

# Regulatory T cells are essential to promote proper CD4 T-cell priming upon mucosal infection

AG Soerens<sup>1,2</sup>, A Da Costa<sup>1,2</sup> and JM Lund<sup>1,2</sup>

Regulatory T cells (Tregs) limit autoimmunity and immunopathology using a variety of suppressive mechanisms, but their roles during pathogen-directed immune responses remain unclear. Following herpes simplex virus-2 (HSV-2) infection, mice lacking Tregs fail to control viral replication, pointing to a role for Tregs in facilitating productive immune responses. Using adoptive transfer of T-cell receptor transgenic CD4 T cells into Treg-sufficient or Treg-depleted mice prior to HSV-2 infection, we found that Tregs are required for timely accumulation of HSV-2-specific CD4 T cells within the infected tissues. Further, Tregs are critical for appropriate trafficking of dendritic cells (DCs) from the vaginal mucosa to the draining lymph nodes, which results in fully effective CD4 T-cell priming, activation, and ultimately migration to the infected tissues. Using CTLA-4 conditional knockout mice, we demonstrate that Tregs impact DC migration through a CTLA-4-mediated mechanism. Together, our data highlight the critical role of Tregs in proper potentiation of adaptive immune responses to microbial infection.

## INTRODUCTION

Regulatory T cells (Tregs) are a subset of CD4 T cells that are essential for maintaining peripheral tolerance,<sup>1,2</sup> yet their precise role during infections remains an active area of investigation.<sup>3-5</sup> In the context of several infections, Tregs are required during the immune response to prevent an overly robust response that causes excessive collateral damage to self-tissue. In these cases, when Tregs are absent during the infection, the immune response is more robust and able to clear the pathogen more quickly, albeit with the risk of elevated immunopathology.<sup>3,6-10</sup> Conversely, in other cases, the removal of Tregs prior to infection results in delayed clearance of the pathogen, suggesting that the presence of Tregs can be beneficial in facilitating an appropriately robust and protective immune response.<sup>11-14</sup> These differing results emphasize that the role played by Tregs during infections is context-dependent.

In the setting of intravaginal (ivag) infection with herpes simplex virus-2 (HSV-2), mice acutely depleted of Tregs suffer from a higher viral burden within the vaginal tissues. The virus also infects the central nervous system more quickly in Treg-depleted mice, causing significantly earlier death.<sup>12</sup> These observations are consistent with a diminished antiviral immune response rather than the overly robust immune response that

would be expected if the primary role of Tregs were to dampen the immune response and limit immunopathology. Consistent with a less effective immune response, Treg-depleted mice showed very early dysregulation of effector cell migration to the infected tissue.<sup>12</sup> However, because a wild-type (WT) HSV-2 infection is rapidly lethal to Treg-depleted mice, the effect of Treg depletion on the adaptive immune response to mucosal infection remains unclear.

In most infection models that have been studied, Tregs do not appear to have a major impact on the initiation of an antigen-specific T-cell response but rather modulate the size and intensity of the T-cell response that develops to target a potential pathogen. Early studies relying on infection with *Leishmania major*, *Pneumocystis carinii*, HSV-1, Friend virus, *Plasmodium yoelii*, and others all found that, when the immune response develops in the absence of Tregs, the resultant effector response is more robust and better able to target the pathogen. These studies collectively supported what has become the dominant model in the field; that Tregs are primarily important during an immune response to limit immunopathology by preventing an excessively robust effector response.<sup>6,7,10,15-18</sup>

In contrast, the adaptive immune response was less effective in mice lacking Tregs following intranasal infection with RSV

<sup>1</sup>Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA and <sup>2</sup>Graduate Program in Pathobiology, Department of Global Health, University of Washington, Seattle, Washington, USA. Correspondence: JM Lund (jlund@fhcrc.org)

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(respiratory syncytial virus) or oral infection with *Candida albicans*. In the case of RSV, CD8 T-cell migration to the infected tissue was delayed in the absence of Tregs, and in the case of *C. albicans*, the T-cell response was not skewed toward the expected T helper type 17 response when Tregs were absent.<sup>11,13,14</sup> In some models, Tregs also limit the engagement of low-affinity clones of CD8 T cells with antigen-bearing dendritic cells (DCs) so that, in the absence of Tregs, a greater share of low-affinity clones are activated.<sup>19</sup> Although these studies differed in their focus, and Tregs may affect both CD4 and CD8 T cells differently, they do suggest that Tregs can also affect qualitative aspects of adaptive immunity in certain contexts.

