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Novel Role for Interleukin-17 in Enhancing Type 1 Helper T Cell Immunity in the Female Genital Tract following Mucosal Herpes Simplex Virus 2 Vaccination

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ABSTRACT It is well established that interferon gamma (IFN-γ) production by $CD4^+$ T cells is critical for antiviral immunity against herpes simplex virus 2 (HSV-2) genital infection. However, the role of interleukin-17A (IL-17A) production by $CD4^+$ T cells in HSV-2 antiviral immunity is yet to be elucidated. Here we demonstrate that IL-17A plays an important role in enhancing antiviral T helper type 1 (T_h1) responses in the female genital tract (FGT) and is essential for effective protection conferred by HSV-2 vaccination. While IL-17A did not play a critical role during primary genital HSV-2 infection, seen by lack of differences in susceptibility between IL-17A-deficient (*IL-17A*$^{-/-}$) and wild-type (WT) C57BL/6 mice, it was critical for mediating antiviral responses after challenge/reexposure. Compared to WT mice, *IL-17A*$^{-/-}$ mice (i) infected intravaginally and reexposed or (ii) vaccinated intranasally and challenged intravaginally demonstrated poor outcomes. Following intravaginal HSV-2 reexposure or challenge, vaccinated *IL-17A*$^{-/-}$ mice had significantly higher mortality, greater disease severity, higher viral shedding, and higher levels of proinflammatory cytokines and chemokines in vaginal secretions. Furthermore, *IL-17A*$^{-/-}$ mice had impaired T_h1 cell responses after challenge/reexposure, with significantly lower proportions of vaginal IFN-γ^+ $CD4^+$ T cells. The impaired T_h1 cell responses in *IL-17A*$^{-/-}$ mice coincided with smaller populations of IFN-γ^+ $CD4^+$ tissue resident memory T (T_{RM}) cells in the genital tract postimmunization. Taken together, these findings describe a novel role for IL-17A in regulating antiviral IFN-γ^+ T_h1 cell immunity in the vaginal tract. This strategy could be exploited to enhance antiviral immunity following HSV-2 vaccination.

IMPORTANCE T helper type 1 (T_h1) immunity, specifically interferon gamma (IFN-γ) production by $CD4^+$ T cells, is critical for protection against genital herpesvirus (HSV-2) infection, and enhancing this response can potentially help improve disease outcomes. Our study demonstrated that interleukin-17A (IL-17A) plays an essential role in enhancing antiviral T_h1 responses in the female genital tract (FGT). We found that in the absence of IL-17A, preexposed and vaccinated mice showed poor disease outcomes and were unable to overcome HSV-2 reexposure/challenge. IL-17A-deficient mice (*IL-17A*$^{-/-}$) had smaller populations of IFN-γ^+ $CD4^+$ tissue resident memory T (T_{RM}) cells in the genital tract postimmunization than did wild-type (WT) mice, which coincided with attenuated T_h1 responses postchallenge. This has important implications for developing effective vaccines against HSV-2, as we propose that strategies inducing IL-17A in the genital tract may promote more effective T_h1 cell immunity and better overall protection.

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Genital herpes, caused primarily by herpes simplex virus 2 (HSV-2), is one of the most predominant viral sexually transmitted infections (STIs) in the world (1). Recent estimates show that 417 million people between the ages of 15 and 49 years are infected with HSV-2 globally, and approximately 19.2 million in this age group become newly infected each year (1). Similar to the case with many other STIs, rates of HSV-2 infection are disproportionally higher in women, with approximately 14.8% of women infected, compared to 8.0% of men, globally (1). Efforts to develop effective vaccines against STIs such as HSV-2 have been unsuccessful, as there is little understanding of factors in the local microenvironment of the female genital tract (FGT) that determine the outcome of exposure to HSV-2, making it difficult to develop preventative interventions (2).

To study the immune mechanisms involved in generating protection against HSV-2 infection, a well-established mouse model of genital HSV-2 infection is commonly used, as it closely recapitulates many aspects of human infection. Mouse studies have demonstrated that both humoral and cell-mediated responses are involved in generating protection against genital HSV-2 infection. Vaccine strategies that can induce robust local antibody responses or passively transfer antibodies from immunized mice can protect mice against subsequent intravaginal HSV-2 challenge (3). However, it is well known that $CD4^+$ T helper 1 (T_h1) cell immune responses and production of interferon gamma (IFN-γ) are critical for protection both following natural resolution of infection and following immunization (4, 5). Therefore, preventative strategies that aim to induce strong T_h1 cell immunity in the genital tract should be able to effectively control HSV-2 infection.

Along with T_h1 cell immunity, genital HSV-2 infection also induces a T_h17 immune response. T_h17 cells are a subset of activated $CD4^+$ T cells, characterized primarily by the secretion of cytokine interleukin-17 (IL-17) as well as IL-21 and IL-22 (6). The IL-17 family of cytokines includes six ligands (IL-17A to IL-17F), and T_h17 cells generally produce IL-17A and IL-17F (6). T_h17 cell immunity and IL-17A production in particular have been shown to play a fundamental role in resolution of fungal and bacterial infections in the genital tract, including infections with *Candida albicans*, *Neisseria gonorrhoeae*, and *Chlamydia trachomatis* (7–12); however, aberrant T_h17 cell responses can lead to autoimmune conditions or chronic inflammatory diseases in other tissues (6, 13, 14). Although IL-17 has been studied in the context of bacterial and fungal infections in the genital tract, the role of IL-17 in genital antiviral responses, particularly during HSV-2 infection, is less understood. Kim et al. reported that here was a significant delay in the deaths of IL-17A-deficient (*IL-17A*$^{-/-}$) mice compared to those of C57BL/6 control mice following primary genital HSV-2 infection, implying that IL-17A may have a pathogenic effect (15). Conversely, we have recently shown that T_h17 cell immunity and IL-17A production may be involved in mediating better protection following intravaginal HSV-2 challenge (16). Evidently, the role of IL-17A in host defense against genital HSV-2 infection remains unresolved, and therefore, it was the focus of the current study.

In addition to antibacterial and antifungal immunity, IL-17 has also been shown to contribute to the generation of efficient T_h1 cell immunity against intracellular pathogens (17–19). In the pulmonary *Mycobacterium tuberculosis* vaccination model, the absence of IL-17 resulted in reduced and delayed IFN-γ responses and, consequently, delayed bacterial clearance (17). Similarly, in IL-17 receptor A (IL-17RA)-deficient mice, genital tract infection with *Chlamydia muridarum* also resulted in reduced IFN-γ production (18). Bai et al. further showed that a reduced *Chlamydia*-specific T_h1 cell response was related to impaired dendritic cell (DC) induction of IFN-γ responses upon IL-17A neutralization (19). Parallel to these studies, we recently reported that enhanced T_h1 cell immunity against HSV-2 in the genital tract involves T_h17 cell responses (16).

We showed that estradiol treatment in mice, which is known to protect against HSV-2 (20–23), enhanced antiviral immunity by priming vaginal DCs to induce T_h17 cell responses following HSV-2 immunization. This resulted in increased production of IL-17A$^+$ CD4$^+$ (T_h17) cells in estradiol-treated mice and coincided with earlier recruitment and increased proportions of IFN-γ^+ CD4$^+$ (T_h1) cells in the genital tract postchallenge (16). This was the first study to suggest that T_h17 responses in the vaginal tract may augment IFN-γ–T_h1 cell immunity. However, it was not determined whether this enhanced antiviral immunity was due to IL-17A alone or the result of other cytokines produced by T_h17 cells, thus emphasizing the importance of examining directly and in more detail the role of IL-17A in the genital tract model of HSV-2 infection.

In light of these findings, the aim of the present study was to investigate the role and mechanism of IL-17A in regulating IFN-γ–T_h1 cell immunity to genital HSV-2 infection. Using IL-17A-deficient (*IL-17A*$^{-/-}$) mice, we found no difference in disease severity from that in wild-type (WT) C57BL/6 mice following primary genital HSV-2 infection. However, following HSV-2 reexposure, *IL-17A*$^{-/-}$ mice had significantly poorer disease outcomes than did WT mice. To further examine the role of IL-17A in postchallenge antiviral responses, *IL-17A*$^{-/-}$ mice were vaccinated intranasally with an attenuated strain of HSV-2 (thymidine kinase deficient [TK$^-$]); they demonstrated poor disease outcomes following intravaginal challenge with WT HSV-2. In addition, *IL-17A*$^{-/-}$ mice had significantly higher levels of proinflammatory cytokines and chemokines in vaginal secretions. Most importantly, *IL-17A*$^{-/-}$ mice showed impaired T_h1 responses following both reexposure and challenge. Upon examination, the attenuated recall responses in *IL-17A*$^{-/-}$ mice were found to correlate with a smaller population of vaginal CD4$^+$ tissue resident memory T (T_{RM}) cells postvaccination. Our data therefore suggest that IL-17A is critical for the induction of optimal T_h1 cell responses and overall protection against genital HSV-2 infection.

RESULTS

IL-17A is not critical during primary genital HSV-2 infection. We recently showed that enhanced protection against HSV-2 involved greater vaginal T_h17 cell responses, which coincided with increased T_h1 cell responses (16); however, it was not established if the protective effect of T_h17 cell immunity was mediated directly by IL-17A. Therefore, we decided to further investigate, in greater detail, the direct role of IL-17A in mediating HSV-2 antiviral immunity in the genital tract.

In order to assess if *IL-17A*$^{-/-}$ mice were more susceptible than WT mice to primary intravaginal infection with HSV-2, ovariectomized (OVX) *IL-17A*$^{-/-}$ and WT mice were inoculated with sublethal doses of WT HSV-2 virus (10^1, 10^2, and 10^3 PFU/mouse). Survival, genital pathology, and viral shedding were monitored daily to determine disease severity and compare susceptibilities to infection. We found that following primary genital infection, there were no significant differences in survival between *IL-17A*$^{-/-}$ and WT mice (Fig. 1A). Comparable rates of survival corresponded with similar severities of genital pathology between *IL-17A*$^{-/-}$ and WT mice (Fig. 1B) and similar numbers of *IL-17A*$^{-/-}$ and WT mice shedding virus postinfection (Fig. 1C). Overall, regardless of the infectious dose used, there were no differences in mortality, disease pathology, and viral shedding between *IL-17A*$^{-/-}$ and WT mice, demonstrating that IL-17A does not appear to play a critical role during primary genital HSV-2 infection.

