ImmunoHorizons

RESEARCH ARTICLE | JULY 18 2019

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Immunohorizons (2019) 3 (7): 317–330.

https://doi.org/10.4049/immunohorizons.1900040

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Innate Immunity

IL-17 Production by $\gamma\delta^+$ T Cells Is Critical for Inducing T_h17 Responses in the Female Genital Tract and Regulated by Estradiol and Microbiota

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ABSTRACT

IL-17 can be produced by adaptive immune cells such as T_h17 cells and by immune cells that produce IL-17 without prior priming. This latter category, which we will refer to as "innate," includes innate cells such as NK cells and innate lymphoid cells and innate-like T cell populations such as NKT cells and $\gamma\delta^+$ T cells. Studies in mucosal tissues have shown that the induction of T_h17 immunity is amplified by innate IL-17 produced within those tissues. However, the role of innate IL-17 and its effect on T_h17 induction in the female genital tract (FGT) is largely unknown. In this study, we characterize the primary source of IL-17–secreting vaginal cells and show that innate IL-17 plays a critical role in priming adaptive T_h17 responses in the FGT. Under homeostatic conditions, $\gamma\delta^+$ T cells were the predominant source of innate IL-17 in the murine FGT, and this population was modulated by both the sex hormone estradiol and the presence of commensal microbiota. Compared with wild-type C57BL/6 mice, vaginal APCs isolated from IL-17A–deficient (*IL-17A*$^{-/-}$) mice were severely impaired at priming T_h17 responses in APC–T cell cocultures. Furthermore, the defect in T_h17 induction in the absence of innate IL-17 was associated with impairment of IL-1β production by vaginal $CD11c^+$ dendritic cells. Overall, our study describes a novel role for IL-17 in the FGT and further demonstrates the importance of factors in the vaginal microenvironment that can influence adaptive immune responses. *ImmunoHorizons*, 2019, 3: 317–330.

INTRODUCTION

IL-17 belongs to a family of cytokines that consists of six related ligands (IL-17A to IL-17F) (1). IL-17 is primarily secreted by $CD4^+$ T cells that differentiate into T_h17 effector cells under the influence of cytokine combinations comprised of IL-6, IL-1β, TGF-β, IL-21, and IL-23 (2–5). The effector response of IL-17 involves the induction of inflammatory mediators such as TNF-α, IL-1, IL-8, G-CSF, and the recruitment of neutrophils for pathogen clearance (6–8). T_h17 immunity and the production of IL-17 have been shown to play a significant role in the resolution of bacterial and fungal infections in mucosal tissues (9, 10), while demonstrating a pathological role in autoimmune and chronic inflammatory diseases (6, 11–13).

Within the female genital tract (FGT), a mucosal site exposed to diverse pathogens, IL-17 has been shown to play an important

Received for publication May 22, 2019. Accepted for publication June 28, 2019.

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This work was supported by operating grants from the Canadian Institutes of Health Research (to C.K.) (Funding Reference Number 93615) and an Applied HIV Research Chair Award (to C.K.) from the Ontario HIV Treatment Network (Applied HIV Research Chair 779). V.C.A. and P.B. were supported by Ontario Graduate Scholarships.

Abbreviations used in this article: DC, dendritic cell; E2, estradiol; ERKO, E2 receptor α knockout; FGT, female genital tract; GF, germ-free; HSV-2, HSV type 2; *IL-17A*$^{-/-}$, IL-17A–deficient; ILC, innate lymphoid cell; KO, knockout; MFI, median fluorescence intensity; OT-II Tg, OVA receptor transgenic mice; OVX, ovariectomized; TC, tissue cell; Tg, transgenic; TK^-, thymidine kinase–deficient; WT, wild-type.

role during bacterial and fungal infections, including *Neisseria gonorrheae*, *Candida albicans*, and *Chlamydia trachomatis* (14–19). However, the role of IL-17 during genital viral infections has not been fully elucidated. We recently described a novel antiviral role for IL-17 in the FGT using a mouse model of genital herpes infection (20, 21). Genital herpes, predominantly caused by HSV type 2 (HSV-2), is one of the most prevalent sexually transmitted viral infections globally and affects more women than men (22). The primary protective response against HSV-2 involves the production of IFN-γ by CD4$^+$ T cells in the FGT (23–25). However, we recently showed that enhanced protection against vaginal HSV-2 infection also coincided with increased levels of IL-17 production by CD4$^+$ T cells (21). We found that mice treated with the female sex hormone estradiol (E2) were completely protected against genital HSV-2 challenge, and protection in these animals was related to increased T_h17 immunity (21). Along with augmented T_h17 responses, mice also demonstrated enhanced T_h1 responses (21). Furthermore, in the absence of T_h17 responses, vaccinated IL-17A–deficient mice (*IL-17A*$^{-/-}$) had impaired IFN-γ$^+$ CD4$^+$ T cell responses as well as poor disease outcomes against HSV-2 challenge compared with wild-type (WT) C57BL/6 mice (20). Collectively, these studies suggest a significant role for T_h17 cells in the immune response to viral infections in the FGT.

Interestingly, although T_h17 cells are often associated with IL-17 production, other immune cells can also produce IL-17 without any prior priming. This production of IL-17, which we will refer to as innate IL-17, comes from sources such as innate cells (NK cells and innate lymphoid cells [ILCs]) and innate-like T cell populations (NKT cells and γδ$^+$ T cells) (26). Although innate and innate-like cells represent a relatively small proportion of the responding lymphocytes to mucosal infections, they are more potent at producing IL-17 compared with T_h17 cells (27). Specifically, innate-like γδ$^+$ T cells are one of the earliest identified sources of IL-17 (28) and have been described as the major source of IL-17 under homeostatic conditions in the FGT (29). They develop early in the fetus and provide immunity prior to the generation of the adaptive immune system (30), and unlike traditional T lymphocytes, γδ$^+$ T cells use their TCR directly as pattern recognition receptors to respond to pathogens (31, 32). γδ$^+$ T cells are predominantly localized in mucosal tissues and rapidly activate upon exposure to pathogens and proinflammatory cytokines released by APCs (32). IL-1, IL-6, IL-18, IL-23, and TGF-β have all been shown to be important for IL-17 production by γδ$^+$ T cells (28).

Production of innate IL-17 under homeostatic conditions has been implicated in the amplification of T_h17 responses in mucosal tissues (33–37). In models of autoimmune encephalomyelitis (37) and colitis (33), IL-17$^+$ γδ$^+$ T cells promoted T_h17 differentiation and amplified IL-17 production by T_h17 cells. In our own studies, we have observed that vaginal cells can also constitutively produce IL-17 without any additional stimulation in vitro (21). This suggests that innate IL-17 may be an important component of the vaginal microenvironment and can potentially affect induction of T_h17 responses. These findings prompted us to further determine the role of IL-17 in the FGT.

In the current study, we examined the importance of innate IL-17 during the induction of adaptive T_h17 responses in the FGT. Similar to previous studies (20, 21), we found that in the absence of IL-17, mice were unable to initiate protective antiviral immunity and could not effectively clear HSV-2 virus in vivo. We then examined the ability of *IL-17A*$^{-/-}$ mice to induce an adaptive T_h17 response in vitro and found that vaginal dendritic cells (DCs) from *IL-17A*$^{-/-}$ mice were significantly impaired at priming T_h17 responses, and this was related to attenuated IL-1β production. We further characterized the innate IL-17–secreting cells and found that under homeostatic conditions, γδ$^+$ T cells were the predominant source of IL-17 in the FGT. Finally, we demonstrated that IL-17 production by γδ$^+$ T cells was modulated by the presence of female sex hormone E2 and commensal microflora. These data suggest that production of IL-17 in the vaginal tract under homeostatic conditions is critical for inducing T_h17 responses, thereby amplifying the overall IL-17 response in the FGT.

MATERIALS AND METHODS

Mice

C57BL/6 mice were obtained from Charles River Laboratories (Saint-Constant, QC, Canada). *IL-17A*$^{-/-}$ mice were kindly provided by Dr. M. Stämpfli (acquired with a Material Transfer Agreement from Dr. Y. Iwakura) (University of Tokyo, Japan) (38, 39), and E2 receptor α knockout (KO) mice (ERKO) were kindly provided by Dr. P. Chambon (University de Strasbourg, France) (40) and bred in the Central Animal Facility (McMaster University, Hamilton, Canada). Germ-free (GF) mice were bred in-house and purchased from the Farncombe Family Axenic-Gnotobiotic Facility (McMaster University). OVA receptor transgenic (Tg) mice (OT-II Tg) were purchased from The Jackson Laboratory (Bar Harbor, ME) (41). All mice were maintained under specific pathogen-free and standard temperature-controlled conditions, which followed a 12-h light/dark cycle. Routine quality assurance was done by serology and PCR to ensure mice remained specific pathogen-free and included testing dirty bedding sentinels, direct resident animals, and exhaust air duct samples of racks. All animal studies were approved by, and in compliance with, the Animal Research Ethics Board at McMaster University.