Despite the early changes that were observed in the immune response following ivag HSV-2 infection in Treg-depleted mice, how Tregs affect the adaptive immune response is not known because ivag infection with WT HSV-2 is quickly lethal to mice, especially those that lack Tregs at the time of infection. The dominant model in the field that Tregs primarily modulate the size and strength of the T-cell response suggests that, in the absence of Tregs, there should be more antigen-specific T cells primed and targeting the virus in the infected tissue. Thus, to investigate the role of Tregs during the adaptive immune response against ivag HSV-2, we used a well-characterized attenuated strain of HSV-2 that mimics the WT virus during the initial infection of epithelial cells but cannot productively infect neurons, allowing for the survival of infected mice.<sup>20</sup> To assess the role of Tregs during the adaptive immune response against attenuated HSV-2, we used *Foxp3<sup>DTR</sup>* mice. *Foxp3<sup>DTR</sup>* mice have been engineered to express the human diphtheria toxin receptor (DTR) under the control of the *Foxp3* promoter, thereby allowing for the targeted depletion of Tregs following administration of diphtheria toxin (DT).<sup>2</sup>

T-cell production of interferon- $\gamma$  (IFN $\gamma$ ) at the infected tissue is the primary mechanism of viral control in the HSV-2 mouse model,<sup>21</sup> with CD4 T cells being the most important cell population for viral control.<sup>21,22</sup> CD4 T-cell priming begins after antigen is carried to the draining lymph nodes (dLNs) by migratory CD11b<sup>+</sup> DCs originating in the infected tissue. Free virus does not travel to the dLNs; therefore, these migratory DCs are fully responsible for CD4 T-cell priming.<sup>23</sup> After priming, CD4 T cells begin entering the infected tissue starting at approximately day 4 and are most abundant 6 days after infection. CD8 T cells do not enter the tissue unless CD4 T cells have already done so, thereby further implicating CD4 T cells as critically essential for viral control.<sup>24</sup> In the infected tissue, inflammatory monocytes process viral antigen and induce IFN $\gamma$  production from antigen-specific T cells. This leads to a characteristic adaptive phase wave of IFN $\gamma$  in the infected vaginal tract beginning at 4 days postinfection.<sup>25</sup>

Using the model of attenuated HSV-2 infection in combination with HSV-2 specific T-cell receptor (TCR) transgenic T cells, we investigated the role of Tregs in the antigen-specific CD4 T-cell response to a mucosal virus infection. Also, as the CD4 T-cell response is dependent on priming by tissue-derived migratory DCs, we examined the role of Tregs on DC migration

from the infected tissues, as well as antigen presentation to CD4 T cells. Here we demonstrate that, contrary to expectations, the antigen-specific CD4 T-cell response in the tissue is severely diminished in the absence of Tregs. Furthermore, this absence seems to be the downstream effect of inefficient priming of the antigen-specific CD4 T-cell response, a phenotype that has not yet been observed in other studies that have addressed the role of Tregs during infection. Overall, our data further the understanding of the role of Tregs in the maintenance of immune homeostasis, particularly within the context of microbial invasion.

## RESULTS

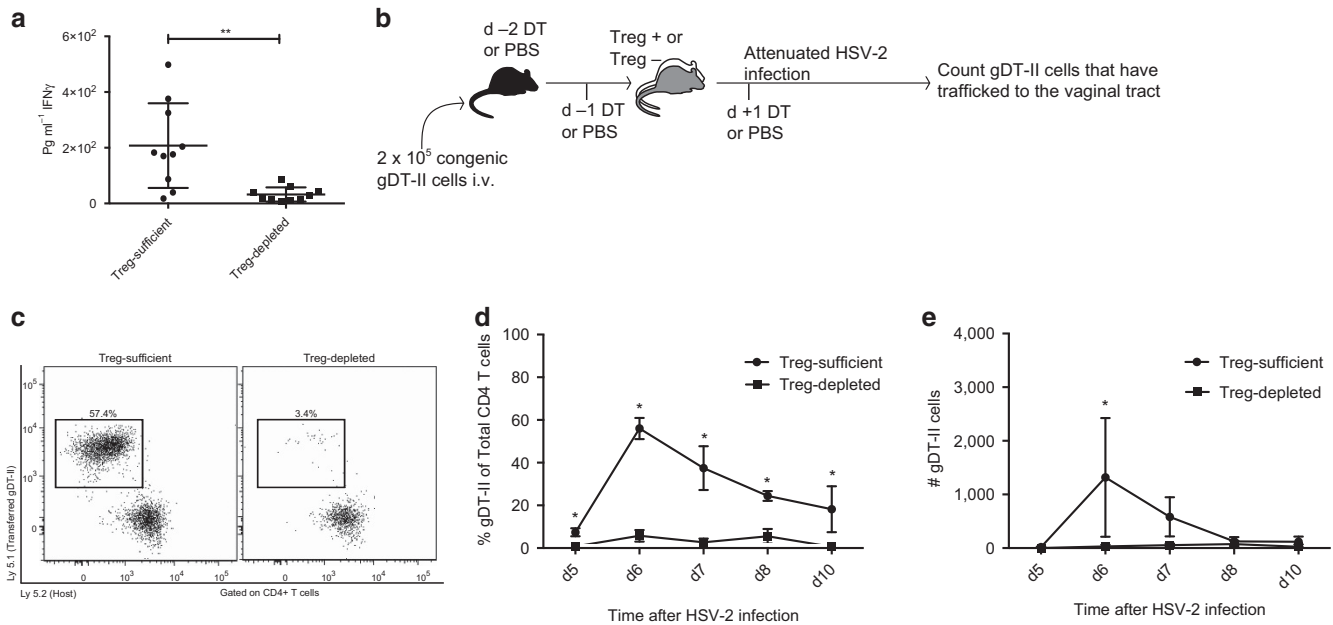
### HSV-2-specific CD4<sup>+</sup> T cells fail to accumulate in the vagina in the absence of Tregs

