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Antigen-Specific CD4+ T Cell-Derived Gamma Interferon Is Both Necessary and Sufficient for Clearing *Chlamydia* from the Small Intestine but Not the Large Intestine

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ABSTRACT The genital tract pathogen *Chlamydia trachomatis* is frequently detected in the gastrointestinal tract, but the host immunity that regulates chlamydial colonization in the gut remains unclear. In a *Chlamydia muridarum*-C57 mouse model, chlamydial organisms are cleared from the genital tract in ~4 weeks, but the genital organisms can spread to the gastrointestinal tract. We found that the gastrointestinal chlamydial organisms were cleared from the small intestine by day 28, paralleling their infection course in the genital tract, but persisted in the large intestine for long periods. Mice deficient in α/β T cells or CD4+ T cells but not CD8+ T cells showed chlamydial persistence in the small intestine, indicating a critical role for CD4+ T cells in clearing *Chlamydia* from the small intestine. The CD4+ T cell-dependent clearance is likely mediated by gamma interferon (IFN-γ), since mice deficient in IFN-γ but not interleukin 22 (IL-22) signaling pathways rescued chlamydial colonization in the small intestine. Furthermore, exogenous IFN-γ was sufficient for clearing *Chlamydia* from the small intestine but not the large intestine. Mice deficient in developing *Chlamydia*-specific Th1 immunity showed chlamydial persistence in the small intestine. Finally, IFN-γ-producing CD4+ but not CD8+ T cells from immunized donor mice were sufficient for eliminating *Chlamydia* from the small intestine but not the large intestine of recipient mice. Thus, we have demonstrated a critical role for Th1 immunity in clearing *Chlamydia* from the small intestine but not the large intestine, indicating that chlamydial colonization in different regions of the gastrointestinal tract is regulated by distinct immune mechanisms.

KEYWORDS *Chlamydia*, gamma interferon, Th1 immunity, genital tract immunity, small intestine immunity

Chlamydia trachomatis is a major sexually transmitted bacterial pathogen which may cause pathologies in the upper genital tract (1–4). The *Chlamydia muridarum* mouse model has been used for investigating *C. trachomatis* pathogenesis due to its ability to induce long-lasting tubal fibrosis/hydrosalpinx in mice following an intravaginal inoculation (5–10). Using this mouse model, it has been demonstrated that a Th1-dominant immunity is required for controlling chlamydial infection in the genital tract (11). In most cases, wild-type C57 mice significantly reduce chlamydial organism burden in the genital tract on day 21 and clear chlamydial infection by day 28 following an intravaginal inoculation. Mice deficient in CD4+ T cells or major histocompatibility

complex (MHC) class II antigen presentation showed significantly prolonged *C. muridarum* infection in the genital tract, while mice deficient in CD8$^+$ T cells or MHC class I antigen presentation cleared *C. muridarum* from the genital tract as robustly as wild-type mice (6). Consistently, mice deficient in interleukin 12 (IL-12) (12) or gamma interferon (IFN-γ) (13), which are compromised in developing Th1 adaptive immunity, showed chlamydial persistence and spreading.

C. trachomatis is also frequently detected in the human gastrointestinal (GI) tract (14–17). Although its medical significance remains unclear, it has been proposed that GI tract *C. trachomatis* may serve as a reservoir for promoting *C. trachomatis* pathogenicity in the genital tract (18, 19). *C. muridarum* has been shown to readily spread to the GI tract via the blood circulatory system (20), since mice wearing Elizabethan collars and singly housed in netted cages to prevent coprophagy still showed spreading of genital *C. muridarum* to the GI tract. Furthermore, hematogenous *C. muridarum* can establish long-lasting colonization in the mouse GI tract (21). The spreading of genital *Chlamydia* to the GI tract has been proposed to induce a 2nd hit to promote chlamydial pathogenicity in the upper genital tract (22). Orally delivered *C. muridarum* is known to colonize the mouse GI tract for long periods (18, 23–26). Although it is unclear whether genital *C. trachomatis* can also spread to the human GI tract, human sexual behavior should be sufficient for orally delivering *C. trachomatis* into the GI tract. Regardless of how *C. trachomatis* is delivered to the GI tract, it is likely that *C. trachomatis* is able to productively colonize the human GI tract, since live *C. trachomatis* organisms have been repeatedly detected on human rectal swabs (15, 16, 27–31). It will be interesting to reveal how *C. trachomatis* tumbles through the human GI tract. Investigating how mouse GI tract immune mechanisms regulate *C. muridarum* colonization may provide useful information for guiding clinical studies.

On the other hand, if a naive mouse is first exposed to *C. muridarum* in the GI tract, the GI *Chlamydia* can act as an oral vaccine to induce protective immunity against subsequent infection with *Chlamydia* in the genital tract (32) or airway (33). It will be interesting to evaluate whether women whose rectal swabs are positive for *C. trachomatis* experience reduced rates of repeated infections in their genital tracts. Thus, revealing the host immune mechanisms responsible for regulating chlamydial colonization in the GI tract may also provide useful information for developing an oral *Chlamydia* vaccine.