Hormone treatment

For hormone experiments, mice were ovariectomized (OVX) to deplete endogenous hormones according to previously published protocols (42, 43). Briefly, mice were administrated an injectable anesthetic preparation of ketamine and xylazine i.p. 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 14 d before the start of experiments. Two weeks later, OVX mice were anesthetized with injectable anesthetic (ketamine and xylazine) and implanted s.c. with 21-d release 17β-E2 (476 ng/mouse per day) or placebo (mock) pellets (Innovative Research of America, Sarasota, FL), according to previously

published protocols (43). The level of serum E2 resulting from the pellets has previously been shown to correspond to that measured during the estrus cycle (44).

Viral infection

OVX mice were immunized intranasally with an attenuated strain of HSV: thymidine kinase–deficient (TK$^-$) HSV-2. Briefly, mice were anesthetized using isoflurane and then inoculated with 5 μl of TK$^-$ HSV-2 (10^2 PFU/mouse) into each nare with a micropipette. Six weeks later, mice were challenged intravaginally with WT HSV-2 (5×10^3 PFU/mouse). Vaginal washes were collected for 6 d postchallenge by pipetting 30 μl of PBS into the vagina five to six times and repeated to collect a total volume of 60 μl.

Genital pathology scoring

Genital pathology was monitored daily based on a five-point scale, as described previously (20, 21): no infection (0), slight redness of external vagina (1), swelling and redness of vagina (2), severe swelling and redness of vagina and surrounding tissues (3), genital ulceration with severe redness and hair loss (4), and severe ulceration extending to surrounding tissues, ruffled hair, hunched back, and lethargy. Animals were sacrificed before they reached stage 5.

Viral titration

Viral shedding in vaginal washes was determined by conducting viral plaque assays on Vero cell (American Type Culture Collection, Manassas, VA) monolayers. Vero cells were grown in α-MEM (Life Technologies, Burlington, ON, Canada) supplemented with 5% FBS (Life Technologies), 1% penicillin-streptomycin (Invitrogen, Burlington, ON, Canada), L-glutamate (BioShop Canada, Burlington, ON, Canada), and 1% HEPES (Invitrogen). Cells were grown to confluency in 12-well plates. Vaginal washes were diluted in α-MEM and then added to monolayers. Infected monolayers were incubated at 37°C for 2 h. Infected monolayers were then overlaid with α-MEM, and infection was allowed to occur for 48 h at 37°C. Cells were then fixed and stained with crystal violet, and viral plaques were enumerated under a microscope. The number of PFU/milliliter was calculated by taking a plaque count for every sample and accounting for the dilution factor.

Tissue isolation and cocultures

Vaginal tissue was enzymatically digested in 15 ml of RPMI 1640 containing 0.00157 g/ml collagenase A (Roche Life Science) at 37°C on a stir plate for 2 h and filtered through a 40-μm filter to obtain a total tissue cell (TC) suspension (45). OT-II Tg spleens were mechanically disrupted, and ammonium-chloride-potassium lysing buffer (Sigma-Aldrich, St. Louis, MO) was used to lyse blood cells. Mononuclear cells were counted, and CD4$^+$ T cells were magnetically sorted using CD4 L3TE microbeads (Miltenyi Biotec, Auburn, CA) based on manufacturer protocols. Sorted CD4$^+$ T cells were stained with 50 μM CFSE (Sigma-Aldrich) according to published protocols (46). Vaginal TC (5×10^5 cells/ml) were incubated in a 96-well plate pulsed with 5 μg/well OVA peptide (323–339) (Biomer Technology, Pleasanton, CA) with sequence ISQAVHAAHAEINEAGR for 16–18 h. CFSE-stained OT-II Tg CD4$^+$ T cells were added at a 1:1 ratio and cocultured for 3.5 d at 37°C in complete RPMI media (47), based on previously standardized protocols (48). During certain experiments, 100 ng/ml rIL-1β or 250 ng/ml rIL-17 (R&D systems, Minneapolis, MN) was added during the peptide-pulse stage or on the first day of coculture.

Flow cytometry

For IL-17A or IL-1β intracellular staining, freshly isolated vaginal TCs or 2-d-old cocultures were treated with 2 μl/ml cell stimulation mixture plus 500× protein transport inhibitors: a mixture of PMA, ionomycin, brefeldin A, and monensin (eBioscience, San Diego, CA) for 16 h at 37°C. Cells were stained with allophycocyanin-ef780 viability dye (eBioscience) for 30 min to exclude dead cells and subsequently stained with the following surface Abs: CD3 BV785 (BioLegend, San Diego, CA), CD4 BV421 (BioLegend), TCR γδ PE (eBioscience), NKp46 PE-cf594 (eBioscience), CD11c PE-Cy7 (BD Biosciences, Mississauga, ON, Canada), or CD11b PE-cf594 (BD Biosciences). Cells were then permeabilized and fixed with BD Pharmingen Transcription Factor Buffer Set (BD Biosciences) according to manufacturer protocols and stained with the following intracellular Abs: IL-17A allophycocyanin (eBioscience), IL-1β PE (R&D Systems), and pro–IL-1β PerCP-ef710 (eBioscience) or control rat IgG1 allophycocyanin (eBioscience), rat IgG1 PE (R&D Systems), and rat IgG1 PerCP-ef710 (eBioscience). Data were acquired on a BD LSR II Flow Cytometer System (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Cytokine analysis

IL-17 levels in coculture supernatants were measured using the Duoset ELISA kit (R&D Systems), according to manufacturer protocols, and plates were analyzed on a Sector Imager 2400 (Meso Scale Discovery, Rockville, MD).

Statistics

Statistical analysis and graphical representations were performed using GraphPad Prism 6.0d (GraphPad Software, San Diego, CA). Data are expressed as mean ± SEM, typically derived from $n = 3$ replicates. Significance was calculated by comparing means using one-way or two-way ANOVA or t tests, as indicated in the figure legends. Statistical significance was defined as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

Ethics statement

All animal use was conducted in accordance to protocols approved by the McMaster University Animal Research Ethics Board as per Animal Utilization Protocol no. 14-09-40 in accordance with Canadian Council of Animal Care guidelines.

RESULTS

Innate IL-17 in the FGT plays an important role in inducing T_h17 responses

Although studies in other mucosal sites, such as the gut, have shown that innate and innate-like sources of IL-17 can help amplify

the overall IL-17 response by enhancing T_h17 responses (33–37), a similar role for IL-17 in the FGT has not been yet reported. Therefore, we wanted to examine the effect of innate IL-17 on T_h17 cell priming by vaginal DCs.

First, to confirm the in vivo significance of vaginal IL-17 in the context of a viral infection, we used a mouse model of HSV-2 immunization and challenge to examine the survival of mice in the absence of IL-17, similar to our previous studies (20, 21). *IL-17A*$^{-/-}$ mice and WT C57BL/6 controls were OVX, immunized intranasally with an attenuated strain of TK$^-$ HSV-2, and then challenged intravaginally with WT HSV-2. Survival and genital pathology were monitored daily, and vaginal washes were collected to measure viral shedding and determine disease severity. Consistent with our previous observations (20, 21), following genital HSV-2 challenge, *IL-17A*$^{-/-}$ mice had poor disease outcomes and demonstrated greater mortality (Fig. 1). Only 17% of *IL-17A*$^{-/-}$ mice survived the viral challenge, compared with 83% of WT controls by day 12 (Fig. 1A). All mice that reached end point developed genital pathology (*IL-17A*$^{-/-}$: 5/6 mice; WT: 1/6 mice) (Fig. 1B). Similarly, viral shedding was also significantly higher in *IL-17A*$^{-/-}$ animals (p = 0.0281) (Fig. 1C), suggesting that IL-17 plays an important role in modulating antiviral responses in the FGT and confirming our previous findings (20, 21).