IL-17A contributes to antiviral responses following genital HSV-2 reexposure. We then examined whether *IL-17A*$^{-/-}$ mice would show compromised antiviral responses following HSV-2 reexposure, as this would determine the role of IL-17A during memory recall responses in the FGT. Previous studies with the intracellular pathogen *M. tuberculosis* have shown that although IL-17 is not critical during primary infection, it is important for augmenting memory responses (17).

Intravaginally preexposed *IL-17A*$^{-/-}$ and WT mice (10^2 PFU/mouse) were reexposed to a higher dose of HSV-2 (5×10^3 PFU/mouse), and survival, genital pathology, and

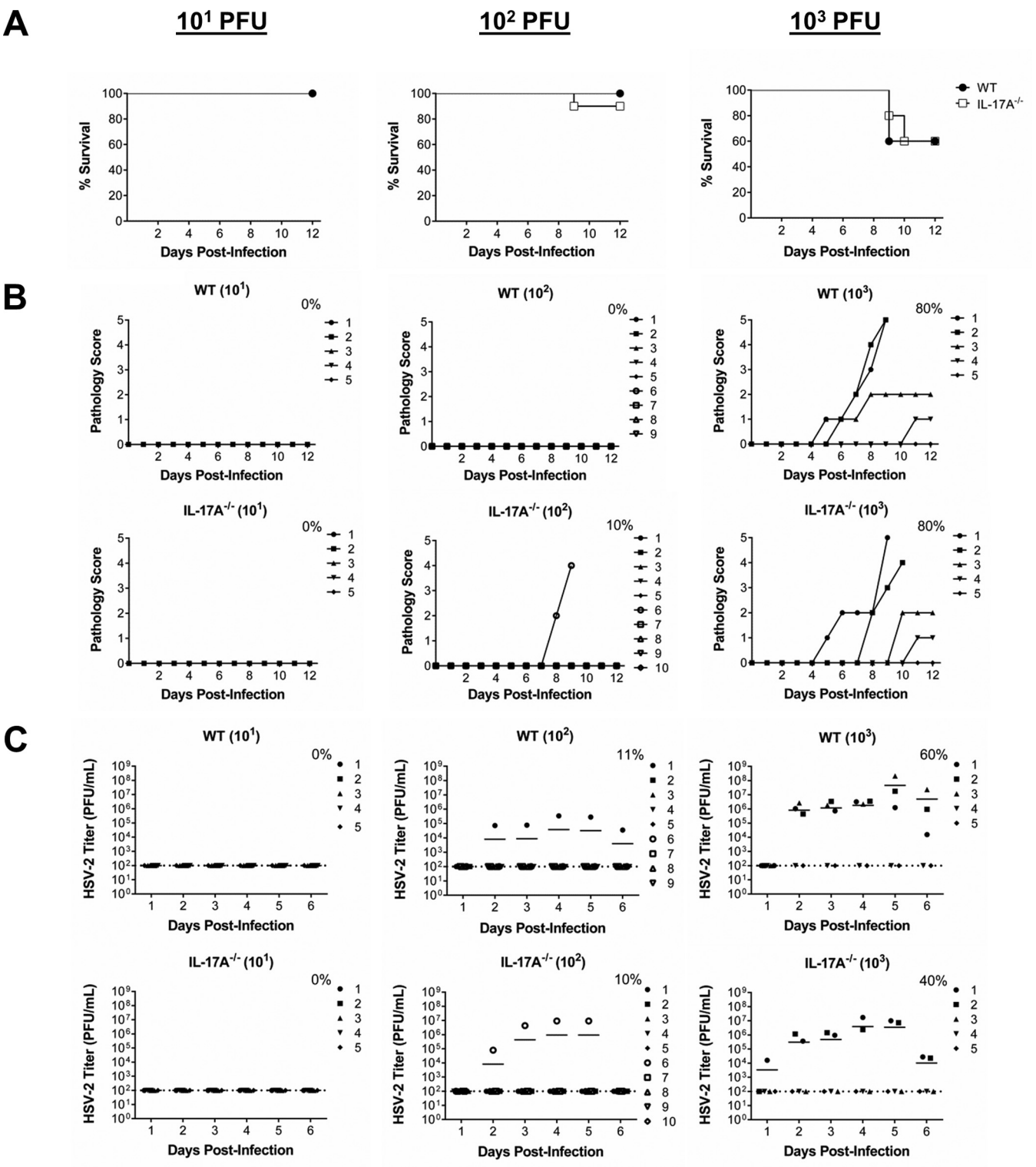

FIG 1 *IL-17A*$^{-/-}$ mice demonstrate no significant difference in susceptibility to primary intravaginal HSV-2 infection. OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice (n = 5 to 10/group) were infected intravaginally with sublethal doses of WT HSV-2 (10^1, 10^2, or 10^3 PFU/mouse). Survival was monitored (A) and pathology scores were recorded on a scale of 0 to 5 (B) for 12 days postinfection. Data points superimposed on the x axes of panel B indicate mice without genital pathology, and the percentages represent maximum numbers of mice that demonstrated pathology. (C) Vaginal washes were collected daily for 6 days postinfection and HSV-2 shedding was assessed using a Vero cell-based assay. The bars in panel C indicate mean PFU per milliliter of shed virus. The dotted lines in panel C indicate the lower detection limit of the assay, and data points on this line indicate undetectable viral shedding. The percentages in panel C represent maximum numbers of mice that shed virus on any given day. Each symbol represents a single animal. The results are representative of those from two independent experiments.

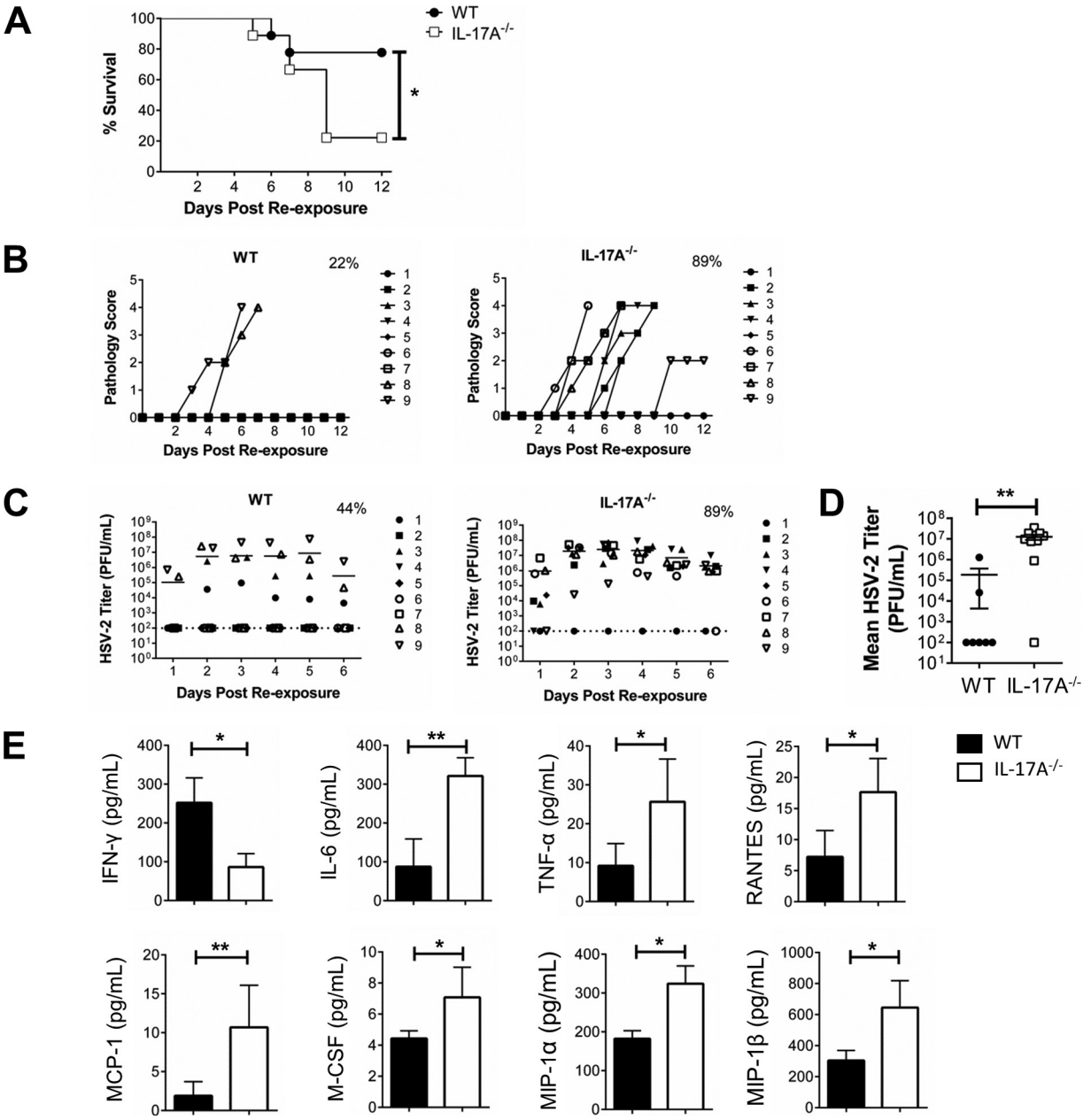

FIG 2 Preexposed *IL-17A*$^{-/-}$ mice are more susceptible to intravaginal HSV-2 reexposure. OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice (n = 9/group) were intravaginally exposed to WT HSV-2 (10^2 PFU/mouse), and 6 weeks later, they were reexposed intravaginally with a higher dose of WT HSV-2 (5×10^3 PFU/mouse). Survival was monitored (A) and pathology scores were recorded on a scale of 0 to 5 (B) for 12 days after reexposure. Significance in difference in survival (A) was calculated using the log rank (Mantel-Cox) test (*, $P < 0.05$). Data points superimposed on the x axes of panel B indicate mice without genital pathology, and the percentages represent maximum numbers of mice that demonstrated pathology. Vaginal washes were collected daily for 6 days after reexposure; HSV-2 viral shedding was calculated using a Vero cell-based assay (C and D), and cytokine and chemokine (IFN-γ, IL-6, TNF-α, RANTES, MCP-1, M-CSF, MIP-1α, and MIP-1β) concentrations were measured by multianalyte assays (E). The bars in panel C indicate mean PFU per milliliter of shed virus. The dotted lines in panel C indicate the lower detection limit of the assay, and data points on this line indicate undetectable viral shedding. The percentages in panel C represent maximum numbers of mice that shed virus on any given day. Data shown in panel D represent the viral loads (means ± SEMs) over 6 days. Each symbol represents a single animal. Data shown in panel E represent the means ± SEMs from two independent experiments, done in duplicate (n = 4 to 7/group). Data were analyzed using the unpaired, nonparametric, two-tailed Mann-Whitney test with 95% confidence interval, with the ROUT method used to identify outliers and the Bonferroni correction used to correct for multiple measures.*, $P < 0.05$; **, $P < 0.01$.

