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J Immunol published online 16 August 2013
http://www.jimmunol.org/content/early/2013/08/16/jimmunol.1301136

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/08/20/jimmunol.1301136.DC1

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**CD4⁺CD25⁺Foxp3⁺ Regulatory T Cells Promote Th17 Responses and Genital Tract Inflammation upon Intracellular Chlamydia muridarum Infection**

Jessica M. Moore-Connors,*,† Robert Fraser,‡,§ Scott A. Halperin,*,† Scott A. Halperin,‡,§,¶ and Jun Wang*,§,¶,∥

The functional role of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) in host responses to intracellular bacterial infection was investigated in an in vitro coculturing system and a murine model of *Chlamydia muridarum* genital tract infection. Remarkably, *C. muridarum* infection subverted the immune suppressive role of CD4⁺CD25⁺Foxp3⁺ Tregs; instead of hampering immune responses, Tregs not only promoted Th17 differentiation from conventional CD4⁺ T cells but also themselves converted into proinflammatory Th17 cells in both in vitro and in vivo settings. Anti-CD25 mAb PC61 treatment to deplete hosts has a major impact on the development responses, Tregs not only promoted Th17 differentiation from conventional CD4⁺ T cells but also themselves converted into proinflammatory Th17 cells in both in vitro and memory phases. Most importantly, Treg-depleted mice displayed significantly attenuated inflammation, neutrophil infiltration, and reduced severity of oviduct pathology upon infection, neutrophil infiltration, and reduced severity of oviduct pathology upon infection with *C. muridarum* genital tract infection to our knowledge, this is the first report demonstrating that the level of pre-existing CD4⁺CD25⁺Foxp3⁺ Tregs in genital infection markedly reduced the frequency and the total number of Th17 but not Th1 CD4⁺ cells at both immune induction and memory phases. In addition, inappropriate CD8⁺ T cell responses (particularly TNF-α-producing CD8⁺ T cells) and production of proinflammatory cytokines downstream of TLR2, such as IL-1β, have also been demonstrated to play important roles in oviduct pathology in mice (7–10). However, a comprehensive understanding of the vital mechanisms controlling *Chlamydia*-caused pathological sequelae remains lacking.

**Chlamydia trachomatis** is an intracellular bacterial pathogen that infects mammalian hosts by targeting mucosal epithelial cells lining the ocular, respiratory, and genital tract (GT). At these various mucosal sites, *Chlamydia*‐caused infections commonly result in chronic inflammation, tissue damage, and fibrosis due to repeated and/or persistent *Chlamydia* infection (1, 2). In the case of *C. trachomatis* GT infection, the most common bacterial sexually transmitted infection, serious sequelae such as pelvic inflammatory disease, ectopic pregnancy, and tubal factor infertility are the primary clinical complications identified in a portion of *Chlamydia*-infected individuals (1, 2). Mouse models of GT infection with *C. muridarum* closely mimic both acute and chronic phases of *Chlamydia* infection in women and have been extensively used in research. A reliable surrogate marker for fibrotic oviduct occlusion and infertility in mice is the development of hydrosalpinx, which is characterized by accumulation of inflammatory exudates and oviduct dilation (3). Histological or cytological examinations of samples collected from mice and human subjects have revealed a profound chronic accumulation of neutrophils associated with *Chlamydia* infections (3–6). Experimental blockade or attenuation of neutrophil infiltration during *C. muridarum* infection results in significantly reduced tissue damage and oviduct pathology without delaying bacterial clearance (4, 6), indicating that prolonged neutrophilic inflammation is an important pathological host response to *Chlamydia* infection. In addition, inappropriate CD8⁺ T cell responses (particularly TNF-α-producing CD8⁺ T cells) and production of proinflammatory cytokines downstream of TLR2, such as IL-1β, have also been demonstrated to play important roles in oviduct pathology in mice (7–10). However, a comprehensive understanding of the vital mechanisms controlling *Chlamydia*-caused pathological sequelae remains lacking.