Next, we wanted to determine the effect of innate IL-17 production on the induction of Ag-specific T_h17 responses in the FGT. We used a previously well-described, in vitro chicken OVA peptide coculture model with OVA-specific OT-II Tg CD4$^+$ T cells (41). Vaginal TC, including APCs, were isolated from *IL-17A*$^{-/-}$ mice and WT C57BL/6 controls, cultured in medium alone or pulsed with OVA peptide, and cocultured with CFSE-labeled OT-II Tg CD4$^+$ T cells (TC+CD4) for 3.5 d, as previously described (21). Using this model, we measured IL-17 levels in TC-alone cultures and found that WT mice produced innate IL-17 (100 ± 43 pg/ml), whereas IL-17 was below detection in TC cultures from *IL-17A*$^{-/-}$ mice (p = 0.003) (Fig. 2A). To determine the role of innate IL-17 on T_h17 differentiation, IL-17 levels were measured in TC+CD4 coculture supernatants. Cultures containing TC from *IL-17A*$^{-/-}$ mice contained 30-fold lower IL-17 compared with those with TC from WT mice (p = 0.001) (Fig. 2A). In addition, we conducted intracellular cytokine staining on day 2 of the cocultures and found that a lower proportion of proliferating CFSE-stained OT-II Tg CD4$^+$ T cells expressed IL-17 in *IL-17A*$^{-/-}$ TC+CD4 cocultures (39%) compared with WT controls (55%) (Fig. 2B). These observations provide further evidence that the absence of innate IL-17 results in diminished T_h17 responses. The small population of CFSE$^-$ cells within WT TC+CD4 cocultures (3.87%) likely

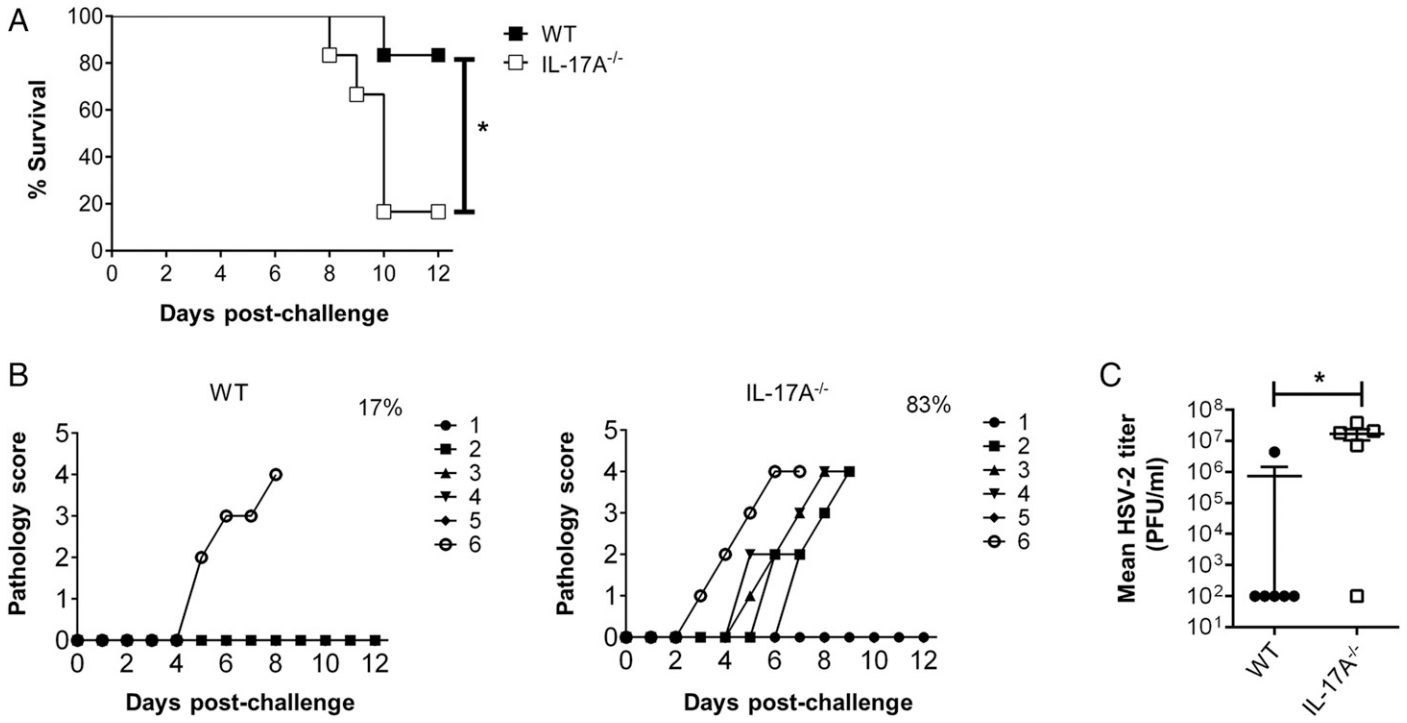

FIGURE 1. *IL-17A*$^{-/-}$ mice are more susceptible to intravaginal HSV-2 challenge following intranasal immunization.
OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice (n = 6 per group) were immunized intranasally with TK$^-$ HSV-2 (10^2 PFU/mouse) and 6 wk later challenged intravaginally with WT HSV-2 (5×10^3 PFU/mouse). Survival and genital pathology were monitored, and vaginal washes were collected daily for 6 d postchallenge. (**A**) Significance in difference in survival was calculated using the log rank (Mantel-Cox) test (*$p < 0.05$). (**B**) Genital pathology scores were recorded on a scale of 0 to 5. Data points superimposed on the x-axes of (B) indicate mice without genital pathology, and the percentages represent maximum numbers of mice that demonstrated pathology. Each symbol represents a single animal. (**C**) HSV-2 shedding was calculated using a Vero cell-based plaque assay, and data represent the viral loads (means ± SEMs) over 6 d. Data were analyzed using an unpaired t test with 95% confidence interval (*$p < 0.05$). Data shown are representative of three independent experiments with similar results.

