IL-4–secreting eosinophils promote endometrial stromal cell proliferation and prevent *Chlamydia*-induced upper genital tract damage

Rodolfo D. Vicetti Miguel[a,1], Nirk E. Quispe Calla[a], Darlene Dixon[b], Robert A. Foster[c], Andrea Gambotto[d], Stephen D. Pavelko[e], Luanne Hall-Stoodley[f], and Thomas L. Cherpes[a]

[a]Department of Comparative Medicine, Stanford University School of Medicine, Stanford, CA 94305; [b]National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709; [c]Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada, N1G 2W1; [d]Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; [e]College of Medicine and Life Sciences, University of Toledo, Toledo, OH 43614; and [f]Department of Microbial Infection and Immunity, Centre for Microbial Interface Biology, College of Medicine, The Ohio State University, Columbus, OH 43210

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Genital *Chlamydia trachomatis* infections in women typically are asymptomatic and do not cause permanent upper genital tract (UGT) damage. Consistent with this presentation, type 2 innate and T_H2 adaptive immune responses associated with dampened inflammation and tissue repair are elicited in the UGT of *Chlamydia*-infected women. Primary *C. trachomatis* infection of mice also causes no genital pathology, but unlike women, does not generate *Chlamydia*-specific T_H2 immunity. Herein, we explored the significance of type 2 innate immunity for restricting UGT tissue damage in *Chlamydia*-infected mice, and in initial studies intravaginally infected wild-type, IL-$10^{-/-}$, IL-$4^{-/-}$, and IL-$4R\alpha^{-/-}$ mice with low-dose *C. trachomatis* inoculums. Whereas *Chlamydia* was comparably cleared in all groups, IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ mice displayed endometrial damage not seen in wild-type or IL-$10^{-/-}$ mice. Congruent with the aberrant tissue repair in mice with deficient IL-4 signaling, we found that IL-4Rα and STAT6 signaling mediated IL-4–induced endometrial stromal cell (ESC) proliferation ex vivo, and that genital administration of an IL-4–expressing adenoviral vector greatly increased in vivo ESC proliferation. Studies with IL-4-IRES-eGFP (4get) reporter mice showed eosinophils were the main IL-4–producing endometrial leukocyte (constitutively and during *Chlamydia* infection), whereas studies with eosinophil-deficient mice identified this innate immune cell as essential for endometrial repair during *Chlamydia* infection. Together, our studies reveal IL-4–producing eosinophils stimulate ESC proliferation and prevent *Chlamydia*-induced endometrial damage. Based on these results, it seems possible that the robust type 2 immunity elicited by *Chlamydia* infection of human genital tissue may analogously promote repair processes that reduce phenotypic disease expression.

Chlamydia trachomatis | endometrium | endometrial stromal cells | eosinophils | interleukin 4

The obligate intracellular Gram-negative bacterium *Chlamydia trachomatis* is sexually transmitted to approximately 130 million individuals each year (1, 2). This high level of populational infectivity is facilitated by predilection of the bacterium to cause asymptomatic and persistent genital infection in women (3, 4). Although *Chlamydia* ascension into upper genital tract (UGT) tissue can elicit pelvic inflammatory disease (PID) and cause Fallopian tube damage that increases risk for ectopic pregnancy and infertility, the large majority of infections have no negative impact on genital tract structure or function (1).

The typical course of clinical disease suggests that *C. trachomatis* infection of the human female genital tract may promote formation of type 2 innate and adaptive immunity, a host defense system elicited in response to persistent foreign antigen stimulation that serves to dampen tissue inflammation and promote wound healing (5). Offering support for this possibility, we found that *C. trachomatis* endometrial infection in women elicited: differentiation of $CD4^+$ T cells that expressed GATA3 and produced IL-4, alternative macrophage activation, enhanced activation of signaling pathways linked to tissue repair, and other host responses characteristic of type 2 immunity (6). Providing additional support for this possibility, an earlier longitudinal study found protection against incident genital *Chlamydia* infection was significantly enhanced among women whose peripheral blood mononuclear cells secreted IL-13 when stimulated ex vivo with chlamydial antigen (7).

Although asymptomatic infection and the scarcity of adverse reproductive sequelae (even after chronic infection) is consistent with a role for type 2 immune responses in women with genital *C. trachomatis* infection, it opposes long-held dogma that maintains type 1 immunity is the predominant and biologically relevant host response (8). However, this dogma was primarily developed in mouse models of genital *Chlamydia muridarum* infection where primary infection is rapidly cleared and no *Chlamydia*-specific T_H2 immunity forms (9, 10). This model, therefore, may underestimate the role of *Chlamydia*-specific T_H2 immunity in the human host response. Moreover, other experimental data implies type 1 immunity may have a two-sided effect—repetitive genital infection of mice and nonhuman primates elicited

Significance

***Chlamydia trachomatis* is the most common sexually transmitted bacterium, but most genital infections in women are asymptomatic. Consistent with this presentation, *Chlamydia* ascension into human endometrial tissue elicits robust innate and adaptive type 2 immunity. Herein, we genitally infected mice with *C. trachomatis* to explore the significance of type 2 innate immunity, finding that IL-4–secreting eosinophils promote endometrial stromal cell proliferation and prevent *Chlamydia* infection from triggering upper genital tract tissue destruction. Such results identify eosinophils as essential for repairing murine genital tissue repair after infectious insult. They also identify a need to define roles played by eosinophils in genital infections of women, and their role in other events associated with endometrial repair, including menstruation, endometritis, and endometriosis.**

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[1]To whom correspondence should be addressed. Email: rodolfo.vicetti@stanford.edu.

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Chlamydia-specific T_H1 immunity that cleared infection but triggered widespread reproductive tract damage (11, 12).

Although mouse model findings with *C. muridarum* were not consistent with typical clinical presentation in women, we posited that genitally infecting mice with low-dose inoculums of an oculogenital *C. trachomatis* serovar would more precisely recapitulate the human host response. Testing this hypothesis, we found instead that clearance of genital *C. trachomatis* infection in mice was also mediated by T_H1 immunity and that infection did not generate *C. trachomatis*-specific T_H2 immunity (12). However, this