In the current study, we have used a *C. muridarum* mouse model to investigate mechanisms of *C. muridarum* colonization in the GI tract. In C57 mice, chlamydial organisms were cleared from the small intestine by day 28 after infection but persisted in the large intestine for long periods. Mice deficient in T cell receptor alpha (TCRα) or CD4$^+$ T cells but not CD8$^+$ T cells showed chlamydial persistence in the small intestine, indicating a critical role for CD4$^+$ T cells in clearing *Chlamydia*. CD4$^+$ T cell-dependent clearance is likely mediated by IFN-γ, since mice deficient in IFN-γ but not IL-22 signaling showed rescue of chlamydial colonization in the small intestine. Furthermore, supplementing TCRα-deficient mice with exogenous IFN-γ or IFN-γ-producing CD4$^+$ but not CD8$^+$ T cells from immunized donor mice was sufficient for eliminating *Chlamydia* from the small intestine but not the large intestine. Thus, we have demonstrated that Th1 immunity is both necessary and sufficient for clearing *Chlamydia* from the small intestine. However, the same Th1 immunity had no impact on chlamydial colonization in the large intestine, suggesting that chlamydial colonization in different regions of the mouse gastrointestinal tract may be regulated by different immune mechanisms. The mechanistic information not only will promote our understanding of how orally delivered *C. trachomatis* interacts with human gastrointestinal tract but also may be useful in developing an oral chlamydial vaccine.

RESULTS

Chlamydia is cleared from the small intestine but persists in the large intestine for a long period. We have previously shown that following an intravaginal inoculation, *C. muridarum* can spread to and colonize in the GI tract for long periods (20),
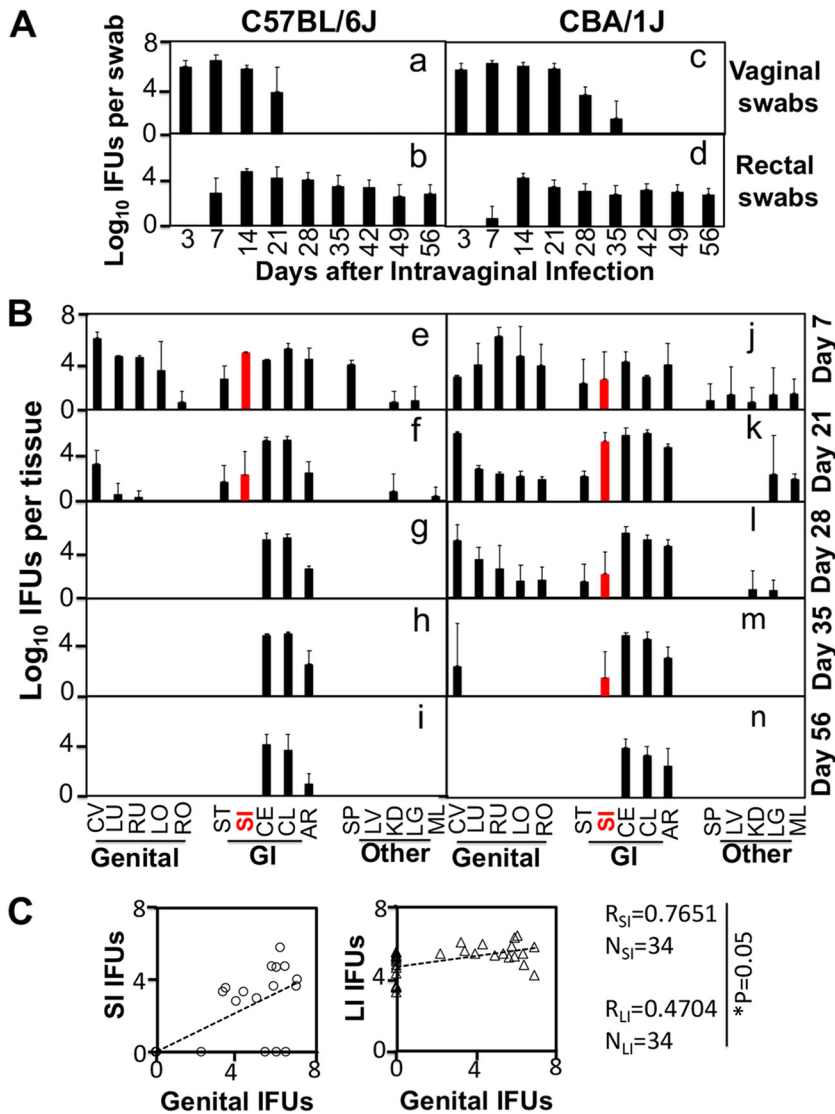
FIG 1 *Chlamydia muridarum* colonization in the small intestine but not large intestine parallels its infection course in the genital tract. (A) C57BL/6J ($n = 5$) or CBA/1J ($n = 6$) mice were intravaginally infected with *C. muridarum* at 2×10^5 inclusion-forming units (IFU) per mouse. At various time points postinfection as indicated on the x axis, both vaginal (a and c) and rectal (b and d) swab specimens were taken for titration of live organisms. The recovered live organisms were expressed as $\log_{10}$ IFU per swab, as displayed on the y axis. Note that vaginal swabs were cleared of IFU on days 28 for C57 and 42 for CBA mice, but rectal swab IFU were present throughout the experiments in both strains. (B) Parallel groups of mice were sacrificed for monitoring live organisms in various tissues on days 7 (e and j), 21 (f and k), 28 (g and l), 35 (h and m), and 56 (i and n) after infection as indicated on the right. The recovered live chlamydial organisms were expressed as $\log_{10}$ IFU per tissue. The assayed organs/tissues include genital tract tissues, i.e., cervix-vagina (CV), left uterine horn (LU), right uterine horn (RU), left oviduct/ovary (LO), and right oviduct/ovary (RO), gastrointestinal (GI) tract tissues, i.e., stomach (ST), small intestine (SI), cecum (CE), colon (CL), and anorectum (AR), and other tissues, such as spleen (SP), liver (LV), kidney (KD), lung (LG), and mesenteric lymph node (ML). $n = 3$ to 5 for each time point, and the data came from three independent experiments. Note that IFU from SI (highlighted in red) parallel IFU from the genital tract in both strains of mice. (C) Total IFU from all genital tissues of a given mouse harvested at a given time point as shown in panel B (genital IFU on the x axis) were correlated with IFU from small intestine tissues (SI IFU on the y axis, left panel) and large intestine tissues (LI IFU on the y axis, right panel) using Spearman's correlation analysis. Note that chlamydial IFU burdens in the genital tract correlate with IFU recovered from the small intestine ($R_{SI} = 0.7651$) significantly better than those from the large intestine ($R_{LI} = 0.4704$). N_{SI} or N_{LI}, number of small intestine or large intestine tissue specimens, respectively.