viral shedding were monitored daily. We found that following intravaginal HSV-2 reexposure, *IL-17A*$^{-/-}$ mice had poor disease outcomes and demonstrated higher rate of mortality (Fig. 2). After reexposure, 78% of WT mice survived, compared to only 22% of *IL-17A*$^{-/-}$ mice (Fig. 2A), and *IL-17A*$^{-/-}$ mice developed more than twice as high

TABLE 1 Cumulative pathology scores for HSV-2-reexposed mice[a]

Group	Pathology score	No. of mice	No. of days	Cumulative pathology	Avg pathology/mouse
WT (5×10^2 PFU)	0	3	15	0	9.6
	4	2	6	48	
IL-17A$^{-/-}$ (5×10^2 PFU)	0	2	15	0	14.4
	4	1	5	20	
	4	1	6	24	
	4	1	7	28	
WT (5×10^3 PFU)	0	7	12	0	5.8
	4	1	7	28	
	4	1	6	24	
IL-17A$^{-/-}$ (5×10^3 PFU)	0	2	0	0	13.3
	4	4	4	64	
	4	2	6	24	
	4	1	8	32	
WT (5×10^4 PFU)	0	4	12	0	0
IL-17A$^{-/-}$ (5×10^4 PFU)	0	2	12	0	7.25
	3	1	3	9	
	4	1	5	20	

[a]Cumulative pathology is calculated by noting the number of mice with their maximum pathology score and the number of days that score was observed. Mice that did not survive the challenge were given highest pathology score for the duration of the experiment to accurately reflect overall pathology for each group. This takes into consideration that each mouse in a group can reach various degrees of pathology through the experiment. The average pathology score per mouse was calculated by dividing the sum of cumulative pathology by total number of mice.

average cumulative genital pathology (*IL-17A*$^{-/-}$, 13.3; WT, 5.8) (Table 1; Fig. 2B). We also collected vaginal washes and found that a greater percentage of *IL-17A*$^{-/-}$ mice than WT mice were shedding virus (*IL-17A*$^{-/-}$, 89%; W, 44%) (Fig. 2C) and had higher viral shedding in the vaginal tract ($P = 0.003$) (Fig. 2D). We repeated these reexposure experiments with other viral doses (primary exposure, 10^1 and 10^3 PFU/mouse; reexposure, 5×10^2 and 5×10^4 PFU/mouse) and consistently found decreased protection in *IL-17A*$^{-/-}$ mice (Table 1). In addition to quantifying viral shedding, cytokine and chemokine concentrations were also measured in vaginal secretions. Forty-eight hours following reexposure, *IL-17A*$^{-/-}$ mice had significantly lower levels (means $\pm$ standard errors of the means [SEMs]) of IFN-γ (*IL-17A*$^{-/-}$, 86.45 $\pm$ 15.34 pg/ml; WT, 252.11 $\pm$ 31.97 pg/ml; $P = 0.016$) (Fig. 2E). Interestingly, levels of proinflammatory cytokines and chemokines, including IL-6 ($P = 0.006$), tumor necrosis factor alpha (TNF-α) ($P = 0.038$), regulated on activation, normal T-cell expressed and secreted (RANTES) ($P = 0.032$), monocyte chemoattractant protein 1 (MCP-1) ($P = 0.009$), macrophage colony-stimulating factor (M-CSF) ($P = 0.038$), and macrophage inflammatory protein 1 alpha (MIP-1α) ($P = 0.017$) and beta (MIP-1β) ($P = 0.017$), were significantly higher in *IL-17A*$^{-/-}$ mice than in WT mice (Fig. 2E). These findings showcase significant differences between WT and *IL-17A*$^{-/-}$ mice upon intravaginal HSV-2 reexposure. In the absence of IL-17A, mice demonstrated higher rates of mortality, more severe genital pathology, and greater viral shedding, along with higher concentrations of proinflammatory factors and lower levels of IFN-γ in vaginal secretions. Together, these results indicate that IL-17A plays an important role in the antiviral responses within the genital tract following HSV-2 reexposure.

IL-17A mediates efficient antiviral T_h1 responses following genital HSV-2 reexposure. Next, we sought to better understand why *IL-17A*$^{-/-}$ mice were more susceptible to HSV-2 reexposure and demonstrated increased mortality (Fig. 2). Since IFN-γ is known to play a key role in clearance of HSV-2 and we saw lower levels of IFN-γ in the vaginal secretions of *IL-17A*$^{-/-}$ mice (Fig. 2E), we decided to examine the *in vivo*

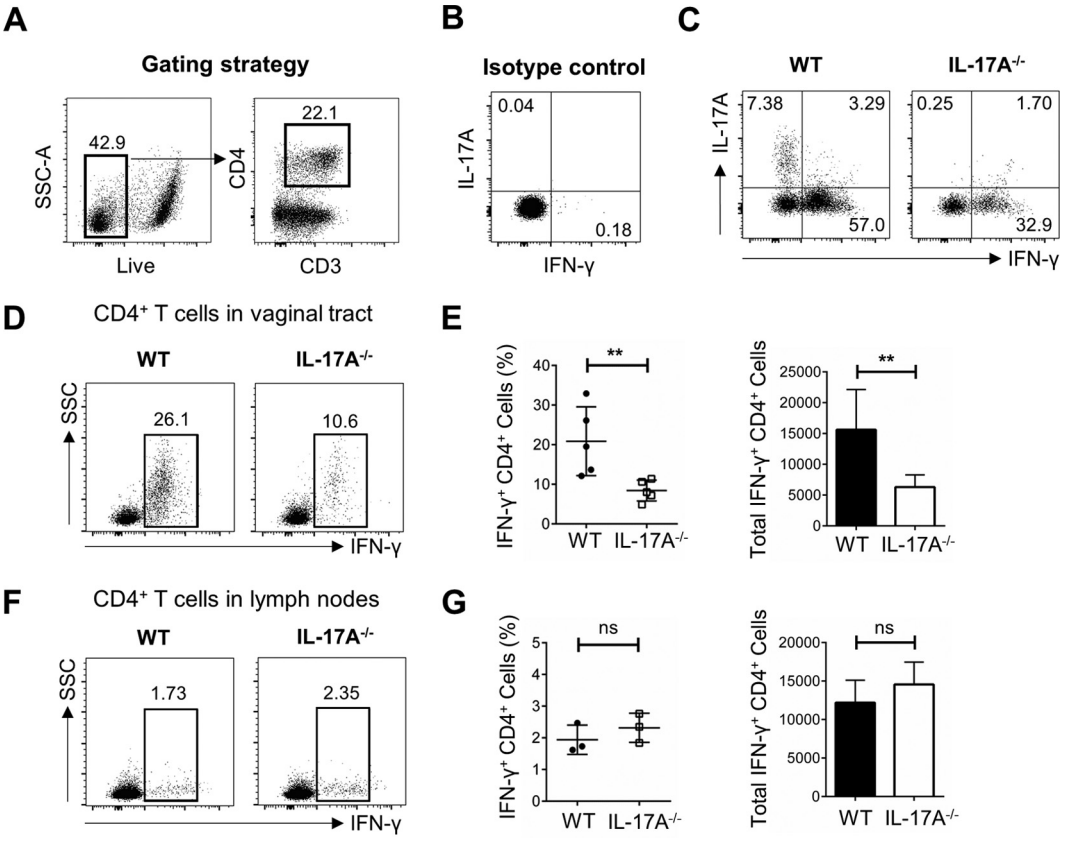

FIG 3 Preexposed *IL-17A*$^{-/-}$ mice have lower proportions of IFN-γ^+ T_h1 cells in the vaginal tract following intravaginal HSV-2 reexposure. OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice (n = 4 to 6/group) were intravaginally exposed to WT HSV-2 (10^2 PFU/mouse) and 6 weeks later reexposed intravaginally with a higher dose of WT HSV-2 (5×10^3 PFU/mouse). Vaginal tissue and lymph nodes were isolated at day 3 following reexposure, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry. (A) $CD4^+$ T cells were gated among total live $CD3^+$ T cells in the vaginal tissue. (B) Isotype controls for intracellular staining of IL-17A and IFN-γ. (C) Vaginal cells were stimulated *in vitro* with a cell stimulation cocktail (CSC) containing Golgi inhibitors and PMA plus ionomycin for 16 h to detect intracellular staining of IL-17A and IFN-γ. (D) Intracellular staining for IFN-γ was used to examine the *in vivo* response to HSV-2 (without *in vitro* stimulation) and the differentiation of $CD4^+$ T cells into T_h1 cells in the vaginal tract. (E) The differences in percentages and total cell numbers of IFN-γ-producing $CD4^+$ T cells after HSV-2 reexposure in the vaginal tract were compared across five independent experiments (n = 4 to 6/group). (F and G) Intracellular staining for IFN-γ (F) and the differences in percentages and total cell numbers of IFN-γ-producing $CD4^+$ T cells after HSV-2 reexposure in the lymph nodes (G) were compared across three independent experiments (n = 5 or 6/group). Data shown in panels E and G represent means ± SEMs. Significant difference in IFN-γ expression was calculated using the unpaired, two-tailed t test with 95% confidence interval. **, $P < 0.01$. ns, no significance.

