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CD4+ T cells are necessary and sufficient to confer protection against *C. trachomatis* infection in the murine upper genital tract

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Abstract

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 where 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. Here we describe a non-invasive transcervical infection model where 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 re-infection.

Introduction

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 (PID), which can result in fallopian tube scarring, infertility, and ectopic pregnancy(2, 3). Better understanding of the interaction of *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 re-infection (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 pathology 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 IFNγ required for bacterial clearance. (9, 16). Infection of $CD4^{-/-}$ mice with *C. trachomatis* leads to higher pathogen load during primary infection, and 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 wildtype and μMT mice demonstrating a requirement for $CD4^+$ T cells in protective immunity to secondary infection

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(17). In contrast, a recent study where antibody 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 antigen 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 pathologies such as hydrosalpinx and infertility (18–21). The use of the *C. muridarum* model has increased our knowledge about *Chlamydia* pathogenesis and immunity however, there are limitations to its use, specifically in the identification of antigens for use in a vaccine to protect against *C. trachomatis* (22–26). To date, there have been no published T cell epitopes shared between *C. trachomatis* and *C. muridarum*(6, 27, 28). Furthermore, *C. muridarum* only models acute phases of human *C. trachomatis* infection and not the chronic phases that are responsible for pathology in humans (5, 6). By identifying new protective T cell antigens and tracking *C. trachomatis*-specific responses to those antigens we may be able to differentiate responses that lead to protective immunity versus those that cause deleterious pathologies (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 immunity, similar to human infections. Here, we describe a model of mouse infection with *C. trachomatis* where 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 to vaginal inoculation with *C. trachomatis*, transcervical inoculation allowed for more efficient colonization and stimulated a more robust and inflammatory antigen specific T cell response in the upper genital tract while also allowing for the consistent development of pathology. Using this model, we characterized the induction of antigen specific memory $CD4^+$ T cells and show that they are necessary and sufficient for protection against re-infection of the murine genital mucosa. This study demonstrates a novel inoculation method that will allow investigators to build on each other's research results by 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*.

Methods and Materials

Mice

C57BL/6, B6.PL-*Thy1a* (CD90.1 congenic), and B6.129S7-*IFNγtm1Agt* (IFN-γ−/−) 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 2.5mg of medroxyprogetrone subcutaneously seven days prior to infection in

order to normalize the murine estrous cycle. All experiments were approved by 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 sonicated to disrupt the inclusion. EBs 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 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 (REF). For titering directly from the genital tract, at the given timepoints the upper genital tract was isolated, homogenized by mechanical disruption, and placed in six-well plates pre-seeded with 5×10^5 McCoy cells and incubated for 36 hours 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. Thirty hours 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-mercaptoethanol, 50 U/ml penicillin, and 50 ug/ml streptomycin. To stimulate the T cells, irradiated feeder splenocytes were pulsed with 5 μM of Cta1 133–152 peptide and cocultured with the CD4-enriched NR1 cells at a stimulator to T cell ratio of 4:1. To polarize T cells towards the Th1 phenotype cells were incubated with 10 ng/ml of IL-12 (Peprotech, Rocky Hill NJ) and 10 μg/ml of anti-IL4 (Biolegend). Cells were stimulated for five days then 10^6 cells were transfered into naïve $CD90.2^+$ IFNγ−/− host mice. Twenty-four hours following transfer, mice were challenged in the uterus with 106 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 Bioscience). For activation marker analysis, we examined anti-CD44 PE-CyChrome 7 (Biolegend), anti-CD62L allophycocyanin-Alexa 750 (Ebioscience), and anti-CD25 allophycocyanin (BD Bioscience). For intracellular staining, the following antibodies were used: anti-IFN-γ PE, anti-IL2- allophycocyanin, anti-IL17 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 Bioscience). The absolute cell numbers in each sample was determined using accucheck counting beads (Invitrogen). Data were collected on a modified FACSCalibur (Cytek Development) or an LSRII (BD Bioscience) and analyzed using Flow Jo (Tree Star).

T cell depletion

For $CD4^+$ T cell depletion experiments, mice were infected with 10^6 IFU *C. trachomatis* or *C. murdidarum* then rested for greater than four weeks. Starting three days prior to secondary challenge, immune mice were intraperitoneally injected with 200μg/mouse anti-CD4 (clone GK1.5) or isotype control (clone LTF-2) everyday. Mice were sacrificed five days 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

Chlamydia 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 NIH tetramer facility (Emory University), purified and tetramerized with streptavidin allophycocyanin or phycoerythrin, 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 30min. Cells were then washed and incubated with anti-PE and anti-APC magnetic beads. Bead-bound cells were then enriched on magnetized columns and a sample was removed for counting. The enriched cells were surface stained with combinations of antibodies listed above.

Statistical analysis

All groups were evaluated for statistical significance through the use of unpaired two-tailed *t* tests. Where 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 on the 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 (NSET device) to bypass the cervix and inject the bacteria directly into the lumen of the uterus (supplemental figure 1). To understand how the bacterial burden in the upper genital tract changes with infection route or *Chlamydia* species, mice were given 10^6, 4.5×10^5, or 10^3 IFU of *C. trachomatis* or *C. muridarum* by either the intravaginal or transcervical method. Three days after infection with 10^6 IFU of *C. trachomatis* transcervically (gray bars) or *C. muridarum* intravaginally (white bars), the murine uteri harbored similar levels of bacteria, (figure 1). In stark contrast, vaginal inoculation with 10^6 IFU of *C. trachomatis* resulted in two 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 days after infection, followed by a precipitous decrease on day 9 and 15 (figure 1). Infection with *C. muridarum* intravaginally resulted in infection levels at day 3 comparable to that 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 days following infection (data not shown).