Our previous studies utilized WT HSV-2, which is lethal in mice and leads to death in Treg-depleted mice as early as day-6 postinfection.<sup>12</sup> Therefore, we first wanted to validate the use of the attenuated HSV-2 186 $\Delta$ kpn as a viable model to study the adaptive immune response following ivag HSV-2 infection. As expected, mice depleted of Tregs prior to infection showed delayed clearance of the virus from the infected tissue (data not shown).

In WT mice, the influx of antigen-specific CD4 T cells into the infected tissue causes localized production of IFN $\gamma$ , a step that is necessary for viral control.<sup>21,24</sup> Earlier work has demonstrated decreased IFN $\gamma$  production in the infected tissue of Treg-depleted mice, despite the presence of bulk CD4 T cells, suggesting that the adaptive immune response may be diminished in Treg-depleted mice.<sup>12</sup> We also saw significantly less IFN $\gamma$  production at 4 days postinfection within the infected tissue of Treg-depleted mice that were infected with the attenuated strain of HSV-2 (**Figure 1a**). Therefore, we used this model to explore the role Tregs have in development of the antigen-specific CD4 T-cell response following ivag HSV-2 infection.

We initially attempted to enumerate HSV-specific T cells by stimulating cells *ex vivo* with inactivated HSV to produce IFN $\gamma$ , but this technique resulted in many T cells from naive, Treg-depleted mice producing cytokines following stimulation with inactivated virus. This high level of background cytokine production in Treg-depleted mice made it impossible to distinguish cells that truly recognize viral antigen from cells that simply produced cytokine because they came out of a strongly pro-inflammatory environment. Thus we instead relied on the transfer of TCR transgenic cells that express TCRs with known specificity.<sup>26</sup>

To assess the migration of HSV-2-specific CD4 T cells to the infected tissue in mice lacking Tregs at the time of infection, we transferred congenically marked, TCR transgenic gDT-II cells that recognize an HSV glycoprotein D epitope<sup>27,28</sup> into *Foxp3<sup>DTR</sup>* mice, treated the mice with DT to deplete Tregs, and subsequently tracked the transferred cells' migration into the infected vaginal tract starting 5 days postinfection (**Figure 1b**). As expected, Treg-sufficient mice showed an influx of CD4 T cells into the vaginal tract following infection and this CD4



**Figure 1** Herpes simplex virus (HSV)-specific CD4 T cells fail to accumulate in the vagina in the absence of regulatory T cells (Tregs). *Foxp3<sup>DTR</sup>* mice were injected with diphtheria toxin (DT) on days  $-2$ ,  $-1$ , and  $+1$  relative to the day of infection. Alternatively, Treg-sufficient mice were injected with phosphate-buffered saline (PBS) instead of DT. Mice were infected intravaginally with (a)  $1 \times 10^6$  or (c–e)  $1.88 \times 10^5$  plaque-forming units of HSV-2 186 $\Delta$ Kpn, whereas naive groups were left uninfected. (a) Concentration of interferon- $\gamma$  (IFN $\gamma$ ) within vaginal washes from mice infected for 4 days with HSV-2. (b) Experimental setup for tracking antigen-specific CD4 T cells to the infected tissue. (c) Representative flow plots showing gDT-II cells in the vaginal tract of Treg-sufficient and Treg-depleted mice 6 days after intravaginal HSV-2 infection. Samples were gated on CD4 T cells and the plots show Ly5.1 (transferred gDT-II cells) vs. Ly5.2 (host cells). (d) Representative data showing the fraction of total vaginal tract CD4 T cells consisting of antigen-specific gDT-II cells at various time points after infection. (e) Representative data showing the number of gDT-II cells recovered from mouse vaginal tracts. Data in a are taken from at least two independent experiments with 4–5 mice per group. Data from day 6 in (d–e) come from or are representative of at least two independent experiments with 4–5 mice per group. Data from days 5, 7, 8, and 10 come from one experiment with four mice per group. Each data set was first screened for outliers using the ROUT test with a  $Q$  value of 0.2%. Statistical significance in a was determined using an unpaired, two-tailed  $t$ -test.  $**P < 0.01$ . Statistical significance in (d–e) was determined using multiple  $t$ -tests with correction for multiple comparisons using the Holm–Sidak method. \*Significance determined with an  $\alpha$  of 0.05. i.v., intravenous.