although the genital *C. muridarum* is cleared within 4 to 5 weeks in most mouse strains (10). We compared *C. muridarum* organism distributions in tissues of both C57BL/6J and CBA/1J mice following intravaginal inoculation (Fig. 1). In C57BL/6J mice, *C. muridarum* live organism burdens in both the genital tract and small intestine were significantly
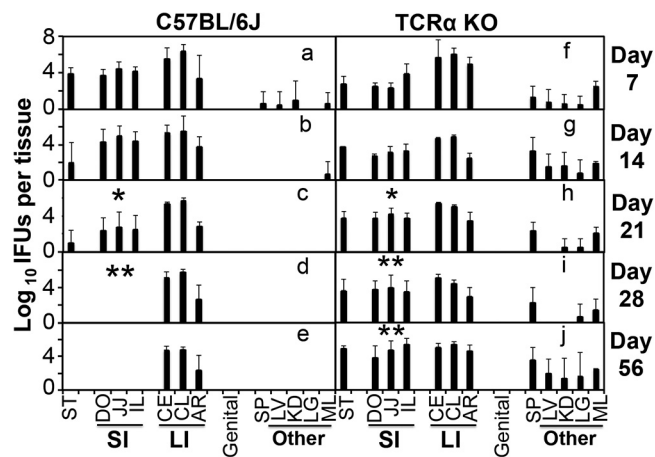
FIG 2 *Chlamydia muridarum* colonization in the gastrointestinal tract of mice deficient in α/β T cells. Mice without (a to e) or with deficiency in the T cell receptor alpha gene (TCRα KO [f to j]) were intragastrically inoculated with *Chlamydia muridarum* at 2×10^5 IFU per mouse. On days 7, 14, 21, 28, and 56 after infection, as indicated on the right side of each panel, groups of 3 to 5 mice were sacrificed for monitoring live chlamydial organisms in various tissues, including stomach, segments of small intestine such as duodenum (DO), jejunum (JJ), and ileum (IL), large intestine tissues such as cecum, colon, and anorectum, genital tract tissue, and other tissues, such as spleen, liver, kidney, lung, and mesenteric lymph node, as indicated on the x axis. The number of live organisms recovered from each tissue sample was expressed as $\log_{10}$ IFU. Note that C57BL/6J mice showed significantly reduced IFU in small intestine by day 21 and cleared IFU by day 28, while TCRα KO mice maintained significant levels of small intestine IFU throughout the experiment. The large intestine IFU remained steadily high in both strains. *, $P < 0.05$; **, $P < 0.01$ (Wilcoxon, panel c versus panel b, panel d versus panel b, panel h versus panel c, panel i versus panel d, and panel j versus panel e). Data were acquired from 3 independent experiments.

reduced on day 21 and completely cleared by day 28. In CBA/1J mice, *C. muridarum* burdens in both the genital tract and small intestine were significantly reduced on day 28 and completely cleared by day 56. However, the *C. muridarum* live organism burdens in the large intestine of both mouse strains remained steady throughout the experiment. These observations suggest that different segments of the GI tract may have evolved distinct mechanisms for controlling chlamydial colonization. The small intestine may use a mechanism similar to that used by the genital tract for controlling chlamydial colonization, while the large intestine is unable to restrict chlamydial colonization at all. Indeed, inclusion-forming units (IFU) recovered from genital tissues correlated significantly better with those from small intestine tissues than large intestine tissues (Fig. 1C). It will be interesting to determine the immune mechanism(s) responsible for the differential clearance of *Chlamydia* from the GI tract.