The relative numbers of *C. trachomatis* present in the upper genital tract were consistent whether we used quantitative PCR (Figure 1) or if we determined IFU by titering (Supplementary Figure 1B). We also examined bacterial load in mice infected with *C. muridarum* by transcervical inoculation. Although there is an even higher load 3 days post-infection (3000 pg/ng), the bacterial burden was similar to intravaginal inoculation for the remainder of the time-course (data not shown). Therefore, we only used intravaginal inoculation of *C. muridarum* for the remainder of the study. When *C. trachomatis* was inoculated via the vaginal route, there was no consistent infection of the upper genital tract. 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 figure 2a/b). Lowering the initial dose led to a corresponding decrease in bacterial burden on day three for both transcervical infections with *C. trachomatis* and vaginal inoculation with *C. muridarum*. As was seen with the higher 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 amount of this organism rises to a similar high level by day six after 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 compared to *C. trachomatis* intravaginal delivery but it never rises to the level seen following infection with *C. muridarum*.

We next examined whether transcervical infection leads to pathology in the upper genital mucosa. Mice were infected intravaginally with *C. trachomatis* or *C. muridarum* or infected transcervically with *C. trachomatis*. Twenty-five days after infection, the genital tract was removed and examined for gross pathology. Overall, pathology was 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 (Figure 1b and (31)). Mice infected transcervically with *C. trachomatis* showed major nodes of inflammation ascending the length of the upper genital tract (Figure 1B). These data indicate that the transcervical model of infection allows the formation of gross pathologies similar to what has been observed with *C. muridarum*. While there have been reports of pathology following *C. trachomatis* infection of innate immune-deficient mice strains, consistent pathology has never been described in wildtype C57Bl/6 mice – a strain restrictive for *C. trachomatis* growth. Furthermore, the pathology in *C. trachomatis* infected mice extends the length of the uterine horn, similar to rare cases described recently following *C. muridarum* infection (32).

Because there was no gross pathology observed on the ovary's following transcervical infection, we subjected several mice infected with C. trachomatis transcervically to histopathology in order 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 figure 1c. Evidence of large neutrophil and macrophage recruitment to the upper genital tract can be seen, 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 pathology similar to *C. muridarum* as well as human infection with *C. trachomatis*.

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

It is well accepted that the IFNγ response between 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 IFNγ response (22, 25). We therefore tested whether the differential effect of IFNγ could explain why the level of *C. muridarum* increased 10-fold from day three to day six, whereas the level of *C. trachomatis* observed following transcervical inoculation did not increase over the same time period. As shown in figure 2, we challenged wild type or IFNγ$^{-/-}$ mice transcervically with *C. trachomatis* and compared the bacterial burden to that obtained through vaginal inoculation with *C. muridarum*. In the mice lacking IFNγ, transcervical infection with *C. trachomatis* led to an expansion of organisms by day 6 that closely resembled the expansion of organisms observed in wild type mice infected with *C. muridarum*. These results suggest that IFNγ restricts the initial growth of *C. trachomatis* rapidly following infection of the upper genital tract. Since this rapid restriction is prior to the peak infiltration of T cells (10), it also leaves open the possibility of other sources of IFNγ capable of restricting *C. trachomatis* growth - such as NK cells resident in the cervical tissues (33). We suspected that the primary effectors of this IFNγ 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 six compared to wild type mice, a level identical to what we observed using IFNγ$^{-/-}$ mice (Figure 2). These data indicate that even though the human adapted *C. trachomatis* is still constrained in its ability to grow due to innate responses mediated by a rapid induction of IFNγ and the murine IRG system, removal of Irgm1/m3 alone allows *C. trachomatis* to become resistant to the murine-specific IFNγ response and to grow similarly to *C. muridarum*.

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 which 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 we used antigen specific CD4$^+$ T cells to directly compare T cell proliferation, activation, cytokine secretion, and recruitment to the genital mucosa between mice infected with *C. trachomatis* intravaginally or transcervically.

We first wanted to determine whether antigen-specific CD4$^+$ T cells are activated following transcervical or intravaginal infection. We transferred CFSE-labeled *C. trachomatis*-specific CD4$^+$ TCR transgenic T cells into naïve mice, which were then challenged with *C. trachomatis* either by the transcervical or vaginal route of infection. As demonstrated in figure 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% vs. 89% becoming CFSE-low). However, the proliferation of *Chlamydia* specific T cells was more robust when infected transcervically 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 there was no difference in the activation state of T cells stimulated by the two routes of infection (Figure 2B).