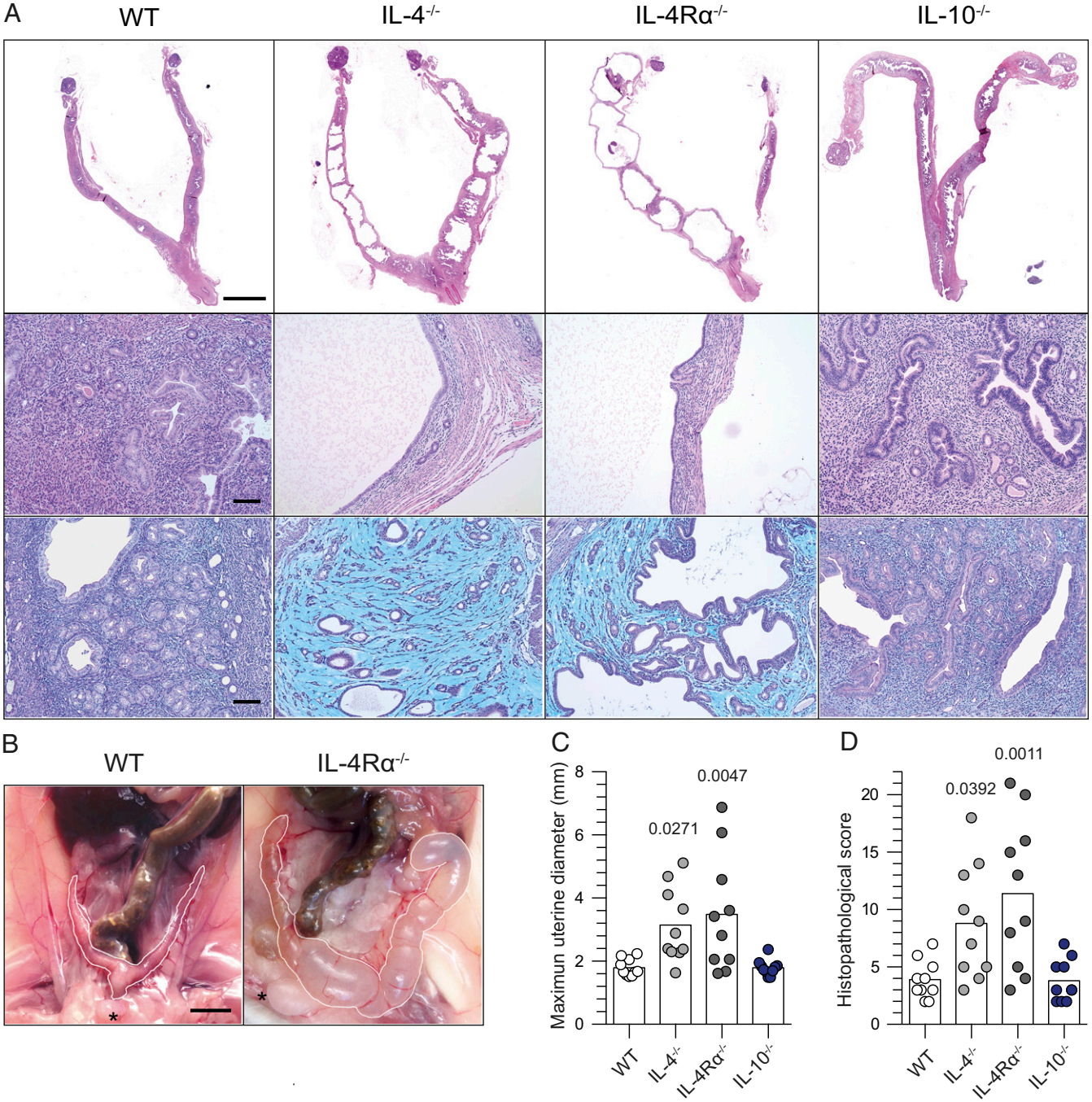

Fig. 1. IL-4 signaling prevents UGT tissue damage after primary genital *C. trachomatis* infection. Six- to eight-week-old female WT, IL-$4^{-/-}$, IL-$4R\alpha^{-/-}$, and IL-$10^{-/-}$ mice were s.c. administered 1 mg of DMPA 5 d before ivag infection with 10^4 IFU of *C. trachomatis* serovar D (infection was daily for 3 consecutive days). Mice were euthanized on 120 dpi, and genital tracts removed en bloc for histopathological evaluation. (*A*) *Top* and *Middle* show representative H&E images of tissue damage detected only in mice with disrupted IL-4 signaling. *Bottom* displays representative images of Alcian blue and periodic acid–Schiff (PAS)-stained UGT sections that show the widespread endometrial stromal myxomatous edema elicited by *Chlamydia* infection of IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ mice. (*B*) Representative macroscopic images show uterine horn dilation seen at 120 dpi only in those mice with disrupted IL-4 signaling (asterisks denote approximate location of pubic symphysis). Quantification of histopathological change was performed by determining the maximum uterine diameter in each animal (*C*) and the composite histopathological score (*D*) (as defined in *Materials and Methods*); (bars denote means, $n = 10$ per group); (all results shown are representative of three independent experiments). (Scale bars: *A, Top,* 4 mm; *A, Middle* and *Bottom*, 100 μm; *B*, 4.5 mm.)

infection did not produce the extensive UGT damage typically seen in mice after primary *C. muridarum* infection (12). These studies thus offered preliminary, albeit indirect, indication that primary genital *C. trachomatis* infection of mice elicits host responses that dampen inflammation and prevent tissue damage.

Because mice did not form *C. trachomatis*-specific T_H2 immunity (12), herein, we used a mouse model of primary *C. trachomatis* infection to delineate the role of type 2 innate immunity in regulating genital inflammation and tissue repair. Our line of investigation led us to increasingly focus on eosinophils, a type 2 innate immune cell shown to secrete preformed cytokines and other immunomodulatory substances that regulate tissue regeneration and wound healing at various tissue sites (13, 14). Although eosinophil function during genital infection was underexplored, these cells were previously shown prevalent in mouse endometrial tissue (15) and were more commonly seen in the endometrium of women with chronic endometritis than women with healthy endometrial tissue (16, 17). Utilization of a murine model of genital *C. trachomatis* infection in the current investigation allowed us to extend earlier findings, revealing that IL-4–secreting eosinophils promote endometrial stromal cell proliferation and are essential for endometrial tissue repair after infectious insult.

Results

IL-4 Signaling Prevents *C. trachomatis* Infection from Inducing UGT Pathology. Although *C. trachomatis* is the most common sexually transmitted bacterium, only a small fraction of women with laparoscopically confirmed PID develop tubal factor infertility or other permanent damage in the reproductive tract (18). Likewise, primary intravaginal (ivag) *C. trachomatis* infection of wild-type (WT) mice elicited acute endometrial inflammation but no permanent UGT damage (12). These findings suggest genital *C. trachomatis* infection may trigger type 2 innate and adaptive immunity, host defenses that modulate tissue inflammation (6). These responses are regulated by cytokines that include IL-4 and IL-10, and are induced during persistent inflammatory states to diminish inflammation and stimulate tissue repair (5). Whereas tissue reparative roles for type 2 immune responses are well established in numerous other conditions (5, 13, 14, 19), their importance in the female genital tract during infectious insult was undefined.

Because primary ivag *C. trachomatis* of WT mice is cleared without permanent tissue damage and without contribution from *Chlamydia*-specific T_H2 immunity (12), it seemed ideally suited to dissect the role of type 2 innate immunity in the female genital tract. To begin, we compared genital pathology in WT, IL-$10^{-/-}$, IL-$4^{-/-}$, and IL-4R$\alpha^{-/-}$ mice 120 d after primary ivag infection with a low-dose *C. trachomatis* inoculum. Although no overt tissue damage was detected in WT and IL-$10^{-/-}$ mice, widespread endometrial pathology developed in IL-$4^{-/-}$ and IL-4R$\alpha^{-/-}$ mice (Fig. 1). Gross changes included hydrometra and prominent unilateral or bilateral uterine horn dilation (Fig. 1 *A*–*C*). UGT histological changes were characterized by endometrial atrophy and stromal myxomatous edema, with no significant inflammatory or fibrotic component (Fig. 1 *A* and *D* and Fig. S1). The tissue damage was confined to the uterine horns, as no oviduct pathology was detected. Because clearance of infection was comparable between WT, IL-$4^{-/-}$, and IL-4R$\alpha^{-/-}$ mice (Fig. 2*A*), and slightly accelerated in IL-$10^{-/-}$ mice (Fig. 2*B*), it seemed unlikely the uterine pathology in mice with deficient IL-4 signaling was caused by impaired control of *C. trachomatis* replication.

Differences in *Chlamydia*-Specific T-cell Immunity Do Not Promote Endometrial Pathology in IL-$4^{-/-}$ and IL-4R$\alpha^{-/-}$ Mice. Although primary *Chlamydia* infection of WT mice caused no overt endometrial damage (Fig. 1), *Chlamydia*-specific T_H1 and T_H17 immune responses to repetitive genital challenge induced UGT destruction

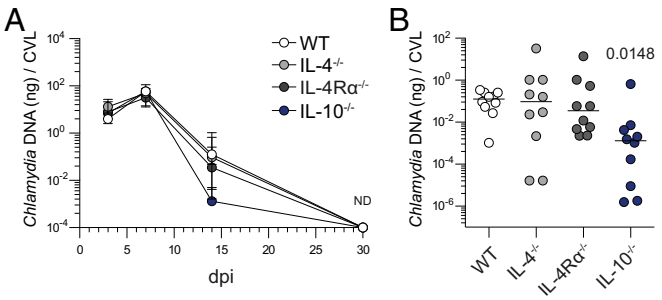

Fig. 2. Endometrial damage seen in mice with disrupted IL-4 signaling is not caused by a reduced ability to clear genital *C. trachomatis* infection. (*A*) Cervicovaginal lavages were collected at indicated dpi from groups of mice detailed in Fig. 1 to quantify *Chlamydia* DNA levels by quantitative real-time (qRT)-PCR (median ± range, $n = 10$); (ND, nondetectable). (*B*) Between-group comparison of *Chlamydia* DNA burden at dpi 14 shows clearance of infection was more rapid in IL-$10^{-/-}$ mice, whereas no differences in clearance were detectable among WT, IL-$4^{-/-}$, and IL-4R$\alpha^{-/-}$ mice (bars denote medians, $n =$ 10 per group; results shown are representative of three independently conducted experiments).

