosted mice had numerically lower hydrosalpinx scores and *Chlamydia* burdens in the ovaries/oviducts than did mice p.o. primed only [PO(L)-PBS mice]. In addition, p.o.-s.c.-immunized mice had significantly lower bacterial burdens in the uteri than did mice that were p.o. primed with live *Chlamydia* only [PO(L)-PBS mice], as well SC(K)-SC(K) mice and unimmunized controls (PBS-PBS mice). Although p.o. infection alone did confer protection against *Chlamydia* challenge, p.o.-s.c. prime-boost immunization resulted in the lowest hydrosalpinx pathology and bacterial burdens in the upper FRT (Fig. 5D to F).

Serum antibodies neutralize *Chlamydia in vitro*. Serum antibodies, dominated by IgG isotypes, were shown to be protective against secondary chlamydial infections (14). To examine the ability of serum and intestinal antibodies to neutralize *Chlamydia*, HeLa cells were infected with EBs that had been preincubated with heat-inactivated serum or fecal extracts of immunized or unimmunized controls. Four weeks after immuniza-

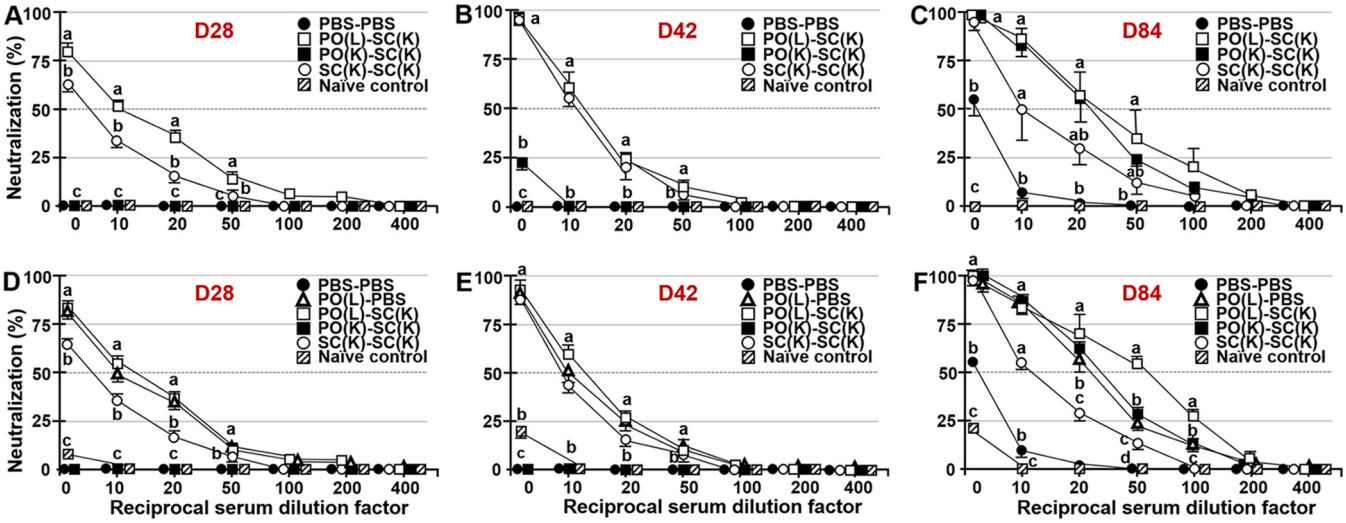

FIG 6 Serum from immunized and p.v. challenged animals neutralizes *Chlamydia* EBs *in vitro*. Neutralizing activity of sera from immunized and unimmunized control mice collected after priming (day 28) (A and D), boosting (day 42) (B and E) and p.v. challenge (C and F) was determined. Pooled serum samples were collected from the first (A to C) and second (D to F) studies. Group means were separated by Tukey's multiple-comparison procedures. Data are shown as means ± SDs and were declared statistically significant at a *P* value of <0.05. Means that do not share a lowercase letter are significantly different from each other.

tion (day 28), sera of mice primed p.o. with live EBs or s.c. with killed EBs had neutralizing activity; however, sera of p.o.-primed mice had significantly higher neutralizing ability (Fig. 6A and D). After s.c. boosting (day 42), there were no differences in neutralization ability among the sera of p.o.- and s.c.-primed mice (Fig. 6B and E). Following the p.v. challenge, sera of all mice exhibited neutralizing ability; however, sera of mice p.o. primed with live or killed EBs had significantly higher neutralizing ability than the sera of SC(K)-SC(K) mice or unimmunized controls (PBS-PBS mice) (Fig. 6C and F). In contrast, fecal extracts of mice from all groups failed to neutralize EBs to any significant extent (neutralization, $<40\%$). The lack of significant *in vitro* neutralizing activity for intestinal IgA might have been due to the extensive dilution of these antibodies in buffers during preparation of fecal extracts. Since *Chlamydia* does infect the GIT for prolonged periods in spite of the presence of IgA, IgA might not play an important role in *Chlamydia* clearance after the GIT is already infected. Moreover, it shows that secreted intestinal IgA does not prevent GIT infection following p.v. challenge, seen in PO(K)-SC(K) animals. This is likely because in these mice FRT-GIT *Chlamydia* spread occurs internally via migration of infected host cells (33).