Recent evidence from multiple studies using mice, non-human primates, and humans has shown convincingly that the quality of a T cell response is a critical factor in defining protective immunity (34). To examine if there is a difference in the quality of the T cell response following different routes of infection, we compared the ability of pathogen-specific T cells to produce multiple cytokines following either transcervical or intravaginal infection with *C. trachomatis*. We transferred *C. trachomatis*-specific $CD4^+$ TCR transgenic T cells into naïve mice, which were then challenged with *C. trachomatis* either by the transcervical or vaginal route of infection. Seven days after infection, we examined the ability of the pathogen specific cells to produce multiple cytokines by flow cytometry. Transcervical inoculation induced over 50% of antigen-specific $CD4^+$ T cells into a "triple producer" phenotype - capable of robustly producing TNFα, IFNγ, and IL-2 simultaneously (Figure 3C -black dots, upper right). These "triple producer" populations have been associated with enhanced protection in other infection models (34). Interestingly, *Chlamydia*-specific T cells from mice infected intravaginally did not contain a high proportion of "triple producing" T cells (black dots vs. gray dots in Figure 3). This suggests that transcervical infection yields a higher quality immune response when compared directly to intravaginal infection.

Our CFSE proliferation data suggested that transcervical infection leads to more proliferation and accumulation of antigen specific T cells (Figure 3A). We next wanted to directly quantify the accumulation of *Chlamydia*-specific T cells in the draining lymph node and genital mucosa. We transferred *C. trachomatis*-specific $CD4^+$ TCR transgenic T cells into naïve mice, and then challenged them with *C. trachomatis* either by the transcervical or vaginal route of infection. Seven days after infection, we measured the absolute number of pathogen-specific T cells in the draining lymph nodes and the genital mucosa. We identified five-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 compared to intravaginally (Figure 3D). These data indicate that transcervical infection leads to enhanced recruitment of antigen specific cells to both the site of activation (the draining lymph node) and the target tissue (the genital mucosa) when compared directly to intravaginal inoculation. 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 the both the draining lymph node and 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* 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 when compared to the vaginal route of inoculation. 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.* showed that following intravaginal inoculation the depletion of $CD4^+$ T cells during the primary immune response did not result in enhanced disease (8). This is in contrast to previous work from our lab showing 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 either infected with 10^6 IFU of *C. trachomatis* transcervically or *C. muridarum* intravaginally, rested for greater than 4 weeks to allow clearance of the initial infection, then re-challenged with the same pathogen. Before re-challenge, the mice were divided into groups treated with either antibody to deplete $CD4^+$ T cells or an isotype control IgG (Figure 4). Depletion was confirmed by flow cytometry, showing more than 1000-fold reduction in the number of $CD4^+$ T cells in the draining lymph nodes and 10-fold in the genital mucosa (supplementary figure 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 naïve control group ($p<0.01$). Immune mice that were depleted of $CD4^+$ T cells did not exhibit any protection and the bacterial burden of *C. trachomatis* was similar to that of naïve mice. In comparison, the bacterial burden in immune mice challenged with *C. muridarum* exhibited a nearly 1000-fold level of protection as compared to the naïve 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 if pathogen specific $CD4^+$ T cells are sufficient to confer protection. Our previous studies have indicated that transfer of 10^7 TH1-skewed antigen-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 here, and therefore might not have accurately reflected the behavior of endogenous antigen-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 pre-activated and skewed towards the TH1 phenotype. Cells were then transferred into mice in numbers ranging from 10^4–0^7. One day after the transfer of T cells, the mice were infected transcervically with *C. trachomatis*. As demonstrated in figure 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 similar protection typical of an immune mouse (Figure 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. For the first time this allows us to estimate the lower limit of antigen specific $CD4^+$ T cells needed to protect the host from infection with *C. trachomatis*. By knowing this lower limit, future vaccines can be designed that offer protection, but where immunopathology can be limited.

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, there is no published report examining the endogenous *Chlamydia*-specific $CD4^+$ T cell population. 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 which recognizes T cells with the same epitope specificity as the T cells from the *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) naïve 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 figure 6a, our tetramers were capable of identifying endogenous *C. trachomatis* specific $CD4^+$ and $CD8^+$ T cells. The number of antigen specific cells purified by the tetramer was enumerated in naïve mice, at the peak of primary infection, and during late memory development (>4 weeks post infection, Figure 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 $CD8^+$ $tetramer^+$ cells in which there was a 100–1000 fold expansion of the cell population during the primary immune response followed by a contraction, leaving a memory population which was 5–10 fold higher than the initial naïve population ($p < 0.05$). These experiments are the first to examine the expansion and contraction of endogenous pathogen-specific T cells following a genital infection. Interestingly, 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 (Figure 5). Therefore using two complimentary techniques we can show that between 5000 and 10000 antigen 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 re-infection.

Discussion

Understanding adaptive immunity to *C. trachomatis* is key to the development of 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.

Here 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 roughly 15% of animals while intravaginal inoculation of *C. trachomatis* led to no obvious pathology (Figure 1B and data not shown). 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 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 antigen specific cells and bystander cells that promote the induction of pathology across several clinically relevant strains of *C. trachomatis*; studies that have been difficult up to this point.