that diminished reproductive fertility (12). It therefore seemed possible that enhanced endometrial damage in IL-$4^{-/-}$ and IL-4R$\alpha^{-/-}$ mice was triggered by altered *Chlamydia*-specific T-cell immunity. However, when T cells from the draining lymph nodes (DLNs) of *C. trachomatis*-infected WT, IL-$4^{-/-}$, and IL-4R$\alpha^{-/-}$ mice were stimulated ex vivo with chlamydial antigen, no significant differences in IFN-γ, TNF, IL-17, or IL-10 production were seen (Fig. 3 *A* and *B*). Conversely, T cells from IL-$10^{-/-}$ mice respectively displayed fivefold and sixfold higher frequencies of IFN-γ– and IL-17–secreting *Chlamydia*-specific $CD4^+$ T cells (Fig. 3 *A* and *B*). Because no UGT pathology was seen in IL-$10^{-/-}$ mice (i.e., animals displaying highly robust *Chlamydia*-specific T_H1 and T_H17 immunity), this result indicated the tissue damage observed in IL-$4^{-/-}$ and IL-4R$\alpha^{-/-}$ mice was not triggered by immunopathological T-cell responses. In response to ex vivo stimulation with chlamydial antigen, T cells from all four mouse groups also failed to secrete detectable levels of IL-4 (Fig. 3*B*). The latter observation was confirmed by using DLNs and UGTs from *Chlamydia*-infected 4get reporter mice (a strain that allows identification of IL-4–producing cells by eGFP expression) (20) (Fig. 3*C*). Taken together, our studies thus far showed primary *C. trachomatis* infection of WT mice was cleared without causing UGT pathology, whereas altered *Chlamydia*-specific T-cell effector function did not cause the pervasive endometrial pathology seen in *Chlamydia*-infected IL-$4^{-/-}$ and IL-4R$\alpha^{-/-}$ mice.

IL-4 Does Not Regulate Differentiation of Uterine Macrophages After Genital *Chlamydia* Infection. Because enhanced UGT pathology in IL-$4^{-/-}$ and IL-4R$\alpha^{-/-}$ mice was not caused by altered *Chlamydia*-specific T-cell effector function, we used our mouse infection model to explore innate immune cells shown to have roles in tissue repair. These cells include macrophages (Mϕs) and eosinophils (5). Mϕs are abundant in endometrial stroma (21) and regulate embryo implantation (22), whereas their differentiation into M2 Mϕs is mediated by IL-4 signaling pathways (5). Likewise, eosinophils are resident in the endometrial stroma of mice (15). Both Mϕs and eosinophils were shown to mediate repair in a variety of tissues (5, 13, 14), but their role in repairing pathogen-induced genital mucosal tissue damage was uncertain.

To begin our investigation of type 2 innate immune cells, we compared uterine myeloid cell recruitment and differentiation in WT and IL-$4^{-/-}$ mice after primary *C. trachomatis* infection (Fig. S2 *A* and *B*). In both, infection induced peak endometrial infiltrates of polymorphonuclear neutrophils (PMNs) and inflammatory monocytes (iMos) at 3 and 7 d after infection (dpi), respectively

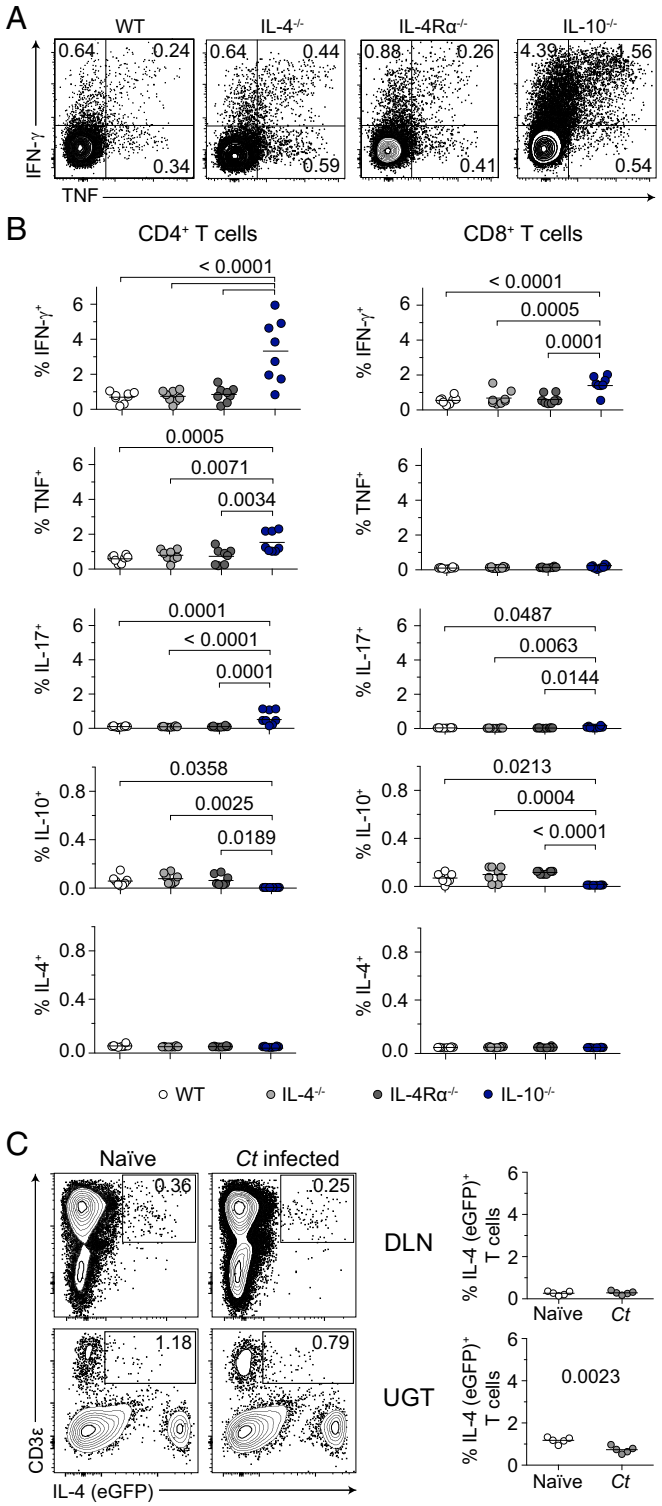

Fig. 3. Endometrial pathology in mice with disrupted IL-4 signaling is not caused by altered development of *Chlamydia*-specific T-cell immunity. Indicated strains of mice were infected as detailed in Fig. 1 and challenged with 10^6 IFU of *C. trachomatis* serovar D at 120 dpi. Mice were euthanized 5 d after challenge (dpc), and DLNs were processed into single-cell suspensions for incubation with inactivated *Chlamydia* EB or media alone. (*A*) Representative contour plots from intracellular cytokine staining that quantified IFN-γ and TNF production by $CD4^+$ T cells stimulated with chlamydial antigen (quadrant numbers denote percentages of cytokine-producing cells). (*B*) Between-group comparisons of percentages of $CD4^+$ and $CD8^+$ T cells producing IFN-γ, TNF, IL-17, IL-10, and IL-4 in response to stimulation with chlamydial antigen (bars indicate means, $n = 8$ per group). (*C*) Using experimental conditions identical to those described in *A*, IL-4-IRES-eGFP (4get) reporter mice were euthanized at 5 dpc. DLN and UGT were excised and immediately processed for flow cytometric assays that identified $IL\text{-}4^+$ T cells (bars denote means, $n = 5$ mice per group; results shown representative of two independently conducted experiments).

(Fig. 4). At 14 dpi, $IL\text{-}4^{-/-}$ mice displayed fewer uterine iMos and Mϕs than WT mice; however, this effect was not sustained at later time points (Fig. 4). Infection also elicited few substantive between-group differences in M1 and M2 marker expression by uterine iMos and Mϕs. At 7 dpi, WT and $IL\text{-}4^{-/-}$ mice displayed comparable percentages of $NOS2^+$ iMos (M1), with uterine Mϕs from both groups of mice primarily exhibiting a M2 phenotype (as defined by CD206, CD200R, CD169, and arginase 1 expression patterns) (Fig. 5*A* and Fig. S2 *C–E*). At 14 dpi, $IL\text{-}4^{-/-}$ mice displayed a Mϕ polarization phenotype comparable to uninfected WT mice (i.e., a higher percentage of M2 uterine Mϕs and less iMos), whereas the UGT of WT mice contained a more prominent $NOS2^+$ iMo population (Fig. 5 *B* and *C*). At 30 dpi (i.e., after resolution of primary infection), both WT and $IL\text{-}4^{-/-}$ mice displayed Mϕ polarization profiles comparable to uninfected mice (Fig. 5*C*). At each indicated time point after genital infection, we also saw no substantial between-group differences in uterine Mϕ expression of M2 markers, CD40, or MHC-II (Fig. 5 *B* and *C* and Fig. S2 *C–E*). Taken together, this examination of WT and $IL\text{-}4^{-/-}$ mice identified no significant differences in endometrial iMo and Mϕ differentiation after primary genital *C. trachomatis* infection.