T cell responses in the vaginal tissue in order to quantify IFN-γ production by $CD4^+$ T cells.

Following intravaginal primary infection and reexposure described above, vaginal tissues and iliac lymph nodes (which drain the genital tract) were collected 3 days later to phenotype and functionally characterize the *in vivo* T cell responses. $CD4^+$ T cells were gated based on total, live $CD3^+$ cells in the vagina (Fig. 3A), and isotype controls for IFN-γ and IL-17A were included (Fig. 3B). To examine IL-17A production by $CD4^+$ T cells, vaginal cells were stimulated *in vitro* with phorbol 12-myristate 13-acetate (PMA) and ionomycin, and higher proportions of total T_h1 and T_h17 cells were seen in WT mice than in *IL-17A*$^{-/-}$ mice (Fig. 3C), with insignificant IL-17A detected in *IL-17A*$^{-/-}$ mice. Following *in vivo* challenge alone, and in the absence of *in vitro* stimulation, vaginal tissue from *IL-17A*$^{-/-}$ mice contained lower proportions of IFN-γ^+ T_h1 cells (10.6% of $CD4^+$ T cells) than that from WT mice (26.1% of $CD4^+$ T cells) (Fig. 3D). Data compiled from multiple independent experiments consistently showed that following reexposure, *IL-17A*$^{-/-}$ mice had significantly lower IFN-γ production by vaginal $CD4^+$ T cells than did WT mice (*IL-17A*$^{-/-}$, 8.42% ± 1.18% of $CD4^+$ T cells; WT, 20.86% ± 3.89% of

$CD4^+$ T cells; $P = 0.016$) (Fig. 3E) and a significantly lower total number of vaginal IFN-γ^+ $CD4^+$ T cells (*IL-17A*$^{-/-}$, 6,304 ± 883; WT, 15,624 ± 2,912; $P = 0.008$) (Fig. 3E). Notably, this difference was not present in the lymph nodes, where *IL-17A*$^{-/-}$ and WT mice had very similar proportions of IFN-γ^+ $CD4^+$ cells (*IL-17A*$^{-/-}$, 2.32% ± 0.27% of $CD4^+$ T cells; WT, 1.94% ± 0.27% of $CD4^+$ T cells; $P = 0.374$) and similar total numbers of IFN-γ^+ $CD4^+$ cells (*IL-17A*$^{-/-}$, 14,562 ± 1,673; WT, 12,194 ± 1,678; $P = 0.400$) (Fig. 3F and G), suggesting that memory $CD4^+$ T cells, which are readily available to induce IFN-γ production, are compartmentalized in the vaginal tract specifically. Together, these data suggest that the inability of *IL-17A*$^{-/-}$ mice to effectively resolve HSV-2 reexposure is likely due to impaired local IFN-γ^+ T_h1 cell responses, indicating that IL-17A plays a critical role in mediating efficient antiviral T_h1 cell immunity in the genital tract.

IL-17A is essential for establishing efficient T_h1 cell responses in the female genital tract following HSV-2 vaccination. Based on the above-described results, which suggested that IL-17A was critical for augmenting T_h1 cell responses (Fig. 3), we wanted to examine whether IL-17A would also play a role in enhancing immune responses following HSV-2 vaccination. To test antiviral immune responses to HSV-2 vaccination, mice were immunized intranasally with an attenuated strain of HSV-2 (TK^- HSV-2), followed by intravaginal challenge with WT HSV-2. Intranasal immunization generates immunity against intravaginal HSV-2 challenge, comparable to local immunization, and has been frequently used by us and others to generate potent immune responses in the genital tract (20, 24–26).

When WT and *IL-17A*$^{-/-}$ mice were immunized intranasally, we found once again that *IL-17A*$^{-/-}$ mice were more susceptible to intravaginal HSV-2 challenge (Fig. 4). There was considerably lower survival in *IL-17A*$^{-/-}$ mice (*IL-17A*$^{-/-}$, 20%; WT, 70%) (Fig. 4A), and this coincided with more than twice as high average cumulative genital pathology (*IL-17A*$^{-/-}$, 14.4; WT, 5.2) (Table 2; Fig. 4B). There were also more *IL-17A*$^{-/-}$ mice shedding virus (*IL-17A*$^{-/-}$, 80%; WT, 30%) (Fig. 4C), with higher viral shedding in the vaginal tract ($P = 0.003$) (Fig. 4D). In vaginal secretions collected postchallenge, *IL-17A*$^{-/-}$ mice had significantly lower levels of IFN-γ (*IL-17A*$^{-/-}$, 129.80 ± 38.94 pg/ml; WT, 438.80 ± 93.77 pg/ml; $P = 0.017$) (Fig. 4E). Levels of other proinflammatory cytokines and chemokines (IL-6, $P = 0.0006$; TNF-α, $P = 0.0003$; RANTES, $P = 0.0002$; MCP-1, $P = 0.029$; M-CSF, $P = 0.029$; MIP-1α, $P = 0.006$; MIP-1β, $P = 0.0006$) were significantly higher in *IL-17A*$^{-/-}$ mice than in WT mice (Fig. 4E). These results confirmed that following intranasal vaccination, IL-17A plays an important role in the antiviral immune response after HSV-2 challenge.

When we examined T cell responses in the vaginal tract postchallenge, we found a clear difference in the T_h1 cell response generated in *IL-17A*$^{-/-}$ mice from that in WT mice. Consistently, there was significantly lower IFN-γ production by $CD4^+$ T cells in the vaginal tissue of immunized *IL-17A*$^{-/-}$ mice postchallenge (*IL-17A*$^{-/-}$, 10.39% ± 1.16% of $CD4^+$ T cells; WT, 26.60% ± 2.01% of $CD4^+$ T cells; $P = 0.002$) (Fig. 5A) and a significantly lower total number of vaginal IFN-γ^+ $CD4^+$ T cells in *IL-17A*$^{-/-}$ mice (*IL-17A*$^{-/-}$, 7,782 ± 872; WT, 19,923 ± 1,504; $P = 0.002$) (Fig. 5B). However, there was no difference in the lymph nodes, where the percentage of IFN-γ^+ $CD4^+$ T cells (*IL-17A*$^{-/-}$, 1.53% ± 0.33% of $CD4^+$ T cells; WT, 1.55% ± 0.23% of $CD4^+$ T cells; $P = 0.963$) and the total number of IFN-γ^+ $CD4^+$ T cells (*IL-17A*$^{-/-}$, 9,596 ± 2,067; WT, 9,722 ± 1,438; $P = 0.963$) were similar between *IL-17A*$^{-/-}$ and WT mice (Fig. 5C and D). Taken together, these results suggest that IL-17A is important for mounting a proficient T_h1 recall response in the FGT that can protect against HSV-2 challenge following vaccination.

The absence of IL-17A is associated with decreased vaginal T_{RM} cells following HSV-2 vaccination. Our results demonstrating more robust $CD4^+$ T cell responses in the vaginal tract than in lymph nodes (Fig. 3 and 5) indicated that the antiviral immune response observed upon HSV-2 reexposure/challenge was compartmentalized in the vaginal tract. These findings suggest that there is a population of $CD4^+$ T cells generated postimmunization which are localized in the vaginal tract and readily