T cell–mediated immunity is essential in host defense against *Chlamydia* infection and has a role in mediating *Chlamydia*-induced inflammation (1, 2, 8, 9). Among the different CD4⁺ Th responses including Th1, Th2 and Th17, IFN-γ–producing CD4⁺ Th1 cells are the primary response induced in mouse models of *Chlamydia* infection and have a well-established protective role. Mice deficient in IFN-γ or IFN-γ receptors are unable to clear genital *C. muridarum* infection and display massive inflammatory responses in the GT (11, 12). Recent work has demonstrated that CD4⁺ Th17 responses are also induced by *Chlamydia* infection (13, 14). Th17 cells are differentiated from conventional αβ CD4⁺ T cells in local draining lymph nodes under the influence of a cytokine milieu containing the immunosuppressive cytokine TGF-β and proinflammatory cytokines IL-1β, IL-6, and IL-23 (15). IL-17A and IL-17F, the major effector cytokines of Th17 cells, are proinflammatory cytokines with potent biological activity in neutrophilic responses via stimulating the production of cytokines and chemokines involved in neutrophil generation, maturation, and mobilization to sites of inflammation (15–17). IL-
17A also promotes the optimum priming of Th1 responses to *C. muridarum* by upregulating dendritic cell (DC) expression of co-stimulatory molecules, class II MHC molecules, and IL-12p70 (13, 18). The role of IL-17/Th17 responses in *Chlamydia* infection is complex. Delivery of neutralizing anti–IL-17A Ab before or shortly after respiratory *C. muridarum* infection markedly attenuates bacterial clearance (13, 19), indicating that IL-17A production is critically required for host defense against *Chlamydia*. However, *Chlamydia*-susceptible mouse strains BALB/c and C3H/HeN are shown to have higher levels of IL-17/IL-17 receptor profiles than *Chlamydia*-resistant C57BL/6 mice (20, 21). Collectively, these studies suggest that IL-17/Th17-mediated responses to *C. muridarum* represent a double-edged sword that can promote bacterial clearance but also potentially damage host tissue when improperly controlled. Elucidating immune mechanisms that effectively regulate IL-17/Th17 responses during *Chlamydia* infection may shed the light on the pathogenesis of *Chlamydia*-associated diseases.

CD4+CD25+Foxp3+ regulatory T (Treg) cells represent 5–10% of peripheral CD4+ T cells in normal hosts and are well documented to prevent autoimmunity by suppressing the activation of autoreactive lymphocytes (22). In addition to their critical immunosuppressive role in self-tolerance and immune homeostasis, accumulating evidence indicates that Tregs also mediate host–pathogen interactions (23). Tregs are classically known to inhibit a wide range of immune responses including proliferation and cytokine production by CD4+ and CD8+ effector T cells, and this suppressive activity has been demonstrated to protect hosts from immunopathology and tissue damage associated with vigorous pathogen-directed immune responses (23, 24). However, Treg-mediated suppression can also facilitate persistent infections by dampening the induction of effective antimicrobial immunity (23, 24). Tregs have been ascribed both beneficial and detrimental roles during infection depending on the nature of the infectious agent (e.g., parasitic, viral, fungal, or bacterial) and/or whether the infection is acute or chronic (23, 24). Emerging evidence suggests that the presence of TLR ligands, inflammatory mediators, and abundant survival cytokines during the acute infection may subvert Treg suppressive function (25, 26). In contrast to having a suppressive role, Tregs have been shown to promote de novo generation of Th17 cells in the presence of TLR ligands in vitro and in vivo (25, 26). More recently, Tregs were demonstrated to enhance host resistance to mucosal fungal infection via the promotion of Th17 responses (27). Although several murine and human studies report that *Chlamydia* infection induces expansion of Foxp3+ Tregs (28–30), our understanding about the role of Tregs during *Chlamydia* infection remains rudimentary.

Given the importance of CD4+ Th responses in *Chlamydia* infection and the known ability of Tregs to mediate effector responses, we investigated the role of Tregs in regulating host responses to *C. muridarum* infection within an in vitro coculture system and in vivo model of GT infection. In this study, we have explicitly demonstrated that Tregs have a divergent role in controlling the induction and differentiation of CD4+ Th responses upon *C. muridarum* infection. Although Tregs had no obvious role in regulating protective Th1 responses, they served as a prominent inducer of and direct contributor to IL-17/Th17 responses during *C. muridarum* infection, which, in turn, facilitated the development of *Chlamydia*-induced immunopathology in vivo.

**Materials and Methods**

**Mice**

Six- to 10- wk-old female C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). Congenic C57BL/6 CD45.1 mice and IL-6-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Foxp3-IREs-GFP knockin (Foxp3-GFP) mice on the C57BL/6 background were kindly provided by Dr. Mohamed Oukka (Harvard Medical School, Boston, MA). MyD88-deficient mice were obtained from Dr. Jean Marshall (Dalhousie University, Nova Scotia, Canada) with the permission from Dr. Shizuo Akira (Osaka University, Osaka, Japan). Mice were housed at the IWK Health Centre animal facility under pathogen-free conditions. All animal procedures were approved by the Ethics Committee according to the Canadian Council for Animal Care guidelines.