p.o. immunization or infection induces IgA secretion in the FRT. Using enzyme-linked immunosorbent assay (ELISA), we failed to detect *Chlamydia*-specific IgA or IgG titers in vaginal washes collected throughout two studies. This could be due to collection of vaginal washes over 4 consecutive days (in 40 μl of PBS each day), which might have led to excessive dilution of mucosal secretions. Instead, Western blot analysis of serum and vaginal washes was used to detect *Chlamydia*-specific or major outer membrane protein (MOMP)-specific antibodies. As shown by ELISA, PO(L)-SC(K) mice developed serum IgA, IgG, and intestinal IgA after priming (day 28), boosting (day 42), and challenge (day 84) (Fig. 7A). Most importantly, these mice also developed *Chlamydia*-specific IgA in vaginal washes at all time points examined (Fig. 7A). PO(K)-SC(K) mice developed significant serum IgA and IgG only after p.v. challenge (day 84); however, they did develop intestinal and FRT IgA, much like PO(L)-SC(K) animals (Fig. 7A and B). SC(K)-SC(K) mice developed serum IgA and IgG at all time points examined (days 28, 42, and 84); however, they developed intestinal IgA only after p.v. challenge (day 84) and failed to develop detectable IgA in vaginal washes (Fig. 7C). Unimmunized control mice (PBS-PBS mice) had *Chlamydia*-specific serum IgA and IgG, as well as intestinal IgA, antibodies after the p.v. challenge (day 84). Similar to SC(K)-SC(K) mice, unimmunized controls had no detectable IgA in vaginal washes at any time point

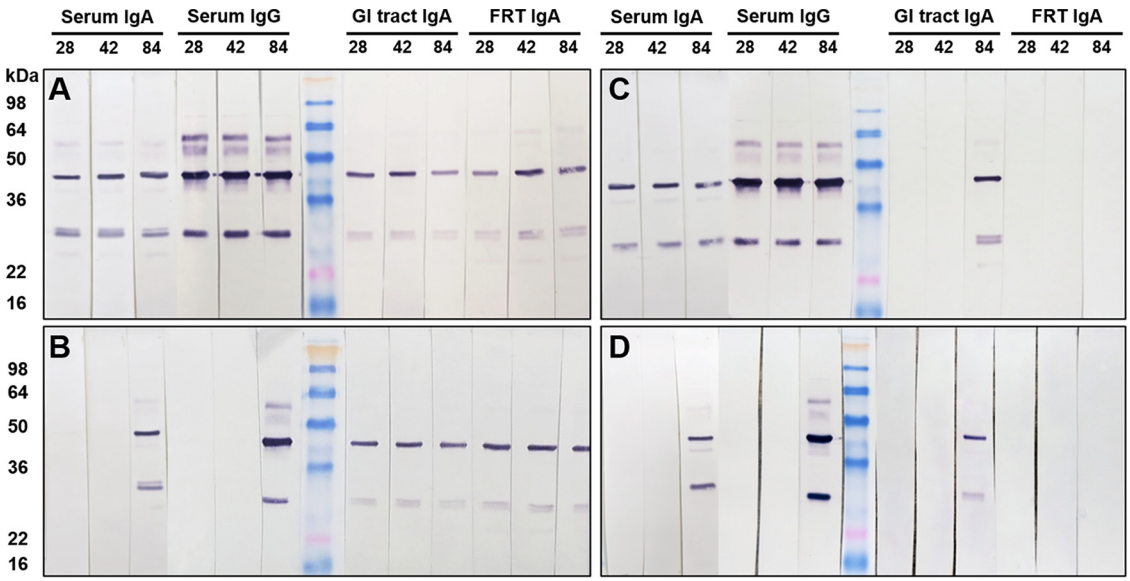

FIG 7 *Chlamydia*-specific serum, intestinal, and FRT antibodies at 4 weeks after priming (day 28), 2 weeks after boosting (day 42), and 6 weeks after p.v. challenge (day 84). For Western blot analysis, nitrocellulose membranes containing whole *Chlamydia* antigen were incubated with pooled serum or vaginal wash samples overnight. Serum or vaginal wash IgG or IgA antibodies bound to *Chlamydia* antigens on the membrane were detected with AP-conjugated rabbit anti-mouse IgG or IgA antibodies.