Our infection timecourse showed that bypassing the cervix alone does not lead to pathogen burden similar to the *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 IFNγ response, as previous reports have shown that the absence of IFNγ leads to higher burden and longer duration of *C. trachomatis* infection (40, 41). IFNγ -mediated restriction of *C. trachomatis* is predominantly driven through the activity of the IRG family of GTPases (22, 25, 42). In contrast to *C. trachomatis*, *C. muridarum* is thought to evade IRG-mediated growth restriction due to the expression of a cytotoxin, which undermines this restriction mechanism (25). When mice deficient in either IFNγ 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 IFNγ -KO mice or IRGm1/m3-KO mice infected transcervically with *C. trachomatis*, or wildtype mice infected with *C. muridarum* (Figure 2). Thus, bypassing the physical restriction of the cervix, in combination with the lack of IRGs, leads to a *C. trachomatis* infection model system where loads and pathologies are comparable to that 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 and (30, 43)). Importantly, neither mouse model allows the 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 IFNγ. Humans lack an IFNγ-inducible IRG response, and instead respond to IFNγ by upregulating the expression of indolamine dioxygenase (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 IFNγ 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 IFNγ seen during infection is the $CD4^+$ T helper cell. However, one recent report called into question the role, if any, $CD4^+$ T cells play in the resolution of, and protection against, *C. trachomatis* during murine infection. Morrison *et al.* showed that clearance of vaginal *C. trachomatis* genital infection is unaltered by depletion of $CD4^+$ T cells in mice deficient for innate immunity (8). The authors concluded that genital infection with *C. trachomatis* in mice does not stimulate an adaptive immune response and are not protected 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 (figure 3a/d). Transcervical infection with *C. trachomatis* also stimulated a robust $CD4^+$ memory response that was essential for protection following re-infection of the genital tract (figure 4). We further characterized the protective capacity of $CD4^+$ T cells, showing that pathogen specific cells skewed towards TH1 are sufficient to protect naïve mice from *C. trachomatis* in a dose dependent manner (figure 5). For the first time we were able to show that only 1000 Chlamydia specific T cells is 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 for the first time to track T cells during a genital tract infection, we demonstrated clonal expansion and memory development of endogenous epitope-specific $CD4^+$ T cells (figure 6). These results showed that during the peak of infection, >1000 antigen specific T

cells are induced, similar to the lowest transfer dose in our protection experiments (Figure 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 pathology 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 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 antigen-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 antigen-specific immune response generated by *C. trachomatis* and *C. muridarum* infections. Only by differentiating antigen specific and bystander immune responses can we determine the role of these populations in development of the immune pathologies 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 IFNγ 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 here will provide a technically easy, non-invasive, 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 pathology vs. protection following infection. These discussions are a critical step in defining the factors that drive *Chlamydia* specific pathogenesis and host defense.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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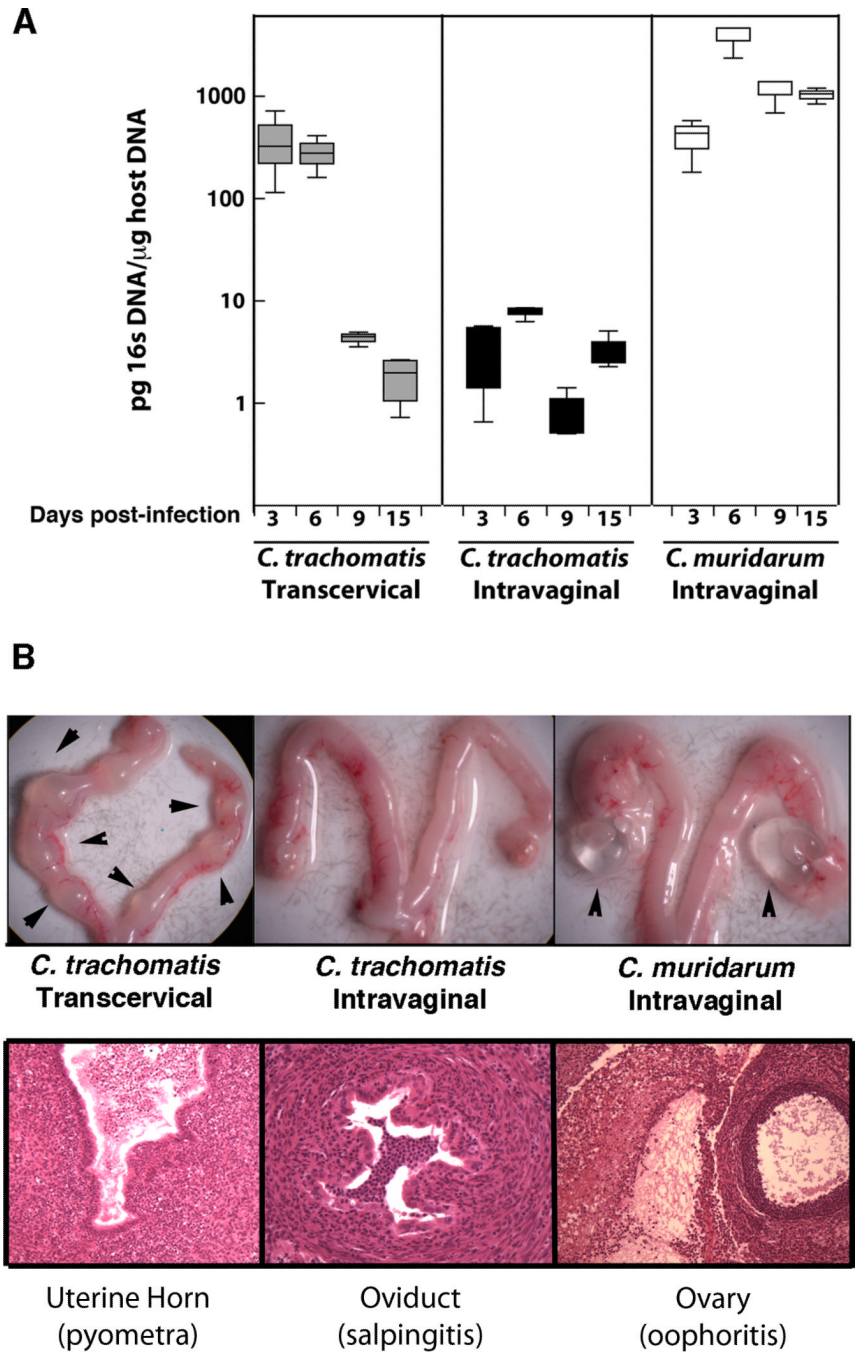