CD4+ but not CD8+ T cells are responsible for clearing *Chlamydia* from the small intestine. When mice deficient in T cell receptor alpha gene (TCRα knockout [KO]) were intragastrically inoculated with *C. muridarum*, the chlamydial organisms remained in the small intestine throughout the experiment (Fig. 2), suggesting that TCRα/β-bearing T lymphocytes are essential for clearing the small intestine of *Chlamydia*. To further define the subsets of T cells required for clearance, we compared mice with and without deficiency in CD4+ or CD8+ T cells for the ability to clear chlamydial colonization in the small intestine on day 28 after intragastric inoculation (Fig. 3). Mice deficient in CD4+ T cells showed a robust colonization by *Chlamydia* in all tissue segments of the small intestine, while both wild-type and CD8+ T cell-deficient mice completely cleared *Chlamydia* from these tissue segments. Furthermore, depletion with an anti-CD4 antibody made the CD8 KO mice susceptible to chlamydial colonization in the small intestine. These observations together demonstrated that CD4+ T cells but not CD8+ T cells are essential for clearing chlamydial colonization from the small intestine. However, regardless of the gene deficiency and/or antibody depletion, chlamydial colonization in the large intestine of the same mice remained steady, suggesting that the large intestine chlamydial organisms are regulated by different immune mechanisms.
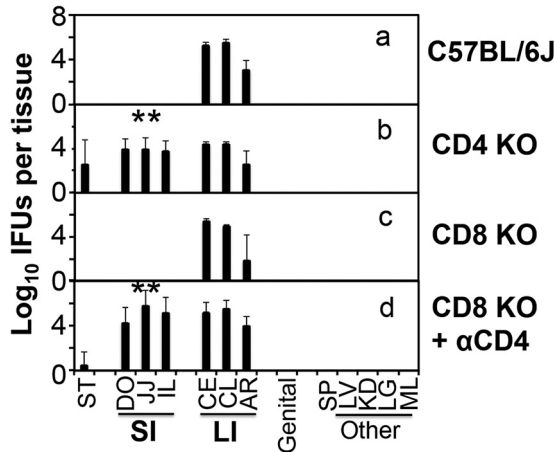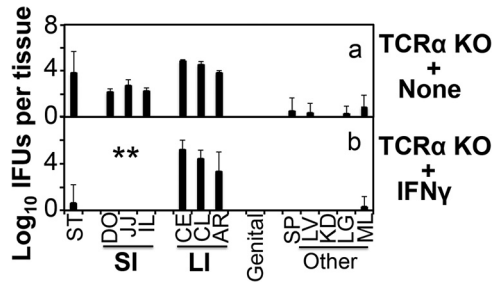
FIG 3 *Chlamydia muridarum* colonization in the gastrointestinal tract of mice deficient in CD4^{+} or CD8^{+} T cells. Mice without (a; $n = 4$) or with deficiency in CD4^{+} T cells (CD4 KO) (b; $n = 3$) or CD8^{+} T cells (CD8 KO) (c and d; $n = 4$ for each panel) were intragastrically inoculated with *Chlamydia muridarum* at 2×10^5 IFU per mouse. Some CD8^{+} KO mice were also treated with an anti-CD4 antibody (d). On day 28 after infection, all mice were sacrificed for monitoring live chlamydial organisms in various tissues as described in the Fig. 2 legend and shown on the x axis. The number of live organisms recovered from each tissue sample was expressed as $\log_{10}$ IFU. Note that mice deficient in CD4^{+} but not CD8^{+} T cells were defective in clearing chlamydial colonization in the small intestine and that depletion with an anti-CD4 antibody made the CD8 KO mice susceptible to chlamydial colonization in the small intestine. **, $P < 0.01$ (Wilcoxon, panel b versus panel a and panel d versus panel a or c). Data were acquired from 3 independent experiments.

IFN-γ-mediated immunity is sufficient for clearing *Chlamydia* from the small intestine but not the large intestine. We next compared mice with and without deficiencies in IFN-γ receptor (IFN-γR) or IL-22 for the ability to clear *Chlamydia* from GI tissues (Fig. 4). The wild-type C57 mice completely cleared chlamydial organisms from the small intestine on day 28 after an intragastrical inoculation with *C. muridarum*. However, mice deficient in IFN-γR were defective in clearing chlamydial organisms from the small intestine and elsewhere, demonstrating that chlamydial clearance is dependent on the IFN-γ signaling pathway. However, mice deficient in IL-22 cleared *Chlamydia* from the small intestine as efficiently as the wild-type mice, indicating that the IL-22 signaling pathway is not required. Furthermore, when exogenous IFN-γ was
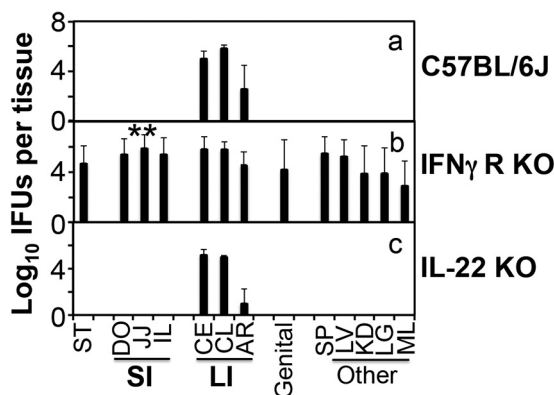
FIG 4 *Chlamydia muridarum* colonization in the gastrointestinal tract of mice deficient in IFN-γ receptor or IL-22. Mice without (a; $n = 4$) or with deficiency in IFN-γ receptor (b; $n = 8$) or IL-22 (c; $n = 4$) were intragastrically inoculated with *C. muridarum* at 2×10^5 IFU per mouse. On day 28 after infection, all mice were sacrificed for monitoring live chlamydial organisms in various tissues as described in the Fig. 2 legend and shown on the x axis. The number of live organisms recovered from each tissue sample was expressed as $\log_{10}$ IFU. Note that mice deficient in IFN-γR but not IL-22 were defective in clearing *Chlamydia*. **, $P < 0.01$ (Wilcoxon, panel b versus panel a or c). Data were acquired from 3 independent experiments.

FIG 5 Inhibition of *Chlamydia muridarum* colonization in the small intestine of TCRα KO mice by exogenous IFN-γ. TCRα KO mice not receiving (panel a, *n* = 4) or receiving injection of exogenous IFN-γ (b; *n* = 5) were intragastrically inoculated with *C. muridarum* at 2×10^5 IFU per mouse. On day 28 after infection, all mice were sacrificed for monitoring live chlamydial organisms as described in the Fig. 2 legend and shown on the *x* axis. The number of live organisms recovered from each tissue sample was expressed as $\log_{10}$ IFU. Note that exogenous IFN-γ cleared IFU from the small intestine but not large intestine of TCRα KO mice. **, *P* < 0.01 (Wilcoxon, panel b versus panel a). Data were acquired from 2 independent experiments.

provided to TCRα-deficient mice, chlamydial organisms were cleared from the small intestine but not large intestine (Fig. 5), demonstrating that IFN-γ is sufficient for eliminating *Chlamydia* from the small but not large intestine.