**Anti-CD25 mAb (PC61) preparation**

Anti-CD25 mAb (rat IgG1, clone PC61) was purified by sequential ammonium sulfate precipitation (50 then 33%) from supernatants of the TIB222 hybridoma culture (American Type Culture Collection, Manassas, VA). After precipitation, the pellet was dissolved in LPS-free PBS and extensively dialyzed against LPS-free PBS solution. The concentration of mAb was determined by bicinechonic acid assay (Thermo Scientific) using purified rat IgG (Sigma-Aldrich, Oakville, Ontario, Canada) as a standard. The LPS content was determined by Limulus amebocyte lysate test (Associates of Cape Cod, East Falmouth, MA). The mAb preparation that had the LPS content <1 endotoxin unit/µg was used for in vivo study. The functional activity of in-house–produced PC61 mAb was confirmed by comparing to commercial product from eBioscience, and the dose of PC61 was determined based on 50% in vivo Treg-depletion rate.

**C. muridarum GT infection**

The mouse pneumonitis biovar *C. muridarum* was propagated in McCoy cells (American Type Culture Collection) according to procedures described previously (21, 21). The infectious elementary bodies were purified by discontinuous density gradient centrifugation using 30% Isovue-370 (Bracco Diagnostics, Princeton, NJ) and 50% sucrose (Sigma-Aldrich). Ten and 3 d prior to infection, mice were injected s.c. with 2.5 mg Depo-provera (Pfizer Canada, Kirkland, Quebec, Canada) to synchronize the estrous cycle. To deplete preexisting Tregs in vivo, ~300 µg PC61 anti-CD25 mAb was administered s.c. in 300 µl sterile PBS 1 d prior to intravaginal infection with *C. muridarum*. Purified rat IgG (Sigma-Aldrich) was used as a control in some experiments. For infection, mice were anesthetized by i.p. injection of ketamine (75 mg/kg) and xylazine (15 mg/kg) and then inoculated intravaginally with 1 × 106 inclusion-forming unit *C. muridarum* in 10 µl sucrose-phosphate–glutamic acid buffer. To monitor bacterial shedding, mice were swabbed vaginally using calcium alginate swabs (Fisher Scientific) at various time points following infection. Swabs were placed in 500 µl sucrose-phosphate–glutamic acid buffer, briefly vortexed with 4-mm sterile glass beads, and rotated at 4˚C for 1 h as described (31). The swabs were then extracted, and the remaining fluid was frozen at −80˚C for bacterial load quantification by quantitative PCR (21).

**Quantitative PCR analysis to determine in vivo *C. muridarum* burden**

To quantify the bacterial burden recovered from vaginal swabs of *C. muridarum*-infected mice, total nucleic acid from 500 µl each swab sample was extracted using DNAzol (Invitrogen, Oakville, Ontario, Canada) according to the manufacturer’s instructions. The level of bacterial burden in each sample was determined by quantitative PCR as previously described (21) with *Chlamydia*-specific primers for 16S rRNA using SYBR Green Supermix (Qiagen) in a 7900HT fast real-time PCR machine (Applied Biosystems, Foster City, CA). Bacterial copy number was calculated using known copy numbers of *C. muridarum* DNA standards extracted from a purified *C. muridarum* preparation using the same procedure and is expressed as log10 value of copies of 16S rRNA per milliliter (21).

**Generation of bone marrow–derived DCs**

To collect bone marrow, the femurs and tibia of naive mice were flushed with RPMI 1640 containing 5% bovine serum (BS). RBCs were lysed by incubation in ACK (0.1 mM EDTA, 0.15 M NH4Cl, and 1 mM KHCO3) buffer for 5 min at room temperature. At day 0, 3 × 106 cells were seeded per 100-mm dish in 10 ml R10 medium (RPMI 1640 supplemented with 10% heat-inactivated FBS [Fisher Scientific], 2 mM l-glutamine [Fisher Scientific], 50 µM 2-ME [Fisher Scientific], 100 U/ml penicillin [Fisher Scientific], streptomycin [Fisher Scientific], and 20 ng/ml [200 U/ml] recombinant mouse GM-CSF [R&D Systems]). Cells were fed with 10 ml R10 medium at day 3. On day 6, loosely adherent cells were used as the source of DCs.

