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$CD4^+$ T Cells Are Necessary and Sufficient To Confer Protection against *Chlamydia trachomatis* Infection in the Murine Upper Genital Tract

David C. Gondek,[1] Andrew J. Olive,[1] Georg Stary, and Michael N. Starnbach

***Chlamydia trachomatis* infection is the most common bacterial sexually transmitted disease in the United States. *Chlamydia* infections that ascend to the upper genital tract can persist, trigger inflammation, and result in serious sequelae such as infertility. However, mouse models in which the vaginal vault is inoculated with *C. trachomatis* do not recapitulate the course of human disease. These intravaginal infections of the mouse do not ascend efficiently to the upper genital tract, do not cause persistent infection, do not induce significant inflammation, and do not induce significant $CD4^+$ T cell infiltration. In this article, we describe a noninvasive transcervical infection model in which we bypass the cervix and directly inoculate *C. trachomatis* into the uterus. We show that direct *C. trachomatis* infection of the murine upper genital tract stimulates a robust *Chlamydia*-specific $CD4^+$ T cell response that is both necessary and sufficient to clear infection and provide protection against reinfection. *The Journal of Immunology*, 2012, 189: 2441–2449.**

The obligate intracellular bacterial pathogen *Chlamydia trachomatis* causes significant morbidity throughout the world (1). The major complications of *C. trachomatis* genital tract infections arise primarily in women and include pelvic inflammatory disease, which can result in fallopian tube scarring, infertility, and ectopic pregnancy (2, 3). Better understanding of the interaction between *C. trachomatis* and the mammalian host is critical for the development of a vaccine to combat the prevalent human diseases caused by this pathogen.

Human infection with *C. trachomatis* stimulates multiple elements of the immune system, but these responses often fail to clear the organism or prevent subsequent reinfection (4–6). The inability to clear chronic *C. trachomatis* infections suggests a failure in adaptive immunity—specifically, the memory responses that should provide long-lasting protection. Studies have shown that mice intravaginally infected with human strains of *C. trachomatis* clear infection quickly and without the inflammation and pathological changes associated with human disease (7–9). Following genital infection with human *C. trachomatis*, $CD4^+$ T cells become activated, proliferate, and are recruited to the genital mucosa (9–15). These $CD4^+$ T cells exhibit a characteristic Th1 response, secreting the high amounts of IFN-γ required for bacterial clearance (9, 16). Infection of $CD4^{-/-}$ mice with *C. trachomatis* leads to higher pathogen load during primary infection, as well as a diminished ability to be protected from secondary infection (9). However, studies examining the protective quality of the $CD4^+$ T cell memory cells induced following *C. trachomatis* infection have been contradictory (8, 9). One investigation examined *C. trachomatis* infection of wild-type and μMT mice, demonstrating a requirement for $CD4^+$ T cells in protective immunity to secondary infection (17). In contrast, a recent study in which Ab was used to deplete $CD4^+$ cells suggested that prior infection of mice with *C. trachomatis* does not yield strong protective immunity and that $CD4^+$ T cells are not critical for the clearance of human strains (8). These contradictory reports highlight the limited understanding of the dynamics of the $CD4^+$ memory T cell response to *C. trachomatis*, particularly in tracking Ag-specific T cell responses over time. One possible reason for the limited data examining $CD4^+$ T cell memory responses is that current small animal models do not accurately recapitulate human infections.

In pursuing mouse models of host defense against *Chlamydia*, investigators have had to choose between infecting mice with *C. trachomatis*, the human pathogen, or *Chlamydia muridarum*, a pathogen isolated from a natural mouse infection. *C. muridarum* has been an attractive option in that infections with this organism persist several days longer than infections with *C. trachomatis,* and are characterized by higher bacterial loads, ascending infections into the upper genital tract, and the development of pathological conditions such as hydrosalpinx and infertility (18–21). The *C. muridarum* model has increased our knowledge about *Chlamydia* pathogenesis and immunity; however, its application is limited, specifically in the identification of Ags for use in a vaccine to protect against *C. trachomatis* (22–26). To date, no T cell epitopes shared between *C. trachomatis* and *C. muridarum* have been published (6, 27, 28). Furthermore, *C. muridarum* models only acute phases of human *C. trachomatis* infection, not the chronic phases responsible for disease in humans (5, 6). By identifying new protective T cell Ags and tracking *C. trachomatis*-specific responses to those Ags, we may be able to differentiate responses that lead to protective immunity from those that cause deleterious pathological conditions (6).

We hypothesized that in the standard vaginal inoculation method of *C. trachomatis* infection, the organism does not reach the upper genital tract and therefore is unable to stimulate robust adaptive

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Abbreviations used in this article: IFU, inclusion-forming unit; IRG, immunity-related GTPase.

immunity, similar to what takes place in human infections. In this article, we describe a model of mouse infection with *C. trachomatis* in which the cervical barrier is bypassed. Using this transcervical infection model, we are able to directly infect the upper genital tract of mice with *C. trachomatis*, colonizing the clinically relevant site. Compared with vaginal inoculation with *C. trachomatis*, transcervical inoculation allowed for more efficient colonization and stimulated a more robust and inflammatory Ag-specific T cell response in the upper genital tract while also allowing for the consistent development of pathological conditions. Using this model, we characterized the induction of Ag-specific memory CD4+ T cells and show that they are necessary and sufficient for protection against reinfection of the murine genital mucosa. This study demonstrates a novel inoculation method that will allow investigators to build on each other's research results, using *C. muridarum* or *C. trachomatis* interchangeably. These data move the field significantly forward with a model system that stimulates immunity, is highly reproducible, and causes disease at the site biologically relevant for human *C. trachomatis* (29). This model system will accelerate our understanding of *Chlamydia* pathogenesis in vivo, help to uniformly define the immune components needed for protection, and enhance the ability to test the capacity of vaccines to protect against infection in the genital mucosa, using *C. trachomatis*.

Materials and Methods

Mice

C57BL/6, B6.PL-*Thy1a* (CD90.1 congenic), and B6.129S7-*IFNγtm1Agt* (IFN-$\gamma^{-/-}$) were purchased from The Jackson Laboratory. *Irgm1/m3*($^{-/-}$) and NR1 mice were described previously (11, 30) and are maintained and cared for within the Harvard Medical School Center for Animal Resources and Comparative Medicine (Boston, MA). All mice were treated with 2.5 mg medroxyprogesterone s.c. 7 d prior to infection to normalize the murine estrous cycle. All experiments were approved by the Institutional Animal Care and Use Committee. In all experiments, four or five mice per group were used.