Antigen-specific Th1 immunity is sufficient for clearing *Chlamydia* from the small intestine but not the large intestine. The observations that CD4+ T cells and IFN-γ were both essential for clearing *Chlamydia* from mouse small intestine suggest a critical role for a Th1-dominant adaptive immunity in the clearance. We next compared mice with and without deficiency in IL-12p35 for the ability to clear *Chlamydia* (Fig. 6). Mice deficient in IL-12p35 are significantly defective in developing Th1 immunity against *C. muridarum* infection in the genital tract (12). We indeed found that IL-12p35-deficient mice were susceptible to chlamydial colonization in the small intestine on day 28 after infection, when *Chlamydia* was completely cleared from the small intestine of wild-type C57 mice. Furthermore, OT2 mice (with TCR on all CD4+ T cells engineered to recognizing an ovalbumin epitope only and unable to develop chlamydial-antigen-specific CD4+ T cells) also failed to clear *Chlamydia* in the small intestine, indicating that chlamydial-antigen-specific CD4+ T cells are required for clearance.

Finally, we used an adoptive transfer approach for testing whether *Chlamydia*-induced IFN-γ-producing CD4+ T cells are sufficient for controlling chlamydial infection
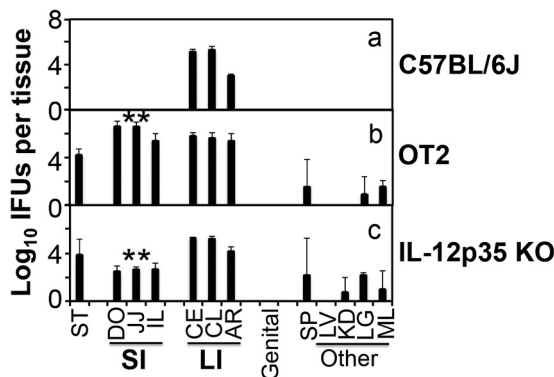
FIG 6 *Chlamydia muridarum* colonization in the small intestine of mice deficient in developing Th1-dominant adaptive immunity. Mice without (a; *n* = 3) or with TCR on all CD4+ T cells engineered to recognize an ovalbumin epitope only (OT2) (b; *n* = 5) or deficient in IL-12p35 subunit gene (c; *n* = 5) were intragastrically inoculated with *C. muridarum* at 2×10^5 IFU per mouse. On day 28 after infection, all mice were sacrificed for monitoring live chlamydial organisms as described in the Fig. 2 legend and shown on the *x* axis. The number of live organisms recovered from each tissue sample was expressed as $\log_{10}$ IFU. Note that both the transgenic OT2 and IL-12p35 KO mice were susceptible to chlamydial colonization in the small intestine. **, *P* < 0.01 (Wilcoxon, panel b or c versus panel a). Data were acquired from 2 independent experiments.
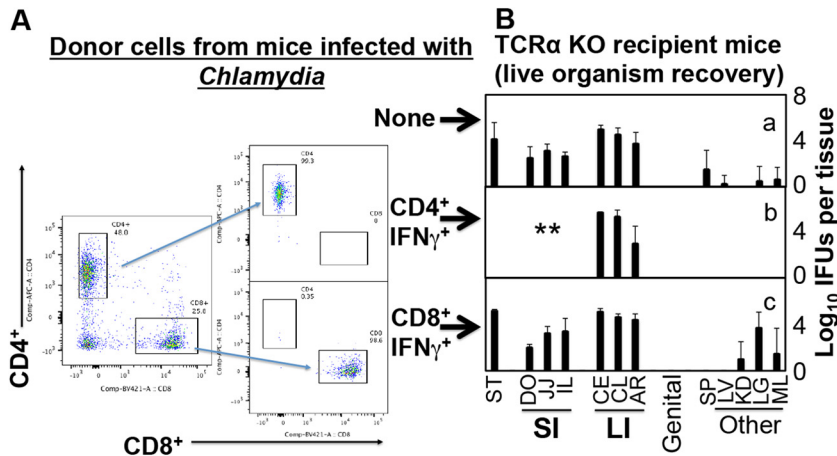
FIG 7 Adoptive transfer of IFN-γ-producing T cells to enhance resistance to *Chlamydia muridarum* colonization in the small intestine. Green fluorescent protein-positive (GFP+) CD4+ (CD4+ IFN-γ+) or GFP+ CD8+ (CD8+ IFN-γ+) T cells were purified from donor mice that express GFP under the control of the IFN-γ promoter on day 10 after the donor mice were intravaginally infected with *C. muridarum*. After flow cytometry sorting, the donor cells reached purity of >98% and were transferred into TCRα KO recipient mice that were intragastrically infected with *C. muridarum*. On day 28 after intragastric infection, recipient mice were sacrificed for monitoring live organisms as described in the Fig. 2 legend and shown on the x axis. Note that recipient TCRα KO mice receiving CD4+ IFN-γ+ but not CD8+ IFN-γ+ T cells cleared *Chlamydia* from the small intestine. **, $P < 0.01$ (Wilcoxon, panel b or c versus panel a). Data were from 2 independent experiments with 4 or 5 mice in each group.

in the small intestine (Fig. 7). To make donor cells, CD4+ or CD8+ T cells that express IFN-γ (designated CD4+ IFN-γ+ or CD8+ IFN-γ+) were sorted from IFN-γ reporter mice (that express enhanced yellow fluorescent protein [eYFP] under the control of the endogenous IFN-γ promoter as a bicistronic IFN-γ–IRES– eYFP mRNA) after the reporter mice were infected with *C. muridarum* for 10 days. The TCRα-deficient mice were used as recipient mice. The recipient mice that received CD4+ IFN-γ+ donor cells cleared *Chlamydia* from the small intestine, while those receiving the same amount of CD8+ IFN-γ+ donor cells failed to do so. The above observations together demonstrate that *Chlamydia*-specific IFN-γ-producing CD4+ (or Th1) but not CD8+ T cells are sufficient for clearing chlamydial colonization in the small intestine. Interestingly, the same Th1 cells did not affect chlamydial colonization in the large intestine, suggesting that the *Chlamydia*-specific Th1 cells were not able to gain access to, or were suppressed from, *Chlamydia*-infected cells in the large intestine.

