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# Innate IFN-γ Is Essential for Systemic *Chlamydia muridarum* Control in Mice, While CD4 T Cell-Dependent IFN-γ Production Is Highly Redundant in the Female Reproductive Tract

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**ABSTRACT** Protective immunity against the obligate intracellular bacterium *Chlamydia* has long been thought to rely on CD4 T cell-dependent gamma interferon (IFN-γ) production. Nevertheless, whether IFN-γ is produced by other cellular sources during *Chlamydia* infection and how CD4 T cell-dependent and -independent IFN-γ contribute differently to host resistance have not been carefully evaluated. In this study, we dissected the requirements of IFN-γ produced by innate immune cells and CD4 T cells for resolution of *Chlamydia muridarum* female reproductive tract (FRT) infection. After *C. muridarum* intravaginal infection, IFN-γ-deficient and T cell-deficient mice exhibited opposite phenotypes for survival and bacterial shedding at the FRT mucosa, demonstrating the distinct requirements for IFN-γ and CD4 T cells in host defense against *Chlamydia*. In *Rag1*-deficient mice, IFN-γ produced by innate lymphocytes (ILCs) accounted for early bacterial control and prolonged survival in the absence of adaptive immunity. Although type I ILCs are potent IFN-γ producers, we found that mature NK cells and ILC1s were not the sole sources of innate IFN-γ in response to *Chlamydia*. By conducting T cell adoptive transfer, we showed definitively that IFN-γ-deficient CD4 T cells were sufficient for effective bacterial killing in the FRT during the first 21 days of infection and reduced bacterial burden more than 1,000-fold, although mice receiving IFN-γ-deficient CD4 T cells failed to completely eradicate the bacteria from the FRT like their counterparts receiving wild-type (WT) CD4 T cells. Together, our results revealed that innate IFN-γ is essential for preventing systemic *Chlamydia* dissemination, whereas IFN-γ produced by CD4 T cells is largely redundant at the FRT mucosa.

**KEYWORDS** IFN-γ, innate, CD4 T cells, infection, *Chlamydia*

*Chlamydia trachomatis* is the obligate intracellular bacterium that causes the most prevalent sexually transmitted infection worldwide. The prevalence of *Chlamydia* infection is partially attributed to its nature as a "silent infection," as most women infected with *C. trachomatis* are asymptomatic and can resolve the infection spontaneously (1). Unfortunately, undiagnosed infections not only facilitate silent disease transmissions but can also lead to severe adverse effects, such as pelvic inflammatory disease, ectopic pregnancy, and infertility (2, 3). There is no licensed human *Chlamydia* vaccine available at present, partially owing to the lack of complete understanding of protective immune mechanisms (4–6).

CD4 T helper 1 (Th1) cell-dependent gamma interferon (IFN-γ) production promotes macrophage activation to eliminate intracellular pathogens within these professional phagocytes. This defense mechanism operates efficiently against bacterial pathogens that exhibit marked infection tropism for macrophages (7, 8). For *Chlamydia*, infection is largely restricted to epithelial cells at barrier tissues such as the eyes, lungs, and reproductive tract. Recent studies have shed light on the mechanisms of cell autonomous immunity against both human pathogen *C. trachomatis* and the mouse-adapted

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pathogen *Chlamydia muridarum* in epithelial cells in response to IFN-$\gamma$ (9–11). Nevertheless, the cellular source for IFN-$\gamma$ at different anatomic sites and their roles at different stages of infection remain to be characterized. Moreover, with increased knowledge of CD4 T cell biology, it is speculated that protective T cell responses to intracellular bacterium such as *Chlamydia* are likely to be more complex than Th1-dependent IFN-$\gamma$ production (12).

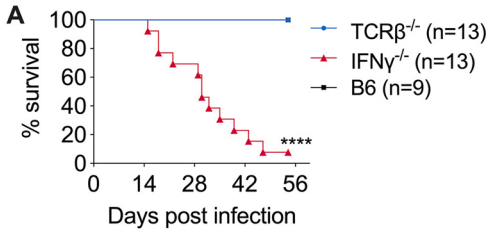
The mouse models of *C. muridarum* infection of the female reproductive tract (FRT) provide an invaluable tool for studying host immunity to *Chlamydia* infection. Research in the field has established a predominant role for CD4 T cells and antibody in host resistance and vaccine-afforded protection against *Chlamydia* (4, 13–15). After *C. muridarum* intravaginal infection, WT B6 mice exhibit a self-limiting infection that resolves within 4 to 5 weeks. Similar courses of infection have also been observed in mice lacking B cells, CD8 T cells, and $\gamma\delta$T cells, demonstrating the redundant roles of these cells in *C. muridarum* clearance from the female reproductive tract (16–18). In contrast, nude mice and mice lacking major histocompatibility complex class II (MHC-II)-restricted CD4 T cells shed persistent high levels of bacteria from the FRT without showing obvious signs of wasting (18–20). While a large body of evidence points to a role for Th1 but not other Th lineage cells in protective immunity against *Chlamydia* (17, 21–26), it remains perplexing that mice lacking the major Th1 effector cytokine IFN-$\gamma$ are capable of eliminating $>$99% of *C. muridarum* from the FRT, although they suffer from multiorgan disseminated infections (17, 27).