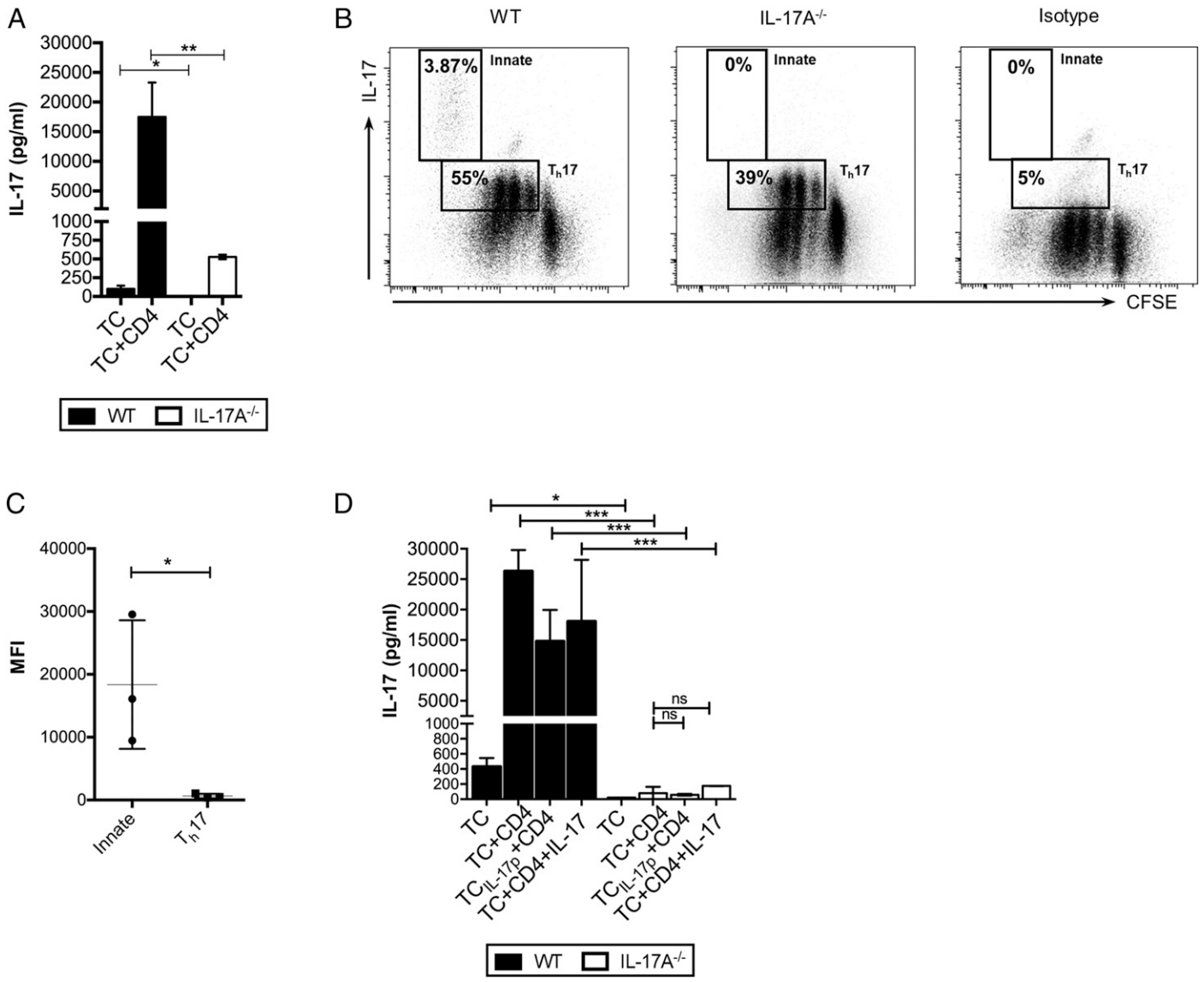

FIGURE 2. IL-17 produced by innate sources in the FGT is critical for potentiating T_h17 responses primed by vaginal APCs.
Vaginal cells from hormone cycle–matched WT (C57BL/6) or *IL-17A*$^{-/-}$ mice (n = 5) were pooled, pulsed with chicken OVA peptide, and cultured alone (TC) or cocultured at a 1:1 ratio with CFSE-stained OT-II Tg CD4$^+$ T cells (TC+CD4) for 3.5 d. (**A**) IL-17 levels in culture supernatants were measured by ELISA. (**B**) On day 2 of coculture, a cell stimulation mixture containing Golgi inhibitors and PMA plus ionomycin was added, and 18 h later, cocultures were stained with Abs against CD3, CD4, and IL-17 and analyzed by flow cytometry to identify IL-17$^+$ cells and measure MFI. Upper box indicates CFSE$^-$, IL-17–producing cells in culture. The lower box indicates CFSE$^+$, IL-17–producing CD4$^+$ T cells, with a lower cut-off that excludes 95% of isotype control CSFE$^+$ cells. (**C**) The difference in MFI was compared between innate and T_h17 sources of IL-17 (n = 3 independent experiments). Data were analyzed using an unpaired t test with 95% confidence interval (*$p < 0.05$). (**D**) Similar cocultures were conducted, with 250 ng/ml of rIL-17 added during the peptide-pulse stage ($TC_{IL\text{-}17p}$+CD4) and washed away before coculture or added on day 1 of coculture (TC+CD4+IL-17) and remained present throughout the duration of the experiment as indicated on the x-axis. IL-17 levels in culture supernatants were measured by ELISA. Data in (A) and (D) are mean ± SEM of three individual wells per coculture condition. Data are representative of three independent experiments with similar results. Significance was calculated by two-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, no significance).

represented the innate IL-17 population in the FGT that was entirely absent in *IL-17A*$^{-/-}$ cocultures (Fig. 2B). The innate IL-17 population had a ~30-fold higher median fluorescence intensity (MFI) compared with the CFSE$^+$ T_h17 cells (p = 0.040), indicating that the CFSE$^-$ population of IL-17–secreting cells was a more potent source of IL-17 than T_h17 cells on a per cell basis (Fig. 2C).

Given the attenuated priming of T_h17 responses demonstrated by *IL-17A*$^{-/-}$ vaginal TCs (Fig. 2A), we wanted to examine

https://doi.org/10.4049/immunohorizons.1900040

whether exogenous addition of rIL-17 could rescue the ability of DCs within the *IL-17A*$^{-/-}$ vaginal TCs to induce T_h17 responses. Vaginal cells from *IL-17A*$^{-/-}$ mice and WT mice were isolated, pulsed with OVA peptide, and cocultured with OT-II Tg CD4$^+$ T cells. To determine whether IL-17 is only required transiently as a pretreatment, 250 pg/ml rIL-17 (a level consistent with that observed in cultures of vaginal cells from WT controls alone [Fig. 2A]) was added during the OVA peptide pulse and removed 12 h later by washing, before coculturing with OT-II Tg CD4$^+$ T cells. Alternatively, to examine whether prolonged exposure of IL-17 is required during the process of T cell differentiation, rIL-17 was added at day 1 of the coculture and remained in culture for the duration of the experiment. In both conditions, rIL-17 was unable to significantly influence T_h17 responses in *IL-17A*$^{-/-}$ cocultures (Fig. 2D), suggesting that short-term restoration of IL-17 may not be sufficient to restore the ability of *IL-17A*$^{-/-}$ vaginal APCs to prime T_h17 responses. Taken together, these results affirm the importance of IL-17 in the antiviral response in the FGT and show that innate IL-17 within the FGT is important for the induction of T_h17 responses.

Innate IL-17 induces vaginal DCs to prime T_h17 responses via an IL-1β–dependent pathway

Various cytokines have been implicated for IL-17 production (28). In our own studies, we showed that within the FGT, adaptive IL-17 production by T_h17 cells was primed by CD11c$^+$ DCs via an IL-1–dependent pathway (21). Given the attenuated T_h17 responses observed in cocultures containing vaginal APCs from *IL-17A*$^{-/-}$ mice (Fig. 2), we wanted to examine whether innate IL-17 plays a role in programming vaginal APCs to produce IL-1.

Vaginal TCs from *IL-17A*$^{-/-}$ mice and WT controls were isolated and cultured overnight (12 h) prior to staining with Abs against CD11c, CD11b, and IL-1β. Dead cells were excluded using a fixable viability dye, CD11c$^+$ DCs were gated (Fig. 3A), and intracellular IL-1β expression was compared by flow cytometry. We found that *IL-17A*$^{-/-}$ mice contained a significantly lower proportion of IL-1β–producing CD11c$^+$ DCs in the FGT compared with WT mice ($p = 0.04$) (Fig. 3B, 3C). In addition, CD11c$^+$ DCs from *IL-17A*$^{-/-}$ mice also contained reduced amounts of IL-1β per cell compared with WT controls, based on MFI (Fig. 3D), although not statistically significant ($p = 0.400$).