(Fig. 7D). Mice that were p.o. infected with *Chlamydia* without an s.c. boost [PO(L)-PBS mice] had serum and mucosal antibody profiles identical to those of PO(L)-SC(K) mice and were not included in the figure. A substantial amount of serum and mucosal antibodies appear to be specific for chlamydial MOMP antigen (molecular weight [MW], ~40 kDa). To confirm this, serum and vaginal washes collected after boosting (day 42) or challenge (day 84) from both studies were analyzed by Western blotting using *C. muridarum* MOMP antigen (37). As shown using whole EBs, PO(L)-SC(K), PO(L)-PBS, and SC(K)-SC(K) mice developed MOMP-specific serum IgG at day 42 and all mice developed serum IgG following a p.v. challenge (Fig. 8A and B). In contrast, all p.o.-primed animals [PO(L)-PBS, PO(L)-SC(K), and PO(K)-SC(K) mice] were positive for MOMP-specific FRT IgA at days 42 and 84, while SC(K)-SC(K) mice and unimmunized controls had no detectable MOMP-specific IgA in the FRT after boosting (day 42) or p.v. challenge (day 84) (Fig. 8C and D).

FRT IgA neutralizes *Chlamydia in vivo* and reduces its FRT to GIT dissemination. To examine the *in vivo* neutralizing ability of secreted IgA, *Chlamydia* EBs were incubated with vaginal washes collected from PO(L)-SC(K), PBS-PBS, and naive (unimmunized and unchallenged) mice and then were used to p.v. infect naive mice. Starting as early as 3 dpi and up to 7 dpi, naive mice infected with EBs incubated with vaginal wash of PO(L)-SC(K) mice exhibited significantly lower vaginal swab titers than did naive mice infected with EBs incubated in vaginal wash of naive or PBS-PBS (challenged) mice (Fig. 9A). Moreover, mice infected with EBs incubated with vaginal wash of PO(L)-SC(K) mice exhibited no enlargement of iliac lymph nodes (ILNs) (Fig. 9B) compared to those of mice infected with EBs incubated with vaginal wash of PBS-PBS and naive mice (Fig. 9C and D), indicating lower infectivity of neutralized EBs in the FRT resulting in reduced *Chlamydia* transport to and burden in the ILNs. Examination of *Chlamydia* titers in the ILNs, spleen, and GIT at 7 dpi revealed that mice infected with EBs in vaginal wash of PO(L)-SC(K) mice had lower *Chlamydia* burdens in the ILNs and spleen (although not statistically significant) and significantly lower *Chlamydia* titers in ceca (Fig. 9E to G). A second study of a similar design revealed that neutralization of EBs with vaginal wash of PO(L)-SC(K) mice reduced *Chlamydia* burden in the FRT of naive mice by ~1 log within 24 h ($P < 0.2$) and by ~1.5 to 2 logs by days 2 ($P < 0.0002$), 3 ($P < 0.0007$), and 7 ($P < 0.0001$) after p.v. challenge compared to EBs neutralized with

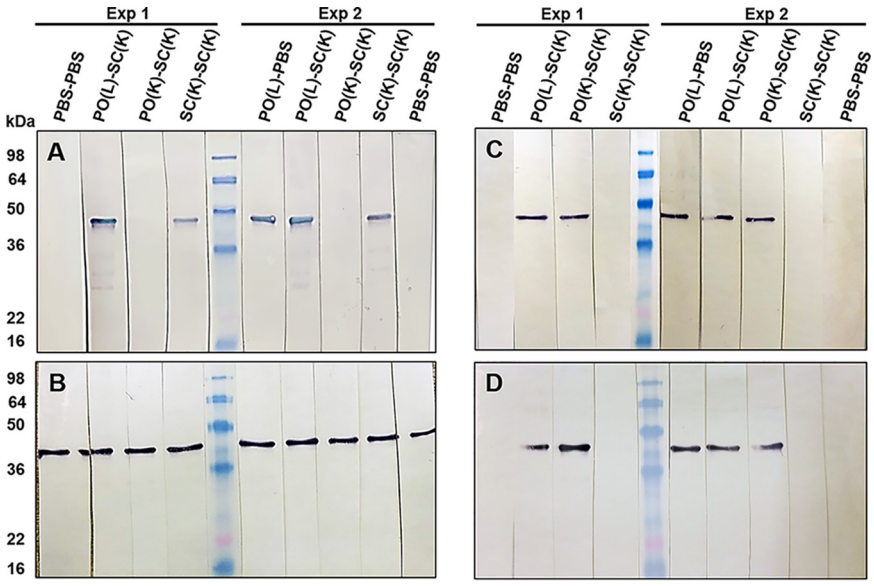

FIG 8 MOMP-specific antibodies in sera and vaginal washes of immunized and control mice 2 weeks after boosting (day 42) and 6 weeks after p.v. challenge (day 84). MOMP-specific serum IgG (A and B) and vaginal wash IgA (C and D) antibodies after boosting immunization (day 42) (A and C) and after p.v. challenge (day 84) (B and D) are shown. Membranes harboring MOMP antigen were incubated overnight with pooled sera or vaginal washes (collected from two studies), and then MOMP-specific IgG and IgA antibodies were detected with AP-conjugated rabbit anti-mouse IgG or IgA antibodies.