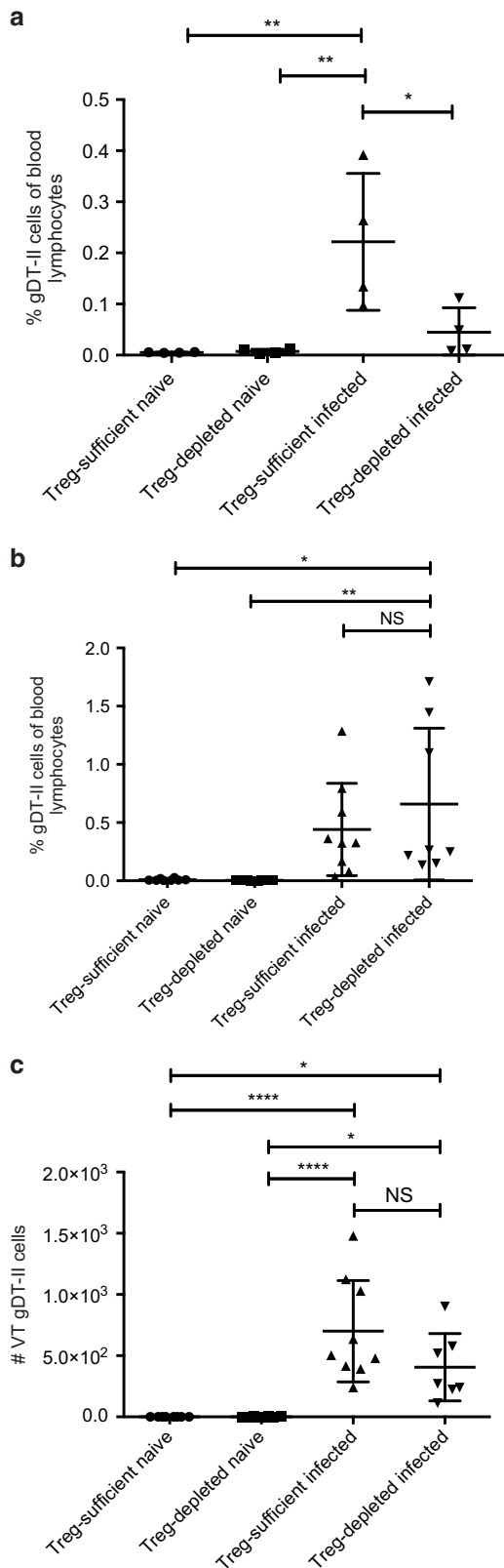
T-cell response was dominated by the congenically labeled gDT-II cells. Consistent with previous research, the greatest infiltration was seen at day-6 postinfection.<sup>24</sup> Conversely, while Treg-depleted mice showed an influx of bulk CD4 T cells into the vaginal tract following infection, the transferred gDT-II cells represented a minute fraction of the CD4 T-cell pool (Figure 1c,d). Similarly, the total number of gDT-II cells that migrated to the infected tissue in Treg-sufficient mice was significantly higher than the number of gDT-II cells that migrated into the infected tissue of Treg-depleted mice (Figure 1e). The number of gDT-II cells in the vaginal tracts of Treg-depleted mice remained low until at least 10 days postinfection, a time when effector cells are contracting in WT mice.

The significant decrease in the number of gDT-II cells entering the infected tissue of Treg-depleted mice supports the possibility that the non-gDT-II CD4 T cells that are present in the infected tissue are not HSV-2-specific T cells, but rather these cells may be polyclonal CD4 T cells activated because of the robust inflammatory conditions following Treg depletion, and then directed toward the vaginal tract owing to virus-induced inflammation. This suggests that mice lacking Tregs at the time of HSV-2 infection are unable to properly combat HSV-2 in the infected tissue with an appropriate CD4 T-cell response.