DISCUSSION

Chlamydia has been frequently detected in the GI tracts of both humans (14–17) and animals (20, 23–25). *C. trachomatis* in the GI tract has been proposed to serve as a reservoir for promoting chlamydial pathogenicity in women's genital tracts (18, 19), while *C. muridarum* has been shown to readily spread to the GI tract (20), which may indirectly promote chlamydial pathogenicity in mouse genital tracts (22). However, when a naive mouse is first exposed to *C. muridarum* in the GI tract, the GI tract *C. muridarum* may act as an oral vaccine to induce transmucosal immunity (32, 33). Thus, it is important to understand how GI *Chlamydia* is regulated by host immune mechanisms.

Although live *C. muridarum* organisms have been shown to last for long periods on rectal swabs, the organisms are cleared from the small intestine as fast as they are eliminated from the genital tract. In the current study, we have demonstrated for the first time that similar to the *C. muridarum* organisms in the mouse genital tract (11), the organisms in the mouse small intestine are eliminated by Th1-dominant immunity. First, mice deficient in TCRα or CD4+ T cells showed chlamydial persistence in the small intestine. Second, mice deficient in CD8+ T cells cleared *Chlamydia* from the small intestine as efficiently as wild-type mice, but depletion of CD4+ T cells rescued

chlamydial colonization in the small intestine of the CD8$^+$ T cell-deficient mice. Third, mice deficient in IFN-γ but not IL-22 signaling rescued chlamydial colonization in the small intestine. Fourth, supplementing TCRα-deficient mice with exogenous IFN-γ was sufficient for clearing *Chlamydia* from the small intestine. Fifth, mice deficient in developing *Chlamydia*-specific CD4$^+$ T cell immunity or Th1 immunity permitted chlamydial persistence in the small intestine. Finally, IFN-γ-producing CD4$^+$ but not CD8$^+$ T cells from immunized donor mice were sufficient for eliminating *Chlamydia* from the small intestine of recipient mice.

Despite the critical role of CD4$^+$ T cell-produced IFN-γ in clearing *C. muridarum* from both the mouse genital tract (13, 34, 35) and small intestine (current study), the precise mechanism for how IFN-γ clears *C. muridarum* remains unknown. Indoleamine dioxygenase (IDO) and nitric oxide (NO) synthase are among the most prominent and well-studied IFN-γ-inducible effectors. IDO works in human cells, while NO works in mouse cells (36). However, blockage of NO synthesis only partially rescued chlamydial growth, suggesting that additional mechanisms remain to be determined. IFN-inducible guanylate binding proteins (GBPs) are known to mediate cell-autonomous host resistance to bacterial pathogens (37). However, *C. muridarum* is resistant to GBP-mediated restrictions on bacterial growth. GBPs neither bind to *C. muridarum* inclusions nor restrict *C. muridarum* growth, although GBPs are still able to promote inflammasome activation in *C. muridarum*-infected cells (38). It is possible that IFN-γ may restrict *C. muridarum* infection by inducing host cell apoptosis since *Chlamydia* is selected to inhibit host cell apoptosis (39, 40). Here we have shown that exogenous IFN-γ is sufficient for eliminating *C. muridarum* from small intestine, which has provided an ideal animal model for further revealing the mechanisms. It is worth noting that besides CD4$^+$ T cells, B cells may also play an important role in anti-*Chlamydia* immunity (34). IFN-γ may also cooperate with other immune components, such as antibodies and neutrophils, for enhancing chlamydial clearance (41, 42).

Once arriving in the large intestine, *C. muridarum* lasts for long periods. The question is why Th1 immunity that is able to clear *C. muridarum* in the small intestine spares *C. muridarum* in the large intestine. There are regional differences in the development and composition of the adaptive immune landscape of the intestine (43). The Th1 immunity identified in the small intestine may not necessarily work in the large intestine. It appears that *C. muridarum* organisms in different segments of the mouse GI tract are regulated by distinct immune mechanisms. A lack of T cells or CD4$^+$ T cells and Th1 immunity did not significantly increase *C. muridarum* colonization in the large intestine. More importantly, supplementation with exogenous IFN-γ or IFN-γ-producing CD4$^+$ T cells from *Chlamydia*-immunized donor mice failed to significantly reduce *C. muridarum* colonization in the large intestine. Since the same immunological conditions significantly inhibited *C. muridarum* colonization in the small intestine of the same mice, we hypothesize that the failure of Th1 immunity to impact the large intestine *Chlamydia* may be due to lack of accessibility to *Chlamydia*-infected cells in the large intestine by the Th1 cells. The large intestine is known to host the highest concentration of microbiota species, and high levels of regulatory T cells (Tregs) have been detected in the large intestine (44–46). *Chlamydia*-infected epithelial cells in the large intestine may be protected from the Th1 immunity by Tregs, which will be investigated in our future studies.