IL-4-Producing Eosinophils Prevent Genital Tissue Damage After *C. trachomatis* Infection. Because genital *C. trachomatis* infection of mice did not induce differentiation of IL-4–secreting T cells (Fig. 2), we alternatively explored whether innate immune cells were a source of IL-4 in the UGT. To facilitate this inquiry, we examined the UGTs from 4get reporter mice. These studies showed that at least 95% of IL-4–expressing cells in uninfected or *Chlamydia*-infected UGTs of mice were eosinophils (SSC^{hi} $CD11b^+$ $Siglec\text{-}F^+$ $CCR3^+$) (Fig. 6 *A* and *B*). The remainder were $CD4^+$ T cells and mast cells (SSC^{hi} $CD49b^-$ $CD11c^-$ $Fc\varepsilon RI^+$) (Fig. S3*A*), whereas IL-4 was undetected in nonhematopoietic cells (Fig. S3*B*). Although relative IL-4 amounts produced by eosinophils were comparable among uninfected and *Chlamydia*-infected mice, we found acute infection increased endometrial eosinophil expression of Ly6C (Fig. 6*B*). We also found that compared with adult murine skin fibroblasts, endometrial stromal cells (ESCs) expressed significantly higher levels of CCL7 and CCL11 (Fig. 6*C*), chemokines regulating eosinophil infiltration of murine endometrial tissue (15). Likewise, we found endometrial eosinophil numbers were significantly increased by *Chlamydia* infection, but that these numbers returned to levels seen in uninfected mice after infection was resolved (Fig. 6*D*). Whereas infection increased endometrial eosinophil numbers in both WT and $IL\text{-}4^{-/-}$ mice, *Chlamydia* infection-induced tissue eosinophilia was blunted in $IL\text{-}4^{-/-}$ mice (Fig. 6*D*). These studies thus identified endometrial eosinophils as a major IL-4 source in the mouse UGT, and suggested that IL-4 secretion helps regulate the infiltration of endometrial eosinophils during *Chlamydia* infection.

Because eosinophils were the primary source of IL-4 in the mouse UGT, we used ΔdblGATA1 mice to define the role of eosinophils in endometrial tissue repair during *C. trachomatis* infection (the ΔdblGATA1 mouse contains a *Gata1* promoter deletion that prevents eosinophil development) (Fig. S3*C*) (23). Comparable to *Chlamydia* eradication by WT, $IL\text{-}4^{-/-}$, and $IL\text{-}4R\alpha^{-/-}$ mice (Fig. 1), we saw WT and ΔdblGATA1 mice similarly clear primary *C. trachomatis* infection (Fig. 6*E*) and display comparable *Chlamydia*-specific T-cell responses (Fig. 6*F*). However,

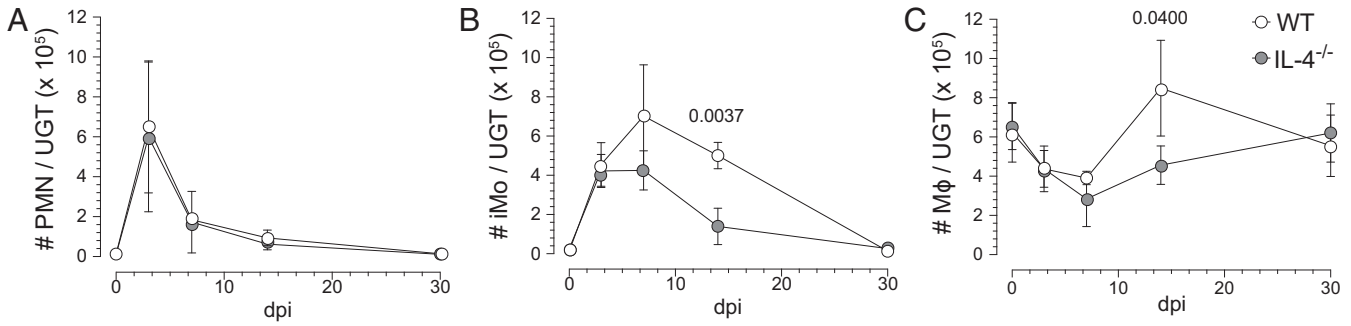

Fig. 4. Disrupted IL-4 signaling does not increase endometrial myeloid cell infiltration after *C. trachomatis* infection. WT and IL-4$^{-/-}$ mice were genitally infected with *C. trachomatis* as specified in Fig. 1. Animals were euthanized at indicated dpi, and UGT were processed for flow cytometric evaluation of myeloid cell infiltration. The absolute numbers of PMNs (*A*), iMos (*B*), and Mϕs (*C*) per UGT are shown, and identifies similar patterns of myeloid cell infiltration in WT and IL-4$^{-/-}$ mice (values are means ± SD, n = 3–6 mice per condition for each time point; results displayed are representative of two independently conducted experiments) (gates defining distinct myeloid cell populations are depicted in Fig. S2).

only ΔdblGATA1 mice developed endometrial pathology, with these mice displaying histopathology that resembled tissue damage seen in mice with disrupted IL-4 signaling (Figs. 1 and 6 *G* and *H*). Together, these studies thus identified IL-4–producing eosinophils as essential for appropriate repair of endometrial tissue after infectious insult.

IL-4 Regulates ESC Proliferation. Earlier studies identified that tissue-resident stromal cells regulate repair by proliferating and differentiating in response to injury (13, 19). Based on an ability to proliferate and migrate through various endometrial layers, it was also proposed that human and murine ESCs similarly contribute to uterine tissue repair (24, 25). However, the exact mechanisms connecting host immune responses to ESC proliferation and endometrial regeneration were not well elucidated. Because we identified that IL-4 signaling was essential for repair of *Chlamydia*-infected endometrial tissue and that eosinophils were the primary source of IL-4 in the mouse endometrium, we performed in vitro and ex vivo experiments to define IL-4–mediated effects on ESC proliferation. Initial studies showed that ESCs express IL-4Rα (CD124) (Fig. 7 *A* and *B* and Figs. S4 and S5), and that isolated ESCs phosphorylate STAT6 in response to IL-4 (Fig. 7*C*). Additional in vitro assays demonstrated that IL-4 increased ESC proliferation (Fig. 7*D*), whereas IL-4 responsiveness was abrogated in ESCs isolated from IL-4Rα$^{-/-}$ or STAT6$^{-/-}$ mice (Fig. 7*D*). Combined, these studies identified that IL-4–mediated increases in ESC proliferation depend on STAT6 phosphorylation.

To resolve whether IL-4–mediated processes comparably enhance ESC proliferation in vivo, WT mice were intrauterinely administered an IL-4–expressing adenoviral vector (Ad.IL-4) (26). This localized IL-4 overexpression increased proliferation of ESCs and luminal endometrial epithelial cells and induced histologic changes that resembled endometrial decidual reaction (Fig. 8 *A* and *B*). It also suggested that IL-4–mediated stimulation of ESC proliferation did not depend on pathogen-induced tissue insult. Administering Ad.IL-4 to IL-4Rα$^{-/-}$ mice or an eGFP-expressing adenoviral vector (Ad.eGFP) to WT mice did not promote ESC proliferation (Fig. 8 and Fig. S6*A*), but Ad.IL-4 administration to STAT6$^{-/-}$ mice stimulated endometrial epithelial cell proliferation without affecting ESC proliferation (Fig. 8*B*). These results indicated IL-4–mediated signaling processes that promote in vivo ESC proliferation are IL-4Rα– and STAT6-dependent, whereas IL-4–mediated signaling processes that stimulate in vivo endometrial epithelial cell proliferation do not depend on STAT6. Finally, we saw no effect on oviduct epithelial or stromal cell proliferation in WT, IL-4Rα$^{-/-}$, or STAT6$^{-/-}$ mice treated with Ad.IL-4 or Ad.eGFP (Fig. S6*B*), implying that IL-4–mediated effects on genital tract stromal cell proliferation were more relevant to endometrial repair processes.