Growth, isolation, and detection of bacteria

C. trachomatis serovar L2 (434/Bu) or *C. muridarum* was propagated within McCoy cell monolayers grown in Eagle's MEM (Invitrogen, Grand Island, NY) supplemented with 10% FCS, 1.5 g/l sodium bicarbonate, 0.1 M nonessential amino acids, and 1 mM sodium pyruvate. Infected monolayers were disassociated from plates, using sterile glass beads, and were sonicated to disrupt the inclusion. Elementary bodies were purified by density gradient centrifugation, as described previously (10). Aliquots were stored at −80°C in medium containing 250 mM sucrose, 10 mM sodium phosphate, and 5 mM L-glutamic acid and were thawed immediately prior to use. To quantify the levels of *C. trachomatis* or *C. muridarum*, we used quantitative PCR with 16S primers specific for *Chlamydia*, as done previously (7). For titering directly from the genital tract, at the given time points the upper genital tract was isolated, homogenized by mechanical disruption, and placed in six-well plates preseeded with 5×10^5 McCoy cells and incubated for 36 h to allow the developmental cycle to finish. Cells were then lysed as described above and titered into 96-well plates containing 1×10^4 McCoy cells. At 30 h post infection, the cells were fixed with methanol and stained using a *Chlamydia* culture diagnostic kit (Roche). Inclusions were then quantified by fluorescence microscopy.

Skewing of NR1 cells and protection assay

CD4+ T cells were purified from NR1 mice, using a mouse CD4 negative isolation kit (Invitrogen) per the manufacturer's directions. The T cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FCS, L-glutamine, HEPES, 50 μM 2-ME, 50 U/ml penicillin, and 50 μg/ml streptomycin. To stimulate the T cells, irradiated feeder splenocytes were pulsed with 5 μM Cta1 133–152 peptide and cocultured with the CD4-enriched NR1 cells at a stimulator/T cell ratio of 4:1. To polarize T cells toward the Th1 phenotype, cells were incubated with 10 ng/ml IL-12 (Peprotech, Rocky Hill, NJ) and 10 μg/ml anti–IL-4 (BioLegend). Cells were stimulated for 5 d; then 10^6 cells were transferred into naive CD90.2+ IFN-$\gamma^{-/-}$ host mice. At 24 h following transfer, mice were challenged in the uterus with 10^6 inclusion-forming units (IFU) of *C. trachomatis* L2.

Flow cytometry

Tissues were mechanically disaggregated and immediately stained for activation markers or stimulated for 5 h with 50 ng/ml PMA (Alexis Biochemical) and 500 ng/ml ionomycin (Calbiochem) in the presence of brefeldin A (GolgiStop; BD Biosciences) for intracellular cytokine staining. Cells were preincubated with anti-FcRγ (Bio X-Cell) before staining with anti-CD4 Pacific Blue (BioLegend) and anti-CD90.1 peridinin chlorophyll-a protein (BD Biosciences). For activation marker analysis, we examined anti-CD44 PE-CyChrome 7 (BioLegend), anti-CD62L allophycocyanin-Alexa 750 (eBioscience), and anti-CD25 allophycocyanin (BD Biosciences). For intracellular staining, the following Abs were used: anti–IFN-γ PE, anti–IL-2–allophycocyanin, anti–IL-17 FITC, and anti–TNF-α PE-CyChrome 7 (BD Biosciences). Cells were permeabilized with the Cytofix/Cytoperm Plus Kit according to the manufacturer's instructions (BD Biosciences). The absolute cell number in each sample was determined using AccuCheck Counting Beads (Invitrogen). Data were collected on a modified FACSCalibur (Cytek Development) or an LSRII (BD Biosciences) and analyzed using FlowJo (Tree Star).

T cell depletion

For CD4+ T cell depletion experiments, mice were infected with 10^6 IFU *C. trachomatis* or *C. muridarum*, then rested for >4 wk. Starting 3 d prior to secondary challenge, each immune mouse was i.p. injected with 200 μg anti-CD4 (clone GK1.5) or isotype control (clone LTF-2) every day. Mice were sacrificed 5 d after challenge, and their lymphocytes were assessed in the spleen, lymph nodes, and uterus. In addition, the bacterial load was determined by quantitative PCR.

Tetramer production, enrichment

C. trachomatis-predicted periplasmic protein Cta1 133–152 (KGIDPQELWVWKKGMPNWEK) biotin-labeled I-A^b molecules and the CrpA 63–71 (ASFVNPIYL) biotin-labeled D^b tetramer were produced by the National Institutes of Health Tetramer Facility (Emory University, Atlanta, GA), purified, and tetramerized with streptavidin allophycocyanin or PE, respectively. Spleen and lymph node cells were prepared, stained with CTA-1:I-Ab–SA-APC at 25°C for 1 h, and then spiked with CrpA:Db-SA-PE at 4°C for 30 min. Cells were next washed and incubated with anti-PE and anti-APC magnetic beads. Bead-bound cells were enriched on magnetized columns, and a sample was removed for counting. The enriched cells were surface stained with combinations of the Abs listed above.

Statistical analysis

All groups were evaluated for statistical significance with unpaired two-tailed *t* tests. When it appeared necessary to highlight significant differences between data points, the level of significance is depicted as $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$.