### Treg presence during T-cell priming is critical for subsequent accumulation of antigen-specific CD4 T cells in the infected tissues

We next examined the blood of Treg-depleted mice to determine whether there is an equal share of circulating gDT-II cells 6 days after infection as compared with Treg-replete mice. Similar to the vaginal tract, mice lacking Tregs at the time of infection had significantly fewer gDT-II cells circulating in the blood (Figure 2a), suggesting that the decreased entry of gDT-II cells into the vaginal tract was likely caused by inefficient activation, clonal expansion, and LN egress of the cells as opposed to the inability to properly target the vaginal tract with activated cells from the blood. To test this idea further, we delayed administration of DT until 3 days after infection. At this time point, T-cell priming is underway, although very few cells have yet migrated to the infected tissue.<sup>24</sup> When we delayed Treg depletion to a time at which T-cell priming and activation is largely completed and cells have entered the migration stage, gDT-II cells were as prevalent in the blood of Treg-depleted mice as in Treg-sufficient controls (Figure 2b). Similarly, the number of gDT-II cells entering the infected tissue was essentially unaffected by the absence of Tregs during this phase (Figure 2c). Together, these data suggest that the curtailed number of gDT-II cells entering the

vaginal tract of mice lacking Tregs at the time of infection (Figure 1) is not due to a migration defect of T cells in Treg-depleted mice but points to the potential necessity of Tregs during the initial T-cell priming phase.