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In vitro co-culture system

For setting up in vitro suppression assay, Tregs (CD4+Foxp3-GFP+) and conventional T cells (Tconv; CD4+Foxp3-GFP−) were sorted from splenocytes of Foxp3-GFP mice using an FACSARia cell sorter (BD Biosciences, San Jose, CA) with purity >94%. Alternatively, Tregs (CD4+CD25+ and Tconv (CD4+CD25−) were sorted from naive splenocytes by CD4 enrichment followed by CD25-positive selection using MACS (Miltenyi Biotec) according to the manufacturer’s instructions. The purity of both populations was consistently >90%. Cells were then cultured in 96-well plates in 200 μl volume containing 2 × 10^4 bone marrow–derived DCs (BMDCs) and 1 × 10^5 sorted naive Tconv with or without titrated ratios (1:1, 1:2, 1:5, or 1:10) of Tregs. To stimulate DCs prior to in vitro coculture experiments, 2 × 10^5 BMDCs were seeded in 96-well plates in 50 μl volume and incubated with media, live C. muridarum (multiplicity of infection 1.0), or LPS (100 ng/ml) for 24 h. Tconv and/or Tregs were then added directly to wells containing BMDCs and stimulated with soluble anti-CD3 (1 μg/ml) for 48–72 h at 37°C 5% CO2. To assess proliferation, Tconv were first labeled with 5 μM fluor670 (eBioScience, San Diego, CA) according to the manufacturer’s instructions, and cellular divi-
sion was determined by flow cytometric analysis following 72 h of co-
culture. In some experiments, purified Tregs alone were cultured with BMDCs and stimulated with soluble anti-CD3 (1 μg/ml) in the presence of IL-2 (10 ng/ml) for 72 h prior to conducting intracellular IL-17A staining.

In vitro restimulation of cells from C. muridarum–infected mice

Single-cell suspensions of spleen and iliac lymph nodes (ILN) were prepared by mechanical disruption and gentle pipetting through 40-

μm cell strainer and washed with RPMI containing 2 mg/ml collagenase II and incubated at 37°C for 20 min. The suspension was then passed through a 40-μm cell strainer and washed with RPMI containing 10% foetal bovine serum (FBS). Cells were cultured in 96-well plates in RPMI 1640 medium containing 10% FBS, 50 U/ml penicillin, 50 g/ml strepto-
mycin, and 2 mmol/LL-glutamine. Cells were heat inactivated by 30 min incubation at 65°C and used as heat-killed crude C. muridarum for 72 h at 37°C. The culture supernatants were used for detecting cytokine amounts of Tregs. Without infection, Tregs sup-
pressed DCs versus noninfected DCs on effector CD4+ T cell proliferation and Th cytokine production in the absence or presence of various amounts of Tregs. Without C. muridarum infection, Tregs sup-
presed Tconv proliferation in a classic dose-dependent manner, with ~50% reduced proliferation at 1:1 ratio of Treg/Tconv compared to Tconv alone (Fig. 1A). Although C. muridarum infection markedly enhanced the overall magnitude of CD4+ T cell proliferation (Fig. 1A), Tregs still displayed a comparable pattern of dose-
dependent suppression in the presence of infection (Fig. 1A). However, substantial differences were observed in CD4+ Th cyto-

kine profiles in this coculture system. Tregs were able to inhibit IFN-γ production in a dose-dependent manner under noninfected conditions, but this inhibitory activity was abolished upon C. mur-
idarum infection (Fig. 1B). In comparison, IL-4 production in the culture supernatants were significantly suppressed by increased densities of Tregs, and C. muridarum infection only altered their overall magnitudes of production (Fig. 1C). In sharp contrast to Th1/ Th2 cytokine profiles, IL-17A production was induced by increasing densities of Tregs and further amplified by C. muridarum infection (Fig. 1D). Using Tregs and Tconv from CD45.2 and CD45.1 congenic C57BL/6 mice, respectively, intracellular IL-17A staining clearly revealed IL-17A–positive staining in Tconv (CD4+CD45.2+). The Th17 frequency was increased from 0.1% of Tconv in the co-
culture with noninfected DCs to 1.2% with C. muridarum–infected DCs and further increased substantially to 6.6% in the presence of Tregs (Fig. 1E). These results clearly demonstrate divergent roles of Tregs in modulating CD4+ Th responses to C. muridarum infection in vitro: upon C. muridarum infection, Tregs lost their regulatory capacity over Th1 responses while serving as a suppressor of Th2 responses and a promoter for Th17 responses.
MyD88-dependent signals in both DCs and Treg themselves are required for Tregs to convert into Th17 cells during *C. muridarum* infection in vitro