vaginal washes of PBS-PBS (unimmunized and challenged) or naive (unimmunized and unchallenged) mice (Fig. 9H). As in the first study, there were no differences in *Chlamydia* titers of ILNs and spleen at 7 dpi among groups (Fig. 9I and J). *Chlamydia* titers in ceca of mice infected with EBs neutralized in vaginal wash of PO(L)-SC(K) mice were significantly lower than titers of other groups (Fig. 9K).

DISCUSSION

The continuously increasing rates of STIs, including chlamydial infections worldwide, have brought a renewed attention and urgency for the development of effective vaccines to target these pathogens (38). Chlamydial infections are acquired mucosally; thus, an effective vaccine that induces mucosal (local) and systemic immunity would be ideal. However, the variability in immune responses in the FRT due to hormonal fluctuations and the lack of organized lymphoid tissues and antigen-sampling M cells render the FRT mucosa a poor site for immunization. Immunization via other mucosal sites, such as i.n. immunization with live EBs, does protect against *Chlamydia* challenge in the FRT and is routinely used as a control in animal vaccine studies (34, 39, 40). i.n. immunizations, however, may have detrimental effects on the central nervous system and may require adjuvants and multiple antigen administrations; thus, they are impractical and likely unsafe (41, 42). Therefore, p.o. immunization may be the most effective and practical route of immunization against this pathogen. In support of this notion are reports that in mice GIT infections with *Chlamydia* confer protection against p.v. and i.n. challenges (27, 43). Here, we show that within a week of i.n. inoculation, *Chlamydia* infects the GIT mucosa, which likely contributes to the protective immunity observed in i.n.-immunized mice (34, 39, 40). As shown by others (26, 27), infection of the GIT by *Chlamydia* does not present with signs of inflammation or pathology, while chlamydial FRT infections result in local inflammation (44), pathogen dissemination to the spleen and the GIT (33, 35, 45), and long-term upper FRT pathology, which is in large part attributed to the induction of "pathogenic" $CD8^+$ T cells (46, 47). The very distinct immune responses that occur at these mucosal sites (GIT versus FRT) led us to hypothesize that the initial encounter of the host with this pathogen determines the long-term outcome of infection. Thus, if following p.v. infection *Chlamydia* disseminates