Results

Transcervical inoculation of C. trachomatis *allows robust and consistent murine upper genital tract infection*

Our primary objective in this study was to compare murine models of *Chlamydia* genital infection upon induction of CD4+ T cell responses. More specifically, we examined the differences between the intravaginal inoculation method and a newly developed transcervical (intrauterine) inoculation method using both *C. trachomatis* and *C. muridarum*. The transcervical infection model uses a thin, flexible probe (nonsurgical embryo transfer device) to bypass the cervix and inject the bacteria directly into the lumen of the uterus (Supplemental Fig. 1). To understand how the bacterial burden in the upper genital tract changes with infection route or *Chlamydia* species, mice were given 10^6, 5×10^4, or 10^3 IFU of *C. trachomatis* or *C. muridarum* by either the intravaginal or the transcervical method. At 3 d post infection with 10^6 IFU of *C. trachomatis* given transcervically (gray bars) or *C. muridarum* given intravaginally (white bars), the murine uteri harbored similar levels of bacteria, (Fig. 1). In stark contrast, vaginal inoculation with 10^6 IFU of *C. trachomatis* resulted in two

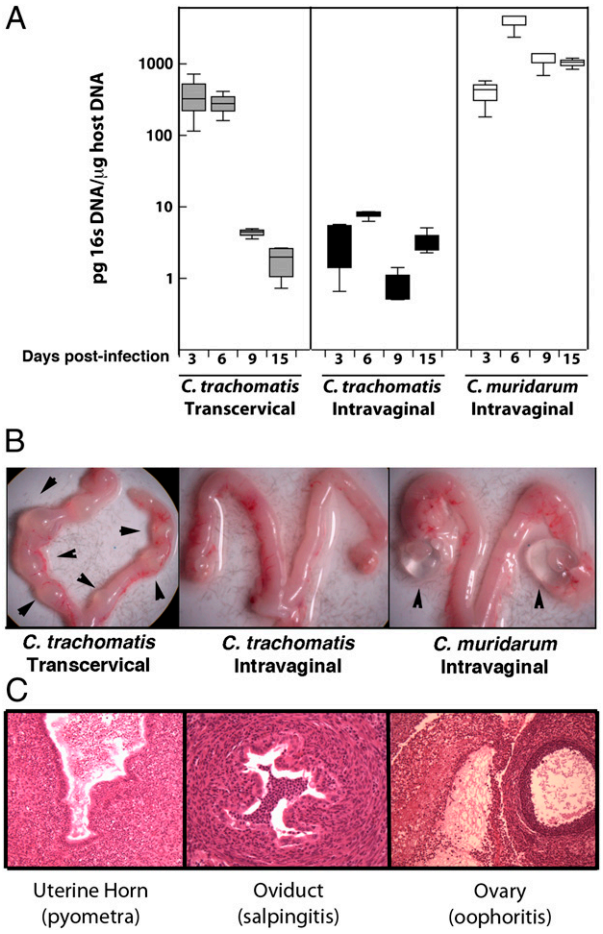

FIGURE 1. Transcervical infection of the genital mucosa with *C. trachomatis* leads to efficient colonization and disease in the upper genital tract. Wild-type mice were infected by either intravaginal or transcervical inoculation with 10^6 IFU of *C. trachomatis* or *C. muridarum*. (**A**) At the indicated time points following infection, genomic DNA was isolated from the upper genital tract. Quantitative PCR was used to calculate the levels of *Chlamydia* 16S DNA relative to levels of host GAPDH. Shown is a box-and-whisker plot from one of two independent experiments. (**B**) At 25 d following infection, the upper genital tract was isolated, photographed, and scored for pathological changes. Arrow heads indicate nodes of inflammation. Shown are representative images from 25 experimental mice per group from two independent experiments. (**C**) Histological sections of the upper genital tract from mice infected transcervically with *C. trachomatis* were stained with H&E. Original magnification ×20 (*left*), ×40 (*middle*), ×20 (*right*). Shown are representative images of the inflammation seen in the uterine horn, oviduct, and ovary following transcervical infection.

logs less bacterial burden in the upper genital tract (black bars). These results suggest that when the physical barrier of the cervix is bypassed, the human-adapted *C. trachomatis* can colonize the murine upper genital tract as efficiently as the murine-adapted *C. muridarum*.

We next examined changes in *Chlamydia* burden over the course of primary infection. For *C. trachomatis* given via the transcervical route, bacterial load remained constant from 3 to 6 d post infection, followed by a precipitous decrease on days 9 and 15 (Fig. 1). Intravaginal infection with *C. muridarum* resulted in infection levels at day 3 comparable to those observed with *C. trachomatis* transcervical infection. By day 6, the *C. muridarum* level had increased 10-fold and then decreased somewhat at days 9 and 15. Infection with either pathogen or route becomes undetectable 21–28 d following infection (data not shown). The relative numbers of *C. trachomatis* present in the upper genital tract were consistent whether we used quantitative PCR (Fig. 1) or whether we determined inclusion-forming units by titering (Supplemental Fig. 1B). We also examined bacterial load in mice infected with *C. muridarum* by transcervical inoculation. Although the load is even higher 3 d post infection (3000 pg/ng), the bacterial burden was similar to that with intravaginal inoculation for the remainder of the time-course (data not shown). Therefore, we used only intravaginal inoculation of *C. muridarum* for the remainder of the study. When *C. trachomatis* was inoculated via the vaginal route, no consistent infection of the upper genital tract was produced. These results show that both *C. trachomatis* and *C. muridarum* are capable of colonizing the upper genital tract, but that *C. trachomatis* must be inoculated across the cervix for colonization to occur.

We also examined the impact of infectious dose on the course of infection (Supplemental Fig. 2A, 2B). Lowering the initial dose led to a corresponding decrease in bacterial burden on day 3 for both transcervical infections with *C. trachomatis* and vaginal inoculation with *C. muridarum*. As was seen with the highest dose (10^6 IFU), each of the lower doses (5×10^4 IFU, 10^3 IFU) resulted in similar burdens of organisms at day 3, using the two models. Regardless of the initial dose of *C. muridarum*, the number of this organism rises to a similar high level by day 6 post infection, and the resolution of infection then follows a similar course, again regardless of inoculation dose. Therefore, transcervical delivery of *C. trachomatis* allows more robust initial infection than does intravaginal delivery of *C. trachomatis*, but it never rises to the level seen following infection with *C. muridarum*.

We next examined whether transcervical infection leads to disease in the upper genital mucosa. Mice were infected intravaginally with *C. trachomatis* or *C. muridarum* or infected transcervically with *C. trachomatis*. At 25 d post infection, the genital tract was removed and examined for gross pathology. Overall, pathological changes were seen in mice infected both intravaginally with *C. muridarum* (~20%) and transcervically with *C. trachomatis* (~15%), but never in mice infected intravaginally with *C. trachomatis* (data not shown). Mice infected with *C. muridarum* showed severe oviduct hydrosalpinx similar to what has been described previously (Fig. 1B and Ref. 31). Mice infected transcervically with *C. trachomatis* showed major nodes of inflammation ascending the length of the upper genital tract (Fig. 1B). These data indicate that the transcervical model of infection allows the formation of gross pathology similar to what has been observed with *C. muridarum*. Although there have been reports of pathological changes following *C. trachomatis* infection of innate immune-deficient mouse strains, consistent disease has never been described in wild-type C57BL/6 mice, a strain restrictive for *C. trachomatis* growth. Furthermore, the pathological changes in *C. trachomatis*-infected mice extend the length of the uterine horn, similar to rare cases described recently following *C. muridarum* infection (32).