We concluded the current study by exploring whether IL-4 directly promotes in vivo ESC proliferation or whether the enhanced ESC proliferation occurs secondary to an IL-4–mediated effect on hematopoietic cells. To explore these possibilities, we intrauterinely administered Ad.IL-4 or Ad.eGFP to WT and IL-4Rα$^{-/-}$ mice reconstituted with WT bone marrow (BM) and WT mice reconstituted with IL-4Rα$^{-/-}$ BM. These BM chimera studies showed that in vivo ESC proliferation was increased in all groups of mice except those mice whose nonhematopoietic cells did not express IL-4Rα (Fig. 8*C*). As a follow-up to these results, we enumerated ESC proliferation at various time points after ivag infection of WT mice, and found highest ESC proliferation at 21 dpi (Fig. S7). Upon comparing levels of ESC proliferation in WT, IL-4$^{-/-}$, or IL-4Rα$^{-/-}$ mice at this same time point after infection, we saw significantly lower ESC proliferation levels in the two groups of mice with defective IL-4 signaling (Fig. 9). Moreover, this difference in ESC proliferation became more profound at 120 dpi (Fig. 9). In combination with our earlier results, these studies showed that IL-4 acts directly on ESCs to increase their proliferation, and this effect helped preserve normal genital tract architecture during infectious insult.

Discussion

To successfully resolve the system perturbations induced by physiologic change or infectious insult, endometrial tissue must display remarkable plasticity. During the human menstrual cycle, tissue homeostasis is restored by coordinated processes that include reepithelialization, ESC remodeling, and endothelial cell proliferation (24, 25). Whereas *C. trachomatis* ascension into endometrial tissue elicits an acute influx of PMNs and other proinflammatory leukocytes, only a small subset of women with laparoscopically confirmed PID develop permanent UGT damage (18). Although mice do not menstruate, their endometrium analogously undergoes cyclical rounds of cell growth and apoptosis during the estrus cycle (27, 28). Primary intravaginal *C. trachomatis* infection of mice likewise elicits robust endometrial inflammation, but causes no permanent UGT damage (12). Our study confirms and extends the latter observation, revealing IL-4–producing eosinophils mediate repair processes during primary *Chlamydia* infection that conserve normal endometrial structure.

Multiple results from our investigation support this conclusion. Our initial work identified IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice as highly susceptible to endometrial damage after primary *C. trachomatis* infection. However, this susceptibility was unrelated to impaired *Chlamydia* control, because genital chlamydial clearance was equivalent among WT mice and mice with deficient IL-4 signaling.

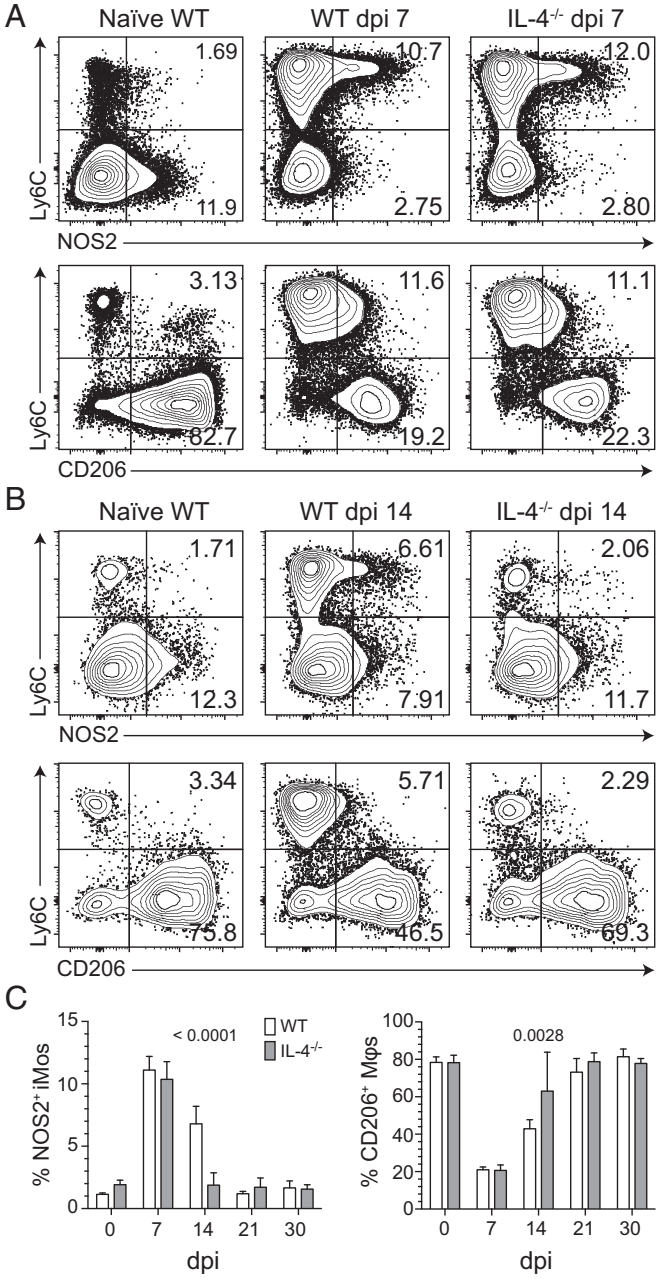

Fig. 5. IL-4 signaling does not influence uterine iMo or Mϕ polarization after primary genital *C. trachomatis* infection. Uninfected WT and IL-4$^{-/-}$ mice remained uninfected or were infected with *C. trachomatis* as detailed in Fig. 1. Mice were euthanized at select dpi, and UGT were processed to evaluate myeloid mononuclear cell polarization by flow cytometry (as defined in *Materials and Methods*). Representative contour plots indicate NOS2 and CD206 expression patterns for iMos and Mϕs in mice at 7 (*A*) and 14 dpi (*B*) compared with uninfected mice. (*C*) Percentages of uterine NOS2$^+$ iMos and CD0206$^+$ Mϕs from live CD11b$^+$ myeloid cells in WT vs. IL-4$^{-/-}$ mice at various dpi appeared distinct only at 14 dpi (bars denote means ± SD, n = 6 mice per condition for each time point; results displayed are representative of two independently conducted experiments).

Likewise, altered T-cell function did not promote immunopathological tissue damage, because WT and IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice showed comparable *Chlamydia*-specific T_H1 and T_H17 effector function. Moreover, and congruent with prior reports (29, 30), IL-10$^{-/-}$ mice cleared infection more rapidly and generated more robust *Chlamydia*-specific T_H1 immunity than WT mice, but the exuberant T_H1 response by IL-10$^{-/-}$ mice to *Chlamydia* infection did not stimulate damage to UGT tissue. Because impaired clearance or altered adaptive immunity did not explain the increased *Chlamydia*-induced endometrial pathology in IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice, we explored the role of innate immune cells in modulating genital tissue repair after infectious insult. Although prior work identified the importance of M2 Mϕs in type 2 immunity and tissue repair (31), our study indicates that uterine Mϕs have a more negligible role in repair of endometrial tissue after primary *C. trachomatis* infection. Conversely, we uncovered an underappreciated significance for eosinophils as host defense against tissue damage during genital infection.