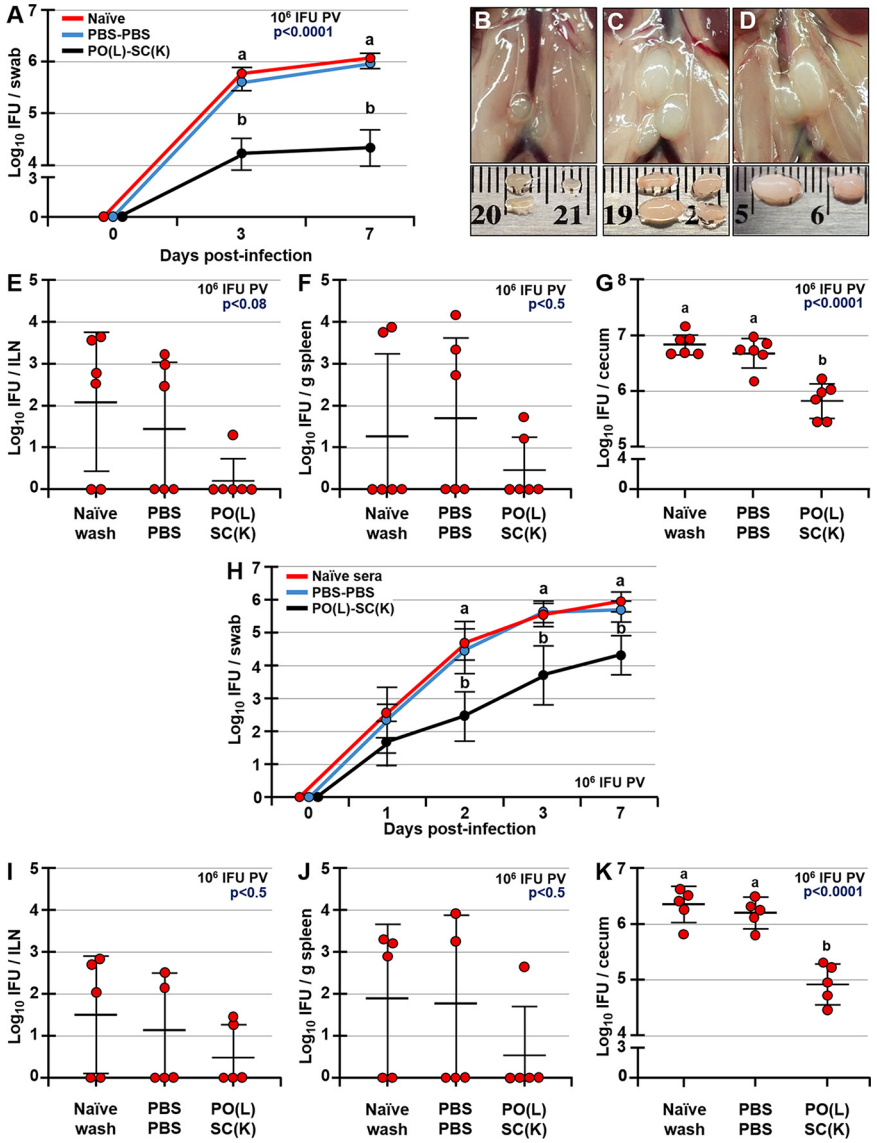

FIG 9 IgA antibodies in vaginal washes of PO-immunized mice neutralize *C. muridarum* EBs and reduce systemic *Chlamydia* spread. (A and H) *Chlamydia* titers in vaginal swabs of naive mice prior to p.v. infection (day 0) and at 1, 2, 3, and 7 dpi p.v. with 10^6 IFU of EBs that were neutralized with either vaginal wash of naive (unimmunized and unchallenged) mice or PBS-PBS (unimmunized and p.v. challenged) and PO(L)-SC(K) mice collected 6 weeks postchallenge (day 84). (B and D) Example images of ILNs of mice infected with EBs neutralized with vaginal washes of PO(L)-SC(K) (B), PBS-PBS (C), or naive (unimmunized and unchallenged) (D) mice collected 6 weeks post-p.v. challenge (day 84). (E to G and I to K) *Chlamydia* titers in ILNs, spleens, and ceca of naive mice at 7 dpi p.v. with neutralized EBs as described for panel A. Data are expressed as means ± SDs. Means were separated using Tukey's multiple-comparison procedures and are declared significantly different at a *P* value of <0.05 ($n = 6$ and $n = 5$). Means that do not share a lowercase letter are significantly different from each other.

systemically, pathogenic $CD8^+$ T cells are generated, which then contribute to the long-term disease sequelae. If, on the other hand, a host's first experience with *Chlamydia* is via the GIT mucosa, then infection of the spleen does not occur and immune responses induced in the GALT are protective in the FRT. Indeed, although GIT infections persist for prolonged periods, *Chlamydia* does not reach the spleen, indicating that the GALT serves as a firewall that limits *Chlamydia* systemic dissemination.

The importance of $CD4^+$ T cells in protection against *Chlamydia* challenge is well established. Mucosa-associated lymphoid tissues are the inducible sites of *Chlamydia*-specific CD4 T cells, which then provide protection to *Chlamydia* challenge in the FRT

(48). The role of B cells and antibodies, on the other hand, is poorly understood. The synergistic action of antibodies and $CD4^+$ T cells was demonstrated in early studies in which depletion of $CD4^+$ T cells prolonged the resolution of a secondary infection, while depletion of $CD4^+$ T cells in B cell-deficient mice resulted in failure resolve the secondary infection (15). B cells have been implicated in the expansion of *Chlamydia*-specific CD4 T cells in the FRT, as well as in *Chlamydia* systemic dissemination (49), which is inhibited by passive transfer of immune serum (50). Based on our previous work (28), we hypothesized that IgA secretion in the FRT is induced in the GALT and protects against primary *Chlamydia* infection and that *Chlamydia* need not be viable to induce immunity in the FRT (29, 30, 51). Prime-boost immunization strategies used in this study were based on reports that mucosal priming followed by systemic boosting confers superior protection against *Chlamydia* challenge (40) and enhances the magnitude and duration of antibody responses (28–30). We show that the type of *Chlamydia* antigen (live or inactivated/killed), the route of infection (p.o. or p.v.), and prime-boost immunization approaches affect the magnitude, diversity, and location of antibody secretion. In agreement with reports that serum antibodies do not protect against primary infection (14), we show that sera of SC(K)-SC(K) mice with an antigen cognition profile and neutralizing ability similar to those of sera of PO(L)-SC(K) animals (Fig. 7) conferred no protection against p.v. challenge. In addition, PO(K)-SC(K) mice had no substantial serum antibodies prior to challenge yet exhibited significantly lower *Chlamydia* burden in the FRT, starting at 3 dpi, which is an insufficient time for induction of serum antibodies.