Because no gross pathology was observed on the ovaries following transcervical infection, we subjected several mice infected with *C. trachomatis* transcervically to histopathological study, to determine whether inflammation ascends the entire length of the upper genital tract, as previously described for *C. trachomatis* infections in humans. The majority of mice examined following transcervical infection with *C. trachomatis* had severe oophoritis (inflammation of the uterine horn, oviduct, and ovary), examples of which can be seen in Fig. 1C. Evidence of large neutrophil and macrophage recruitment to the upper genital tract can be observed, as well as fibrin/mucus suggesting fluid buildup in these tissues. Importantly, we observed massive inflammation of the oviduct,

a hallmark of murine infections with *C. muridarum*. Taken together, our transcervical model of *C. trachomatis* infection occurs throughout the upper genital tract and yields pathological changes similar to those with *C. muridarum* as well as human infection with *C. trachomatis*.

IFN-γ restricts the initial growth of C. trachomatis *in the murine upper genital tract following transcervical infection*

It is well accepted that the IFN-γ response in mice and humans is quite different and that *C. muridarum* has adapted to evade this response in its murine host (22, 25). In contrast, the human pathogen *C. trachomatis* is highly susceptible to the murine IFN-γ response (22, 25). We therefore tested whether the differential effect of IFN-γ could explain why the level of *C. muridarum* increased 10-fold from day 3 to day 6, whereas the level of *C. trachomatis* observed following transcervical inoculation did not increase over the same time period. As shown in Fig. 2, we challenged wild-type or IFN-$\gamma^{-/-}$ mice transcervically with *C. trachomatis* and compared the bacterial burden with that obtained through vaginal inoculation with *C. muridarum*. In mice lacking IFN-γ, transcervical infection with *C. trachomatis* led to an expansion of organisms by day 6 closely resembling that observed in wild-type mice infected with *C. muridarum*. These results suggest that IFN-γ restricts the initial growth of *C. trachomatis* rapidly following infection of the upper genital tract. Because this rapid restriction occurs prior to the peak infiltration of T cells (10), it also leaves open the possibility of other sources of IFN-γ capable of restricting *C. trachomatis* growth, such as NK cells resident in the cervical tissues (33). We suspected that the primary effectors of this IFN-γ–mediated restriction of *C. trachomatis* in mice were the immunity-related GTPases (IRGs). When we examined the peak burden of *C. trachomatis* after transcervical infection of IRGm1/m3-deficient mice, we found a higher level of bacteria on day 6, compared with that in wild-type mice, a level identical to what we observed using IFN-$\gamma^{-/-}$ mice (Fig. 2). These data indicate that even though human-adapted *C. trachomatis* is still constrained in its ability to grow owing to innate responses mediated by a rapid induction of IFN-γ and the murine IRG system, removal of IRGm1/m3 alone allows *C. trachomatis* to become resistant to the murine-specific IFN-γ response and to grow similarly to *C. muridarum*.

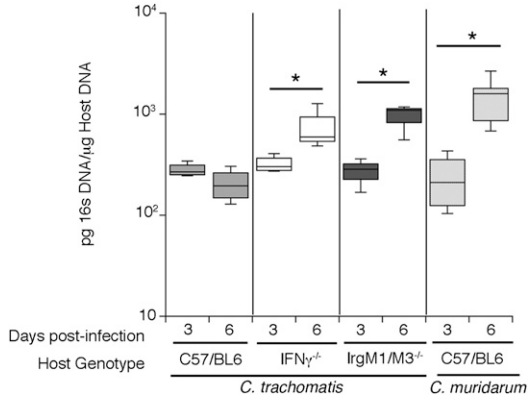

FIGURE 2. IFN-γ restricts *C. trachomatis* rapidly following infection of the upper genital mucosa. Wild-type, IFN-$\gamma^{-/-}$, or IRGm1/m3$^{-/-}$ mice were infected transcervically with 10^6 IFU of *C. trachomatis.* A separate group of wild-type mice were infected with 10^6 IFU of *C. muridarum.* At 3 and 6 d following infection, genomic DNA was isolated from the upper genital tract. Quantitative PCR was used to calculate the levels of *Chlamydia* 16S DNA relative to levels of host GAPDH. Shown is a box-and-whisker plot from one of two independent experiments. *$p < 0.05$.

Transcervical inoculation of C. trachomatis *leads to a robust primary immune response*

Previous reports have shown that CD4+ T cells specific for *C. trachomatis* can protect against systemic infection (11, 16). However, a recent report by Morrison et al. suggests that CD4+ T cells are dispensable for protection against genital infection with *C. trachomatis*. In their experiments, Morrison et al. inoculated mice in the vaginal vault, a method we have now shown does not promote efficient infection of the upper genital tract by *C. trachomatis*. This led us to suspect that Morrison's finding resulted from a lack of colonization of the target tissue when comparing the two *Chlamydia* species. We hypothesized that if transcervical infection was used to directly infect the upper genital tract with *C. trachomatis* we would observe a significant role for CD4+ T cells in immunity to this organism. To test this idea, we used Ag-specific CD4+ T cells to directly compare T cell proliferation, activation, cytokine secretion, and recruitment to the genital mucosa in mice intravaginally infected with *C. trachomatis* with those infected transcervically.

We first wanted to determine whether Ag-specific CD4+ T cells are activated following transcervical or intravaginal infection. We transferred CFSE-labeled *C. trachomatis*-specific CD4+ TCR transgenic T cells into naive mice, which were then challenged with *C. trachomatis* by either the transcervical or the vaginal route of infection. As demonstrated in Fig. 3A, these *C. trachomatis*-specific T cells were capable of recognizing the infecting bacteria and proliferated to a similar degree regardless of the route of infection (93% versus 89% becoming CFSE low). However, the proliferation of *Chlamydia*-specific T cells was more robust when the animals were infected transcervically rather than intravaginally, as seen by the marked difference in their accumulation following division. We also examined the phenotype of these pathogen-specific T cells based on activation markers CD44 and CD62L, and found that no difference could be found in the activation state of T cells stimulated by the two routes of infection (Fig. 2B).