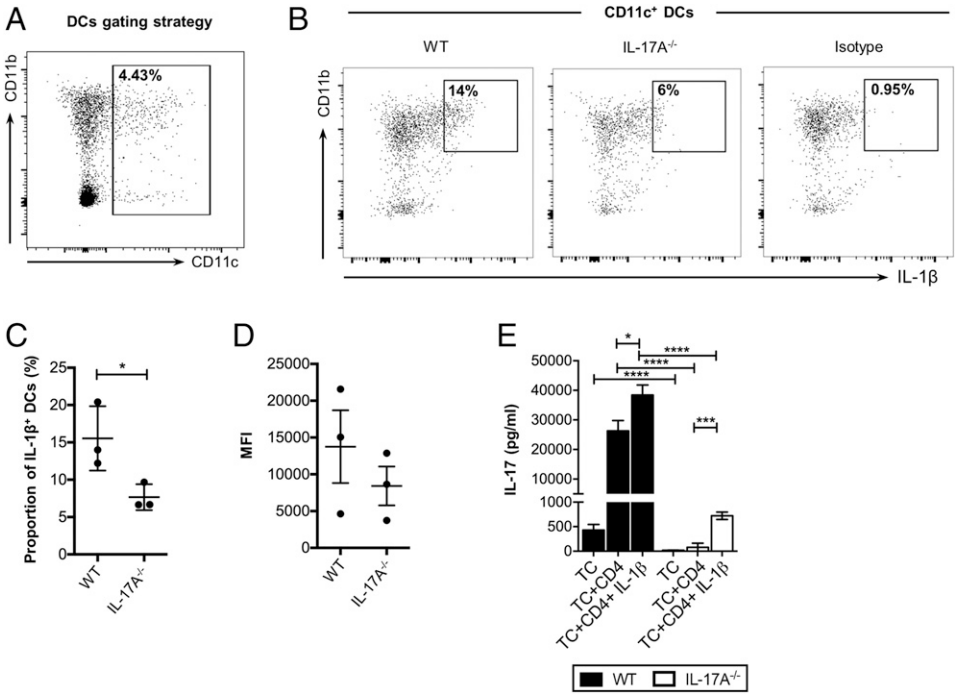

FIGURE 3. Vaginal CD11c$^+$ DCs from *IL-17A*$^{-/-}$ mice are impaired in potentiating T_h17 responses because of diminished IL-1β production. Vaginal cells from hormone cycle–matched WT or *IL-17A*$^{-/-}$ mice ($n = 5$) were pooled, cultured overnight without any additional stimulation, stained with Abs against CD11c, CD11b, and IL-1β, and analyzed by flow cytometry to identify IL-1β$^+$ cells, with dead cells excluded. (**A**) CD11c$^+$ DCs were gated as shown, and (**B**) IL-1β expression by vaginal CD11c$^+$ CD11b$^+$ DCs from WT and *IL-17A*$^{-/-}$ mice was compared with isotype control. (**C**) The differences in percentage and (**D**) MFI of IL-1β$^+$ DCs were compared between vaginal cells from WT and *IL-17A*$^{-/-}$ mice ($n = 3$ independent experiments). Data were analyzed using an unpaired *t* test with 95% confidence interval (*$p < 0.05$). (**E**) Vaginal cells from hormone cycle–matched WT or *IL-17A*$^{-/-}$ mice ($n = 5$) were pooled, pulsed with chicken OVA peptide, and cultured alone (TC) or cocultured at a 1:1 ratio with OT-II Tg CD4$^+$ T cells (TC+CD4) for 3.5 d. Alternately, 100 ng/ml of rIL-1β was added on day 1 of coculture (TC+CD4+IL-1β). IL-17 levels in culture supernatants were measured by ELISA. Data are mean ± SEM of three individual wells per coculture condition. Data are representative of three independent experiments with similar results. Significance was calculated by two-way ANOVA (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$).

Given this observation, we wanted to examine whether the addition of exogenous IL-1β would restore T_h17 responses in *IL-17A*$^{-/-}$ cocultures. Addition of rIL-1β to *IL-17A*$^{-/-}$ cocultures on day 1 (TC+CD4+IL-1β) was able to significantly ($p = 0.0005$) enhance T_h17 responses almost 10-fold compared with cultures without IL-1β (Fig. 3E). However, IL-17 levels were still significantly lower than those seen in cultures with WT vaginal TCs (Fig. 3E).

Overall, these results showcase an inherent deficit in the ability of vaginal DCs from *IL-17A*$^{-/-}$ mice to produce IL-1β, thereby suggesting that the priming of T_h17 responses by innate IL-17 is dependent on IL-1β. Even with the addition of exogenous IL-1β, there was only a partial rescue of T_h17 responses primed by *IL-17A*$^{-/-}$ vaginal DCs. Together, this suggests that innate IL-17 is important for programming vaginal DCs to produce IL-1β, which in turn is critical for priming vaginal T_h17 responses in the FGT.

γδ$^+$ T cells are the predominant source of innate IL-17 in the FGT

Having established an important role for innate IL-17 in the priming of T_h17 responses in the FGT, we next wanted to characterize the primary sources of IL-17 production. IL-17 in mucosal tissues can be produced by a variety of innate lymphocytes (NK cells and ILCs) (26), innate-like lymphocytes (NKT and γδ$^+$ T cells) (26), and adaptive sources (T_h17 cells) (49), and so we focused on examining these lymphocyte populations.

Vaginal cells isolated from C57BL/6 mice were stained with a panel of Abs against CD3, CD4, TCR γδ, IL-17, and NKp46 to identify these lymphocyte populations (29, 50). Dead cells were excluded using a fixable viability dye, and single cells were gated to identify total IL-17$^+$ populations using flow cytometry (Fig. 4A). We found that IL-17$^+$ cells did not express NKp46 (Fig. 4A) and could instead be divided into two major populations based on their CD3 and CD4 expression (CD3$^+$ CD4$^+$ and CD3$^+$ CD4$^-$) (Fig. 4B). These were further separated based on TCR γδ expression (Fig. 4B), and we observed that γδ$^+$ T cells (CD3$^+$ CD4$^-$ γδ$^+$) were the primary source of IL-17 (Fig. 4C, 4D). The γδ$^+$ T cell population accounted for 75% of total IL-17–producing cells, which was significantly higher than both CD4$^+$ T cells (21%) and other sources (4%) of IL-17 (Fig. 4C, 4D).

We also examined TCR γδ expression by vaginal cells from *IL-17A*$^{-/-}$ mice and found frequencies of γδ$^+$ cells comparable to those in WT animals (Fig. 4E) (not significant; $p = 0.243$), suggesting no inherent defect in the development of the γδ$^+$ population in the absence of IL-17. Similar to previous reports (29), these findings confirm that innate-like γδ$^+$ T cells represent the primary source of IL-17 in the FGT under homeostatic conditions.

E2 enhances IL-17 production by γδ$^+$ T cells in the FGT

The FGT is a complex tissue, regulated by a variety of factors within the local microenvironment. One major component that influences immune cell populations and responses in the FGT is the presence of female sex hormones. Specifically, we have shown that the female sex hormone E2 is protective against viral infections in the FGT and can enhance T_h17 responses primed by vaginal CD11c$^+$ DCs (21). Therefore, we wanted to examine whether E2 can also influence innate IL-17$^+$ populations in the FGT.

To determine the effect of E2 on the primary IL-17–secreting cell population (γδ$^+$ T cells), C57BL/6 mice were OVX to remove endogenous sources of reproductive hormones and implanted with prolonged-release E2 or placebo (mock) pellets. Two weeks later, vaginal cells were isolated and stained for flow cytometry. We found that E2 treatment consistently and significantly ($p = 0.05$) induced greater proportions of IL-17$^+$ cells in the FGT compared with mock-treated or ERKO controls (Fig. 5A, 5B). Furthermore, IL-17$^+$ cells from E2-treated mice produced significantly greater IL-17 per cell ($p = 0.007$) compared with controls, based on MFI levels (Fig. 5C). When we characterized the overall IL-17$^+$ population based on CD3 and CD4 expression, and further separated these populations based on TCR γδ expression as done previously (Fig. 4), we found that the predominant source of IL-17 in E2-treated animals was the γδ$^+$ T cell population (Fig. 5D–F) described earlier (Fig. 4). However, this population was largely absent or diminished in mock-treated mice (E2: 64 ± 12%; mock: 16 ± 6%; $p = 0.02$) (Fig. 5F).

Taken together, these findings demonstrate that factors in the microenvironment of the FGT, such as the presence of E2, can influence innate levels of IL-17. Specifically, E2 plays a critical role in upregulating IL-17 production by γδ$^+$ T cells.

Commensal microbiota enhances IL-17 production by γδ$^+$ T cells in the FGT

Another factor shown to influence IL-17 production in mucosal tissue is the presence of the commensal microflora. In the gut, the local microbiota has been reported to drive IL-17 production by both γδ$^+$ T cells (51, 52) and T_h17 cells (53). Similarly, the vaginal microflora has also been shown to regulate immune responses in the FGT (54). As a result, we investigated whether the vaginal microbiota could also influence innate IL-17 in the FGT.

To determine the effect of the microbiota on the primary IL-17–secreting cell population (γδ$^+$ T cells), vaginal cells from hormone cycle–matched C57BL/6 GF mice and WT mice were isolated, and IL-17 production was compared by flow cytometry. We consistently found that GF mice had significantly lower proportions of total IL-17$^+$ cells compared with WT controls ($p = 0.028$) (Fig. 6A, 6B) as well as lower IL-17 produced per cell ($p = 0.002$) (Fig. 6C). Subsequently, a distribution of IL-17–producing cell subtypes based on CD3 and CD4 expression (Fig. 6D), followed by TCR γδ expression (Fig. 6E), showed that IL-17$^+$ γδ$^+$ T cells were significantly decreased in GF mice (GF: 26 ± 4%; WT: 54 ± 8%; $p = 0.02$) (Fig. 6F). Overall, these results demonstrate that presence of microbiota enhances IL-17 production by γδ$^+$ T cells in the FGT.