As expected, all p.o.-primed mice developed *Chlamydia*-specific intestinal IgA (26, 27), which did not clear *Chlamydia* from the GIT, as there were no differences in cecal *Chlamydia* titers among groups (Fig. 4A and B). In contrast, unimmunized controls and SC(K)-SC(K) mice developed intestinal IgA only after p.v. challenge (Fig. 3, 7, and 8). Most importantly, all p.o.-immunized mice also had *Chlamydia*-specific IgA in vaginal washes after priming, and in contrast, unimmunized and SC(K)-SC(K) mice had no detectable *Chlamydia*-specific IgA at any time point (Fig. 7 and 8). Others reported the presence of *Chlamydia*-specific IgA in vaginal washes within 1 to 4 weeks of infection (52–54), which is in apparent disagreement with our results. This could be because in these studies, ELISAs were used for measuring IgA (53, 54), which in our hands lacked the sensitivity to detect low IgA concentrations in diluted vaginal secretions and was unreliable. While in one study (54), the presence of *Chlamydia*-specific IgA in vaginal washes was shown by Western blotting at 50 dpi p.v., others did not examine the specificity of IgA in p.v.-infected mice (52); thus, it is difficult to compare our results with the results of these particular studies. If *Chlamydia*-specific IgA is present in the FRT a few weeks after an infection, its concentrations are likely very low and biologically insignificant for protection against primary infection. Western blot analysis, used in this study, allows detection of small amounts of antibodies, as well as the specificity of antibodies to *Chlamydia* antigens. Importantly, *Chlamydia*-specific IgA profiles in the FRT prior to p.v. challenge mirror the intestinal IgA profiles, indicating that GALT is the inductive site for IgA secretion in the FRT. Our report that p.v. antigen administration induces intestinal, but not FRT, IgA antibodies (28), as observed in SC(K)-SC(K) and unimmunized mice after p.v. challenge, supports this notion. In unimmunized controls, *Chlamydia* disseminates internally to the GIT (33), thus inducing serum IgA and IgG, as well as IgA secretion in the GIT. Serum IgA is not the source of intestinal IgA, as SC(K)-SC(K) mice developed serum IgA after priming yet exhibited intestinal IgA only after p.v. *Chlamydia* challenge (Fig. 7C). The lack of detectable IgA in FRT secretions of SC(K)-SC(K) mice, in spite of IgA presence in serum, and the presence of IgA in FRT secretions of PO(K)-SC(K) mice, in spite of the lack of serum IgA, indicates that IgA is produced by local B cells in the FRT. Others have shown that expression of polymeric immunoglobulin receptor (pIgR), IgA, and IgG secretion in the FRT is upregulated during *Chlamydia* infection (52). However, since the specificity of IgA and IgG antibodies was not examined, their significance for *Chlamydia* neutralization or clearance is unclear. The lack of *Chlamydia*-specific IgA in vaginal washes of unimmunized mice

after p.v. infection additionally explains why B cell- and IgA-deficient mice clear primary *Chlamydia* infection at rates similar to those of wild-type mice (15, 20).

We argue that secreted *Chlamydia*-specific IgA in the FRT is critical for protection against *Chlamydia* infection. Neutralization of *Chlamydia* EBs in the FRT by IgA is the likely reason for the drastic reduction of the infectious burden in naive mice as early as 1 to 3 dpi. In addition, neutralization of EBs hinders systemic dissemination of *Chlamydia*, reflected in significantly reduced *Chlamydia* titers in ceca at 7 dpi. Neutralization of *Chlamydia* in the FRT by secreted IgA can additionally inhibit *Chlamydia* ascension to the upper FRT, thus diminishing or preventing the inflammatory response and tissue damage. In women, *C. trachomatis*-specific IgA found in FRT secretions is critically important for pathogen clearance (22), which is in agreement with our findings. This is not to suggest that $CD4^+$ T cells are not critical for protective immunity, including humoral immunity provided by B cells in the form of antibodies.

Our findings can be summed up in four main conclusions: (i) infection via the GIT or the FRT mucosa induces *Chlamydia*-specific serum IgG, with p.o. infection inducing primarily IgG2c and p.v. infection inducing IgG1 and IgG2c; (ii) p.o. immunization or infection induces IgA secretion in the GIT and the FRT; (iii) p.v. infection induces serum and intestinal IgA but fails to induce IgA secretion in the FRT; and (iv) secreted FRT IgA antibodies neutralize *Chlamydia* and are critically important for protection against *Chlamydia* challenge. Mucosal sites differ in their physiological functions, and consequently, the immune responses reflect these functions. Unlike the FRT (33), GALT halts the systemic dissemination of *Chlamydia* and induces IgA secretion locally (in the GIT) and at a distant site (the FRT). Thus, GALT appears to be the bona fide mucosal site for induction of IgA secretion in the FRT. Secreted IgA neutralizes *Chlamydia* in the FRT, thus lowering its infectivity, accelerating its clearance, and reducing its systemic spread. Importantly, infection with and replication of *Chlamydia* in the GIT are not necessary for induction of protective immunity in the FRT. This work will open the doors for the development of p.o. subunit vaccines to target sexually transmitted pathogens, as well as for beginning to understand how the GALT regulates the immune responses in the FRT.