Recent evidence from multiple studies using mice, nonhuman primates, and humans has shown convincingly that the quality of a T cell response is critical in defining protective immunity (34). To examine whether the quality of the T cell response differs following different routes of infection, we compared the ability of pathogen-specific T cells to produce multiple cytokines after either transcervical or intravaginal infection with *C. trachomatis*. We transferred *C. trachomatis*-specific CD4+ TCR transgenic T cells into naive mice, which were then challenged with *C. trachomatis* by either the transcervical or the vaginal route of infection. At 7 d post infection, we examined by flow cytometry the ability of the pathogen-specific cells to produce multiple cytokines. Transcervical inoculation induced >50% of Ag-specific CD4+ T cells into a "triple producer" phenotype capable of robustly producing TNF-α, IFN-γ, and IL-2 simultaneously (Fig. 3C, black dots, *upper right*). These "triple producer" populations have been associated with enhanced protection in other infection models (34). Of interest, *Chlamydia*-specific T cells from mice infected intravaginally did not contain a high proportion of "triple-producing" T cells (black dots versus gray dots in Fig. 3). This observation suggests that transcervical infection yields a higher quality immune response when compared directly with intravaginal infection.

Our CFSE proliferation data suggested that transcervical infection leads to more proliferation and accumulation of Ag-specific T cells (Fig. 3A). We next wanted to directly quantify the accumulation of *Chlamydia*-specific T cells in the draining lymph node

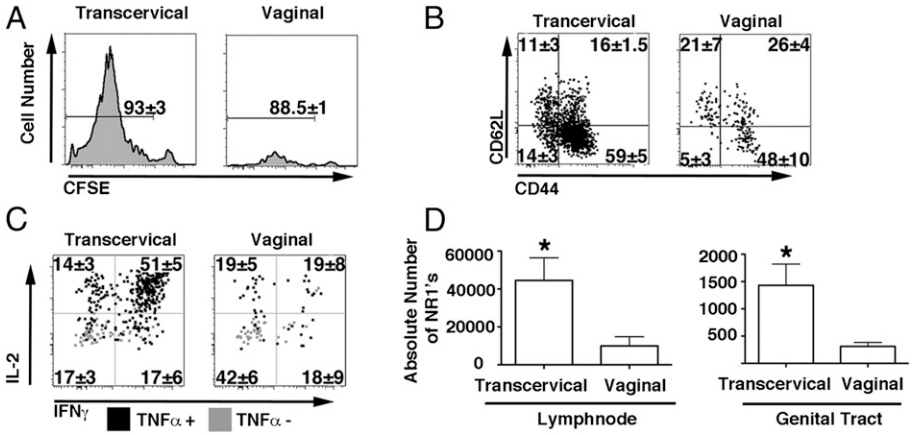

FIGURE 3. Transcervical infection with *C. trachomatis* leads to accumulation of cytokine-secreting pathogen-specific CD4$^+$ T cells. Wild-type mice were infected by either intravaginal or transcervical inoculation with 10^6 IFU of *C. trachomatis* or *C. muridarum*. Wild-type CD90.1$^+$ transgenic CD4$^+$ T cells were CFSE labeled and transferred into CD90.2 hosts 1 d before they were inoculated transcervically or intravaginally with 10^6 IFU of *C. trachomatis*. At 7 d following infection, the draining lymph nodes were harvested and cells were prepared for flow cytometry. Ag-specific CD4$^+$ T cells (CD90.1$^+$ CD4$^+$) were analyzed for CFSE dilution (**A**) and the surface levels of the activation markers CD62L and CD44 (**B**). (**C**) Wild-type CD90.1$^+$ transgenic CD4$^+$ T cells were isolated 7 d following infection, restimulated in vitro, and prepared for intracellular cytokine staining. Gray or black dots indicate whether cells are positive or negative for TNF-α, respectively. (**D**) Wild-type CD90.1$^+$ transgenic CD4$^+$ T cells were transferred into CD90.2 hosts 1 d before they were inoculated transcervically or intravaginally with 10^6 IFU of *C. trachomatis.* At 7 d following infection, the draining lymph nodes and the genital tract were isolated and prepared for flow cytometry. The absolute number of *Chlamydia*-specific CD4$^+$ T cells in each tissue was enumerated. Shown are representative plots from three independent experiments. $*p < 0.05$.

and genital mucosa. We transferred *C. trachomatis*-specific CD4$^+$ TCR transgenic T cells into naive mice, and then challenged them with *C. trachomatis* by either the transcervical or the vaginal route of infection. At 7 d post infection, we measured the absolute number of pathogen-specific T cells in the draining lymph nodes and the genital mucosa. We identified 5-fold more *Chlamydia*-specific CD4$^+$ T cells in the draining lymph node ($p < 0.03$) and upper genital tract ($p < 0.02$) when mice were infected transcervically rather than intravaginally (Fig. 3D). These data indicate that transcervical infection, in comparison with intravaginal inoculation, leads to enhanced recruitment of Ag-specific cells to both the site of activation (the draining lymph node) and the target tissue (the genital mucosa). Together these data suggest that although vaginal infection of mice with *C. trachomatis* elicits a pathogen-specific immune response, the quality of the response is minimal and the pathogen-specific CD4$^+$ T cells are not robustly activated. Conversely, when the physical barrier of the cervix is bypassed using transcervical inoculation, *C. trachomatis*-specific CD4$^+$ T cells are stimulated, the response of those T cells is more potent, and the cells accumulate in both the draining lymph node and the genital mucosa to promote clearance.