Figure 1. Transcervical infection of the genital mucosa with *C. trachomatis* leads to efficient colonization and pathology in the upper genital tract

Wildtype mice were infected by either intravaginal or trancervical 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) 25 days following infection the upper genital tract was isolated, photographed, and scored for pathology. 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 hematoxylin and eosin. Shown are representative images of the inflammation seen in the uterine horn, oviduct, and ovary following transcervical infection.

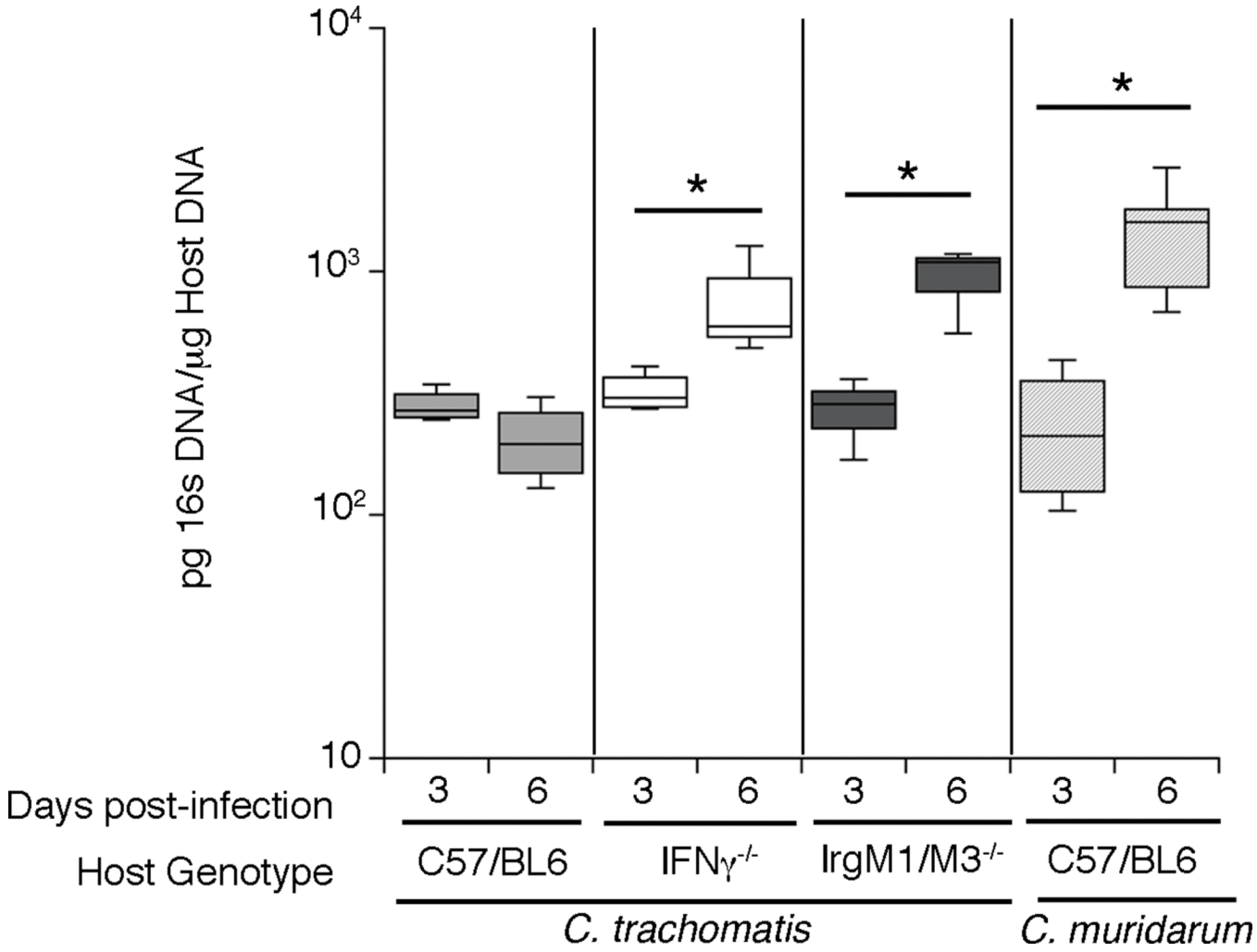

Figure 2. IFNγ restricts *C. trachomatis* rapidly following infection of the upper genital mucosa

Wild-type, IFNγ 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.* Three and six days 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<.05.