We noticed a clear IL-17A–positive population of Tregs (CD4+ CD45.2+) in our coculture system (Fig. 1E). To further verify that IL-17A was not produced by any contaminating Tconv in the Treg preparation, we sorted Tregs from Foxp3-GFP mice and directly stimulated these Tregs with anti-CD3 in the presence of noninfected DCs, *C. muridarum*–infected DCs, or LPS-treated DCs. As shown in Fig. 2A, both LPS and *C. muridarum*–treated DCs stimulated clear IL-17A production from Tregs, with ∼4% of Tregs becoming IL-17A+Foxp3-GFP+. Because Tregs express a spectrum of TLRs and the engagement of TLRs has been reported to directly modulate Treg function (26, 32, 33), we wondered whether IL-17A production by Foxp3+ Tregs is a direct or indirect effect of TLR signaling in Tregs. To this end, we used Tregs and DCs derived from C57BL/6 mice or MyD88-deficient mice and set up a combinatorial coculture system. We found that the absence of MyD88-dependent signaling in either Tregs or DCs completely abolished IL-17A production upon *C. muridarum* infection or LPS stimulation (Fig. 2B). Accordingly, we only detected little to no proinflammatory cytokines such as IL-6, IL-1β, and TNF-α in MyD88-deficient DC cultures upon *C. muridarum* infection (Fig. 2C). Taken together, our in vitro results suggest that Tregs are not only a potent inducer of Th17 differentiation but are themselves a significant contributor to Th17 populations responding to *C. muridarum* infection. Moreover, the process of Treg to Th17 conversion requires activation of MyD88-dependent signaling pathways in both DCs and Tregs.

*C. muridarum* infection induces expansion of Tregs that precedes Th17 differentiation in vivo

Given the potent role of Tregs in promoting Th17 differentiation from both Tconv and themselves upon *C. muridarum* infection in vitro, we next examined the kinetics of Foxp3+ cells and Th17 responses at immune induction sites (i.e., the draining ILN and spleen) as well as the genital infection site during *C. muridarum* GT infection in Foxp3-GFP mice. We observed a rapid expansion of Foxp3+CD4+ cells in the ILN as early as day 3 postinfection, which continued to increase at day 5 and returned back to naive levels by day 10 postinfection (Fig. 3B). Consistent with our in vitro data, in addition to conventional CD4+ Th17 cells, a portion of IL-17A–producing CD4+ T cells were also Foxp3+Foxp3- (Fig. 3C, 3D). Unlike the early expansion of Foxp3+Foxp3- Tregs, however, both Foxp3+Foxp3- and Foxp3+Foxp3- Th17 populations as well as total CD4+ T cells were not significantly increased relative to naive mice until day 5 postinfection (Fig. 3A, 3C). Thus, it appears that the expansion of Tregs precedes the induction of Th17 response in vivo and, in conjunction with our in vitro observation, suggests that a certain threshold for the number or...
relative density of Tregs over Tconv may be required to effectively induce Th17 responses to \textit{C. muridarum} infection. The kinetics of Tregs and Th17 responses in the spleen appeared to concurrently peak at day 5 postinfection (Fig. 3D). Contrary to the lymphoid organs, the number of total CD4+ T cells in the GT was very low in naive mice and gradually increased, peaking by day 10 post-infection. Along with increased numbers of effector CD4+ T cells, both Tregs and Th17 cells accumulated in the infected GT with comparable frequencies throughout infection. In comparison, the Foxp3-GFP+IL-17+ double-positive Th17 cells were present in the GT at a very low level (Fig. 3D).