CD4$^+$ T cells are necessary and sufficient to confer protection against C. trachomatis *infection in mice*

As shown above, transcervical inoculation of *C. trachomatis*, when compared with the vaginal route, leads to higher bacterial loads in the upper genital tract throughout infection, causes the development of gross pathology, and induces a higher quality *Chlamydia*-specific CD4$^+$ T cell response. With these data, we clearly show that intravaginal inoculation of mice with *C. trachomatis* is a poor model of human infection. Therefore, as we continued these studies, we used only the transcervical infection model to directly examine the role of CD4$^+$ T cells in protection against *C. trachomatis*. Morrison et al. (8) showed that following intravaginal inoculation the depletion of CD4$^+$ T cells during the primary immune response did not result in enhanced disease. This finding is in contrast to previous work from our laboratory revealing that depletion of CD4$^+$ T cells during primary infection leads to increased bacterial loads (30). To rectify the disparity in these findings, we examined whether protection from a secondary challenge with *C. trachomatis* would require a competent CD4$^+$ T cell compartment. Mice were infected with 10^6 IFU of either *C. trachomatis* given transcervically or *C. muridarum* given intravaginally, rested for >4 wk to allow clearance of the initial infection, and then rechallenged with the same pathogen. Before rechallenge, mice were divided into groups treated with either Ab to deplete CD4$^+$ T cells or an isotype control IgG (Fig. 4). Depletion was confirmed by flow cytometry, showing >1000-fold reduction in the number of CD4$^+$ T cells in the draining lymph nodes and 10-fold reduction in the genital mucosa (Supplemental

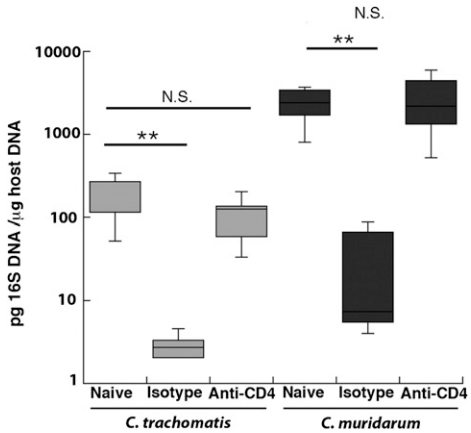

FIGURE 4. CD4$^+$ T cells protect the genital mucosa from reinfection with *C. trachomatis*. Wild-type mice were infected transcervically with 10^6 IFU of *C. trachomatis* or intravaginally with 10^6 IFU of *C. muridarum*. At 5 wk after primary infection, mice were injected with anti-CD4 or isotype control Ab. Naive and immune mice were then infected with 10^6 IFU of *C. trachomatis* or *C. muridarum.* At 6 d after challenge, genomic DNA was isolated from the upper genital tract. Quantitative PCR was used to calculate the levels of *Chlamydia* 16S DNA relative to levels of host GAPDH. Shown is a box-and-whisker plot from one of three independent experiments. $**p < 0.01$.

Fig. 3). After secondary challenge of the group infected with *C. trachomatis*, we examined the level of organisms present in the genital tract. The immune mice were protected 100-fold more than the naive control group ($p < 0.01$). Immune mice depleted of $CD4^+$ T cells did not exhibit any protection, and the bacterial burden of *C. trachomatis* was similar to that of naive mice. In comparison, the bacterial burden in immune mice challenged with *C. muridarum* exhibited a nearly 1000-fold level of protection, compared with the naive control. This protection was completely eliminated if *C. muridarum* immune mice were depleted of $CD4^+$ T cells before secondary challenge. These results demonstrate that $CD4^+$ T cells are necessary for protection in the genital tract, whether mice are infected with *C. muridarum* or *C. trachomatis*.

We next addressed whether pathogen-specific $CD4^+$ T cells are sufficient to confer protection. Our previous studies have indicated that transfer of 10^7 Th1-skewed Ag-specific cells could confer protection against *C. trachomatis* if the mice were challenged either intravenously (16) or transcervically (10, 30). However, these studies involved transfer of larger numbers of CD4 T cells than we use in our study, and therefore might not have accurately reflected the behavior of endogenous Ag-specific cells (35). We wanted to determine the minimum number of transferred pathogen-specific $CD4^+$ T cells capable of conferring protection against *C. trachomatis*. *C. trachomatis*-specific T cells were first preactivated and skewed toward the Th1 phenotype. Cells were then transferred into mice in numbers ranging from 10^4 to 10^7. At 1 d after the transfer of T cells, the mice were infected transcervically with *C. trachomatis*. As demonstrated in Fig. 5, all doses of Th1-skewed pathogen-specific T cells were capable of conferring protection. However, the level of protection provided by those cells was dose dependent, with any number greater than 10^5 transferred cells conferring protection typical of an immune mouse (Fig. 4). It has been well described that following transfer of transgenic T cells, only 10–15% of the transferred population takes hold in the new host (35). Therefore, these data suggest that only 10^4 pathogen-specific $CD4^+$ T cells are sufficient to confer protection against *C. trachomatis* in the genital mucosa. To our knowledge, for the first time this allows us to estimate the lower limit of Ag-specific $CD4^+$ T cells needed to protect the host from infection with *C. trachomatis*. By knowing this lower limit, we can design future vaccines that offer protection but limit immunopathological changes.

C. trachomatis *infection stimulates the activation and memory development of endogenous* C. trachomatis*-specific* $CD4^+$ *T cells*

Many of the experiments described above depend on the response of TCR transgenic cells transferred into mice. As yet, no published report examining the endogenous *Chlamydia*-specific $CD4^+$ T cell population is available. Such data would be helpful in determining the biological relevance of the TCR transgenic transfer system. To address the capacity of *C. trachomatis* to elicit a response of endogenous pathogen-specific $CD4^+$ T cells in mice, we used a MHC class II tetramer that recognizes T cells with the same epitope specificity as the T cells from *C. trachomatis*-specific TCR transgenic mice. Using the MHC class II tetramer, we isolated endogenous *C. trachomatis*-specific $CD4^+$ T cells from the peripheral lymphoid tissues of 1) naive mice, 2) mice responding to a primary infection with *C. trachomatis*, and 3) mice that had recovered from infection and therefore harbored populations of memory T cells. For comparison, we also purified endogenous *C. trachomatis*-specific $CD8^+$ T cells from the same groups of mice, using a tetramer that binds CrpA-specific T cells. As demonstrated in Fig. 6A, our tetramers were capable of identifying endogenous *C. trachomatis*-specific $CD4^+$ and $CD8^+$ T cells. The number of Ag-specific cells purified by the tetramer was enumerated in naive mice, at the peak of primary infection, and