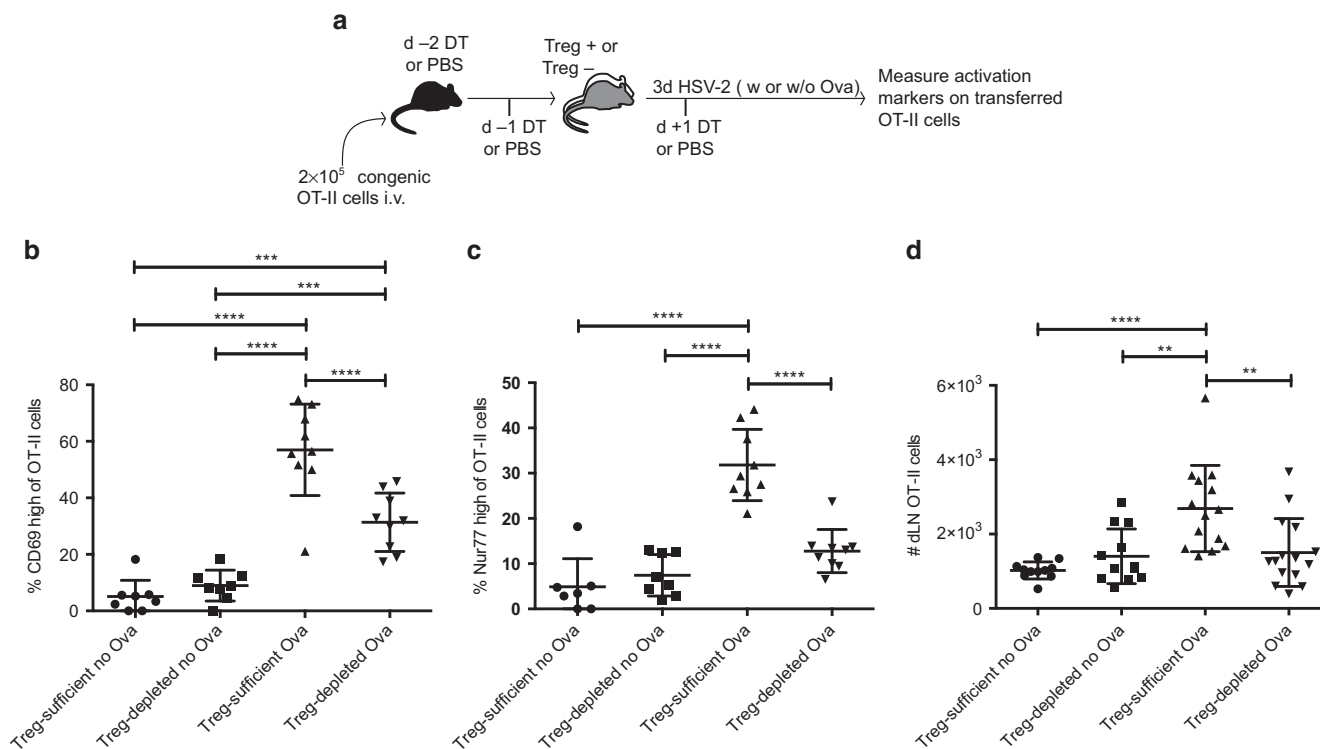


To test this hypothesis, we transferred antigen-specific CD4 T cells into  $\text{Foxp3}^{\text{DTR}}$  mice, depleted the mice of Tregs, infected the mice ivag with HSV-2 and then measured the activation of the transferred cells 3 days postinfection, a time when T-cell priming has just begun in this model<sup>23</sup> (Figure 3a). In these experiments, we used TCR transgenic OT-II cells, which recognize a peptide from the chicken ovalbumin (OVA) protein, and infected the mice with a strain of attenuated HSV-2 that expresses OVA.<sup>29</sup> As a control, attenuated HSV-2 lacking OVA expression was used for infection (“no OVA” group), enabling comparison of OT-II activation in the absence of cognate antigen but under otherwise similar *in vivo* conditions. OT-II cells transferred into Treg-sufficient mice infected with HSV-2-OVA showed increased expression of both CD69 and Nur77 3 days postinfection, suggesting that antigen recognition had occurred, thereby triggering T-cell activation (Figure 3b,c). OT-II cells transferred into Treg-depleted, HSV-2-OVA-infected mice, however, showed very little upregulation of these markers. These data suggest that the transferred OT-II cells in the Treg-depleted mice did not “see” antigen and therefore were not activated as efficiently as the OT-II cells in Treg-sufficient mice. In addition, we found significantly more OT-II cells in the dLNs of Treg-sufficient mice as compared with Treg-depleted mice 3 days postinfection, likely because the OT-II cells in the Treg-sufficient mice had begun to divide more efficiently than the same cells in the Treg-depleted mice (Figure 3d). Together, these data suggest impaired CD4 T-cell priming in Treg-depleted mice and are consistent with the diminished entry of antigen-specific cells into the infected tissue (Figure 1) and subsequent delay in viral clearance.

#### Tregs are critical for appropriate trafficking of DCs from the vaginal mucosa to the dLNs

CD11b<sup>+</sup> sub-mucosal DCs that migrate from the HSV-2-infected tissues to the dLNs present antigen to naive CD4 T cells and mediate effector T-cell activation.<sup>23</sup> As antigen-specific CD4 T cells in Treg-depleted mice did not activate efficiently in response to infection, we hypothesized that this could be, in

**Figure 2** Absence of regulatory T cells (Tregs) in the postpriming phase does not diminish the antigen-specific CD4 T-cell accumulation in the vagina. Mice were depleted of Tregs at (a) days  $-2$ ,  $-1$ , and  $+1$  or at (b, c) days  $+3$  and  $+4$  relative to the day of infection. Alternatively, Treg-sufficient mice were injected with phosphate-buffered saline instead of diphtheria toxin (DT). Mice were infected intravaginally with  $1.88 \times 10^5$  plaque-forming units of herpes simplex virus-2 186 $\Delta$ Kpn, whereas naive groups were left uninfected. (a) The fraction of gDT-II cells in the blood as a percentage of total blood lymphocytes 6 days after infection when Tregs were depleted prior to infection. (b) The fraction of gDT-II cells in the blood as a percentage of total blood lymphocytes 6 days after infection when Tregs were depleted during the postpriming, migration phase of the immune response. (c) The number of gDT-II cells recovered from the vaginal tracts of mice 6 days after infection when Tregs were depleted during the postpriming, migration phase of the immune response. All data come from or are representative of at least two independent experiments with 4–5 mice in each group. Each data set was first screened for outliers using the ROUT test with a  $Q$  value of 0.2%. Statistical significance was then determined using an ordinary one-way analysis of variance followed by Tukey’s test to compare the mean of each group with the mean of all other groups. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\*\* $P \leq 0.0001$ . Error bars show s.d. NS, not significant.