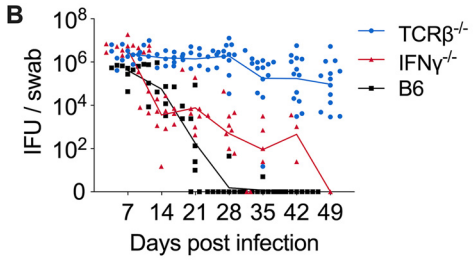
The distinct phenotypes of CD4 T cell-deficient and IFN-$\gamma$-deficient mice after *C. muridarum* infection prompted us to investigate the definitive contributions of CD4 T cell-dependent and -independent IFN-$\gamma$ production in host defense against *Chlamydia*. We hypothesized that IFN-$\gamma$ produced during innate immune response prevents lethal disseminated infection, while CD4-dependent IFN-$\gamma$ production is largely dispensable at the FRT mucosa. We tested these hypotheses using loss- and gain-of-function approaches in gene-deficient mouse models in which contributions of innate and adaptive IFN-$\gamma$ production can be dissected separately.

## RESULTS

**IFN-$\gamma$-deficient and T cell-deficient mice exhibit opposite phenotypes after *Chlamydia muridarum* intravaginal infection.** In order to directly compare the phenotypes of IFN-$\gamma$-deficient and T cell-deficient mice, we infected IFN-$\gamma^{-/-}$ and TCR$\beta^{-/-}$ mice intravaginally with *C. muridarum* strain Nigg II and compared survival and bacterial shedding during primary infections. Consistent with previous findings (17), 100% of TCR$\beta^{-/-}$ mice survived the infection with no obvious sign of wasting disease (Fig. 1A). Meanwhile, these mice manifested high-grade, persistent bacterial shedding with an average of $>10^5$ bacteria recovered from the FRT for at least 60 days (Fig. 1A and B). In contrast, over 90% of IFN-$\gamma^{-/-}$ mice succumbed to infection between days 15 and 47 after infection (Fig. 1A). Notably, IFN-$\gamma^{-/-}$ mice that survived by day 35 postinfection exhibited an ~10,000-fold reduction in FRT bacterial burden compared to day 7 (Fig. 1B). These opposite phenotypes of T cell-deficient and IFN-$\gamma$-deficient mice suggest that distinct host defense mechanisms are involved in IFN-$\gamma$- and T cell-dependent *Chlamydia* containment in systemic versus mucosal tissues. Importantly, a non-$\alpha\beta$ T cell source of IFN-$\gamma$ in TCR$\beta^{-/-}$ mice must have contributed to systemic *Chlamydia* control for their long-term survival.

**ILCs are essential for systemic bacterial control and prolonged survival of *Rag1*$^{-/-}$ mice.** The nonlethal phenotype of TCR$\beta^{-/-}$ mice led us to hypothesize that innate lymphocytes (ILCs) are essential for preventing lethal *Chlamydia* dissemination. To test this hypothesis, we infected *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice intravaginally with *C. muridarum* and monitored survival. *Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice quickly succumbed to infection, with a median survival time of 12 days. This is significantly shorter than the average 30 days of survival in *Rag1*$^{-/-}$ mice (Fig. 2A). Fast weight loss was observed in *Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice starting from day 5 postinfection (dpi), but not in *Rag1*$^{-/-}$ mice (Fig. 2B). By the time *Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice were moribund (10 to 13 dpi), we detected




| Days post infection | 7 | 14 | 21 | 28 | 35 | 42 |
|---|---|---|---|---|---|---|
| IFNγ$^{-/-}$ vs B6 | **** | ** | * | *** | * | ns |
| TCRβ$^{-/-}$ vs B6 | *** | *** | *** | **** | **** | **** |
| IFNγ$^{-/-}$ vs TCRβ$^{-/-}$ | ns | **** | *** | **** | *** | ** |