DISCUSSION

Although IL-17 is commonly referred to as a CD4$^+$ T cell–secreted cytokine, the majority of IL-17 released constitutively under homeostatic conditions is produced by CD4$^-$ innate and innate-like immune cells. The presence of innate IL-17 in mucosal tissues

https://doi.org/10.4049/immunohorizons.1900040

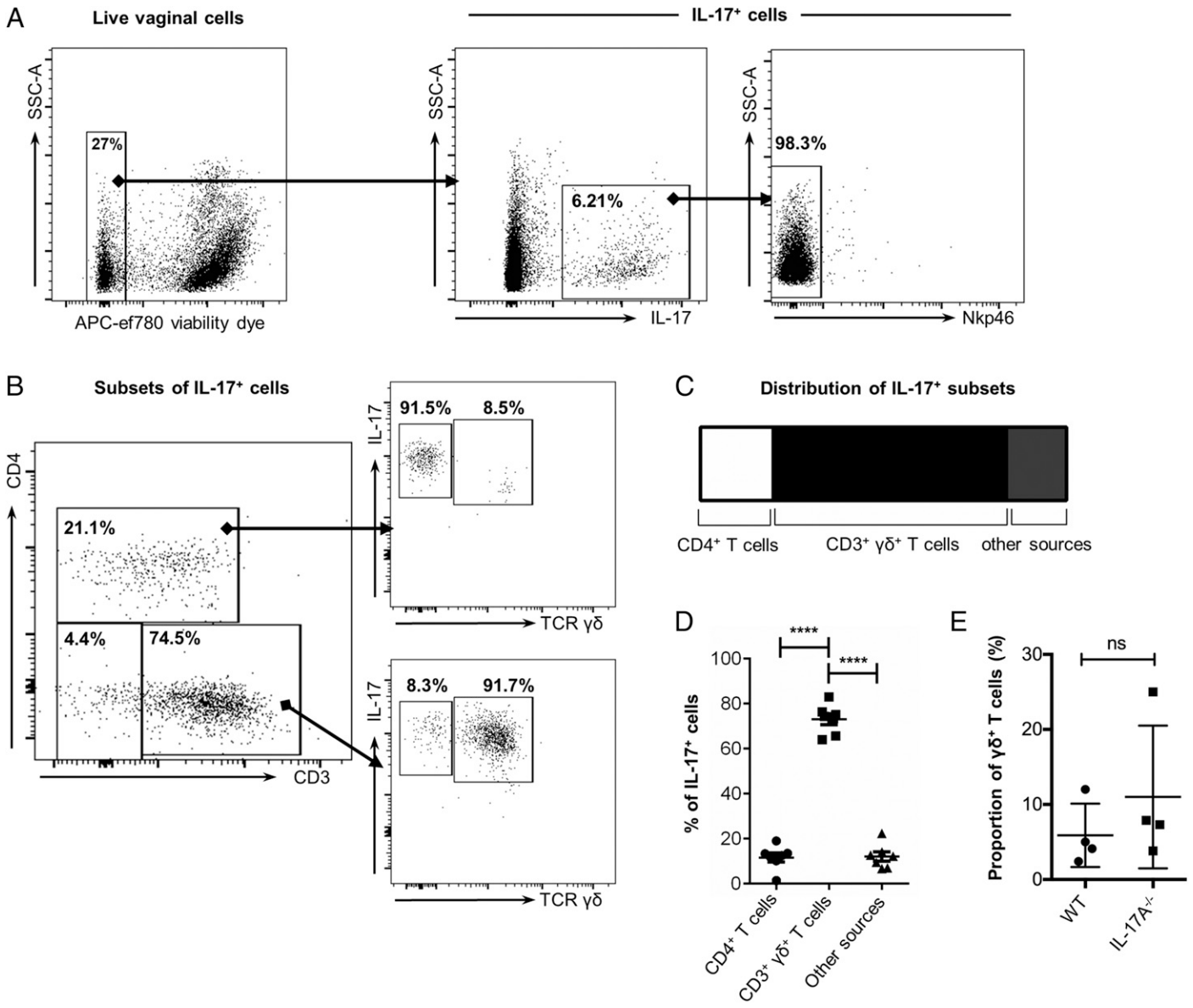

FIGURE 4. IL-17 in the FGT is predominantly produced by γδ$^+$ T cells.

Vaginal cells from hormone cycle–matched WT (C57BL/6) or *IL-17A*$^{-/-}$ mice (n = 5–7) were isolated, pooled, and stimulated with a cell stimulation mixture containing Golgi inhibitors and PMA plus ionomycin for 18 h. Cells were stained with Abs against CD3, CD4, NKp46, TCR γδ, and IL-17 and analyzed by flow cytometry to identify IL-17$^+$ cells. (**A**) Dead cells were excluded, and total IL-17$^+$ cells were gated. (**B**) Subsets of IL-17$^+$ cells were further gated based on CD3 and CD4 expression, and each subset was examined for TCR γδ expression. (**C**) The distribution of cell subsets among all IL-17–producing cells were examined and (**D**) quantified in five independent experiments. Data are mean ± SEM, and significance was calculated by one-way ANOVA (****$p < 0.0001$). (**E**) The difference in percentage of γδ$^+$ cells was compared between WT and *IL-17A*$^{-/-}$ mice from four independent experiments. Data were analyzed using an unpaired t test with 95% confidence interval. Data are representative of four independent experiments with similar results. ns, no significance.

has been shown to be important for potentiating T_h17 immunity and thereby playing an important role in protecting against pathogen exposure and disease (33–37). In the FGT, T_h17 responses have shown to be protective against bacterial, fungal, and viral infections (14–19). Recent work from our laboratory has focused on the role of T_h17 responses during genital HSV-2 infection (20, 21). We have shown that vaginal DCs are critical for priming T_h17 responses, which leads to enhanced T_h1 responses (21), and that IL-17 plays a critical role in mediating T_h1 responses during in vivo HSV-2 challenge (20, 21). However, the significance of IL-17 produced by innate or innate-like sources in the FGT and its influence on T_h17 immunity is less understood. In the current study, we investigated the role of innate IL-17 in the induction of T_h17 responses in the FGT and found that innate IL-17 was