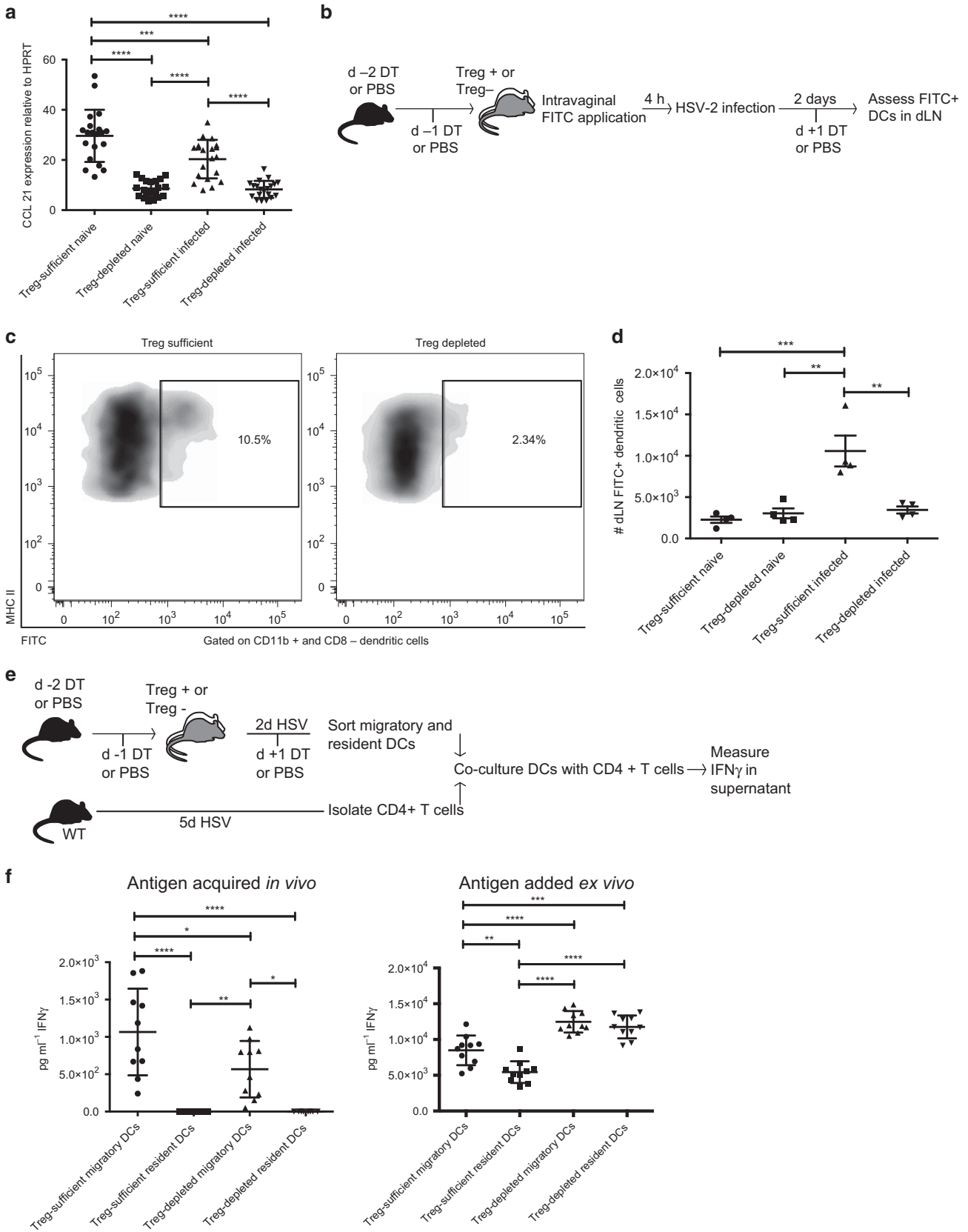
**Figure 3** Antigen-specific CD4 T-cell activation and priming following herpes simplex virus-2 (HSV-2) infection are compromised in the absence of regulatory T cells (Tregs). **(a)** Experimental setup. *Foxp3<sup>DTR</sup>* mice were injected with diphtheria toxin (DT) on days -2, -1, and +1 relative to the day of infection. Alternatively, Treg-sufficient mice were injected with phosphate-buffered saline (PBS) instead of DT. Mice were infected intravaginally with  $1.88 \times 10^5$  plaque-forming units of HSV-2 186ΔKpn expressing ovalbumin (OVA; OVA groups), whereas no OVA groups were infected with the same quantity of HSV-2 186ΔKpn. **(b)** Percentage of transferred OT-II cells showing increased expression of CD69 in the draining lymph nodes (dLNs) of 3-day infected mice. **(c)** Percentage of transferred OT-II cells showing increased expression of Nur77 in the dLNs of 3-day infected mice. **(d)** The number of OT-II cells recovered from the dLNs of mice 3 days after infection. All data come from two to three experiments with 4–5 mice in each group. Each data set was first screened for outliers using the ROUT test with a *Q* value of 0.2%. Statistical significance was then determined using an ordinary one-way analysis of variance followed by Tukey's test to compare the mean of each group with the mean of all other groups. \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ . Error bars show s.d. i.v., intravenous.

part, caused by a dysregulation in the migration of the antigen-bearing sub-mucosal DCs. This possibility was further supported by our previous work demonstrating an early drop in dLN production of C-C motif chemokine ligand 21 (CCL21) following Treg depletion.<sup>12</sup> Migratory DCs express C-C motif chemokine receptor 7 to follow a gradient of CCL21 into the T-cell zone of a dLN, thereby enabling them to present cognate antigen to T cells.<sup>30,31</sup> As such, we reasoned that altered CCL21 production in the absence of Tregs could affect DC migration subsequent to HSV-2 infection. Thus we confirmed that CCL21 production in the dLNs was decreased in Treg-depleted mice using attenuated TK-HSV-2.<sup>12,32</sup> Indeed, quantitative reverse transcriptase-PCR analysis revealed a significant drop in CCL21 transcript production in response to Treg depletion or HSV-2 infection within 2 days of the infection (**Figure 4a**).