Although our study identifies that endometrial eosinophils provide an essential host defense during infectious insult, the capacity of IL-4–secreting eosinophils to promote ESC proliferation and tissue repair in the *Chlamydia*-infected endometrium is highly congruent with established roles for eosinophils in other tissues. To promote muscle regeneration, eosinophils stimulate fibro/adipogenic progenitors, bipotential cells capable of differentiating into fibroblasts and adipocytes (13, 14). IL-4–secreting eosinophils in mice are also rapidly recruited to sites of toxin-mediated liver damage, and stimulate tissue regeneration by directly promoting proliferation of quiescent hepatocytes (13, 14). In a mouse model of experimental colitis, resident mucosal eosinophils also exert protective effects by secreting antiinflammatory lipid mediators (32). Likewise, the detection of increased numbers of activated eosinophils in individuals with inactive ulcerative colitis suggests these cells may facilitate repair of colonic mucosal epithelium in humans (33). When pulmonary mucosal tissue of mice is repetitively challenged with a highly immunogenic allergen, airway hyperresponsiveness and lung inflammation is resolved by IL-10–secreting eosinophils (34). Consistent with the eosinophil-mediated repair of *Chlamydia*-infected endometrial tissue identified herein, eosinophils may also regulate noninfectious processes in the mouse endometrium. As an example, when mice are depleted of granulocytes with an anti–Gr-1 monoclonal antibody (an antibody that also binds and systemically depletes eosinophils) (35), altered patterns of uterine bleeding and tissue remodeling were detected (36, 37). Our study provides an important extension of these prior studies, demonstrating that IL-4–producing eosinophils are essential to prevent genital pathogen-induced destruction of UGT tissue.

To uncover this function for endometrial eosinophils, we used a murine model of genital *C. trachomatis* infection. Prior studies, including our own, failed to detect significant T_H2 responses in the UGT of *Chlamydia*-infected mice (12, 29, 38), and we exploited this observation in the current investigation to interrogate the role of type 2 innate immunity in pathogen clearance and tissue repair. However, *C. trachomatis* is exclusively a human pathogen (12, 39), a factor that appears to limit the ability of murine *C. trachomatis* infection to fully model human host disease. Whereas phenotypic expression of disease in women typically involves Fallopian tube damage, we found that primary ivag *C. trachomatis* infection of IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice elicits pervasive endometrial damage that is oviduct-sparing. Despite the dissimilar expression of *C. trachomatis*-induced UGT pathology, it seems likely our findings will inform processes of endometrial repair in women. In the first place, there is significant functional overlap between human and mouse eosinophils (40), including the production and secretion of preformed IL-4 (40-43). The recruitment of eosinophils to the endometrium also appears similar, because CCL11 and CCR3 expression by human and mice eosinophils regulates endometrial trafficking (44–48). Congruent with these observations, we found mouse ESCs express high levels of CCL11 (Fig. 6*C*), an eosinophil chemoattractant similarly secreted by IL-4–stimulated human endometriotic stromal cells (49). Unlike mice, eosinophils may not be a resident population in the human endometrium, but large numbers of these cells are known to infiltrate the genital tract of

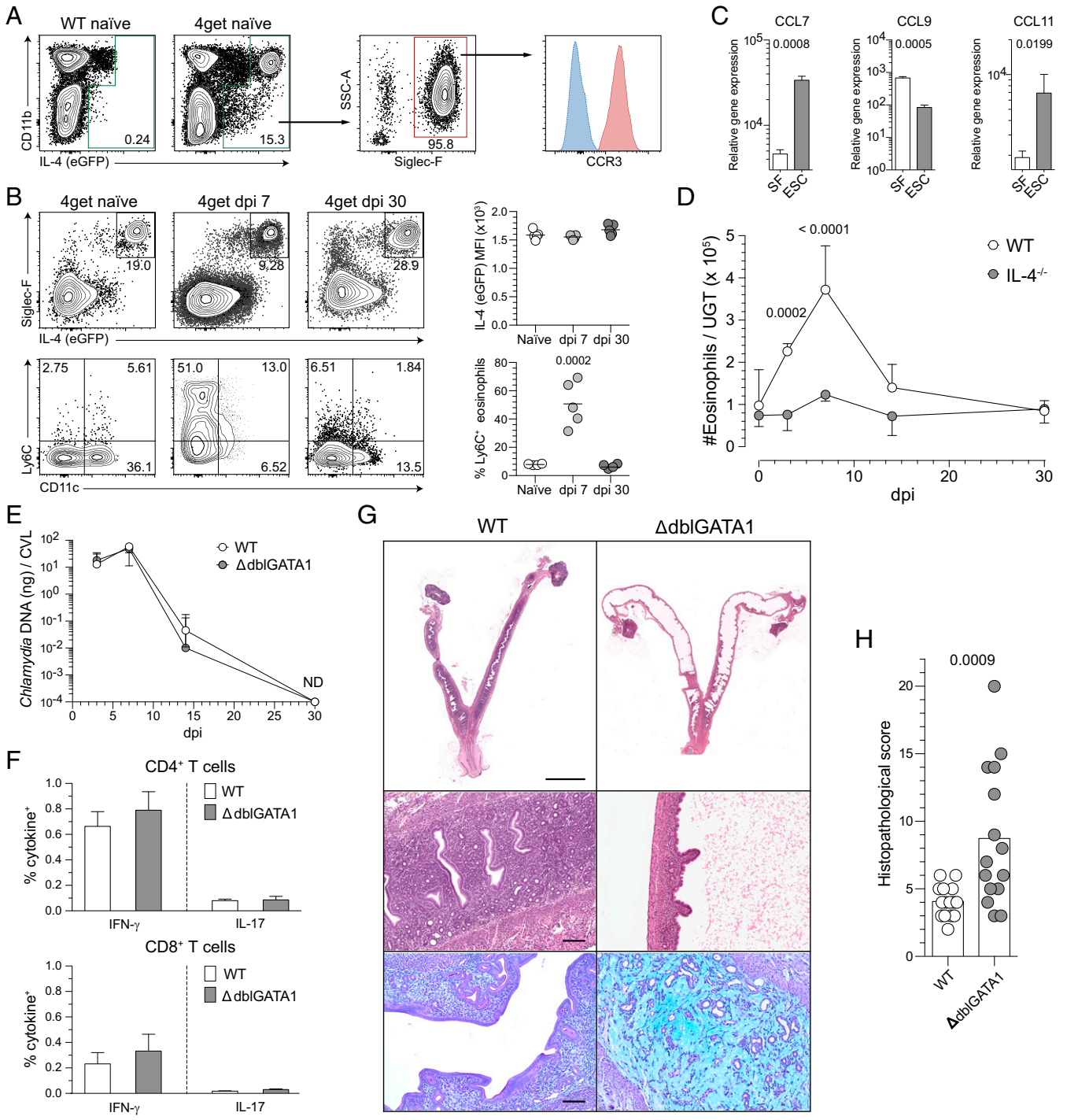

Fig. 6. IL-4–producing eosinophils regulate repair of UGT tissue after *C. trachomatis* infection. Uterine horns from 4get reporter mice were processed as described in Fig. 4 to identify IL-4–producing cells by flow cytometry. (*A*) Representative contour plots show most IL-4–producing cells were of myeloid origin (gated on live total CD45$^+$ cells from whole UGT), and at least 95% of these cells were eosinophils. Histogram displays representative CCR3 expression (blue line indicates FMO control). (*B*) Interestingly, eosinophils remained the primary cellular source of IL-4 throughout primary *Chlamydia* infection (*Upper*, gated on live CD11b$^+$ myeloid cells), and displayed elevated levels of Ly6C during acute infection (*Lower*, gated on live SSC^{hi} CD11b$^+$ Siglec-F$^+$ cells; $n = 5$ in all conditions). (*C*) CCL7, CCL9, and CCL11 gene expression levels as assessed from isolated murine skin fibroblasts (SF) or ESCs (bars indicate means ± SD, $n = 3$ per group). (*D*) WT and IL-4$^{-/-}$ mice were infected as described in Fig. 4, and UGTs were collected and processed at indicated dpi to enumerate eosinophils via flow cytometry ($n = 5$ mice/condition/time point). (*E*) Six- to eight-week-old WT and ΔdblGATA1 mice were genitally infected with *C. trachomatis* as described in Fig. 1, and chlamydial clearance was defined as detailed in Fig. 2 (median ± range, $n = 5$ per group). (*F*) WT and ΔdblGATA1 mice were treated as indicated in Fig. 3, and DLNs were processed to evaluate *Chlamydia*-specific T-cell effector function ($n = 7$ per group; bars indicate means ± SD; each result displayed representative of 2–3 independent experiments). (*G*) Representative UGT histology from WT and ΔdblGATA1 mice 120 d after primary ivag *C. trachomatis* infection: *Top* and *Middle* display representative H&E images of tissue pathology detected in ΔdblGATA1 mice. *Lower* shows representative Alcian blue and PAS images of endometrial stromal myxomatous edema in infected ΔdblGATA1 mice that resembled pathology seen in *Chlamydia*-infected mice with disrupted IL-4 signaling. (Scale bars: *Top*, 4 mm; *Middle* and *Bottom*, 100 μm). (*H*) Semiquantitative scoring system defined in *Materials and Methods* showed significantly increased UGT pathology in ΔdblGATA1 mice (bars denote means, 15 mice per group) (results pooled from two independent experiments).