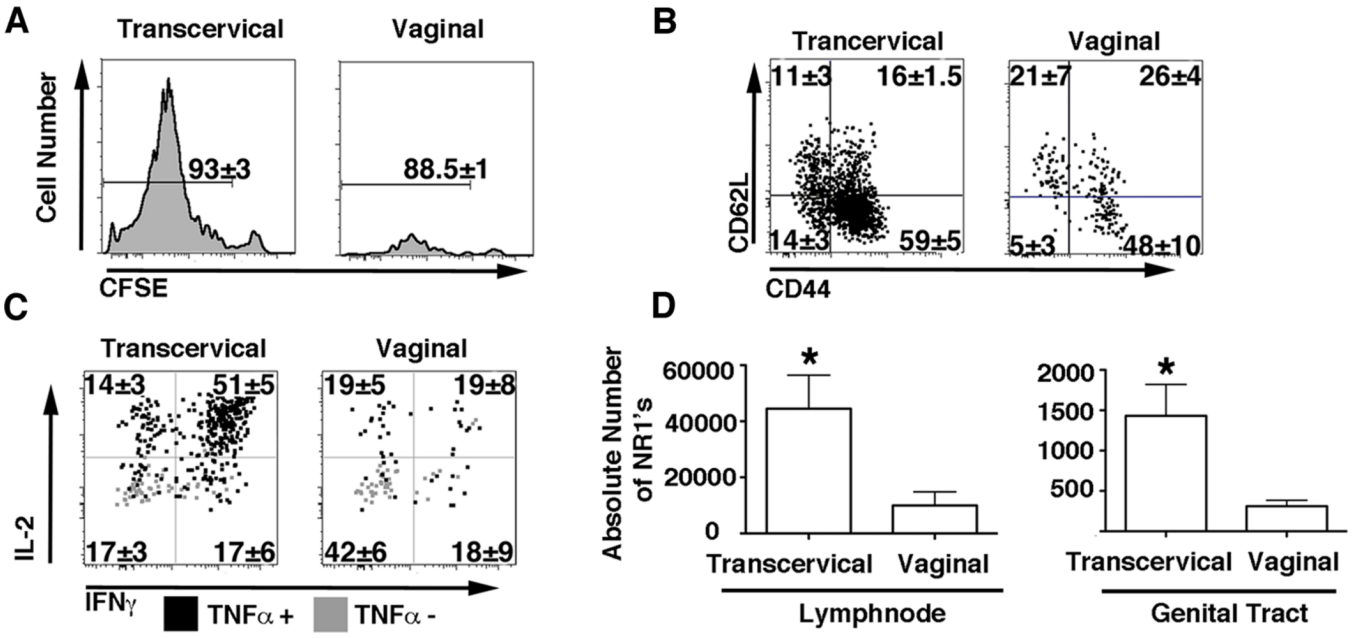

Figure 3. Transcervical infection with *C. trachomatis* leads to the accumulation of cytokine secreting pathogen specific CD4$^+$ T cells

Wildtype mice were infected by either intravaginal or trancervical 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 one day before they were inoculated transcervically or intravaginally with 10^6 IFU of *C. trachomatis*. Seven days following infection the draining lymph nodes were harvested and cells were prepared for flow cytometry. Antigen 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 seven days following infection, re-stimulated in vitro, and prepared for intracellular cytokine staining. Gray or Black dots indicate whether cells are positive or negative for TNFα. repectively. (D) Wild-type CD90.1$^+$ transgenic CD4$^+$ T cells were transferred into CD90.2 hosts one day before they were inoculated transcervically or intravaginally with 10^6 IFU of *C. trachomatis*. Seven days 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.