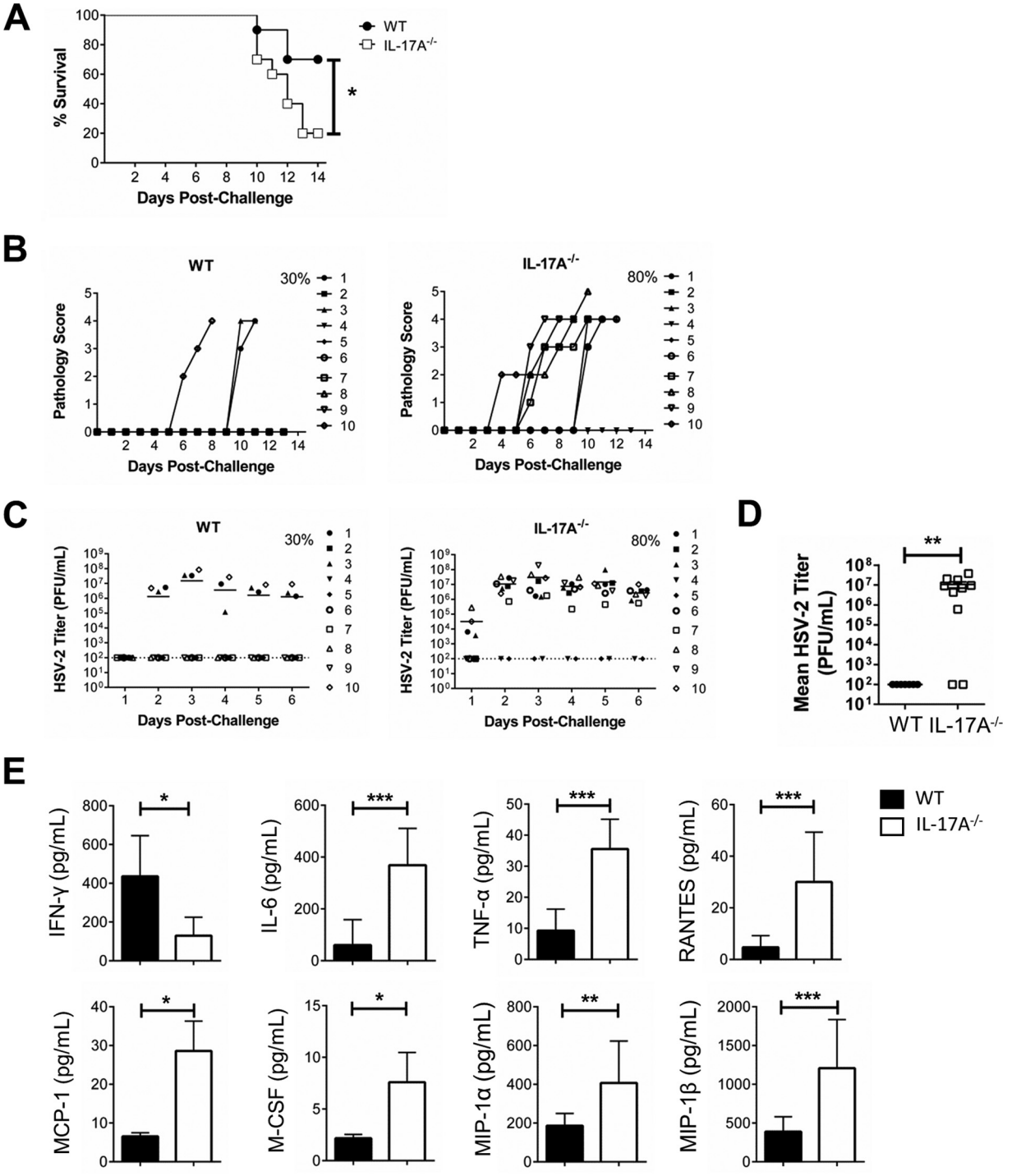

FIG 4 Intranasally immunized *IL-17A*$^{-/-}$ mice are more susceptible to intravaginal HSV-2 challenge. OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice (n = 10/group) were immunized intranasally with TK$^-$ HSV-2 (10^2 PFU/mouse) and 6 weeks later challenged intravaginally with WT HSV-2 (5×10^3 PFU/mouse). Survival was monitored (A) and pathology scores were recorded on a scale of 0 to 5 (B) for 14 days postchallenge. Significance in difference in survival (A) was calculated using the log rank (Mantel-Cox) test (*, $P < 0.05$). Data points superimposed on the x axes of panel B indicate mice without genital pathology, and the percentages represent maximum numbers of mice that demonstrated pathology. Vaginal washes were collected daily for 6 days postchallenge; HSV-2 shedding was calculated using a Vero cell-based assay (C and D), and cytokine and chemokine (IFN-γ, IL-6, TNF-α, RANTES, MCP-1, M-CSF, MIP-1α, and MIP-1β) concentrations were measured by multianalyte assays (E). The bars in panel C indicate mean PFU per milliliter of shed virus. The dotted lines in panel C indicate the lower detection limit of the assay, and data points on this line indicate undetectable viral shedding. The percentages in panel C represent maximum numbers of mice that shed virus on any given day. Data shown in panel D represent the viral loads (means ± SEMs) over 6 days. Each symbol represents a single animal. Data shown in panel E represents means ± SEMs from two independent experiments, done in duplicate (n = 4 to 7/group). Data were analyzed using the unpaired, nonparametric, two-tailed Mann-Whitney test with 95% confidence interval, with the ROUT method used to identify outliers and the Bonferroni correction used to correct for multiple measures. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TABLE 2 Cumulative pathology scores for HSV-2 (5×10^3 PFU)-challenged mice[a]

Group	Pathology score	No. of mice	No. of days	Cumulative pathology	Avg pathology/mouse
WT (5×10^3 PFU)	0	7	12	0	5.2
	4	1	6	24	
	4	1	4	15	
	4	1	3	12	
IL-17A$^{-/-}$ (5×10^3 PFU)	0	2	13	0	14.4
	4	2	3	24	
	4	3	4	48	
	4	1	6	24	
	4	1	7	28	
	5	1	4	20	

[a]Cumulative pathology is calculated by noting the number of mice with their maximum pathology score and the number of days that score was observed. Mice that did not survive the challenge were given highest pathology score for the duration of the experiment to accurately reflect overall pathology for each group. This takes into consideration that each mouse in a group can reach various degrees of pathology through the experiment. Average pathology score per mouse was calculated by dividing the sum of cumulative pathology by total number of mice.

available to respond to future HSV-2 exposure. Therefore, we next focused on examining the tissue-resident memory T cell (T_{RM}) population established in the vaginal tract postimmunization. We wanted to see if the IFN-γ-producing CD4$^+$ cells we had observed were consistent with the phenotype of CD4$^+$ T_{RM} cells and if absence of IL-17A would affect the induction of this population, thereby explaining the subsequent inefficient T_h1 response generated postchallenge.

Following vaccination with TK$^-$ HSV-2, vaginal cells were isolated, pooled, and stimulated to assess IFN-γ production. CD4$^+$ T cells were identified in the vaginal tract

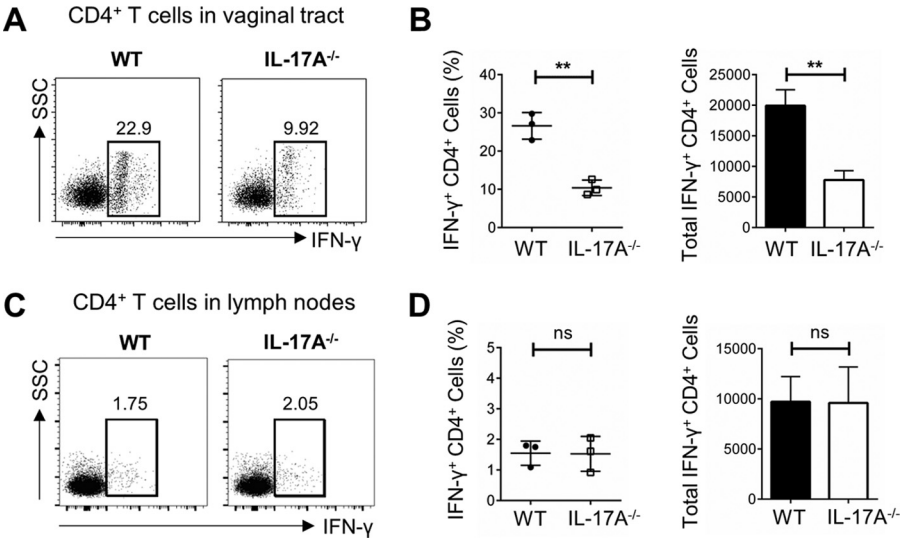

FIG 5 Intranasally immunized *IL-17A*$^{-/-}$ mice have lower proportions of IFN-γ$^+$ T_h1 cells in the vaginal tract following intravaginal HSV-2 challenge. OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice ($n = 4$ to 6/group) were immunized intranasally with TK$^-$ HSV-2 (10^2 PFU/mouse) and 6 weeks later challenged intravaginally with WT HSV-2 (5×10^3 PFU/mouse). Vaginal tissues and lymph nodes were isolated at day 3 postchallenge, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry. Intracellular staining for IFN-γ was used to examine the *in vivo* response to HSV-2. (A and B) The differentiation of CD4$^+$ T cells into T_h1 cells in the vaginal tract (A) and the differences in percentages and total numbers of IFN-γ-producing CD4$^+$ T cells post-HSV-2 challenge in the vaginal tract (B) were compared across three independent experiments ($n = 4$ to 6/group). (C and D) The differentiation of CD4$^+$ T cells into T_h1 cells (C) and the differences in percentages and total numbers of IFN-γ-producing CD4$^+$ T cells after HSV-2 challenge in the lymph nodes (D) were compared across three independent experiments ($n = 4$ to 6/group). Data shown in panels B and D represent means ± SEMs. Significant difference in IFN-γ expression was calculated using the unpaired, two-tailed *t* test with 95% confidence interval. **, $P < 0.01$.