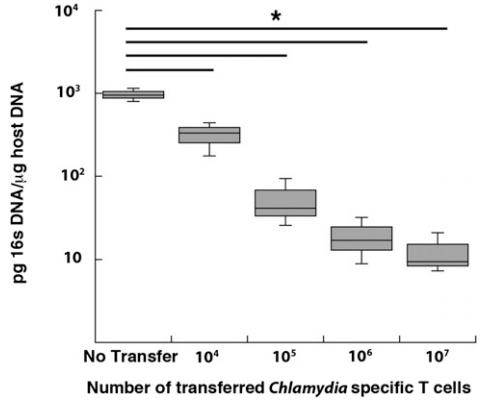

FIGURE 5. Ag-specific Th1 cells are sufficient to protect the genital mucosa from infection with *C. trachomatis*. Wild-type *Chlamydia*-specific $CD4^+$ T cells were skewed in vitro to the Th1 phenotype for 5 d. These preactivated T cells were then transferred into IFN-$\gamma^{-/-}$ host mice. The following day, the mice were challenged transcervically with 10^6 IFU *C. trachomatis*. At 6 d post infection, the genital tract was harvested and genomic DNA was isolated. We used quantitative PCR to compare the levels of *Chlamydia* 16S DNA with host GAPDH. Shown is a box-and-whisker plot from one representative experiment of three independent experiments. *$p < 0.05$.

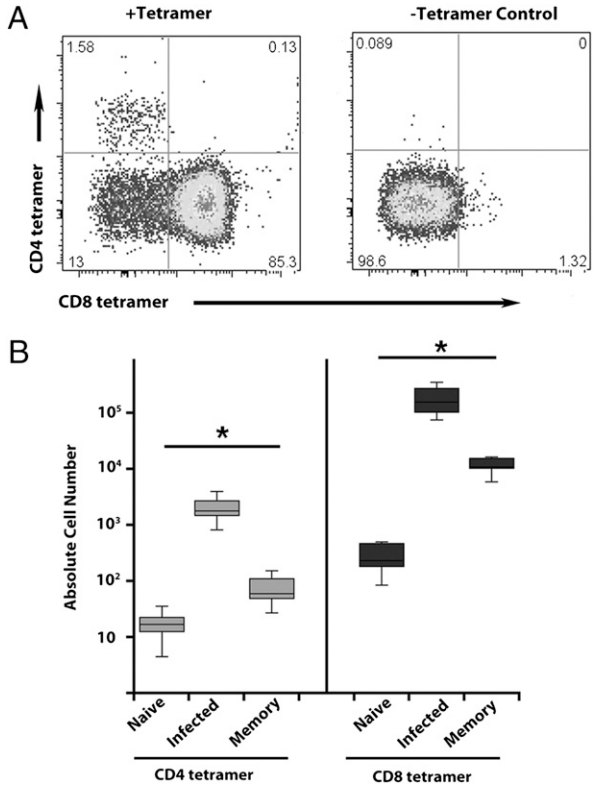

FIGURE 6. Endogenous T cell response to *C. trachomatis* infection. T cells specific for *C. trachomatis* were isolated from naive, infected, or memory mice via magnetic isolation and tetramer pulldown. (**A**) Representative plot for isolation of tetramer-specific and negative control at the peak of primary infection. The axis identifies $CD4^+$ and $CD8^+$ T cells specific for CTA-1 and CrpA, respectively. (**B**) Absolute number of pathogen-specific tetramer-positive $CD4^+$ and $CD8^+$ T cells isolated from naive, primary infected, and memory mice. *$p < 0.05$.

during late memory development (>4 wk post infection, Fig. 6C). The endogenous epitope-specific $CD8^+$ T cells vastly outnumbered the epitope-specific $CD4^+$ T cells at all time points. However, a similar trend was observed with both $CD4^+$ and $CD8^+$ tetramer-positive cells in which the cell population expanded 100- to 1000-fold during the primary immune response, followed by a contraction, leaving a memory population 5- to 10-fold higher than the initial naive population ($p < 0.05$). To our knowledge, these experiments are the first to examine the expansion and contraction of endogenous pathogen-specific T cells following *Chlamydia* infection. Of interest, the absolute number of class II tetramer-positive cells identified during the peak of infection is similar to the minimum number of transgenic T cells needed for protection (Fig. 5). Therefore, using two complementary techniques we can show that between 5000 and 10,000 Ag-specific $CD4^+$ T cells are necessary for protection in the genital mucosa. Collectively, these data indicate that endogenous $CD4^+$ T cell immunity is primed following transcervical infection with *C. trachomatis* and a memory $CD4^+$ T cell pool develops to protect against reinfection.

Discussion

Understanding adaptive immunity to *C. trachomatis* is key to developing an effective vaccine against this pathogen. Following vaginal infection in humans, the bacteria can ascend to the upper genital tract, where persistent infection results in inflammation and tissue damage. In contrast to human infection, vaginal infection of mice with *C. trachomatis* does not result in significant upper genital tract infection or pathology (28). The mouse-specific *Chlamydia* species, *C. muridarum*, is able to ascend from the vagina to the upper genital tract, causing robust inflammation but not persistent infection (36). Deciphering any differences in how the immune system responds to these organisms will allow both to be used in studies of disease pathogenesis and to develop vaccines.

In this article, we sought to compare the ability of the mouse to mount a $CD4^+$ T cell response against both *Chlamydia* species and measure the level of protection provided by that immunity. One of the most significant differences between the two species is the inability of *C. trachomatis* to ascend from the vagina/ectocervix to the upper genital tract in mice. This deficiency may prevent *C. trachomatis* from stimulating robust protective immunity in the vaginal vault, as previous reports have suggested that the lower genital tract is an immune-suppressive environment (37, 38). We hypothesized that the cervix is the primary physical barrier to upper genital tract infection with *C. trachomatis*, and that if we bypassed this restriction we might allow for an immune response to this human-adapted pathogen. We show there is a marked difference in the infection and the resulting immune response between transcervical and vaginal delivery of *C. trachomatis*. Transcervical infection also led to gross pathology in ~15% of animals, whereas intravaginal inoculation of *C. trachomatis* led to no obvious pathological changes (Fig. 1B; data not shown). To our knowledge, this is the first fully immune competent mouse model to consistently demonstrate gross pathology after *C. trachomatis* infection (39). One important consideration with this study is the use of the LGV strain of *C. trachomatis*. Future studies will need to broadly examine the transcervical infection model along with other genital strains of *C. trachomatis* that are of high public health interest. These studies will allow us to begin examining the characteristics of both Ag-specific cells and bystander cells that promote the induction of disease across several clinically relevant strains of *C. trachomatis*—studies that have been difficult up to this point.