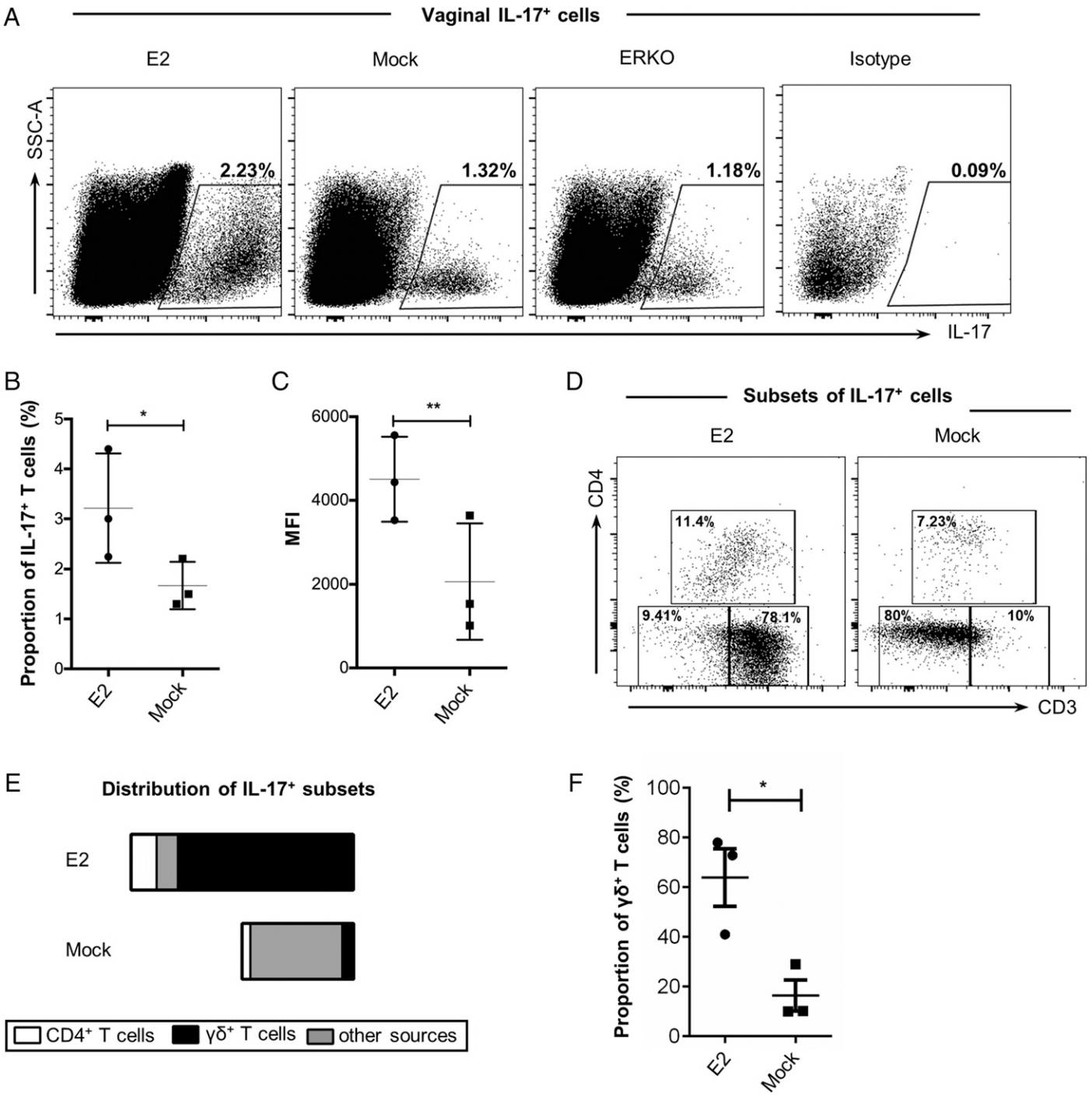

FIGURE 5. E2 induces IL-17–producing γδ$^+$ T cells in the FGT.

Vaginal cells from OVX WT (C57BL/6) mice treated with E2 or placebo (mock) pellets (n = 5) were isolated, pooled, and stimulated with a cell stimulation mixture containing Golgi inhibitors and PMA plus ionomycin for 18 h. (**A**) Cells were stained with Abs against CD3, CD4, and IL-17 and analyzed by flow cytometry. ERKO mice were used as additional controls. Dead cells were excluded, and total IL-17$^+$ cells were gated. (**B**) The differences in percentage and (**C**) MFI of IL-17$^+$ cells were compared between E2 and mock mice from three independent experiments. Data were analyzed using the unpaired t test with 95% confidence interval (*$p < 0.05$, **$p < 0.01$). (**D**) Subsets of IL-17$^+$ cells were further identified based on CD3 and CD4 expression. (**E**) The distributions of the cell subsets among all IL-17–producing cells comparing E2-treated and mock-treated mice. (**F**) The difference in percentage of IL-17–producing γδ$^+$ cells was compared between E2 and mock mice from three independent experiments. Data are mean ± SEM, and significance was calculated using an unpaired t test with 95% confidence interval (*$p < 0.05$). Data are representative of three independent experiments with similar results.

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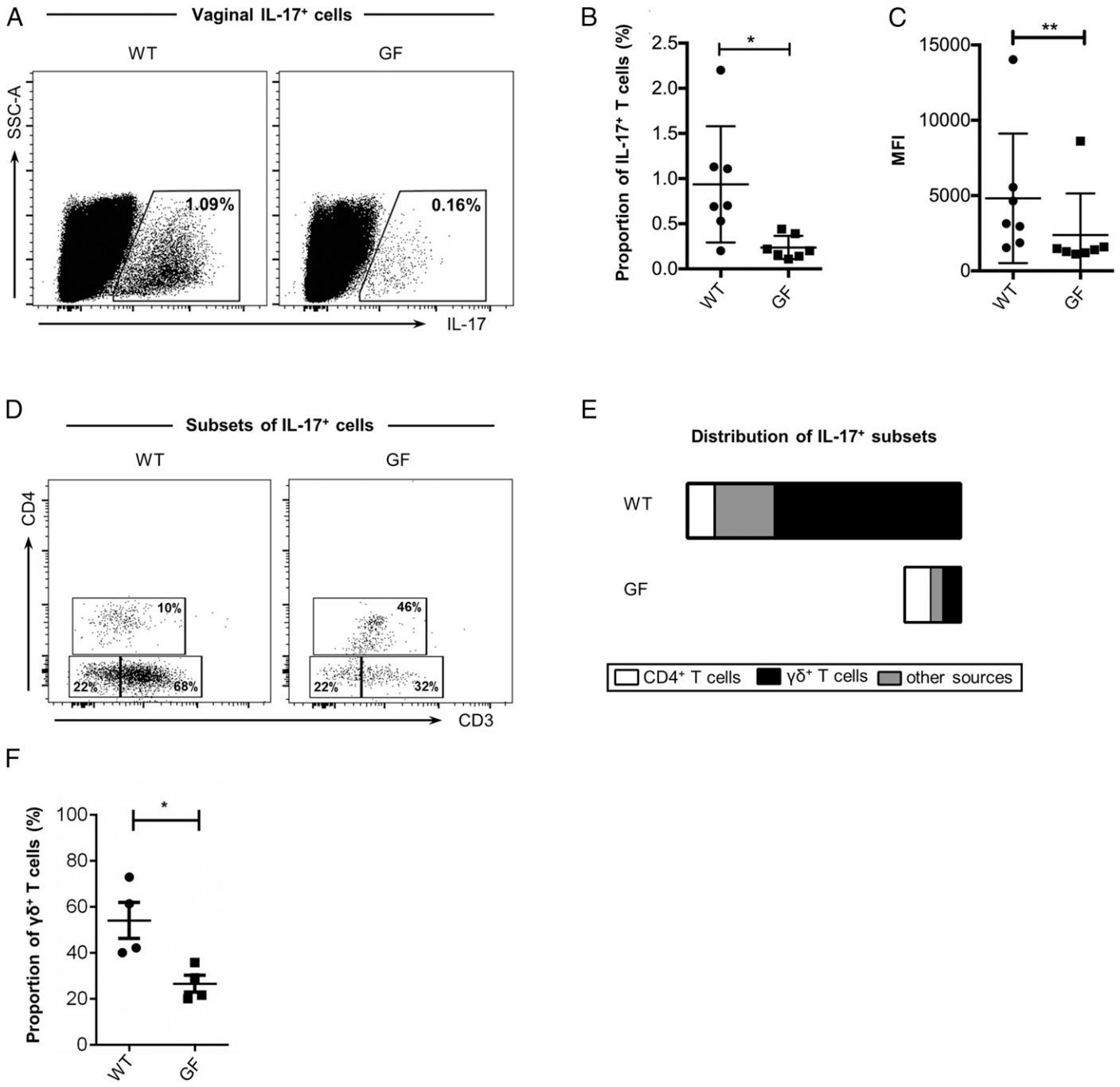

FIGURE 6. Microbiota induces IL-17–producing γδ$^+$ T cells in the FGT.

Vaginal cells from hormone cycle–matched WT (C57BL/6) or GF mice (n = 5) were isolated, pooled, and stimulated with a cell stimulation mixture containing Golgi inhibitors and PMA plus ionomycin for 18 h. (**A**) Cells were stained with Abs against CD3, CD4, and IL-17 and analyzed by flow cytometry. Dead cells were excluded, and total IL-17$^+$ cells were gated. (**B**) The differences in percentage and (**C**) MFI of IL-17$^+$ cells were compared between GF and WT mice from seven independent experiments. Data were analyzed using the unpaired t test with 95% confidence interval, with the ROUT method used to identify outliers (*$p < 0.05$, **$p < 0.01$). (**D**) Subsets of IL-17$^+$ cells were further identified based on CD3 and CD4 expression. (**E**) The distributions of the cell subsets among all IL-17–producing cells comparing WT and GF mice. (**F**) The difference in percentage of IL-17–producing γδ$^+$ cells was compared between WT and GF mice from four independent experiments. Data are mean ± SEM, and significance was calculated using an unpaired t test with 95% confidence interval (*$p < 0.05$). Data are representative of four independent experiments with similar results.

important for inducing vaginal T_h17 responses. This was evident, as vaginal APCs from *IL-17A*$^{-/-}$ mice were severely impaired at priming T_h17 responses in APC–T cell cocultures (Fig. 2). In addition, vaginal DCs from these mice also produced significantly lower levels of IL-1β (Fig. 3), which is critical for priming T_h17 responses in the FGT (21). We further characterized the IL-17–secreting cells in the FGT and discovered that the predominant source was innate-like γδ$^+$ T cells (Fig. 4). Furthermore, IL-17 production by γδ$^+$ T cells was shown to be influenced by factors such as sex hormones (E2) and the microbiota (Figs. 5, 6). To the best of our knowledge, this is the first study demonstrating that innate IL-17 plays a critical role in potentiating T_h17 immunity in the FGT and examining the factors that regulate it.