**FIG 1** IFN-γ-deficient mice and αβ T cell-deficient mice exhibit opposite phenotypes after *Chlamydia muridarum* intravaginal infection. B6, IFN-$\gamma^{-/-}$, and TCR$\beta^{-/-}$ mice were infected intravaginally with $1 \times 10^5$ *C. muridarum*. Survival (A) and bacterial shedding (B) from the lower female reproductive tract (FRT) were monitored by vaginal swabs. Data are combined results of three independent experiments with 9 to 13 mice per group. Each data point represents an individual mouse. Lines represent mean $\log_{10}$-transformed values. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

widespread bacterial dissemination in these mice, with high bacterial burdens in systemic tissues, including spleen, kidney, lung, ball bladder, and peritoneal cavity, as well as vaginal and rectal mucosa (Fig. 2C). Notably, while all systemic tissues of *Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice exhibit 1- to 4-log-higher bacteremia than *Rag1*$^{-/-}$ mice, *Chlamydia* burdens at the FRT mucosa were not significantly different between the two strains (Fig. 2C). These results demonstrate that ILCs are essential for systemic *Chlamydia* control but are redundant for host resistance to *Chlamydia* at the FRT mucosa in the absence of adaptive immunity.

**Innate IFN-γ production, partially by NK cells and ILC1s, is essential for early control of *Chlamydia* dissemination.** We next investigated whether IFN-γ production by ILCs, in particular the group 1 ILCs, including NK cells and ILC1s, accounts for the key innate effector mechanism for early control of *C. muridarum* systemic dissemination. To do this, we depleted either IFN-γ or NK1.1$^+$ cells from *Rag1*$^{-/-}$ mice and monitored survival. As shown in Fig. 3A, both IFN-γ- and NK1.1-depleted groups displayed accelerated death compared to the phosphate-buffered-saline (PBS)-treated group, with average survival times of 16.5 and 21 days, respectively. At day 14 postinfection, bacterial burdens in the lower FRT and on rectal swabs were not affected by either depletion, while significantly more *C. muridarum* organisms were detected in both IFN-γ- and NK1.1-depleted groups in the upper FRT (Fig. 3B). Anti-IFN-γ treatment resulted in more than a 1.5-log increase in *C. muridarum* burdens in all systemic tissues, including spleen, kidney, and lung. The anti-NK1.1-treated group showed trends of higher systemic bacteria burdens than PBS-treated group, but such differences did not reach statistical significance at day 14, which is 1 week earlier than their average survival time (Fig. 3B). IFN-γ secretion by mature group 1 ILCs (CD11b$^+$ NK1.1$^+$) were readily detectable in the spleens of *Rag1*$^{-/-}$ mice throughout the course of infection but were not evident in wild-type (WT) B6 controls (Fig. 3C and D). Unexpectedly, circulating IFN-γ levels in *Rag1*$^{-/-}$ mice were not significantly affected by NK1.1 depletion at both days 7 and 14 postinfection (Fig. 3E). Together, these findings led us to conclude that both innate IFN-γ and group 1 ILCs are essential for early containment of *Chlamydia* dissemination. While NK1.1$^+$ group 1 ILCs are potent IFN-γ producers, they do not

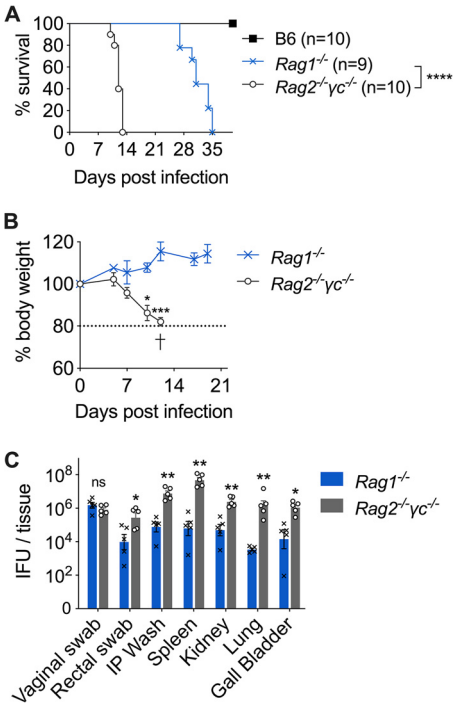



**FIG 2** Innate lymphocytes (ILCs) are essential for systemic bacterial control and prolonged survival of *Rag1*-deficient mice. B6, *Rag1*$^{-/-}$, and *Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice were infected intravaginally with $1 \times 10^5$ *C. muridarum* organisms. (A) Survival. (B) Body weight. (C) Bacterial burdens in vaginal swabs, rectal swabs, and systemic organs determined at 12 days postinfection. Data shown are combined results of two independent experiments with 9 or 10 mice per group (A) or representative results of two independent experiments with 3 to 5 mice per group in each experiment (B and C). Each data point represents an individual mouse. Bars and error bars represent means and standard errors of the means (SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