PC61 treatment prior to \textit{C. muridarum} GT infection significantly attenuates Th17 responses without affecting Th1 responses in vivo

The rapid expansion of CD4+Foxp3+CD25+ Tregs in the ILN upon \textit{C. muridarum} GT infection led us to hypothesize that the majority of these expanded Tregs originate from pre-existing endogenous Tregs and that their frequency may directly affect the magnitude of Th17 responses to \textit{C. muridarum} infection. To test this hypothesis, we used PC61 anti-CD25 mAb to deplete pre-existing Tregs in naive mice 24 h prior to \textit{C. muridarum} GT infection. We found that PC61, but not control rat IgG, reduced the frequency of pre-existing Foxp3+ cells in peripheral blood by ~40 and >50% in ILN 24 h postinjection, and Treg levels remained low in PC61-treated mice until day 13 postinjection (Fig. 4A). Although PC61 treatment has been previously reported to deplete recently activated CD4+ Tconv (34), the total number of CD4+Foxp3− Tconv was markedly induced in the ILNs of both PC61-treated mice and control mice at day 5 postinfection compared with their naive counterparts, and the level of CD4+Foxp3+ Tconv induction was perhaps even greater in the PC61-treated group (Fig. 4B, 4C). In contrast, the total number of CD4+Foxp3+ Tregs in PC61-treated mice was ~50% of that observed in control mice, both naive and at day 5 postinfection (Fig. 4B, 4C). PC61 treatment in our model therefore resulted in prominent depletion of CD4+Foxp3+ Tregs with no apparent effect on CD4+Foxp3+ Tconv. In parallel with the reduced frequency of Tregs in PC61-treated mice, we observed a significantly reduced percentage and total number of Th17 cells at day 5 postinfection in the ILN of PC61-treated mice compared to control (Fig. 4D, 4E). In contrast to the Th17 profile, the frequency and total number of Th1 cells were not altered by PC61 treatment at the same time point. Although no difference in Th1 or Th17 profiles was observed between groups at day 10 postinfection, PC61 treatment did result in significantly lower levels of Th17 memory responses in the spleen at day 46 postinfection (Fig. 4F). Taken together, our in vitro and in vivo data collectively demonstrate a prominent role for Tregs in the regulation of Th17, but not Th1, responses to \textit{C. muridarum} infection.

PC61 treatment prior to \textit{C. muridarum} genital infection markedly reduces oviduct pathology without altering the level of bacterial shedding in the GT

Having characterized the role of Tregs in controlling different CD4+ Th responses, we further examined the impact of PC61 treatment in bacterial clearance and inflammatory responses during \textit{C. muridarum} infection. Remarkably, PC61-treated mice displayed less acute inflammatory infiltration in the oviduct (pyosalpinx) by
day 10 postinfection and significantly reduced oviduct dilation (hydrosalpinx) by day 46 postinfection (Fig. 5A, 5B). Furthermore, the incidence of grossly apparent hydrosalpinx in PC61-treated versus control mice was ∼16 and ∼57%, respectively (n = 6 to 7 mice/group) at day 46 postinfection. In sharp contrast to apparent amelioration of acute-phase inflammation and chronic-phase oviduct pathology in PC61-treated mice, the level of bacterial shedding was comparable between groups throughout infection (Fig. 5C), indicating that Treg-mediated immune mechanisms involved in the development of oviduct pathology have no obvious role in controlling the genital bacterial shedding.

To further understand the impact of PC61-mediated Treg depletion on acute-phase inflammation in the GT, we performed UL to characterize cells infiltrating the uterus. PC61-treated mice had significantly fewer overall cells recovered by UL compared with control mice at days 4 and 7 postinfection (Fig. 5D). The majority (~90%) of UL cells from control mice at day 4 postinfection had a CD11b+Gr1+ neutrophil phenotype, whereas only ~66% of UL cells from PC61-treated mice were CD11b+Gr1+, which translated into an ~5-fold reduction in the absolute numbers of neutrophils in UL samples from PC61-treated mice (Fig. 5E). Immunohistochemical anti-Gr1 staining also revealed a significantly reduced number of neutrophils in PC61-treated mice compared with controls at day 10 postinfection (Fig. 5F). Consistent with a well-established role of IL-17/Th17 responses in stimulating production of neutrophil chemoattractants such as CXCL2 to mobilize neutrophils, IL-17A and CXCL2 levels in GT homogenates were positively correlated at day 5 postinfection (Fig. 5G), and soluble IL-17A levels in PC61-treated mice were significantly lower compared with control mice (Fig. 5H). Collectively, these data suggest that modulation of IL-17/Th17 is a potential mechanism through which PC61-mediated Treg depletion attenuated neutrophilic inflammation during C. muridarum infection.