Our infection time course showed that bypassing the cervix alone does not lead to a pathogen burden similar to that with *C. muridarum*. The 10-fold increase in bacterial burden from day 3 to day 6 following *C. muridarum* infection was not seen with *C. trachomatis*. We next examined whether the lack of expansion was due to the suppression of *C. trachomatis* growth by the murine IFN-γ response, as previous reports have shown that absence of IFN-γ leads to higher burden and longer duration of *C. trachomatis* infection (40, 41). IFN-γ–mediated restriction of *C. trachomatis* is predominantly driven through IRGs (22, 25, 42). In contrast to *C. trachomatis*, *C. muridarum* is thought to evade IRG-mediated growth restriction owing to the expression of a cytotoxin, which undermines this restriction mechanism (25). When mice deficient in either IFN-γ or IRGs (IRGm1/m3) are infected with *C. trachomatis*, we observe a burst of bacterial growth from day 3 to day 6. These enhanced bacterial loads are identical, whether it is IFN-γ–knockout mice or IRGm1/m3-knockout mice infected transcervically with *C. trachomatis*, or wild-type mice infected with *C. muridarum* (Fig. 2). Thus, bypassing the physical restriction of the cervix, in combination with the lack of IRGs, leads to a *C. trachomatis* infection model system in which loads and pathological changes are comparable to those observed with *C. muridarum* vaginal infection. Both *C. trachomatis* transcervical infections and *C. muridarum* intravaginal infections are highly inflammatory, as measured by the significant influx of neutrophils and the response of $CD4^+$ T cells that ultimately are able to clear infection with either species (data not shown; Refs. 30, 43). Importantly, neither mouse model allows development of the chronic infections observed in humans (30, 40). One explanation for the lack of persistent infection in mice is that mice and humans have different cell-autonomous mechanisms of responding to IFN-γ. Humans lack an IFN-γ–inducible IRG response, and instead respond to IFN-γ by upregulating the expression of IDO (7, 44). IDO induces tryptophan catabolism, resulting in a persistent form of *C. trachomatis* that does not grow rapidly and is not cleared. It is this critical difference in the response to IFN-γ that may prevent *C. trachomatis* and *C. muridarum* from causing persistent infections in a murine model of genital infections. We are now examining whether transcervical infection of mice in which IRGm1 and IRGm3 are knocked out, and human IDO is knocked in, might allow infection with *C. trachomatis* that more closely models the persistent infections seen in humans.

It is well accepted that the primary source of IFN-γ seen during infection is the $CD4^+$ Th cell. However, one recent report called into question the role, if any, that $CD4^+$ T cells play in the resolution of, and protection against, *C. trachomatis* during murine infection. Morrison et al. (8) showed that clearance of vaginal *C. trachomatis* genital infection is unaltered by depletion of $CD4^+$ T cells in mice deficient in innate immunity. The authors concluded that genital infection with *C. trachomatis* in mice does not stimulate an adaptive immune response and does not protect from subsequent infection. We hypothesized that the lack of adaptive immunity observed using their model was due to the inability of *C. trachomatis* to access the upper genital tract. Transcervical infection resulted in a significant enhancement in the ability of *C. trachomatis* to prime pathogen-specific T cells and recruit them to the upper genital mucosa (Fig. 3A, 3D). Transcervical infection with *C. trachomatis* also stimulated a robust $CD4^+$ memory response that was essential for protection following reinfection of the genital tract (Fig. 4). We further characterized the protective capacity of $CD4^+$ T cells, showing that pathogen-specific cells skewed toward Th1 are sufficient to protect naive mice from *C. trachomatis* in a dose-dependent manner (Fig. 5). To our knowledge, for the first time we were able to show that only

1000 *Chlamydia*-specific T cells constitute the lower limit needed for significant protection (Fig. 5). Finally, we tested whether transcervical inoculation was capable of stimulating endogenous *C. trachomatis*-specific T cells. Using a MHC II tetramer to track T cells during a genital tract infection, we demonstrated clonal expansion and memory development of endogenous epitope-specific CD4+ T cells (Fig. 6). These results showed that during the peak of infection, >1000 Ag-specific T cells are induced, similar to the lowest transfer dose in our protection experiments (Fig. 5). By quantifying the lower limit needed for protection, we now have a baseline that allows us to tune the infiltration of different cell types to reduce pathological changes while still promoting protection. Together, our studies illustrate our unique ability to examine physiological levels of *Chlamydia*-specific T cell responses, using a combination of TCR transgenic T cells and class I and class II tetramers. To date, there is a lack of data using TCR transgenic T cells or MHC II tetramers to determine the extent to which *Chlamydia*-specific CD4+ T cells are recruited to the genital mucosa following *C. muridarum* infection. This shortcoming in the literature has made it impossible to know whether the T cells responding to *C. muridarum* infection are Ag specific or whether many of them are bystander cells that merely follow the chemokine/cytokine gradients resulting from inflammation. Studying total CD4+ T cell infiltration may not reflect the Ag-specific immune response generated by *C. trachomatis* and *C. muridarum* infections. Only by differentiating Ag-specific and bystander immune responses can we determine the role of these populations in development of the immune disorders seen clinically.

This study demonstrates that infection of the upper genital tract with either *C. trachomatis* or *C. muridarum* stimulates protective immune responses as well as gross pathology. Immunity to both species is dependent on CD4+ T cells, and only 1000 *Chlamydia*-specific CD4+ T cells are sufficient to confer protection. Importantly, in the absence of the murine IRG or IFN-γ response, *C. trachomatis* and *C. muridarum* colonize the murine upper genital tract at similar levels, yet neither species causes persistent infections. The novel transcervical inoculation technique described in this article will provide a technically easy, noninvasive, highly reproducible, biologically relevant system for vaccine development (29). The model allows investigators to transcend discussions of relevant model system and focus on issues of disease versus protection following infection. These discussions are a critical step in defining the factors that drive *Chlamydia*-specific pathogenesis and host defense.

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Disclosures

The authors have no financial conflicts of interest.

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