seem to be the only source for IFN-$\gamma$ derived from innate immune responses following *C. muridarum* intravaginal infection.

**IFN-$\gamma$ production by CD4 T cells is largely redundant at the FRT mucosa.** The complete lack of IFN-$\gamma$ production in IFN-$\gamma^{-/-}$ mice allows the evaluation of only the global effect of this cytokine, which derives from numerous cellular sources. To specifically define the contribution of CD4 T cell-dependent IFN-$\gamma$ production to host resistance to *Chlamydia*, we conducted T cell adoptive transfer experiments in which we transferred either IFN-$\gamma$-sufficient (WT) or IFN-$\gamma$-deficient (IFN-$\gamma^{-/-}$) CD4 T cells into innate immunity-intact TCR$\beta^{-/-}$ mice and challenged the recipients intravaginally with *C. muridarum* (Fig. 4A). Compared to the WT CD4 T cell transfer group, TCR$\beta^{-/-}$ mice receiving IFN-$\gamma$-deficient CD4 T cells exhibited similar rates of bacterial containment for the first 21 days (fold change in $\log_{10}$, 3.1 $\pm$ 1.8 in WT CD4 transfer versus 2.7 $\pm$ 1.2 in IFN-$\gamma^{-/-}$ CD4 transfer; $P = 0.21$) (Fig. 4B). *Chlamydia* burden continued to decrease another 10-fold in the IFN-$\gamma^{-/-}$ CD4 T cell transfer group before these mice entered the chronic, low-grade shedding phase around day 35 (Fig. 4B). The cumulative ~5,000-fold decrease in bacterial burden demonstrated that IFN-$\gamma$ produced by CD4 T cells is dispensable for eliminating vast majority of the pathogen from the FRT. Last, as a result of the functional innate immunity in TCR$\beta^{-/-}$ recipient mice, the lethal dissemination phenotype was completely rescued in this adoptive transfer model compared to IFN-$\gamma^{-/-}$ mice, reinforcing the idea that innate IFN-$\gamma$ is sufficient for systemic *Chlamydia* containment (Fig. 4C).

## DISCUSSION

The ability of T cells to produce IFN-$\gamma$ in response to infection is an important readout for their antigen specificity and effector function. Although Th1 cells are essential for combating intracellular parasites, the reliance on IFN-$\gamma$ to confer protection appears