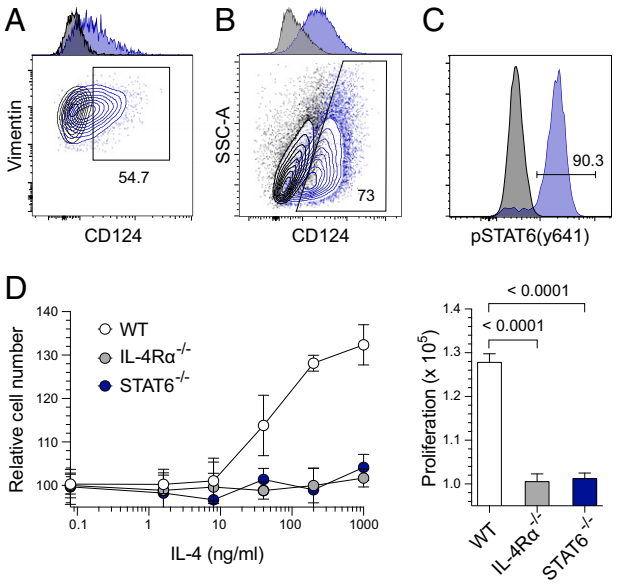

Fig. 7. IL-4 induces STAT6-dependent in vitro proliferation of endometrial stromal cells (ESCs). (*A*) Uninfected WT (blue lines) and IL-4Rα$^{-/-}$ (black lines) mice were euthanized, and uteri were processed to evaluate CD124 (IL-4Rα) expression by ESCs directly ex vivo. (*B*) ESCs isolated from WT (blue lines) and IL-4Rα$^{-/-}$ (black lines) mice were similarly assessed for CD124 expression (ESCs were live CD45^{-} vimentin^{+} CD90^{+} cells); *A* and *B* display representative contour plots and adjunct histograms. (*C*) Representative histograms of isolated ESCs from uninfected WT mice were treated with IL-4 (blue line) or vehicle (black line) for 15 min, fixed and permeabilized, and stained for pSTAT6 (Y641) expression. (*D*) ESCs isolated from WT, IL-4Rα$^{-/-}$, and STAT6$^{-/-}$ mice were plated in serum-free media and treated with vehicle or specified IL-4 concentrations. Three days later, relative cell numbers were compared by using PrestoBlue cell viability reagent (*Left*). AUC analyses (*Right*) revealed that IL-4 stimulates ESC proliferation in an IL-4Rα and STAT6-dependent manner (values are means ± SD, $n = 3$ per group; results displayed are representative of 3–4 independent experiments).

women during menstruation and cervical ripening and the endometrium of women with endometriosis and endometrial adenocarcinoma (50–53). Because these clinical conditions are all associated with tissue remodeling and activation of signaling pathways linked to tissue regeneration (54–57), it seems plausible that human and mouse endometrial eosinophils play analogous roles in repair of genital tissue. Further supporting the clinical relevance of our findings, retrospective studies found eosinophils more common in the endometrium of women with chronic endometritis vs. healthy controls (16, 17). Because increased eosinophil numbers appear less common in the endometrium of women with acute endometritis, and danger signals released from damaged genital epithelial cells are shown to active eosinophils (58), findings from these retrospective clinical studies imply that eosinophils also participate in endometrial tissue repair processes in humans.

In addition to revealing a role for eosinophils in endometrial tissue repair after infectious insult, our study identifies IL-4–mediated promotion of ESC proliferation as a mechanism by which eosinophils regulate this repair. The latter finding is congruent with prior experimental models that showed eosinophils and other type 2 innate immune cells regulate stromal cell-mediated tissue repair (13, 19). Earlier studies likewise showed human and mouse ESCs play key roles in the tissue repair necessitated by cyclic menstruation or parturition (24, 25). During endometrial regeneration, ESCs were also shown to provide a source of epithelial cells by undergoing mesenchymal-epithelial transition (28, 59). Although IL-4 was previously shown to promote in vitro proliferation of endometriotic stromal cells (60), our study establishes that IL-4 also promotes in vivo ESC proliferation, an essential reparative process in several animal models of endometrial tissue repair (24, 25). By identifying that no hematopoietic cell intermediaries are required to promote in vivo ESC proliferation (Fig. 8), our study also establishes that enhanced ESC proliferation is a direct effect of IL-4. Because genital *C. trachomatis* infection induced uterine pathology in IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice (while uninfected mice of these same genotypes developed no pathology), our study provides strong evidence IL-4–secreting eosinophils promote ESC proliferation that repairs genital pathogen-induced endometrial tissue damage.

While establishing that eosinophils, a key type 2 innate immune cell, prevent UGT tissue damage in a mouse model of genital infection, we speculate that our study may also have implications for *Chlamydia* vaccine development. Genital *C. trachomatis* infection in women is often chronic, rarely causes

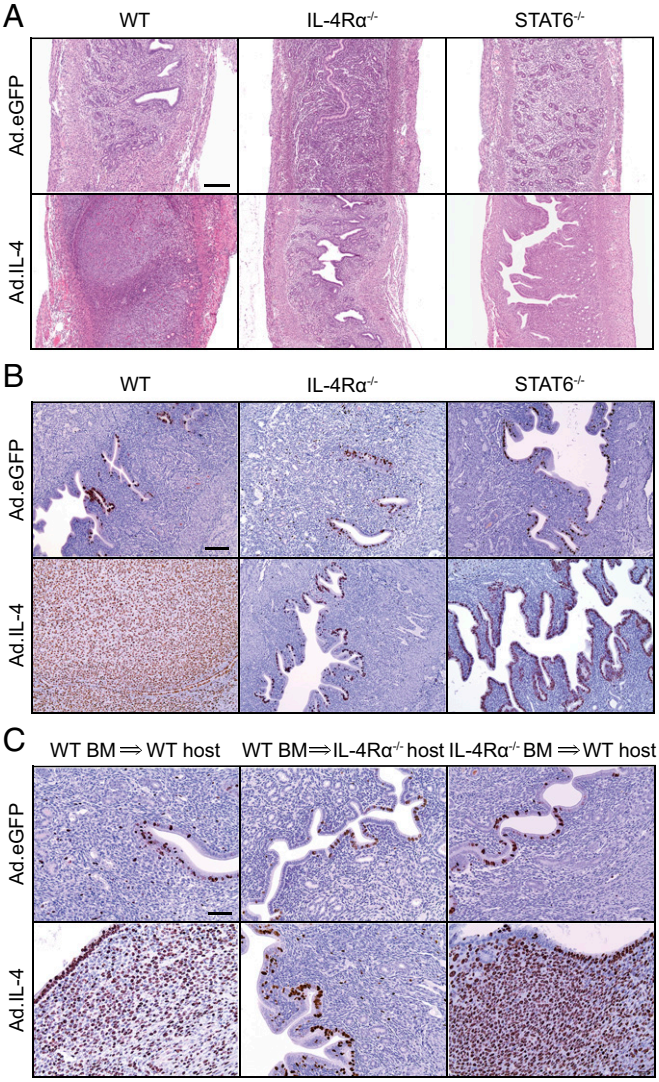

Fig. 8. IL-4 overexpression induces robust in vivo ESC proliferation that depends on IL-4Rα expression by nonhematopoietic cells. Uninfected WT, IL-4Rα$^{-/-}$, or STAT6$^{-/-}$ mice were intrauterinely administered 10^{10} viral particles of Ad.eGFP or Ad.IL-4 via a nonsurgical embryo transfer device. Animals were euthanized 3 d later, and UGT excised and paraffin-embedded for histopathological evaluation (*A*) and immunohistochemical Ki-67 staining (*B*) (representative images shown; $n = 3$ per group). (*C*) As described in *Materials and Methods*, lethally irradiated WT or IL-4Rα$^{-/-}$ mice were reconstituted with BM cells from WT or IL-4Rα$^{-/-}$ mice (as indicated) 2 mo before treatment with Ad.eGFP or Ad.IL-4 (representative images displayed; $n = 3$ per group) (results representative of 2–3 independent experiments). (Scale bars: *A*, 400 μm; *B* = 100 μm; *C*, 50 μm.)