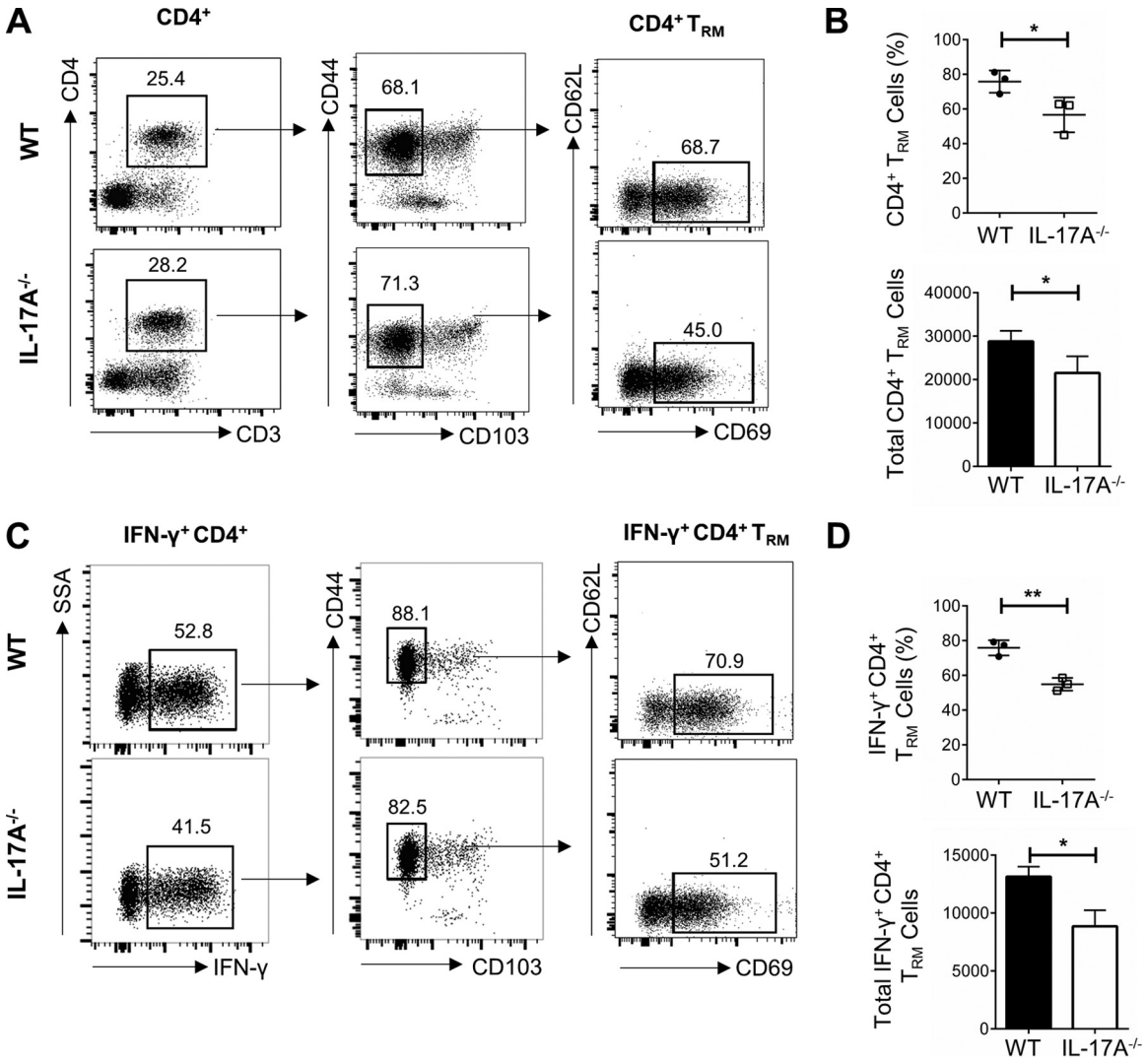

FIG 6 Phenotypic and functional characteristics of $CD4^+$ T_{RM} cells localized in the vaginal tracts of WT and *IL-17A*$^{-/-}$ mice following HSV-2 vaccination. OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice ($n = 5$ to 7/group) were vaccinated intravaginally with TK^- HSV-2 (10^4 PFU/mouse), and 3 weeks later, vaginal tissues were collected, pooled, and processed. Vaginal cells were stimulated *in vitro* with a cell stimulation cocktail (CSC) containing Golgi inhibitors and PMA plus ionomycin for 16 h, stained with a panel of antibodies, and examined by flow cytometry. (A) $CD4^+$ T cells were gated among total live $CD3^+$ T cells in the vaginal tissue, and $CD4^+$ T_{RM} cells were detected using surface markers CD44, CD103, CD69, and CD62L. $CD4^+$ T_{RM} cells were defined as $CD4^+$ $CD44^+$ $CD103^-$ $CD69^+$ $CD62L^-$. (B) The differences in percentages and total numbers of $CD4^+$ T_{RM} cells postimmunization in the vaginal tract were compared across three independent experiments ($n = 5$ to 7/group). (C) IFN-γ^+ $CD4^+$ T_{RM} cells were further gated based on detection of surface markers associated with T_{RM} cells. (D) The differences in percentages and total numbers of IFN-γ^+ $CD4^+$ T_{RM} cells postimmunization in the vaginal tract were compared across three independent experiments ($n = 5$ to 7/group). Data shown in panels B and D represent means ± SEMs. Significant difference in IFN-γ expression was calculated using the unpaired, two-tailed *t* test with 95% confidence interval. *, $P < 0.05$; **, $P < 0.01$.

and further classified based on previously reported cell surface markers associated with vaginal T_{RM} populations (27). Following gating strategies previously described in the literature, $CD4^+$ T cells were gated based on live $CD3^+$ cells, followed by gating based on the positive expression of adhesion molecule CD44 and negative expression of the retention marker CD103 ($CD44^+$ $CD103^-$) and, finally, gating based on the positive expression of T cell activation and retention molecule CD69 and negative expression of the adhesion molecule CD62L ($CD69^+$ $CD62L^-$). We found that postimmunization, *IL-17A*$^{-/-}$ mice had a smaller population of $CD4^+$ T_{RM} cells ($CD4^+$ $CD44^+$ $CD103^-$ $CD69^+$ $CD62L^-$) than did WT mice (*IL-17A*$^{-/-}$, 45.0%; WT, 68.7%) (Fig. 6A). Data compiled from multiple independent experiments consistently showed that postimmunization, *IL-17A*$^{-/-}$ mice had significantly smaller populations of $CD4^+$ T_{RM} cells

than did WT mice (*IL-17A*$^{-/-}$, 56.53% ± 5.82%; WT, 75.77% ± 3.70%; $P = 0.05$) (Fig. 6B) and a significantly lower total number of vaginal CD4$^+$ T_{RM} cells (*IL-17A*$^{-/-}$, 21,521 ± 2,212; WT, 28,791 ± 1,406; $P = 0.05$) (Fig. 6B). Furthermore, when focusing specifically on the population of CD4$^+$ T cells producing IFN-γ, using the same gating strategy, we found that there was a smaller population of IFN-γ$^+$ CD4$^+$ T_{RM} cells (CD4$^+$ IFN-γ$^+$ CD44$^+$ CD103$^-$ CD69$^+$ CD62L$^-$) in the vaginal tract of *IL-17A*$^{-/-}$ mice (*IL-17A*$^{-/-}$, 51.2%; WT, 70.9%) (Fig. 6C). This was true across multiple independent experiments, where *IL-17A*$^{-/-}$ mice consistently had significantly lower proportions (*IL-17A*$^{-/-}$, 54.90% ± 2.11%; WT, 75.83% ± 2.52%; $P = 0.003$) and total numbers (*IL-17A*$^{-/-}$, 8,862 ± 801; WT, 13,149 ± 497; $P = 0.01$) of IFN-γ$^+$ CD4$^+$ T_{RM} cells (Fig. 6D). Overall, these results suggest that IL-17A helps establish a proficient IFN-γ$^+$ CD4$^+$ T_{RM} cell population postimmunization and, consequently, mediates efficient antiviral T_h1 responses postchallenge.

DISCUSSION

The generation of robust IFN-γ-T_h1 cell responses is critical for protective immunity against HSV-2 infection, and recent studies suggest that IL-17 potentiates T_h1 immunity, thereby playing an important role in controlling infections with intracellular pathogens (17–19, 28, 29). In the current study, we investigated the role of IL-17A in host defense against genital HSV-2 infection. We found that in the absence of IL-17A, protection against both intravaginal HSV-2 reexposure following primary genital infection and intravaginal HSV-2 challenge following intranasal immunization was significantly decreased. This was evident because *IL-17A*$^{-/-}$ mice had higher rates of mortality and viral shedding, and more severe disease pathology, than did WT mice. In addition, there were higher levels of inflammatory cytokines and chemokines in the vaginal secretions of *IL-17A*$^{-/-}$ mice. Furthermore, poor disease outcomes in the absence of IL-17A also coincided with lower T_h1 responses than in WT mice, with significantly less IFN-γ production by vaginal CD4$^+$ T cells following reexposure as well as postchallenge in immunized mice. Finally, IL-17A was shown to be important for establishing proficient IFN-γ$^+$ CD4$^+$ T_{RM} cell populations in the FGT postimmunization. To the best of our knowledge, this is the first study demonstrating that IL-17A plays a critical role in mediating efficient antiviral T_h1 responses in the female genital tract, thereby improving HSV-2 vaccination efficacy.

The function of IL-17A, a cytokine primarily produced by T_h17 cells, in reproductive tract infections initiated by exposure to fungal and bacterial pathogens has been reported by several studies. IL-17A can help control overgrowth of the fungus *C. albicans* by upregulating proinflammatory cytokines and antimicrobial peptides and recruiting neutrophils through the secretion of neutrophil-recruiting chemokines (10, 30). In addition, T_h17 deficiency is associated with chronic candidiasis (31). Similarly, IL-17A also plays a protective role in defense against bacterial STIs. During murine genital tract infection with *N. gonorrhoeae*, IL-17A is important for the recruitment of neutrophils and resulting clearance of infection (11), while the absence of IL-17A leads to prolonged infection (7). However, the role of IL-17A during viral infections in the genital tract has not been well described. A study by Kim et al. reported that the deaths of *IL-17A*$^{-/-}$ mice were significantly delayed following intravaginal infection with HSV-2 compared to the deaths of WT mice, suggesting that IL-17A-producing T cell receptor (TCR) γδ$^+$ CD4$^-$ CD8$^-$ T cells play a pathogenic role during HSV-2 infection (15). However, that study only examined the role of IL-17A in the context of a primary infection with a lethal dose of virus, focusing on IL-17A$^+$ TCRγδ$^+$ T cells, an approach starkly different than the one used in the current study. In this study, we examined the role of IL-17A in closer detail following HSV-2 challenge, as opposed to only after primary infection, as *IL-17A*$^{-/-}$ mice showed no differences in disease outcome compared to WT mice following primary infection with nonlethal doses of virus (Fig. 1). However, following intravaginal challenge, *IL-17A*$^{-/-}$ mice consistently demonstrated greater mortality and genital pathology, along with higher viral shedding, than WT mice (Fig. 2 and 4; Tables 1 and 2). Studies examining the role of IL-17 during infection with other intracellular pathogens, such as *M. tuberculosis*, show similar results, where

although IL-17 is dispensable during the primary infection, it appears to play a critical role in inducing immune responses following vaccination (17, 28). Similar to the role of IL-17 in the *M. tuberculosis* model, our results also show that IL-17 is critical for inducing efficient immune response to HSV-2 postimmunization.

In addition, the study by Kim et al. used a lethal dose of HSV-2 (15), and it is possible that this may mask any effect that IL-17A has in terms of the efficiency in the immune response generated. When administered such a high dose of virus, mice may be able to compensate for the lack IL-17A by producing greater amounts of other protective cytokines and immune factors, making it difficult to assess the efficiency of the immune response generated. Interestingly, we observed significantly increased proinflammatory cytokine and chemokine production in vaginal washes collected from *IL-17A*$^{-/-}$ mice, even while using lower viral doses (Fig. 2D and 4D). It is possible that the increased production of proinflammatory cytokines in the absence of IL-17A is a compensatory mechanism which is implemented to help protect the mice against HSV-2 infection, and using even higher viral doses further amplifies this compensatory effect. Similarly, a study examining the role of IL-17 during genital *C. muridarum* infection found that IL-17RA knockout (KO) mice exhibited increased TNF-α production, which acted as a compensatory mechanism to effectively control chlamydial genital infection (18). Furthermore, other studies, including that by Kim et al., utilize medroxyprogesterone acetate (MPA) to make mice susceptible to HSV-2. MPA is an injectable progestin-based formulation that thins the vaginal epithelium and makes mice susceptible to infection. Several studies, including our own, have shown that MPA can significantly dampen mucosal antiviral responses to HSV-2 (32, 33). Specifically, prolonged exposure to MPA has been shown to decrease levels of HSV-2-specific mucosal immune responses following intravaginal immunization. Therefore, the use of MPA may have impacted the results of the study by Kim et al., resulting in an inaccurate assessment of the role of IL-17A during HSV-2 infection. To avoid compromised immune responses as a result of MPA treatment and the presence of endogenous hormones, we use an alternative model in which mice undergo an ovariectomy in order to remove the source of endogenous hormones altogether. This procedure naturally thins the vaginal epithelium, making the mice susceptible to intravaginal HSV-2 infection. This allows us to study antiviral immune responses in the female genital tract under no-hormone or clearly defined hormonal conditions, and it is the technique that was used in the present study.