The information acquired from mouse model study may be useful in guiding clinical studies on *C. trachomatis* colonization in the gut. *C. trachomatis* is frequently detected on rectal swabs (15, 16, 27–31), suggesting that *C. trachomatis* is introduced into the human GI tract. It will be worth testing whether *C. trachomatis* is also efficiently cleared from human small intestine but persists in the large intestine for a long period. More importantly, we have recently shown that oral delivery of *C. muridarum* is nonpathogenic while inducing transmucosal immunity against subsequent challenge infection in the mouse genital tract (32). Investigating the mechanisms of chlamydial colonization in the GI tract would provide mechanistic information useful in developing an oral chlamydial vaccine. The current study has revealed that robust Th1-dominant immunity is induced by the orally delivered *Chlamydia*, which efficiently cleared the chlamydial organ-

isms from the small intestine within 4 weeks, demonstrating the capacity of orally delivered chlamydial organisms to induce protective immunity against genital tract infection. It is well established that a Th1-dominant response is protective against chlamydial infection in the genital tract (34). The question is whether the gastrointestinal *C. trachomatis* can induce protective immunity in human genital tracts, which is testable by evaluating whether humans whose rectal swabs are positive for *C. trachomatis* can develop *Chlamydia*-specific Th1 immunity and display lower rates of repeated infections. Thus, mouse studies can provide mechanistic information for guiding clinical investigations.

We are aware of the distinction between *C. trachomatis* and *C. muridarum*. *C. trachomatis* is naturally transmitted among humans sexually, while *C. muridarum* may be naturally transmitted among mice via the oral-fecal route (instead of sexually). This is because the *C. muridarum* plasmid or plasmid-encoded proteins or some chromosomal proteins have been shown to be more important for *C. muridarum* to colonize the GI tract than to infect the genital tract (47, 48). In addition, the *C. muridarum* genome still maintains three copies of a full-length cytotoxin gene with significant homology to genes for large clostridial cytotoxins (49). *C. muridarum* may have acquired the cytotoxin gene from other enteric bacteria, and maintaining the full-length cytotoxins may be necessary for *C. muridarum* to compete against other enteric bacteria in the GI tract. However, *C. trachomatis* has been transmitted sexually among humans, forcing *C. trachomatis* to adapt to the human genital mucosa and allowing the cytotoxin genes to be lost or significantly shortened in *C. trachomatis* (49). Nevertheless, *C. trachomatis* has been frequently detected in the GI tract in humans but without any significant association with GI pathologies (14–17, 50). *C. trachomatis* may have experienced selection pressures from both the genital and GI tracts. It will be worth testing whether *C. trachomatis* is regulated by different immune mechanisms in different regions of the human GI tract.

MATERIALS AND METHODS

Chlamydial organism growth. *Chlamydia muridarum* used in the current study was clone G13.32.1 (51), which was derived from strain Nigg3 (GenBank accession number CP009760.1). The *C. muridarum* organisms were propagated in HeLa cells (human cervical carcinoma epithelial cells; ATCC catalog number CCL2.1) and purified as elementary bodies (EBs) as reported previously (20, 39). Aliquots of purified EBs were stored at −80°C until use. The storage buffer (SPG) consisted of 220 mM sucrose, 12.5 mM phosphate, and 4 mM L-glutamic acid (pH 7.5).

Mouse infection. Mouse experiments were carried out in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (52). The protocol was approved by the Committee on the Ethics of Laboratory Animal Experiments of the University of Texas Health Science Center at San Antonio.

C. muridarum EBs were used to inoculate 6- to 7-week-old female mice (Jackson Laboratories, Inc., Bar Harbor, ME) intravaginally or intragastrically as described previously (10, 20, 26, 48). The following mice were used in the current study. The two wild-type strains were CBA1/J (stock number 000656) and C57BL/6J (0006640). Gene knockout (KO) strains were CD4 KO (002663 [B6.129S2-*Cd4tm1Mak*/J]), CD8 KO (002665 [B6.129S2-*Cd8atm1Mak*/J]), IFN-γR KO (003288 [B6.129S7-*Ifngr1tm1Agt*/J]), IL-22 KO (027524 [C57BL/6-*Il22tm1.1(icre)Stck*/J]), IL-12p35 KO (002692 [B6.129S1-*Il12atm1Jm*/J]), and TCRα KO (002116 [B6.129S2-*Tcratm1Mom*/J]). The transgenic mouse strains were OT2 {004194 [B6.Cg-Tg(TcraTcrb)425Cbn/J]} and IFN-γ reporter (017581 [B6.129S4-*Ifngtm3.1Lky*/J]). For intravaginal inoculation, stock EBs diluted in 10 μl of SPG that contained 2×10^5 IFU or the desired IFU as indicated for individual experiments were delivered to the ectocervix area using a 20-μl micropipette tip. Five days prior to inoculation, each mouse was injected subcutaneously with 2.5 mg of Depo-Provera (Pharmacia Upjohn, Kalamazoo, MI) suspended in sterile phosphate-buffered saline (PBS). For intragastric inoculation, EBs diluted in 100 μl of SPG that contained 2×10^5 IFU or the desired number of IFU as indicated for individual experiments were delivered to the stomach using a straight-balled end needle designed for mouse oral gavage (N-PK 020; Braintree Scientific Inc., Braintree, MA). Following the initial inoculation, both vaginal and rectal swabs were taken periodically or organs/tissues were harvested (after mice were sacrificed) for titration of viable organisms as described previously (20, 21, 26). In some experiments, mice were treated with an anti-CD4 antibody, while in others, exogenous molecules or donor T cells were provided as described below. At designated time points after inoculation, mice were sacrificed for titration of viable organisms as described below.