**Discussion**

In this study, we have demonstrated that Tregs have a divergent role in controlling CD4+ Th responses during intracellular C. muridarum GT infection. In contrast to their inability to control Th1 responses to C. muridarum in vitro and in vivo, Tregs displayed a novel innate-like property by potently promoting Th17 differentiation from Tconv and by themselves converting into IL-17 producers. Correspondingly, in vivo depletion of pre-existing Tregs PROMOTE CHLAMYDIA-INDUCED Th17 AND TISSUE INFLAMMATION

**FIGURE 3.** Foxp3+ Treg expansion precedes Th17 differentiation in draining lymph nodes upon C. muridarum genital infection. Foxp3-GFP mice were intravaginally infected with C. muridarum. The absolute number of CD4+ T cells and CD4+Foxp3+ Tregs (n = 4–10 mice/time point) (A, B) and the absolute numbers of Foxp3+ and Foxp3− Th17 cells (n = 3–5 mice/time point) (C) in the ILN at days 0, 3, 5, 10, and 20 postinfection were determined by FACS. Data are presented as mean ± SEM and are pooled data of two independent experiments. (D) Representative dot plots of Foxp3-GFP expression and IL-17A staining on CD4-gated cells in the ILN, spleen, and GT. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 versus day 0 using one-way ANOVA test. p.i., Postinfection.
Tregs by PC61 anti-CD25 mAb resulted in diminished Th17 responses to *C. muridarum* GT infection. Consistent with reduced IL-17/Th17 responses, PC61-treated mice displayed striking amelioration of neutrophil infiltration and oviduct pathology in GT tissue relative to control mice, despite comparable bacterial shedding throughout the acute and chronic phases of infection.

Tregs have previously been correlated with increased Th17 levels in various models of inflammation (35–38), but the relevance of Tregs in the induction of Th17 responses to intracellular bacterial infection is unclear. In this study, we have demonstrated a prominent role for Tregs in promoting Th17 responses during *C. muridarum* infection in vitro and in vivo. Mechanistically, Tregs may simply serve as a cellular source of TGF-β, a key Th17-promoting cytokine, to promote conventional Th17 differentiation under the influence of a proinflammatory cytokine-enriched environment upon infection (26). Several Th17-promoting proinflammatory cytokines such as IL-6, IL-1β, and IL-23 were readily detected in *C. muridarum*-infected DC in a dose-dependent manner (Supplemental Fig. 1). Accordingly, IL-17 production by Tregs and Tconv was markedly reduced in in vitro cocultures using IL-6-deficient DCs compared with the wild-type control DCs upon *Chlamydia* or LPS stimulation, highlighting a critical role for proinflammatory cytokines such as IL-6 in promoting Th17 responses by Tregs (Supplemental Fig. 2). However, recent work indicates that Tregs are dispensable as a source of TGF-β for Th17 differentiation in vitro and in vivo (36, 39). Alternatively, Tregs may promote Th17 development by virtue of constitutive high CD25 (IL-2Rα) expression to actively consume IL-2 (36). Tregs form stable, long-lasting associations with lymph node DCs in vivo (40), and the high density of Tregs residing in proximity of Tconv can deplete IL-2 availability to activated Tconv, thereby facilitating Th17 development because IL-2–activated STAT5 inhibits IL-17A production (36, 41). In agreement with our observation, a recent study also demonstrated that reducing Treg numbers in vivo via PC61-mediated Treg depletion led to significantly decreased Th17 responses to *C. albicans* infection and reduced neutrophil influx into the infected mucosa (27). Notably, although reduced Th17 responses and attenuated neutrophil mo-
bilitation ameliorated immunopathology during C. muridarum infection, these cellular changes severely impaired host resistance to C. albicans infection, indicating that the Th17-promoting activity of Tregs can lead to completely disparate biological outcomes depending on the specific pathogen.

Of importance, a significant portion of Th17 cells induced by C. muridarum infection were Foxp3+IL-17A+ and indeed originated from Tregs themselves. Consistent with this observation, Foxp3+IL-17A+ cells have also been observed in vivo during C. albicans and Leishmania major infections (27, 42). Our data suggest that the process of Treg to Th17 conversion requires activation of signaling pathways in both DCs and Tregs that are mediated by MyD88, a common adaptor protein shared by TLR and IL-1 signaling pathways (43). It is conceivable that the MyD88–TLR pathway in DCs is required to create proinflammatory cytokine environment to initiate the conversion process; however, whether the MyD88–TLR pathway and/or MyD88–IL-1 pathway in Tregs themselves are involved in Treg to Th17 conversion is unclear. On one hand, TLR2 ligation has been shown to directly promote Treg proliferation and Th17 conversion by human Foxp3+ Tregs (44, 45). On the other hand, the MyD88–IL-1 pathway has also been shown to potently promote Th17 conversion from Tregs due to the high level of IL-1R expression by Tregs (25). Regardless of how Tregs convert into Th17 cells, the Foxp3+IL-17A+ population observed in C. muridarum–infected mice was most prominent in the primary immune induction site (i.e., the ILN), but not peripheral genital infection site, which supports the idea that these double-positive cells represent a transitional stage of Foxp3+ Tregs converting to Th17 (46). As such, it remains to be determined whether and how Treg-derived Th17 cells contribute to Th17-mediated inflammation during Chlamydia infection in vivo.