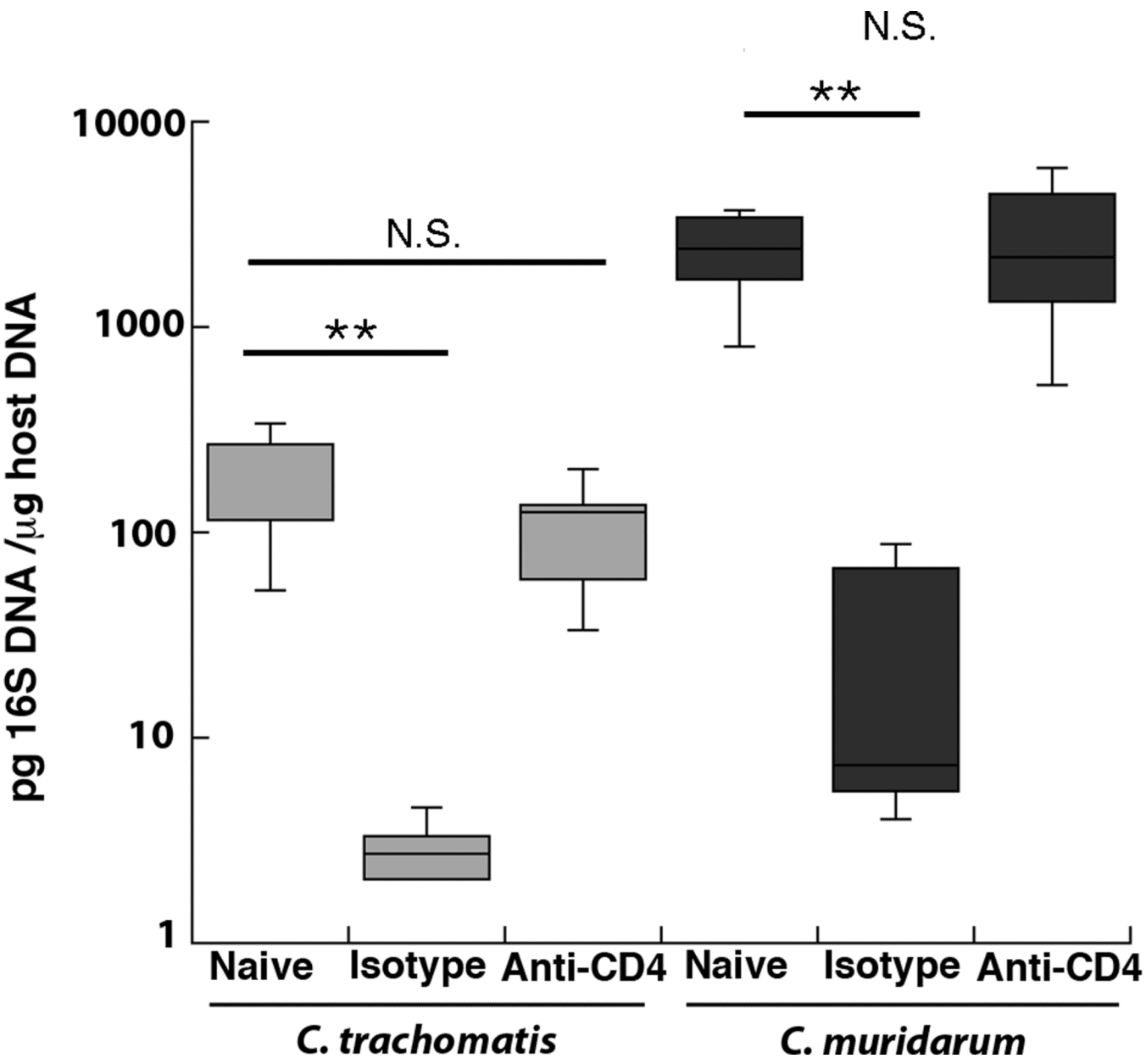

Figure 4. CD4$^+$ T cells protect the genital mucosa from re-infection with *C. trachomatis*

Wildtype mice were infected transcervically with 10^6 IFU of *C. trachomatis* or intravaginally with 10^6 IFU of *C. muridarum*. Five weeks after primary infection mice were injected with anti-CD4 or isotype control antibody. Naïve and immune mice were then infected with 10^6 IFU of *C. trachomatis* or *C. muridarum.* Six days 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<.01.

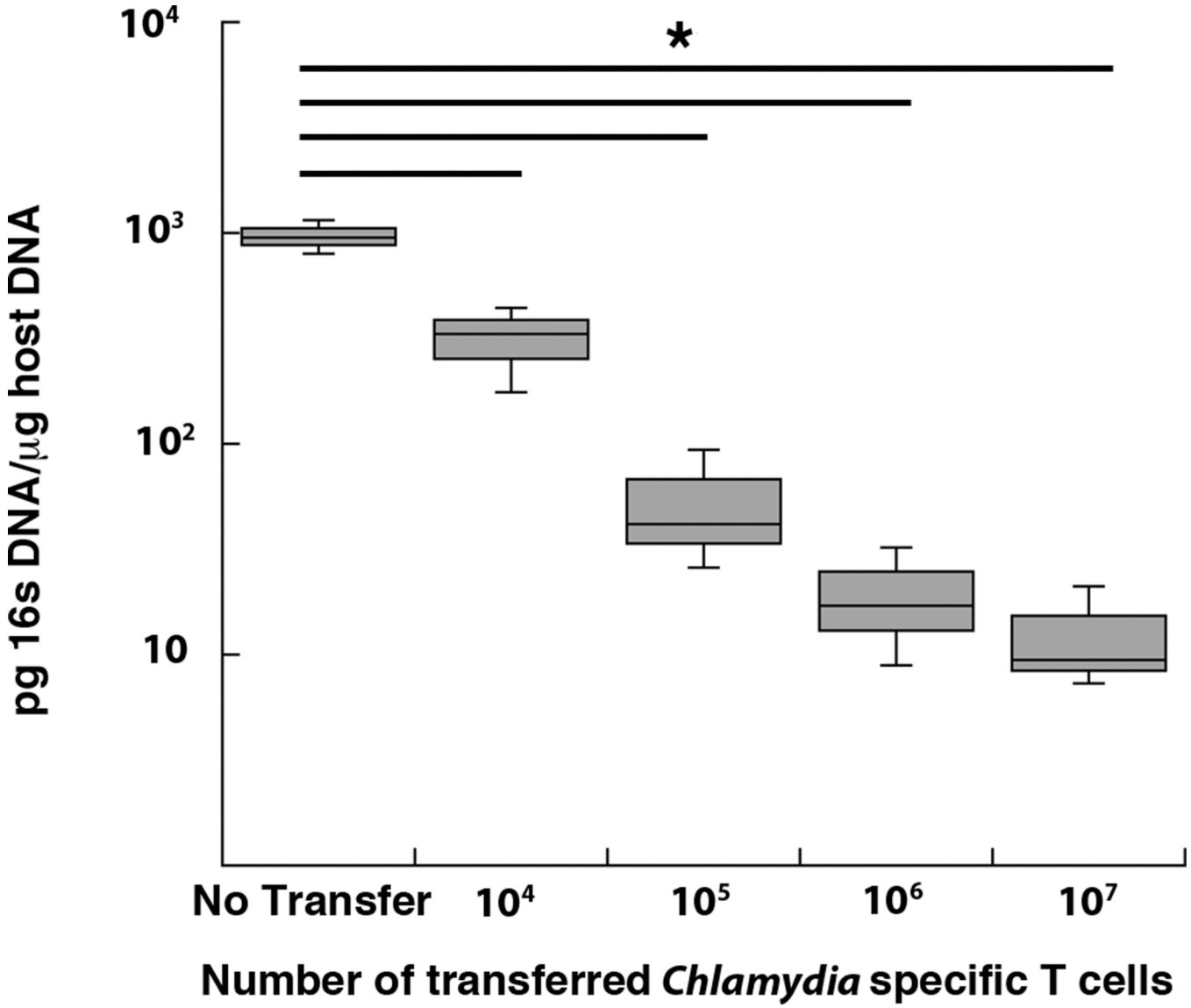

Figure 5. Antigen-specific TH1 cells are sufficient to protect the genital mucosa from infection with *C. trachomatis*