MATERIALS AND METHODS

Ethics statement. All animal experiments were carried out in strict accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals* (55). All procedures involving animals were approved by the Southern Illinois University's Institutional Animal Care and Use Committee (protocols 15-035 and 15-041).

Animals and housing. Six- to 8-week-old C57BL/6 female mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in ventilated cages under specific-pathogen-free conditions with food and water provided *ad libitum*. To prevent infection of unimmunized controls and mice p.o. immunized with killed EBs, cages were changed one at a time under a biosafety flow hood.

Cell and bacterial culture conditions. HeLa 229 cells (human cervical carcinoma epithelial cells) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (DMEM-10) and 50 μg/ml of gentamicin in a humidified incubator at 37°C and 5% CO_2. *Chlamydia muridarum* strain Nigg was propagated in and purified from HeLa 229 cells as described previously (56). Confluent HeLa cell monolayers were infected with *C. muridarum* by centrifugation for 1 h at 545 $\times$ *g* and room temperature (RT) (or by rocking for 2 h at 37°C) and incubated in infection medium (DMEM-10 supplemented with nonessential amino acids and 1 μg/ml of cycloheximide). At 40 to 42 h postinfection, HeLa cells were ruptured by brief sonication and cell debris was pelleted by low-speed centrifugation (500 $\times$ *g* for 10 min at 4°C). EBs were pelleted from the supernatant by high-speed centrifugation (10,000 $\times$ *g* for 30 min at 4°C) and purified on a Percoll gradient by centrifugation at 30,000 $\times$ *g*, as described previously (57). All *C. muridarum* EB stocks were stored at −80°C in sucrose-phosphate-buffered glutamic acid (SPG) until used.

Inactivation of *C. muridarum*. Gradient-purified *C. muridarum* EBs were thawed on ice and then fixed in 4% paraformaldehyde (PFA) for 15 min. Fixed EBs were pelleted by high-speed centrifugation (13,000 $\times$ *g*) and washed three times in PBS. After the final wash, EBs were resuspended in PBS and sonicated on ice in order to generate smaller nanoparticle-sized antigen. To confirm that EBs were not infectious, aliquots of 200 μl (containing 1×10^8 IFU) taken from the prepared stock (which had 5×10^8 IFU/ml) were used to infect HeLa cell monolayers in triplicates, and the infection was expanded six times. No IFU were detected in HeLa cell monolayers infected with PFA-inactivated *C. muridarum* EBs.

Immunization of mice with *C. muridarum*. Two studies were conducted in which mice were p.o. or s.c. primed with either viable (live) or inactivated (killed) *C. muridarum* EBs. In the first study, mice were fasted for 4 h and p.o. fed 1×10^6 IFU of live or 1×10^7 IFUs of killed *C. muridarum* in 20 μl of SPG (via

a pipette tip). In the second study the same doses of live or killed *Chlamydia* EBs were administered via a gastric gavage using a round-tip needle to ensure immunization via GALT and not sublingually. For s.c. immunizations, mice were injected with 1×10^7 IFU of killed *Chlamydia* on their backs. To examine the dynamics of GIT infection, separate groups of mice were infected via gastric gavage with 1×10^7 or i.n. with 1×10^4 IFU of *Chlamydia*. For i.n. infections, mice were lightly anesthetized and infected with a total volume of 5 μl by applying 2.5 μl of the inoculum to each nare with a pipette tip.

Per-vaginal *C. muridarum* challenge. In order to synchronize their estrus cycles, mice were s.c. injected with 2.5 mg of medroxyprogesterone acetate (MPA; Henry Schein) 5 days prior to p.v. challenge with 2×10^5 *C. muridarum* IFU delivered in 10 μl of SPG (at day 42 post-priming immunization). To monitor *Chlamydia* shedding, vaginal swabs were collected prior to p.v. infection, 3 days after the infection, and weekly thereafter until the study was terminated. To ensure consistency in immunization and p.v. challenge doses, all animals were primed, boosted, and p.v. challenged from a single, pretitered *C. muridarum* stock.

Collection of sera, fecal extracts, and vaginal washes. Blood, fecal extracts, and vaginal washes were collected prior to immunization and weekly thereafter for the duration of the studies. Blood was collected via the tail vein, and plasma was separated by centrifugation. Fecal extracts were prepared as described previously (28, 29) and stored at −20°C until analysis. Vaginal washes were collected in 40 μl of PBS daily over 4 days. Samples were first collected from unimmunized controls (PBS-PBS mice), PO(K)-SC(K) mice, and then from groups that were p.o. infected with live *Chlamydia* [PO(L)-PBS and PO(L)-SC(K) mice]. This was done to preclude accidental infection of unimmunized controls (PBS-PBS mice) and PO(K)-SC(K) mice prior to p.v. challenge.