Tregs are generally viewed as a suppressor of Th1 immune responses. Nonetheless, Treg depletion prior to infection in our model did not significantly alter Th1 responses to C. muridarum infection. Th1-polarizing and -promoting cytokines IL-12p70 and IL-27 were detected in supernatants from C. muridarum–infected DCs (Supplemental Fig. 1); thus, C. muridarum–infected DCs might subvert the immunosuppressive functions of Tregs by providing a cytokine environment that favors the induction of Th1 responses. Consistent with our observation, Treg depletion in other intracellular bacterial infection or immunization models using Mycobacterium bovis bacillus Calmette-Guérin has also been shown to have very limited impact on Th1 responses (47, 48). However, in an experiment in which we adoptively trans-
ferred additional Tregs into the host 24 h prior C. muridarum infection, we indeed observed a significant reduction of Th1 responses in these mice in ILN at day 5 postinfection compared with control mice. Accordingly, the effect of Tregs in promoting Th17 was delayed to day 18 postinfection (Supplemental Fig. 3). Therefore, the level of Tregs present in the host at the moment of C. muridarum infection dictates the overall kinetics of Th1 and Th17 responses. The observed inability of Tregs to regulate Th1 responses in our setting and some other studies may be due to a relatively low level of endogenous Tregs in C57BL/6 mice (49). Overall, it is conceivable that Tregs have divergent roles as a suppressor of Th1 and promoter of Th17 responses.

In this study, we have employed the widely used strategy of PC61 anti-CD25 mAb treatment to deplete CD4+Foxp3+ Tregs prior to infection in vivo. PC61 has been shown to cause a significant reduction in Foxp3+ Tregs by targeting these cells for elimination by phagocytes via Ab-dependent cellular cytotoxicity (50). Although PC61 does not result in complete depletion of Foxp3+ Tregs, the partial elimination achieved by this method has been found to adequately alter the immunological balance in order to examine the role of Tregs (50). The ability of PC61 to deplete activated effector T cells has been the major concern for using this Ab. However, recent studies have demonstrated that the impact of PC61 on non-Tregs varies from model to model depending on the nature of immune responses (51). In our model, we did not see any impact of PC61 treatment on the number of Tconv; rather, depletion was restricted to the Foxp3+ Treg compartment. Importantly, anti-CD25 mAb has actually been speculated to enhance Th17 differentiation by binding IL-2R on the surface of activated Tconv and blocking IL-2 signaling. However, we and others (27, 52) indeed observe PC61 to have the opposite effect on Th17 responses. Thus, we conclude that PC61 did not significantly target CD25 on Tconv in our study and, instead, exerted its effect mainly by depleting Foxp3+ Tregs. With this approach, our data for the first time, to our knowledge, suggest that a higher level of pre-existing CD4+Foxp3+ Tregs in Chlamydia-infected hosts may predispose the development of Chlamydia-associated diseases. This notion may help conclude some observations in the field. For instance, Chlamydia-resistant C57BL/6 mice indeed carry significantly fewer endogenous Tregs relative to Chlamydia-susceptible BALB/c mice (49). Furthermore, TLR2-deficient mice, which have ~50% fewer endogenous Tregs than their wild-type C57BL/6 counterparts (45), also develop significantly less oviduct pathology during C. muridarum infection compared with wild-type mice, despite having comparable courses of infection (7). A recent follow-up study indicated that IL-1β, a Th17-promoting cytokine, is the key molecule downstream of TLR2 responsible for this phenomenon (10). In our view, therefore, the magnitude of Treg responses dictates the intensity of Th17 responses as well as the extent of acute and chronic inflammation in the GT. Although high levels of IL-17A are strongly implicated in tissue damage, IL-17A extent of acute and chronic inflammation in the GT. Although high

Disclosures
The authors have no financial conflicts of interest.

References