Numerous studies have shown that innate and innate-like lymphocytes can influence the differentiation and function of adaptive lymphocytes (55). We previously observed that vaginal cells can produce modest amounts of IL-17 in culture without any stimulation (21), and we wanted to further examine the influence of this innate IL-17 on the priming of T_h17 responses by vaginal DCs. We consistently found that vaginal cells from *IL-17A*$^{-/-}$ mice were impaired at priming T_h17 responses in vitro (Fig. 2A, 2D), suggesting that the presence of innate IL-17 can influence adaptive immunity primed by vaginal APCs. Interestingly, rIL-17 (250 pg/ml) did not restore the functional ability of the DCs in priming these responses (Fig. 2D). This is consistent with the observations made in a study by Kumar et al. (56) that provided evidence demonstrating that T_h17 differentiation is not directly dependent on IL-17RA signaling and thereby suggests that IL-17 affects T_h17 differentiation in an indirect manner. However, other in vivo studies suggest that the absence of IL-17 may lead to inherent defects in DC-mediated priming of CD4$^+$ T cell responses (57). Bai et al. (57) demonstrated, using a lung *C. muridarum* infection model, that neutralization of IL-17 significantly impaired DC function, including lower levels of IL-12 production and lower expression of MHC class II and CD40. Furthermore, the mice produced higher levels of IL-10 and IL-4 and skewed the immune responses toward a T_h2 phenotype instead of the typical T_h1 response seen in controls (57). Likewise, we have previously shown that in the absence of IL-17 (*IL-17A*$^{-/-}$ mice), there was a 7-fold lower T_h1 response in the FGT compared with WT mice (20, 21). Our current findings further expand on the idea that under homeostatic conditions, IL-17 plays a critical role in priming CD4$^+$ T cell responses in the FGT.

The production of IL-17 has been shown to depend on different cytokines, including IL-1, IL-6, IL-21, IL-23, and TGF-β (2–5, 28). However, although γδ$^+$ T cells from IL-6 KO and TGF-β KO mice produced comparable or attenuated levels of IL-17, respectively, those from IL-1R KO mice failed to secrete any IL-17 in response to IL-23 and TLR agonists (37, 58). This suggests that IL-1 may play a critical role in the production of IL-17 by γδ$^+$ T cells. Likewise, we previously showed that IL-1β, but not IL-6, production by vaginal CD11c$^+$ DCs is critical for priming T_h17 responses in the FGT (21). Therefore, we wanted to examine whether the attenuated T_h17 responses seen in *IL-17A*$^{-/-}$ cocultures was due to a defect in the ability of *IL-17A*$^{-/-}$ mice to produce IL-1β. We found that vaginal DCs from *IL-17A*$^{-/-}$ mice produced lower amounts of IL-1β (Fig. 3B, 3C) compared with WT DCs. These observations are directly consistent with prior literature indicating that IL-17 can induce IL-1β production in mucosal APCs (59, 60). Nakae et al. (39) observed lower IL-1β and impaired CD4$^+$ T cell responses by Langerhans cells from *IL-17A*$^{-/-}$ mice. Furthermore, although the addition of rIL-1β increased T_h17 differentiation in *IL-17A*$^{-/-}$ cocultures (Fig. 3E), these levels were still significantly lower than those observed in WT cultures. Given that there are numerous stimulatory and inhibitory factors involved in priming T_h17 responses (61), we suggest that there may be other intrinsic defects in *IL-17A*$^{-/-}$ DCs related to signal transduction, activation markers (56), or cytokine processing (39) that cannot be restored by the transient addition of rIL-1β. Overall, our findings demonstrate that IL-17 in the FGT microenvironment may have an important effect on the function of vaginal DCs and their ability to prime adaptive T_h17 responses via an IL-1β–dependent pathway.

Although innate IL-17 can be produced by a mixed population of lymphocytes under homeostatic conditions, consistent with a previous report by Kim et al. (29), we found that γδ$^+$ cells are the primary source of innate IL-17 in the FGT (Fig. 4D). Additionally, we looked at γδ$^+$ cells in the FGT of *IL-17A*$^{-/-}$ mice to determine if there was a defect in the γδ$^+$ T cell population in the absence of IL-17 and found no significant difference as compared with WT mice (Fig. 4E). We also identified other sources of innate IL-17–producing cells (62). Although the CD3$^+$ CD4$^+$ IL-17$^+$ cells are typically identified as T_h17 cells, subpopulations in this subset can also represent innate cell types such as fetal lymphoid tissue inducer cells or NK cell receptor negative type 3 ILCs (50). Because of the lack of CD4 expression in CD3$^+$ CD4$^-$ γδ$^-$ IL-17$^+$ cells, these may represent NK cell receptor negative type 3 ILCs. However, given the previous observations by Serafini et al. (63) showing that GATA3 is important for type 3 ILCs, further studies with GATA3 KO mice may be required to conclusively identify these cells in the FGT. Irrespective of these other sources of IL-17, our findings demonstrate that γδ$^+$ T cells are the primary source of innate IL-17 in the FGT.

Having identified the primary source of IL-17–producing cells critical for inducing adaptive T_h17 responses, we examined how two important factors in the local FGT microenvironment, sex hormones, and the microbiota can influence the presence and function of these cells. We have previously shown that E2 can enhance T_h1 and T_h17 responses in the FGT (20, 21). However, the effect of E2 on levels of innate IL-17 has not been reported. We found that E2-treated mice contained a higher proportion of overall IL-17$^+$ cells in the FGT compared with mock or ERKO control animals (Fig. 5A–C). Also, the primary source of this IL-17 in the FGT, γδ$^+$ T cells, was almost completely absent in E2-depleted mice (Fig. 5D–F). These results suggest that E2 is involved in modulating and enhancing the production of innate IL-17.

There is also cross-talk between the microbiota and hormones present in the FGT, and E2 has been shown to be important for a healthy vaginal microflora (64, 65). Interestingly, the gut microbiota has been linked to IL-1β–mediated T_h17 responses primed by mucosal DCs (66), and the microbiome has also been linked to

IL-17 production by innate and innate-like lymphocytes in mucosal tissues (36, 67); however, the influence of the vaginal microbiota on IL-17 production is largely unknown. We examined whether the microbiota is important for IL-17 production in the FGT by using GF mice devoid of commensal bacteria, and additionally, we used stage-matched animals to ensure there was limited influence of hormones on the immune responses measured. We observed a significant reduction in the overall proportion of IL-17$^+$ cells in the FGT of GF mice compared with WT controls (Fig. 6A–C), and there was also a marked decrease in the proportion of γδ$^+$ T cells in these mice (Fig. 6E, 6F). These findings are in contrast to a previous report by Kim et al. (29) in which they concluded that the vaginal microflora does not affect IL-17 production by genital γδ$^+$ T cells. However, they used antibiotics via drinking water to deplete commensal bacteria, whereas we used GF mice that were completely devoid of microflora. The effects of the temporary depletion compared with complete absence of bacteria during development of mucosal immunity may explain the discrepancy between our findings. Together, our findings in regard to the effects of E2 and microbiota demonstrate the degree to which the microenvironment can influence the induction of immune responses and the development, self-renewal, or maintenance of IL-17–producing γδ$^+$ T cells in the FGT.

In summary, our study expands on previous work regarding the importance of antiviral T_h17 immunity in the FGT (20, 21) and provides insight regarding a novel role for IL-17 in the vaginal microenvironment. We have shown that innate IL-17 is primarily secreted by γδ$^+$ T cells in the FGT and is modulated by both E2 and the microbiota. This IL-17–rich environment conditions vaginal DCs to prime potent T_h17 responses, likely via an IL-1β–dependent pathway. To our knowledge, this is the first report to show the importance of IL-17 produced by γδ$^+$ T cells in the induction of T_h17 immunity in the FGT as well as demonstrate an association between E2, microbiota, and IL-17–producing γδ$^+$ T cells. Our findings showcase the complexity of the vaginal microenvironment and highlight the importance of understanding how endogenous factors (IL-17, hormones, microbiota) can regulate the priming of adaptive immune responses by vaginal DCs and thereby influence overall immunity in the FGT.

DISCLOSURES

The authors have no financial conflicts of interest.

ACKNOWLEDGMENTS

We thank Kristen Mueller for providing technical assistance.

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