Several studies show that IL-17 potentiates T_h1 cell immune responses and plays a critical role in controlling infections with intracellular pathogens (17–19, 28, 29). The contribution of IL-17 to the development of T_h1 cell immunity against intracellular pathogens has been shown during infection with pulmonary *M. tuberculosis*, *Francisella tularensis*, and *C. muridarum*, as well as genital infection with *C. muridarum*. Khader et al. determined, using the pulmonary *M. tuberculosis* vaccination model, in which, like anti-HSV-2 immunity, IFN-γ^+ CD4$^+$ T cell responses play a key role in generating effective immunity, that T_h17 responses induced in the lungs following immunization play a key role in accelerating T_h1 cell responses (17). This resulted in the early resolution of bacteria postchallenge, and in the absence of IL-17, IFN-γ responses and clearance of the bacteria were significantly delayed. Similarly, a study examining the role of T_h17 cell immunity in host resistance to the intracellular bacterium *F. tularensis* showed that T_h1 cell immunity was compromised in the absence of IL-17A (29). In addition, studies looking at chlamydial infection found that IL-17A is important for the development of T_h1 responses in both the lungs and genital tract. Scurlock et al. found that IL-17RA mice demonstrated reduced IFN-γ production within the vaginal tract following genital *C. muridarum* infection (18). Furthermore, Bai et al. reported that mice treated with anti-IL-17A antibodies showed significantly delayed clearance of *C. muridarum* and increased disease severity in the lung. This corresponded with significantly reduced *Chlamydia*-specific T_h1 responses and was related to impaired DC induction of IFN-γ responses in the absence of IL-17A (19). Parallel to these studies, we recently showed that IL-17A in the vaginal tract may augment antiviral IFN-γ-T_h1 cell responses

in the vaginal tract (16). We found that HSV-2 vaccination under the influence of the female sex hormone estradiol resulted in increased production of IL-17A by $CD4^+$ cells, which coincided with earlier recruitment and increased proportions of vaginal IFN-γ^+ $CD4^+$ cells postchallenge (16); however, it was not established if IL-17A was directly responsible for the increase in IFN-γ. Interestingly, similar to what was shown by Bai et al. in the *C. muridarum* model, we also found that in *in vitro* cocultures conducted with vaginal cells from *IL-17A*$^{-/-}$ mice and ovalbumin (OVA)-specific OT-II transgenic $CD4^+$ T cells, vaginal antigen-presenting cells (APCs) from *IL-17A*$^{-/-}$ mice were significantly impaired at inducing IFN-γ production compared to vaginal APCs from WT mice, suggesting that there is an intrinsic impairment in the priming of T_h1 cell responses by vaginal DCs in *IL-17A*$^{-/-}$ mice (16).

The present study advances our understanding regarding the *in vivo* role of IL-17A, by focusing on the ability of *IL-17A*$^{-/-}$ mice to initiate an IFN-γ^+ $CD4^+$ T cell response *in vivo* in the context of HSV-2 infection. To assess the differentiation of $CD4^+$ T cells into IFN-γ^+ T_h1 cells, vaginal cells were isolated following *in vivo* HSV-2 challenge and left unstimulated to measure spontaneous cytokine production. We have previously shown that levels of cytokine production, including that of IFN-γ, by vaginal $CD4^+$ T cells are comparable between cells which have been stimulated with UV-inactivated HSV-2 *in vitro* postchallenge and cells grown in culture following *in vivo* HSV-2 challenge, without further *in vitro* antigen challenge (34). This is likely because at 3 days after *in vivo* challenge, T cells in the vaginal tract are already activated. Therefore, we consider T cell responses following *in vivo* challenge to be good surrogate measures of antigen-specific T cell responses. Similar to the reported *in vitro* results regarding impaired T cell priming in the absence of IL-17A (16), we show that there is a significant decrease in IFN-γ production by vaginal $CD4^+$ T cells *in vivo* following HSV-2 challenge (Fig. 3 and 5). Evidently, lower IFN-γ production in the absence of IL-17A is enough to critically impact survival and disease outcomes, as having an approximately 2-fold-lower IFN-γ^+ $CD4^+$ T cell response in the vaginal tract after reexposure (Fig. 3) was sufficient to lower survival from 78% in WT mice to 22% in *IL-17A*$^{-/-}$ mice (Fig. 2). Comparable trends were also seen in vaccinated *IL-17A*$^{-/-}$ mice, as a loss in IFN-γ^+ $CD4^+$ T cell efficiency postchallenge (Fig. 5) resulted in survival decreasing from 70% in WT mice to only 20% in *IL-17A*$^{-/-}$ mice (Fig. 4). Since IFN-γ plays a critical role in host protection against HSV-2 in the mouse model of infection, decreased IFN-γ production in the vaginal tract can help explain the inability of *IL-17A*$^{-/-}$ mice to overcome HSV-2 challenge.

Our findings suggest that IL-17A plays an in important role in mediating efficient antiviral immune responses in the vaginal tract following HSV-2 vaccination, and based on previous work, this appears to be linked to a defect in the priming of T_h1 cell responses in the absence of IL-17A (16). Interestingly, IL-17A also been shown to play a role in augmenting long-lasting memory responses in the lung (17). In the current study, we found that the protective IFN-γ^+ $CD4^+$ T cell response was compartmentalized in the vaginal tract and that in the absence of IL-17A, there was an impaired IFN-γ response in vaccinated mice postchallenge (Fig. 3 and 5). To better understand how IL-17A may be involved in generating efficient recall responses, we examined the repertoire of T cells in the vaginal tracts of *IL-17A*$^{-/-}$ and WT mice following HSV-2 vaccination, in order to compare the phenotype and functional characteristics of the T cells which localize to the vaginal tract postimmunization. Specifically, we looked at $CD4^+$ T_{RM} cells, a newly described subset of memory T cells, to examine if IL-17A is involved in establishing long-lasting, efficient tissue resident populations. T_{RM} cells are clonally expanded memory T cells which enter peripheral tissues, where they are retained long-term and are able to survive without depending on replenishment from circulating T cells (35). More importantly, T_{RM} cells have the ability to respond rapidly to infection and thereby represent a critical subset of cells involved in immunological protection against invading pathogens. $CD8^+$ and $CD4^+$ T_{RM} cells established in the vaginal tract postimmunization have been shown to be involved in mediating better protection against HSV-2 challenge than that with circulating memory T cells on their

own (27, 36); however, this is a new area of research in the field, and very little is known about these populations in the genital mucosa. In particular, it has been shown that vaginal CD4$^+$ T_{RM} cells are established in extremely low numbers, making them especially difficult to detect and characterize. For this reason, postimmunization, we stimulated our cells *in vitro* with a cell stimulation cocktail including PMA and ionomycin in order to assess maximum IFN-γ production by CD4$^+$ T_{RM} cells. Although this stimulation process may have resulted in T cell responses which are not entirely HSV-2 specific, it allowed us to measure the maximum ability of the vaginal cells to induce cytokine responses, which closely mimic those produced in response to HSV-2. Based on the limited published data, we utilized markers (CD44, CD103, CD69, and CD62L) which have been shown to identify tissue resident memory populations specifically in the vaginal tract (27, 36). CD44 is a cell adhesion molecule and cell activation marker that is highly expressed by T_{RM} cells in most mucosal tissues. CD69 is another activation marker found to be expressed by T_{RM} cells and is thought to play a role in T_{RM} cell retention within periphery tissues, while CD62L is a lymph node homing receptor that is expressed by central memory T cells and not T_{RM} cells. Unlike in other mucosal tissues, vaginal CD4$^+$ T_{RM} cells do not highly express the conventional memory and mucosal homing marker, CD103, as CD103 expression was shown to be detected in only 10% of CD4$^+$ T_{RM} cells in the vagina following infection with TK$^-$ HSV-2 (27). We thereby characterized CD4$^+$ T_{RM} cells as follows: CD4$^+$ CD44$^+$ CD103$^-$ CD69$^+$ CD62L$^-$. Interestingly, we found that following HSV-2 vaccination, there was a smaller population of CD4$^+$ T_{RM} cells in the vaginal tract of *IL-17A*$^{-/-}$ mice than in that of WT mice (Fig. 6A and B). Furthermore, the population of vaginal IFN-γ^+ CD4$^+$ T_{RM} cells was also smaller in *IL-17A*$^{-/-}$ mice (Fig. 6C and C). This suggests that in the absence of IL-17A, mice are also impaired in the ability to generate a proficient pool of local, vaginal CD4$^+$ T_{RM} cells following HSV-2 vaccination. Since it has been shown that T_{RM} cells are the primary source of IFN-γ early on during HSV-2 challenge (27), this can help explain why *IL-17A*$^{-/-}$ mice have a less efficient T-cell response upon subsequent HSV-2 exposure. However, with the limited knowledge available regarding the phenotype and the functional characteristics of vaginal CD4$^+$ T_{RM} cells, further work is ongoing to better understand the importance of this T cell population and how IL-17A may be involved in the establishment of these tissue memory cells. In ongoing studies, we are optimizing a model using transgenic mice expressing CD4$^+$ T cell receptors specific for the HSV-2 glycoprotein D-derived epitope, which will allow us to better study HSV-2-specific CD4$^+$ T cell responses and bypass the use of stimulatory markers, which may influence the expression of our T_{RM} cell markers.