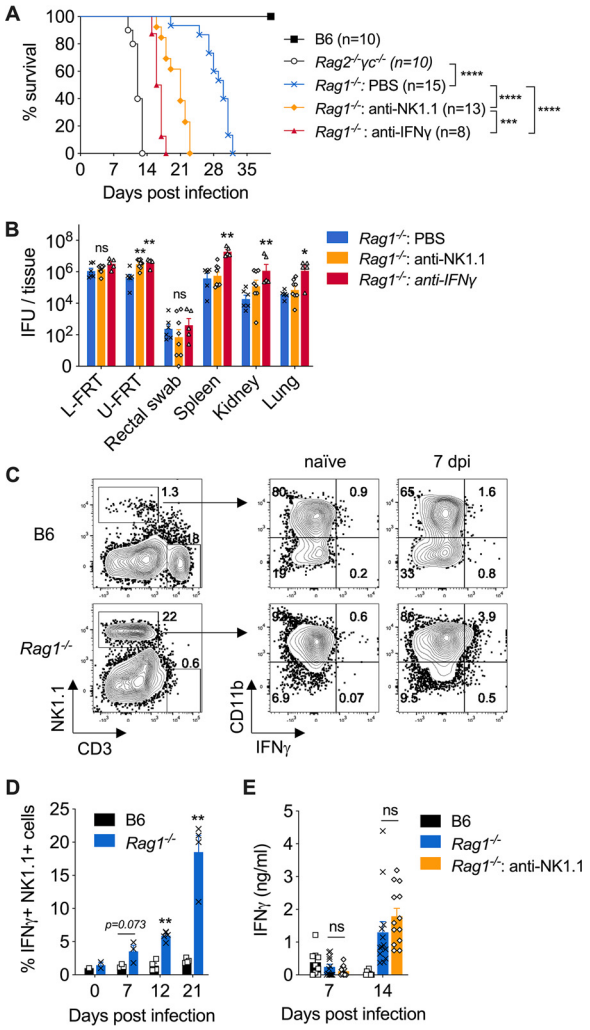
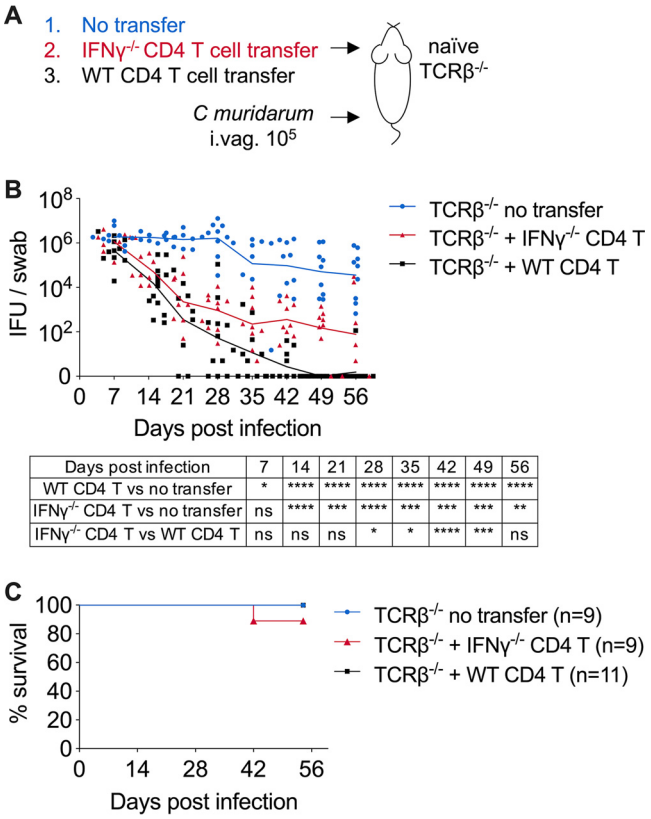



**FIG 3** Innate IFN-γ and group 1 ILCs contribute to host resistance to lethal *Chlamydia* dissemination in *Rag1*$^{-/-}$ mice. B6, *Rag1*$^{-/-}$, and *Rag2*$^{-/-}$ γc$^{-/-}$ mice were infected intravaginally with $1 \times 10^5$ *C. muridarum* organisms. Groups of *Rag1*$^{-/-}$ mice were treated with either anti-IFN-γ or anti-NK1.1 depleting Abs throughout the infection. (A) Survival. (B) Bacterial burdens in lower FRT (L-FRT), upper FRT (U-FRT), rectal swabs, and systemic organs determined at 14 days postinfection. (C) Representative flow cytometry plots showing IFN-γ secretion by CD11b$^+$ NK1.1$^+$ cells detected by IFN-γ secretion assay. (D) Percentages of IFN-γ-producing CD11b$^+$ NK1.1$^+$ cells quantified based on the flow cytometry analysis in panel C. (E) Serum IFN-γ level on days 7 and 14 after infection, as measured by IFN-γ cytokine enzyme-linked immunosorbent assay (ELISA). Data in panels A, B, D, and E are combined results of at least two independent experiments with 3 to 5 mice per group in each experiment. Each data point in panels B, D, and E represents an individual mouse. Bars and error bars represent means and SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

to be pathogen specific. Mice lacking the IFN-γ receptor or the Th1 transcription factor T-bet are highly susceptible to *Salmonella* infection (28, 29). In contrast, effective control of *Mycobacterium tuberculosis* infection depends on Th1 cells but has a minimal requirement of IFN-γ in the lung (30–32). The importance of CD4 T cells in host resistance to *Chlamydia* is highlighted by the absolute requirement of MHC-II and αβTCR for bacterial control in the FRT mucosa, while the most prominent phenotype of IFN-γ$^{-/-}$ mice after *C. muridarum* intravaginal infection is systemic bacterial dissemination (17, 18, 27). By conducting side-by-side comparison of TCRβ$^{-/-}$ and IFN-γ$^{-/-}$ mice in this study, we confirmed these previous findings and demonstrated unambiguously that hosts had distinct requirements for CD4 T cells and IFN-γ for *Chlamydia* resistance.