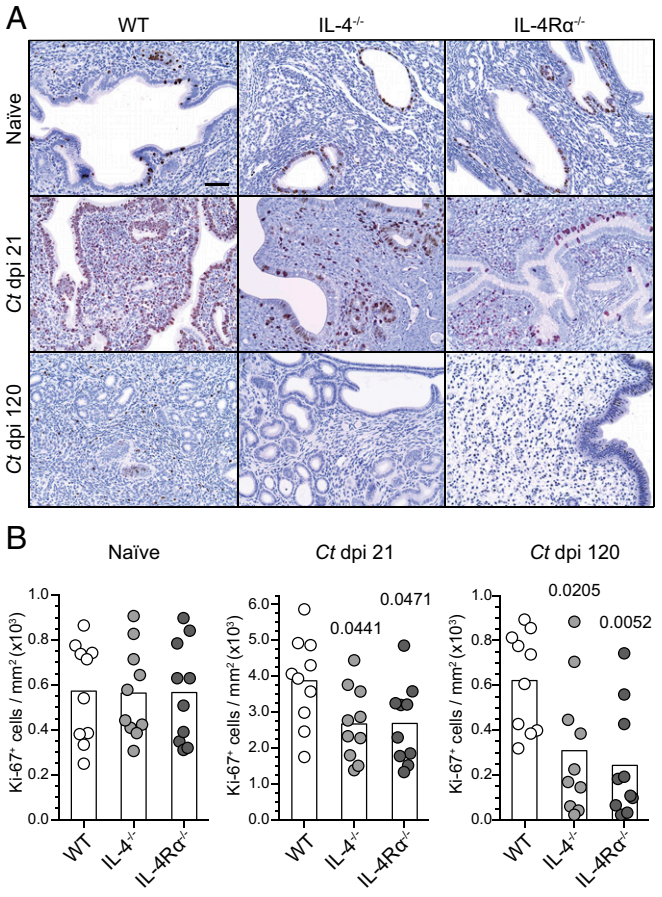

Fig. 9. IL-4 signaling promotes ESC proliferation after *C. trachomatis* infection. (*A*) Uninfected female WT, IL-4$^{-/-}$, or IL-4Rα$^{-/-}$ mice remained uninfected or were ivag infected with *C. trachomatis* as detailed in Fig. 1. Animals were euthanized 21 and 120 d later to assess expression of Ki-67 by endometrial cells (representative images displayed). (Scale bar: 50 μm.) (*B*) Quantification of Ki-67^{+} endometrial cells from animals described in *A* (bars denote means, $n = 10$ per group); results representative of three independent experiments).

phenotypic disease expression, and elicits robust type 2 innate and adaptive immunity (6, 7). This clinical presentation implies immune tolerance, not just immune-driven resistance mechanisms were selected by evolution to combat this ubiquitous human pathogen (61). Whereas tolerance exerts marginal influence on pathogen load, it is more capable than immune-driven resistance mechanisms alone to maintain immunological equilibrium and dampen immunopathological tissue damage (61). By demonstrating the significance of type 2 innate immunity during genital *Chlamydia* infection, our current findings support this possibility. Although inducing *Chlamydia*-specific type 1 immunity has been a long-term goal of *Chlamydia* vaccine research (62), candidate vaccines that elicit only T_H1-type responses have potential to promote *Chlamydia* clearance and tissue immunopathology (11, 12). In other words, for a T_H1-based *Chlamydia* vaccine to be both effective and safe, it may also need to induce T_H2 responses that promote genital tissue repair and resolve the recurrent bouts of inflammation induced by repetitive exposure to the bacterium during sexual intercourse. Incorporating responses that promote tissue repair may be especially useful for *Chlamydia* vaccines, because memory T_H2 responses are critical for preventing tissue damage induced by multicellular pathogens (63, 64). As another potential advantage of *Chlamydia* vaccines that induce T_H2 immunity, permanent tissue damage is most effectively avoided when tissue repair is initiated shortly after inflammation onset (31). Whereas increasing the number of IL-4–secreting eosinophils in the UGT may not offer a mechanism by which *Chlamydia* vaccines minimize the risk of immunopathological tissue damage, our study highlights the significance of type 2 immunity during infectious insult in the female genital tract and the need to delineate the significance of type 2 immunity as human host defense against *C. trachomatis* and other genital pathogens.

Materials and Methods

Mice. Mouse experiments were approved by Institutional Animal Care and Use Committees at The Ohio State University and the University of Pittsburgh. Experiments were performed per institutional guidelines and in accordance with the *Guide for the Care and Use of Laboratory Animals* (65). As indicated, 6- to 10-wk-old female BALB/cJ, BALB/c-Il4^{tm2Nnt}/J (IL-4$^{-/-}$), BALB/c-Il4ra^{tm1Sz}/J (IL-4Rα$^{-/-}$), C.129P2(B6)-Il10^{tm1Cgn}/J (IL-10$^{-/-}$), C.129-Il4^{tm1Lky}/J (4get), C.129S2-Stat6^{tm1Gru}/J (STAT6$^{-/-}$), and C.129S1(B6)-Gata1^{tm6Sho}/J (ΔdblGATA1) mice (all from Jackson Laboratories) were housed in specific pathogen-free conditions before use. To generate bone marrow chimera mice, bone marrow was transferred from female WT BALB/cJ or IL-4Rα$^{-/-}$ mice into lethally irradiated female WT or IL-4Rα$^{-/-}$ mice as described (66), but with the modifications that mice were reconstituted with 5×10^6 bone marrow cells and used 2 mo after reconstitution.

***C. trachomatis* Infection.** Because mouse susceptibility to genital *C. trachomatis* infection is estrus cycle stage-dependent (67), mice were s.c. injected with 1 mg of depot-medroxyprogesterone acetate (DMPA) (Upjohn) 5 d before ivag infection. This dose produces pharmacologically relevant serum MPA levels (68) and ensures productive infection (12). When uninfected mice were used in experiments, they were similarly treated with DMPA. Murine ivag infection with 10^4 inclusion-forming units (IFUs) of oculogenital strain UW-3/Cx of *C. trachomatis* serovar D (ATCC VR885) (American Type Culture Collection) was performed as described (12). This infectious inoculum is consistent with *C. trachomatis* levels detected in human biological specimens (69). At various time points after infection, IFUs were measured as described (70).

Adenoviral Vector Administration and Confocal Microscopy. The adenoviral type 5-derived E1- and E3-deleted replication-deficient vectors expressing eGFP (Ad.eGFP) or murine IL-4 (Ad.IL-4) used in our studies are described in detail (26). Briefly, as DMPA impairs genital mucosal barrier protection and increases susceptibility to genital viral infection (71), mice received 1 mg of this synthetic progestin 5 d before genital mucosal administration of any adenoviral vector. For in vivo transduction using adenoviral vectors, DMPA-treated mice were intrauterinely administered 10^{10} viral particles of indicated adenoviral vector via a nonsurgical embryo transfer device (NSET) (ParaTechs Corporation). Ivag administration of Ad.eGFP did not induce significant protein expression in the murine endometrium (Fig. S8). To define eGFP expression after Ad.eGFP ivag or intrauterine administration, genital tissues were harvested 1 d later, fixed with 4% methanol-free formaldehyde (Thermo Fisher Scientific), and embedded in 6% low melting point agarose (Life Technologies). Two hundred fifty-micrometer sections were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) solution in PBS, stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich), embedded in mounting media (Vector Laboratories), and evaluated for reporter gene expression with a FV1000 spectral confocal microscope system (Olympus). As shown in Fig. S8, intrauterine Ad.eGFP was the only administration route that provided significant transduction of eGFP in the murine endometrium. To evaluate the effect of adenoviral-mediated transduction of eGFP or IL-4 on in vivo ESC proliferation, adenoviral vector-treated mice were killed 3 d after administration of recombinant adenovirus, and whole UGTs were excised for histopathological and immunohistochemical analysis (described in detail in *SI Materials and Methods*).

Detailed descriptions of methods used for *C. trachomatis* quantification, histopathologic and immunohistochemical analysis, flow cytometric analysis, ESC isolation and evaluation of ESC function, RNA gene expression analysis, and statistical considerations are in the *SI Materials and Methods*.

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