Antibody depletion, providing exogenous molecules or cells (adoptive transfer). In some experiments, mice were treated with anti-CD4 antibody (clone number GK1.5, rat IgG2b, κ, purified as described previously [41] or purchased from Bio X Cell, West Lebanon, NH). The antibody treatment began on day 5 after intragastric inoculation and then was continued twice weekly throughout experiments. Each injection was administered intraperitoneally with 400 μg of IgG in 400 μl of PBS as described previously (41, 53).

For injecting exogenous IFN-γ into mice, IFN-γ (catalog no. 575308; Biolegend, Inc., San Diego, CA) was diluted in sterile PBS and injected intraperitoneally at 16 μg/injection in a 200-μl volume. The injection started on day 7 after chlamydial infection and continued once every 2 days throughout the experiments.

For adoptive-transfer experiments, both the spleen and mesenteric lymph nodes were harvested from IFN-γ reporter mice on day 10 after chlamydial infection. Single cell suspensions were labeled with the following antibodies: anti-CD4 (conjugated with allophycocyanin [APC], clone number GK1.5, rat IgG2b, κ, catalog number 100404; Biolegend, Inc., San Diego, CA), anti-CD8 (conjugated with Efluor 450, clone number eBioH35-17.2, catalog number 12-0083-81; Thermo Fisher Scientific, Inc., Waltham, MA), and anti-CD3 (conjugated with phycoerythrin [PE], clone number 17A2, rat IgG2b, κ, catalog number 100206; Biolegend). Donor cells were then purified using flow cytometry sorting (BD FACSAria II, catalog number 642886; BD Biosciences San Jose, CA), gating CD3 and YFP doubly positive cells and then sorting for CD4$^+$ or CD8$^+$ cells. The sorted donors were designated IFN-γ^+ CD4$^+$ or IFN-γ^+ CD8$^+$ T cells. The flow cytometry sorting ensured >98% purity for both donor cell populations prior to adoptive transfer. For adoptive transfer, each recipient mouse received 2 $\times$ 10^6 corresponding donor T cells three times on days 7, 14, and 21 after the recipient mice were infected with *C. muridarum*. Retro-orbital injection was used to deliver the donor cells.

Titrating live chlamydial organisms recovered from swabs and tissue homogenates. To quantitate live chlamydial organisms on vaginal or rectal swabs, each swab was soaked in 0.5 ml of SPG and vortexed with glass beads, and the chlamydial organisms released into the supernatants were titrated on HeLa cell monolayers in duplicate. The infected cultures were processed for immunofluorescence assay as described previously (54, 55) and below. Inclusions were counted in five random fields per coverslip under a fluorescence microscope. For coverslips with less than one IFU per field, entire coverslips were counted. Coverslips showing obvious cytotoxicity of HeLa cells were excluded. The total number of IFU per swab was calculated based on the mean IFU per view, the ratio of the view area to that of the well, dilution factor, and inoculation volumes. Where possible, a mean IFU/swab was derived from the serially diluted and duplicate samples for any given swab. The total number of IFU/swab was converted into log$_{10}$, which was used to calculate the mean and standard deviation across mice of the same group at each time point.

For quantitating live organisms from mouse organs and tissue segments, each organ or tissue segment was transferred to a tube containing 0.5 to 5 ml of SPG depending the sizes of the organs. Each GI tract was cut into different segments/portions as indicated for individual experiments, including stomach (ST), small intestine (SI), cecum (CE), colon (CL), and anorectum (AR). In some experiments, SI was further divided into duodenum, jejunum, and ileum. Each genital tract was divided into segments, including vagina/cervix (CV), left uterine horn (LU), right uterine horn (RU), left oviduct/ovary (LO), and right oviduct/ovary (RO). Other tissues, including spleen (SP), liver (LV), kidney (KD), lung (LG), and mesenteric lymph node (ML), were also measured. The organs and tissue segments were homogenized in cold SPG using a 2-ml tissue grinder (K885300-0002; Fisher Scientific, Pittsburgh, PA) or an automatic homogenizer (TH115; Omni International, Kennesaw, GA). The homogenates were briefly sonicated and spun at 3000 rpm for 5 min to pellet remaining large debris. The supernatants were titrated for live *C. muridarum* organisms on HeLa cells as described above. The results were expressed as log$_{10}$ IFU per organ or tissue segment.

Immunofluorescence assay. The immunofluorescence assay used for titration of live organisms was carried out as described previously (39, 56). A rabbit antibody (designated R1604, raised with purified *C. muridarum* EBs) was used as a primary antibody to label all *C. muridarum* organisms in HeLa cells, which were visualized with a goat anti-rabbit IgG conjugated with Cy2 (green, catalog number 111-225-144; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The DNA dye Hoechst 3328 (blue; Sigma-Aldrich, St. Louis, MO) was used to visualize nuclei. The doubly labeled samples were used for counting *C. muridarum* organisms under a fluorescence microscope (AX70; Olympus) equipped with a charge-coupled-device (CCD) camera (Hamamatsu).

Statistical analyses. The time courses of live organism shedding (area under the curve [AUC]) or IFU from individual tissues were compared between two groups using the Wilcoxon rank sum test (an in-house Excel sheet), while category data, including number of mice positive for live organism shedding, were analyzed using Fisher's exact test (http://vassarstats.net/tab2x2.html). Correlations of chlamydial colonization in different tissues were analyzed by calculating Spearman rank order correlation coefficients (http://vassarstats.net/corr_rank.html). Furthermore, the significance of the difference between two correlation coefficients was also calculated (http://vassarstats.net/rdiff.html).

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