Wildtype *Chlamydia* specific $CD4^+$ T cells were skewed *in vitro* to the TH1 phenotype for 5 days. These pre-activated T cells were then transferred into IFNγ host mice. The following day the mice were challenged transcervically with 10^6 IFU *C. trachomatis*. Six days after infection, the genital tract was harvested and genomic DNA was isolated. We used quantitative PCR to compare the levels of *Chlamydia* 16s DNA to host GAPDH. Shown is a box-and-whisker plot from one representative experiment of three independent experiments. *P<.05

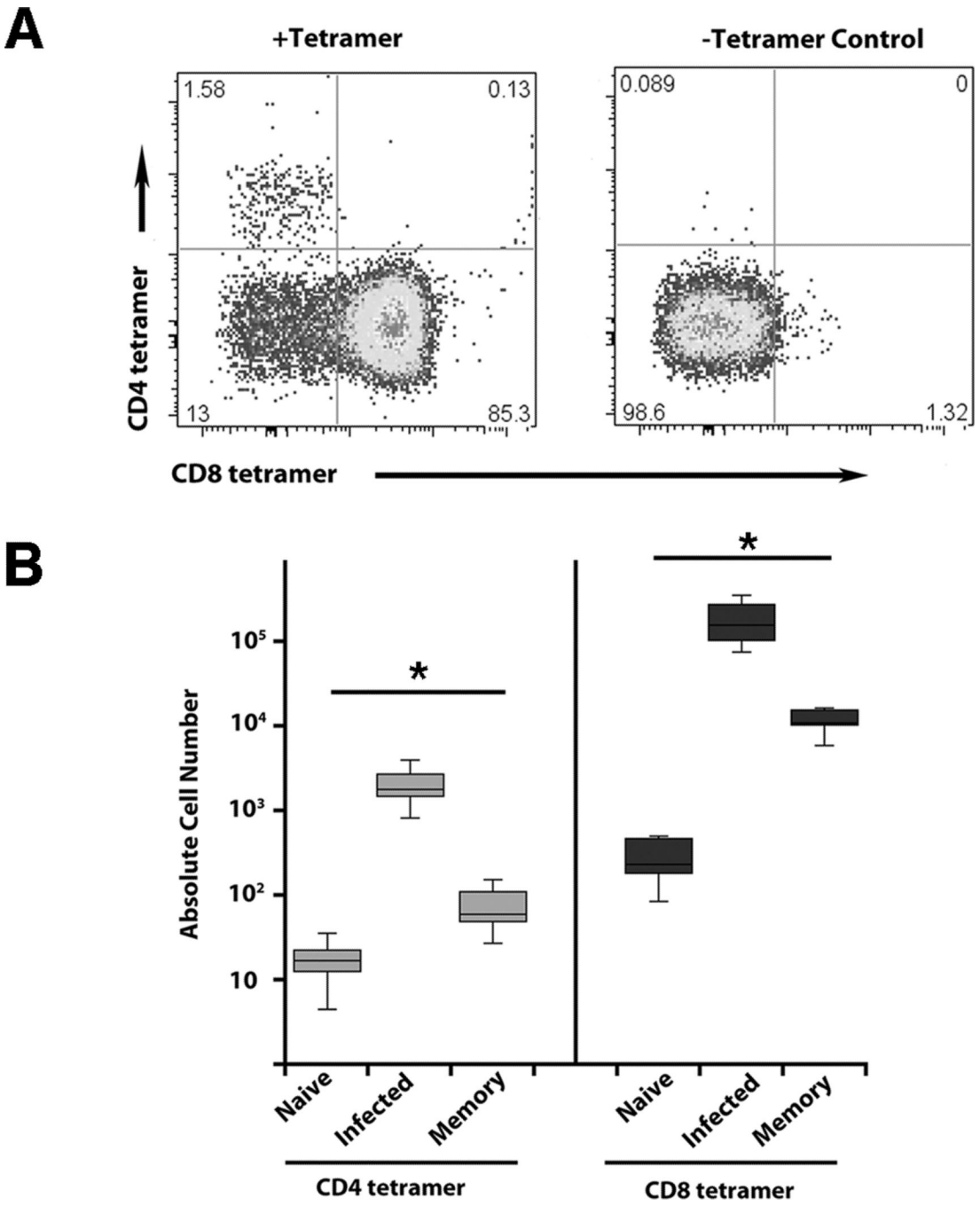

Figure 6. Endogenous T cell response to *C. trachomatis* infection

T cells specific for *C. trachomatis* were isolated from naïve, infected, or memory mice via magnetic isolation and tetramer pull down. 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 naïve, primary infected, and memory mice. *P<.05