In summary, our study provides insight regarding a novel antiviral role for IL-17A in the genital tract, in which IL-17A improves mucosal vaccination efficacy by mediating efficient T_h1 cell immunity. While it is well established that IFN-γ^+ CD4$^+$ T cells play a critical role against HSV-2 infection, little is known about the contribution of IL-17A$^+$ CD4$^+$ T cells. We show for the first time that IL-17A contributes significantly to the generation of protective antiviral T_h1 cell immunity in the genital tract. In the absence of IL-17A, there is a compromised antiviral T_h1 cell response following intravaginal HSV-2 reexposure and challenge, resulting in poor disease outcomes. This has important implications in terms of HSV-2 vaccine development, as a hallmark of an effective vaccination strategy is the ability to generate a rapid, robust local effector response, and we propose that HSV-2 vaccine strategies which induce IL-17A production in the genital tract may promote more effective T_h1 cell immunity.

MATERIALS AND METHODS

Animals. Six- to 8-week-old female C57BL/6 mice were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada). *IL-17A*$^{-/-}$ mice (C57BL/6 background) were generated by Yoichiro Iwakura (University of Tokyo, Tokyo, Japan) (37) and bred internally in the Central Animal Facility at McMaster University. All mice were maintained under specific-pathogen-free (SPF) and standard temperature-controlled conditions that followed a 12-h light/dark cycle, and they were given low-fat mouse chow. Routine quality assurance was done by serology and PCR to ensure that mice remained SPF and included testing dirty bedding sentinels, direct resident animals, and exhaust air duct samples of

racks. All animal studies performed were approved by, and in compliance with, the Animal Research Ethics Board (AREB) at McMaster University.

OVX. Endogenous hormones in mice were depleted by ovariectomy (OVX). Prior to OVX, mice were administered analgesic (Temgesic) subcutaneously, and after 30 min, they received an injectable anesthetic preparation of ketamine and xylazine intraperitoneally. Ovaries were removed by making two bilateral incisions, followed by small incisions through the peritoneal wall, and excised through the incisions. Incisions were closed using surgical clips, and mice recovered for 10 to 14 days before the start of experiments.

Viral infection. For primary HSV-2 infection, OVX mice were anesthetized intraperitoneally and intravaginally infected with 10 μl of WT HSV-2 strain 333 (10^1, 10^2, or 10^3 PFU/mouse). After inoculation, mice were placed on their backs for approximately 30 to 45 min to allow for the inoculum to infect the vaginal tract. For reexposure experiments, mice were reexposed intravaginally 6 weeks later with a higher dose of WT HSV-2 (5×10^2, 5×10^3, or 5×10^4 PFU/mouse). For intranasal immunization experiments, OVX mice were anesthetized using isoflurane. The mice were then immunized intranasally with 5 μl of thymidine kinase-deficient (TK^-) HSV-2 strain 333 (10^2 PFU/mouse) into each nare with a micropipette, for a total of 10 μl. Six weeks following intranasal immunization, mice were challenged intravaginally with WT HSV-2 (5×10^3 PFU/mouse). To examine T_{RM} cell populations following vaccination, mice were immunized intravaginally as described above with TK^- HSV-2 strain 333 (10^4 PFU/mouse).

Collection of vaginal washes. Vaginal washes were collected daily for up to 6 consecutive days after HSV-2 reexposure or challenge by pipetting 30 μl of phosphate-buffered saline (PBS) into the vagina 5 or 6 times. This was repeated to give a total volume of approximately 60 μl, and washes were stored at -70°C until use.

Genital pathology. Following infection with HSV-2, genital pathology was monitored daily and scored on a 5-point scale: 0, no infection; 1, slight redness of external vagina; 2, swelling and redness of external vagina; 3, severe swelling and redness of both vagina and surrounding tissue and hair loss in genital area; 4, genital ulceration with severe redness; and 5, severe genital ulceration extending to surrounding tissue. Animals were euthanized by cervical dislocation when they reached stage 4/5. To compare groups, cumulative pathology scores were determined by tabulating the number of mice with the highest score of pathology they achieved and the number of days that score was observed. Mice that did not survive the challenge were given the highest pathology score for the duration of the experiment to accurately reflect overall pathology for each group. The sum of these scores for all the mice was the total level of pathology for each group, and the average pathology score per mouse for each group was then calculated.

Viral titration. Viral titers in vaginal washes were determined by viral plaque assay on Vero cell (ATCC, Manassas, VA) monolayers. Vero cells were grown in supplemented α-minimum essential medium (α-MEM) (Gibco Laboratories, Burlington, Canada) supplemented with 5% fetal bovine serum (FBS; Gibco Laboratories), 1% penicillin-streptomycin (Invitrogen, Burlington, Canada), L-glutamate (BioShop Canada Inc., Burlington, Canada), and 1% HEPES (Invitrogen). For plaque assays, Vero cells were grown to confluence in 12-well plates. Vaginal lavage samples were removed from -70°C and thawed on ice. Samples were diluted (10^{-2} to 10^{-7}) in α-MEM and added to monolayers. Infected monolayers were incubated at 37°C for 2 h and were rocked every 15 min to facilitate viral absorption. Infected monolayers were then overlaid with α-MEM to stop viral adsorption. Infection was allowed to occur for 48 h at 37°C. Monolayers were then fixed and stained with crystal violet, and viral plaques were counted under a light microscope. The number of PFU per milliliter was calculated by taking a plaque count for every sample and accounting for the dilution factors.

Multiplex cytokine and chemokine assay. Vaginal washes collected from mice after reexposure and postchallenge were analyzed for cytokines and chemokines, using the 31-Plex Mouse Cytokine/Chemokine Discovery Luminex assay from Eve Technologies (Calgary, Canada) as per the manufacturer's protocol.

Single-cell preparation and cultures. Three days postchallenge, iliac lymph nodes (LN), which drain the genital tract, were removed and a single cell suspension was prepared by mechanically disrupting the tissues. Debris was allowed to settle, and the supernatant containing single cells was collected and centrifuged for 5 min (1,500 rpm) at 4°C. Cells then were resuspended in 1 ml of RPMI 1640 medium supplemented with 10% FBS, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 1% L-glutamine, 0.1% 2-mercaptoethanol, 1× nonessential amino acids, and 1× sodium pyruvate (Gibco Life Technologies, Burlington, Canada). Finally, mononuclear cells were counted and cell preparations were seeded in a 24-well plate at 3×10^6 cells/ml in previously described supplemented RPMI 1640 medium.

Vaginal tracts were removed, pooled, cut lengthwise, washed to remove mucus, and minced into 2- to 4-mm pieces. The vaginal tissue pieces were digested in 15 ml of RPMI 1640 medium containing 0.00157 g/ml of collagenase A (Roche Life Science, USA) at 37°C on a stir plate, stirring with a magnetic bar for 1 h. Following the first digestion, supernatants were collected, and the tissues were digested for a second time in another 15 ml of collagenase A in RPMI 1640 medium for 1 h. At the end of the second digestion, supernatants were collected. The remaining tissue was passed through a 40-μm filter (Small Parts, Miami Lakes, FL), and then all collected supernatants were passed through a 40-μm filter into a 50-ml Falcon tube. Vaginal cell samples were then centrifuged for 10 min (1,200 rpm) at 4°C. Cells were resuspended in 1 ml of previously described supplemented RPMI 1640 medium, mononuclear cells were counted, and cell preparations were seeded in a 24-well plate at a density of 5×10^5 to 1×10^6 cells/well in previously described supplemented RPMI 1640 medium. Cells either were left unstimulated (brefeldin A and monensin) or underwent *in vitro* stimulation with 2 μl/ml of cell stimulation cocktail (CSC) plus

protein transport inhibitors (500×) (cocktail of PMA, ionomycin, brefeldin A, and monensin [eBioscience, San Diego, CA]) for 16 h at 37°C.

Flow cytometry. Following 16 h of incubation at 37°C with or without CSC, cells were collected and stained with allophycocyanin (APC)-ef780 viability dye (eBioscience) for 30 min. Cells were washed and incubated for 5 to 10 min with 2 μl of Fc block (anti-mouse CD16/32; eBioscience) to reduce nonspecific Fc receptor staining. Cells were then stained for cell surface markers using the following antibodies at concentrations based on manufacturer specification sheets: peridinin chlorophyll protein (PerCP)-labeled anti-mouse CD4, phycoerythrin (PE)-CF594-labeled hamster anti-mouse CD3, brilliant violet 510 (BV510)-labeled rat anti-mouse CD103, brilliant blue 515 (BB515)-labeled rat anti-mouse CD62L, PE-labeled hamster anti-mouse CD69 (BD Biosciences, Mississauga, Canada), BV421-labeled anti-mouse CD4, and Alexa Fluor 700 (AF700)-labeled anti-mouse CD44 (BioLegend, San Diego, CA). Cells were incubated with these antibodies for 30 min and then permeabilized and fixed using a transcription factor buffer set (BD Biosciences) by following the manufacturer's protocol. Cells were then stained for intracellular markers using the following antibodies: BV421- and fluorescein isothiocyanate (FITC)-labeled rat anti-mouse IFN-γ or BV421- and FITC-labeled rat IgG1 isotype control (BD Biosciences), and APC-labeled anti-mouse IL-17A or APC-labeled rat IgG2 isotype control (eBioscience). The validity of intracellular staining was verified by fluorescence-minus-one (FMO) controls and/or appropriate isotype controls. Data were collected by flow cytometric analysis using a BD LSRII flow cytometer system (BD Bioscience Pharmingen), and results were analyzed using FlowJo software.

Statistical analysis. Statistical analysis and graphical representation were performed using GraphPad Prism 6.0d (GraphPad Software, San Diego, CA). The Mantel-Cox log rank test was used to calculate significant differences in survival. Differences between the groups were identified using the unpaired, nonparametric, two-tailed *t* test and multiple-comparison Mann-Whitney test with 95% confidence interval, with the ROUT method used to identify outliers. The Bonferroni correction was used to correct for multiple measures in the cytokine analysis. Data are expressed as means ± standard errors of the means.

Ethics statement. All animals in this study were housed at the McMaster Central Animal facility, and all protocols used were approved by the McMaster University Animal Research Ethics Board (AREB) as per AUP number 14-09-40 in accordance with Canadian Council of Animal Care (CCAC) guidelines.

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P.B. and C.K. conceived and designed the experiments. P.B., V.C.A., P.V.N., and D.V. performed the experiments. P.B., V.C.A., and C.K. analyzed the data. M.R.S. provided materials and assisted with data analysis. P.B. and C.K. wrote the manuscript.

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