Determination of *Chlamydia*-specific serum and mucosal antibody titers and Western blot analysis of antibody specificity. Antibody titers in sera, fecal extracts, and vaginal washes of mice were measured using ELISAs. EBs were rendered nonviable as described above, and flat-bottomed 96-well plates were coated with EB antigen at 10 μg/ml suspended in ELISA coating buffer. Coated plates were incubated overnight at 4°C. Unbound *Chlamydia* antigen was then removed from the wells, and plates were blocked for 1 h at 37°C with 200 μl of ELISA blocking buffer (0.2% porcine gelatin in 1× PBS). Plates were then washed with ELISA wash buffer before the addition of samples (serum, vaginal washes, or fecal extracts). Following an overnight incubation, plates were washed and monoclonal alkaline phosphatase (AP)-conjugated goat anti-mouse IgG1, IgG2c, or IgA antibodies diluted in blocking buffer were added and allowed to incubate at RT for 2 h. Plates were washed again in ELISA wash buffer, and AP activity was determined as described previously (28, 29, 58). Antibody titers are expressed as $\log_{10}$ values of the highest serial dilution that yielded an optical density at 405 nm (OD_{405}) that was twice that of negative controls. The presence and specificity of antibodies in sera, fecal extracts, and vaginal washes were also determined by Western blot analysis, for which 10 μg of gradient-purified *Chlamydia* EB protein was loaded per well. Similarly, sera, intestinal extracts, and vaginal washes were examined for the presence of *C. muridarum* MOMP.

Determination of *C. muridarum* titers in tissues. Tissues were excised aseptically from mice at different times postinfection and placed in sterile tubes containing SPG. Tissues were then processed and *Chlamydia* titers determined as previously described (26, 33, 59). Titers are expressed as $\log_{10}$ IFU per vaginal swab, uterus, ovary/oviduct, cecum, or iliac lymph node (ILN) or per g of spleen. *Chlamydia*-negative samples were expanded for six passages to ensure that low titers could be detected. Negative cultures at passage 1 remained negative throughout the 6 expansions. All reported titers are from the first passage.

Evaluation of FRT pathology. The FRTs were excised aseptically, imaged, and scored in order to evaluate the extent of pathology according to an ordinal scale as described previously (60). Hydrosalpinx severity scores are reported as average bilateral scores per mouse in all treatment groups.

***In vitro C. muridarum* neutralization assays.** Plasma and fecal extracts from immunized and naive mice were assayed for *in vitro* antibody-mediated neutralization. In order to exclude any fecal debris, fecal extracts were passed through a premoistened 0.45-μm filter. Briefly, fecal extracts or complement-inactivated sera were incubated with 1×10^3 *C. muridarum* IFU for 1 h at 37°C and 5% CO_2 prior to infection of monolayers in 96-well plates (61). At 24 h postinfection (hpi), cells were fixed in ice-cold absolute methanol and IFU were numerated. Percentage neutralization is reported as percent reduction of IFU by a test serum compared to naive sera or fecal extracts.

***In vivo C. muridarum* neutralization.** To examine whether antibodies found in vaginal washes can neutralize *Chlamydia in vivo*, EBs were incubated on ice for 30 min with pooled vaginal washes of naive mice (unimmunized and unchallenged), PBS-PBS mice (unimmunized and challenged) or PO(L)-SC(K) mice, collected at 6 weeks postchallenge (day 84). After incubation, 10 μl of EB and vaginal wash mixture containing 1×10^6 IFU of *Chlamydia* was used to p.v. infect groups of 6 naive mice. Vaginal swabs were collected at 3 and 7 days postinfection (dpi), at which time tissues were collected for examining whether neutralization affected *Chlamydia* dissemination to the GIT. For this study, mice were housed in wire mesh cages and were fitted with neck collars in order to preclude GIT infection via grooming as described previously (33). An identical study was conducted to examine the *in vivo C. muridarum* neutralization at early stages of infection (days 1 to 7).

Staining and analysis of frozen tissue sections by IFM. Excised tissues were snap-frozen in Tissue-Tek OCT compound (Sakura Finetek) on dry ice and stored at −80°C. Cryosections (5 to 7 μm thick) were fixed in 4% PFA and stained with monoclonal antibodies and actin-binding phalloidin-Alexa Fluor 350 for 1 to 2 h. All antibodies were used at a dilution of 1:100. Stained tissue sections were washed in PBS and mounted using Fluoromount-G (Southern Biotech), and images were acquired and analyzed as described previously (30, 62).

Statistical analysis. All data were analyzed using analysis of variance (ANOVA) procedures and SAS software. Population means were separated using Student's *t* test or Tukey's multiple-comparison procedures and were declared significantly different at a *P* value of <0.05.

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