TCRβ$^{-/-}$ mice lack αβ T cells but retain relatively intact innate immunity and several adaptive immune components, such as γδ T cells and T-independent antibody, all




| Days post infection | 7 | 14 | 21 | 28 | 35 | 42 | 49 | 56 |
|---|---|---|---|---|---|---|---|---|
| WT CD4 T vs no transfer | * | **** | **** | **** | **** | **** | **** | **** |
| IFNγ$^{-/-}$ CD4 T vs no transfer | ns | **** | *** | **** | *** | *** | *** | ** |
| IFNγ$^{-/-}$ CD4 T vs WT CD4 T | ns | ns | ns | * | * | **** | *** | ns |

**FIG 4** CD4 T cell-dependent IFN-$\gamma$ production is largely redundant at the FRT mucosa. CD4 T cells isolated from WT B6 or IFN-$\gamma^{-/-}$ mice were adoptively transferred to TCR$\beta^{-/-}$ mice. Recipient TCR$\beta^{-/-}$ mice were infected intravaginally with $1 \times 10^5$ *C. muridarum* organisms. (A) Schematic depicting the TCR$\beta^{-/-}$ adoptive transfer experimental setup. (B and C) Bacterial shedding from the FRT (B) and survival of TCR$\beta^{-/-}$ recipient mice (C) after receiving naive CD4 T cells from either WT or IFN-$\gamma^{-/-}$ donors and intravaginal infection. Data are combined results of three independent experiments with 6 to 11 mice per group. Each data point in panels B and C represents an individual mouse. Lines represent mean $\log_{10}$-transformed values. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

of which may contribute to the long-term survival of the hosts. As part of the innate immune system, ILCs are known for functions that parallel their T cell counterparts but differ from T cells in their embryonic origin, tissue residency, lack of rearranged receptor for Ag recognition, and unique contribution to tissue integrity (33). Immune responses of the ILCs are critical for early control of pathogen replication before the host launches an effective adaptive immune response. This defense mechanism could be particularly important for containing mucosal pathogens like *Chlamydia*, since the target FRT tissue lacks defined lymphoid structure, and consequently, effective adaptive immune responses are significantly delayed (34, 35). Using the *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ $\gamma c^{-/-}$ models, we showed in this study that removing ILCs from the innate immune system had a detrimental effect on host resistance to *Chlamydia*, as *Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice quickly succumbed to disseminated infection with high-grade bacteremia. In contrast, ILC-sufficient *Rag1*$^{-/-}$ mice exhibited significantly longer survival and stable body weights. These observations made it evident that ILCs are essential for preventing early systemic *Chlamydia* dissemination in the absence of adaptive immunity. Given the early responses of ILCs, it is unlikely that ILCs are completely redundant with adaptive responses to prevent early bacterial dissemination in immunocompetent hosts (36), although this notion needs to be confirmed experimentally.

Our IFN-$\gamma$ and NK1.1 depletion experiment directly addressed the idea that both innate IFN-$\gamma$ and NK1.1$^+$ group 1 ILCs contribute to host resistance to lethal *Chlamydia* dissemination in *Rag1*$^{-/-}$ mice. Group I ILCs, including cytotoxic NK cells and

noncytotoxic ILC1s, are the major source of IFN-$\gamma$ during innate immune responses, along with neutrophils, macrophages, dendritic cells (DCs), and a subset of ILC3s (37–39). In immunocompetent hosts, IFN-$\gamma$ production by NK cells can be detected as early as 4 h after intravaginal *C. muridarum* infection (40). The early response of NK cells dictates CD4 T cell differentiation and memory responses during *C. muridarum* lung infections (41, 42). Recently, Poston et al. showed that T cell-independent IFN-$\gamma$ cooperates with B cells to prevent lethal dissemination caused by the highly virulent *C. muridarum* strain CM001 (20). Our data add to previous findings by showing *ex vivo* IFN-$\gamma$ secretion on the surface of CD11b$^+$ NK1.1$^+$ cells, indicating that group 1 ILCs carry out a protective function, as least in part, by their production of IFN-$\gamma$ in the absence of T cells. It should be noted that both IFN-$\gamma$-depleted and NK1.1-depleted *Rag1*$^{-/-}$ mice exhibited slightly longer survival times than *Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice, indicating that additional cellular sources, perhaps ILC3s, may also produce IFN-$\gamma$ and confer protection against *Chlamydia*, as shown in mouse endometrium tissue in recent studies (43, 44). Additionally, other protective mechanisms independent of IFN-$\gamma$ may be involved in ILC-mediated protection. It is intriguing to observe that serum IFN-$\gamma$ was not significantly affected by anti-NK1.1 treatment in *Rag1*$^{-/-}$ mice and was minimally detected in WT animals. These results suggest two non-mutually exclusive probabilities. First, circulating IFN-$\gamma$ is neither necessary nor sufficient for protection. Instead, close proximity between IFN-$\gamma$-secreting cells and responders are essential for bacterial control (45). Second, circulating IFN-$\gamma$ is more efficiently removed by IFN-$\gamma$ receptor-expressing cells in immune-sufficient mice (46). Future experiments are required to address these issues directly.

The sustained *Chlamydia* replication in the FRT of TCR$\beta^{-/-}$ mice suggested that innate immune cells, $\gamma\delta$ T cells, and T-independent antibodies (Abs) are incapable of restraining *Chlamydia* growth in the FRT mucosa. In contrast, adoptive transfer of CD4 T cells, regardless of their ability to produce IFN-$\gamma$, reduced bacterial burden to less than 0.1% of the peak burden within 3 weeks. These results are in agreement with previous studies using IFN-$\gamma^{-/-}$ mice, but this study differs from them by retaining intact innate IFN-$\gamma$ in our system, thereby preventing lethal disseminated infection (17, 27). By solely manipulating the CD4 T cell compartment, we showed that CD4 T cells are both necessary and sufficient for *C. muridarum* control in the FRT. More importantly, we showed unequivocally that CD4 T cell-derived IFN-$\gamma$ is largely redundant for protective immunity in the FRT, at least during the early phase of bacterial replication.

Numerous studies in the field have firmly established the importance of the pleiotropic cytokine IFN-$\gamma$ in *C. muridarum* and *C. trachomatis* infection, *in vitro* and *in vivo* (10, 47–57). Our study, along with many others, has demonstrated that early IFN-$\gamma$ production by ILCs is essential for preventing bacterial dissemination and achieving complete eradication of *C. muridarum* from the FRT at a late stage of infection relies on CD4 T cell-derived IFN-$\gamma$ (17, 27). Non-lymphogranuloma venereum (LGV) strains of *C. trachomatis* rarely cause disseminated infection in humans. In contrast, low-grade *C. trachomatis* shedding is commonly observed in otherwise healthy individuals (58). Therefore, it is reasonable to speculate that IFN-$\gamma$ effectively blocks *Chlamydia* dissemination in humans, whereas a further evolved adaptive immune response is likely required to overcome *C. trachomatis* evasion of IFN-$\gamma$-induced cell-autonomous immunity in FRT epithelium (10). In line with this argument, our CD4 T cell adoptive transfer studies in mice emphasized that a highly effective mechanism of T helper cell-mediated protection against mucosal *Chlamydia* infection independent of IFN-$\gamma$ is yet to be discovered. Efforts to search for such a protective mechanism are urgently needed, as precise understanding of protective immunity is fundamental to the rational design of a much-needed *Chlamydia* vaccine.

Fortunately, recent studies have started to shed light on several important aspects of CD4 T cell biology related to host protective responses beyond IFN-$\gamma$ production. Yu et al. showed that vaccination using live or dead *C. muridarum* EB elicits different degrees of protection that correlates with the frequency of multifunction Th1 cells

(23). Likewise, a recently developed *C. muridarum* TCR-transgenic model has revealed that the ability of monoclonal Ag-specific CD4 T cells to coproduce IFN-$\gamma$, tumor necrosis factor alpha (TNF-$\alpha$), and interleukin 2 (IL-2) is essential for their protective efficacy (59). Using MHC-II tetramers, we demonstrated that *C. muridarum* infection induces a highly heterogenous T helper response dominated by Th1 and accompanied by fractions of *Chlamydia*-specific Treg and Th17 cells in lymphoid and mucosal tissues (35). Other Th lineages, such as an IFN-$\gamma$- and IL-13-producing CD4 T cell clone, and a Th2-dominant response in human *C. trachomatis* infection have also been documented (60, 61). Finally, an elegant *C. trachomatis* vaccine study conducted by Stary et al. revealed that T cell activation, effector functions, and formation of tissue-resident memory should all be taken into consideration when protective efficacy afforded by a vaccine is evaluated (62). Our knowledge of CD4 T cell differentiation is evolving quickly as a result of groundbreaking technologies. It is proposed that a continuum of CD4 T cell states will likely replace our traditional understanding of defined CD4 T cell lineages (63). With the increased knowledge and higher resolution tool to understand CD4 T cell biology, a refreshed notion of protective immunity against *Chlamydia* will likely emerge.

## MATERIALS AND METHODS

**Mice.** C57BL/6 (B6), IFN-$\gamma^{-/-}$ (B6.129S7-*Ifng*$^{tm1Ts}$/J), *Rag1*$^{-/-}$ (B6.129S7-*Rag1*$^{tm1Mom}$/J), and TCR$\beta^{-/-}$ (B6. 129P2-*Tcrb*$^{tm1Mom}$/J) mice were purchased from The Jackson Laboratory. *Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice were purchased from Taconic Biosciences. All mice used for experiments were 6 to 16 weeks old, unless otherwise noted. Mice were maintained under specific-pathogen-free (SPF) conditions, and all mouse experiments were approved by the University of Arkansas for Medical Sciences (UAMS) Institutional Animal Care and Use Committee (IACUC).

**Bacteria.** *Chlamydia muridarum* strain Nigg II was originally purchased from ATCC (VR-123; Manassas, VA). The organism was propagated in HeLa 229 cells. Elementary bodies (EBs) were purified by renografin discontinuous density gradient centrifugation, aliquoted, and stored at −80°C until use. EBs were titrated on HeLa 229 cells as previously described (35).

**Infection and bacteria enumeration.** Mice were synchronized for estrus by subcutaneous injection of 2.5 mg Depo-Provera (Greenstone, NJ), 5 to 7 days prior to intravaginal infection. For intravaginal infection, $1 \times 10^5$ *C. muridarum* organisms in sucrose-phosphate-glutamic acid (SPG) buffer were deposited directly into the vaginal vault using a pipet tip. To enumerate bacterial shedding from the FRT, vaginal swabs were collected, suspended in SPG buffer, and disrupted with glass beads. Inclusion-forming units (IFUs) were determined by plating serial dilutions of swab samples on HeLa 229 cells, staining with anti-major outer membrane porin (MOMP) monoclonal antibody (MAb) and counting under microscope. To enumerate bacteria burden within tissues, intraperitoneal (i.p.) wash fluid was collected in SPG buffer, and spleens, kidneys, lungs, and gallbladders were homogenized in SPG buffer. Tissue homogenates were disrupted with glass beads and centrifuged at 500 $\times$ *g* for 10 min, supernatants were collected, and serial dilutions were plated on HeLa 229 cells for IFU counts.

**Ab-mediated depletion.** IFN-$\gamma$ *in vivo* depletion was performed by i.p. injection of 0.25 mg anti-IFN-$\gamma$ (XMG1.2; BioXcell) on days −1 and 1 postinfection and every 3 days thereafter. NK cells were depleted by i.p. injection of 0.3 mg anti-NK1.1 (purified MAb from PK136 hybridoma; gift from Richard Morrison, UAMS) on days −3, −1, and 1 postinfection and every 3 days thereafter.

**CD4 T cell adoptive transfer.** Total CD4 T cells from donor mice were purified from spleens using a STEMCELL EasySep CD4 T cell isolation kit according to the manufacturer's instructions (STEMCELL Technologies). Depending on individual experiment, 5 to 20 million purified CD4 T cells were transferred intravenously into recipient mice via the tail vein.

**IFN-$\gamma$ secretion assay and flow cytometry.** An IFN-$\gamma$ secretion assay was conducted according to the manufacturer's instructions (mouse IFN-$\gamma$ secretion assay detection kit; Miltenyi). Briefly, spleens were harvested, and single-cell suspensions were prepared in RPMI with 5% fetal calf serum (FCS). Cells were labeled with IFN-$\gamma$ catch reagents for 5 min on ice and incubated for 45 min at 37°C. IFN-$\gamma$-producing cells were stained with IFN-$\gamma$ detection antibody in conjunction with cell surface markers (listed below) and analyzed on an LSRFortessa flow cytometer (BD Biosciences). Antibodies used included CD3e (145-2C11), CD11b (M1/70), and NK1.1 (PK136) (BioLegend). Data were analyzed using FlowJo software (Tree Star).

**Statistical analysis.** Statistical analysis was performed with GraphPad Prism 8. An unpaired *t* test was used for normally distributed continuous-variable comparisons; a Mann-Whitney U test was used for nonparametric comparisons. The log-rank Mantel-Cox test was used for survival curves.

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