To test the possibility that fewer migratory DCs trafficked from the infected tissue to the dLNs, we labeled DCs within the vaginal tract by applying a solution of fluorescein isothiocyanate (FITC) to the vaginal canal prior to infection with HSV-2. As previously demonstrated, this procedure does not result in drainage of FITC itself to the dLNs.<sup>33</sup> The mice were infected for 2 days, and their dLNs were analyzed for FITC+ DCs that

had originated in the vaginal tract (**Figure 4b**). Significantly fewer FITC+ sub-mucosal DCs entered the dLNs following infection in Treg-depleted mice as compared with Treg-sufficient mice, suggesting that the migration of antigen-bearing DCs from the infected tissues to the dLNs is dysregulated in the absence of Tregs (**Figure 4c,d**).

To further assess the entry of viral antigen into the dLNs of Treg-depleted mice, we tested the ability of migratory DCs from the dLNs of infected mice to present viral antigen to CD4 T cells. Mice were infected for 2 days with HSV-2 prior to isolating major histocompatibility complex (MHC) II-high migratory DCs from the dLNs. The sorted migratory DCs were then co-cultured *ex vivo* with CD4 T cells isolated from the dLNs of WT mice infected with HSV-2 for 5 days and thus enriched for endogenous HSV-2-specific CD4 T cells. Subsequent to *ex vivo* co-culture, levels of IFN $\gamma$  in the culture supernatant were measured by enzyme-linked immunosorbent assay as a readout for the ability of the sorted DCs to present *in vivo*-acquired antigen to the anti-HSV-2 CD4 T cells (**Figure 4e**). Consistent with the FITC-labeling experiment, migratory DCs sorted from Treg-depleted mice induced less IFN $\gamma$  production from anti-HSV-2 CD4 T cells, despite equal



numbers of these cells used in culture (Figure 4f). As expected, only MHC II-high, migratory DCs were capable of inducing  $\text{IFN}\gamma$  *ex vivo* (Figure 4f).<sup>23</sup> When the culture was supplemented with heat-inactivated HSV-2 virus *ex vivo*, DCs from both Treg-depleted, as well as Treg-sufficient dLNs, were fully capable of presenting antigen to anti-HSV-2 CD4 T cells (Figure 4f). Indeed, consistent with previous data showing increased expression of co-stimulatory molecules such as CD80 on DCs in the LNs of Treg-depleted mice,<sup>2</sup> DCs sorted from Treg-depleted mice that were supplemented with HSV-2 antigen *ex vivo* induced more  $\text{IFN}\gamma$  production than the same population of cells sorted from Treg-sufficient mice (Figure 4f). This suggests that the decreased production of  $\text{IFN}\gamma$  in the co-cultures where antigen was acquired *in vivo* was not the result of functionally impaired DCs in the Treg-depleted group but rather that the population of DCs sorted from Treg-depleted mice contained a smaller fraction of HSV-2-antigen-bearing cells that had entered the dLNs following the infection.

#### CTLA-4 expression by Tregs is critical to promote proper DC migration from the infected tissues in the context of HSV-2 infection

We next turned to the functional mechanism by which Tregs facilitate entry of antigen-bearing DCs into the dLNs following vaginal HSV-2 infection. CTLA-4 is constitutively expressed by Tregs and is well known to be involved in multiple pathways of Treg-mediated suppression. Specifically, CTLA-4 can compete with CD28 for co-stimulatory molecule binding to antigen-presenting cells, as well as physically remove CD80 and CD86 from the surface of antigen-presenting cells, thereby limiting co-stimulation available for T cells.<sup>34</sup>  $\text{CTLA-4}^{\text{flox/flox}} \times \text{Foxp3}^{\text{Cre}}$  mice begin to show signs of multi-organ lymphocyte infiltration and disease at 7 weeks of age,<sup>35</sup> demonstrating not only the importance of CTLA-4 expression by Tregs for the maintenance of immune homeostasis but also presenting a challenge in using these mice in studies with further manipulations owing to their early spontaneous disease. Thus we used a mixed bone marrow chimera strategy: lethally irradiated  $\text{Foxp3}^{\text{DTR}}$  mice were re-constituted with equal parts bone marrow from  $\text{Foxp3}^{\text{DTR}}$  mice and either  $\text{CTLA-4}^{\text{flox/flox}} \times \text{Foxp3}^{\text{Cre}}$  mice or  $\text{CTLA-4}^{\text{WT/WT}} \times \text{Foxp3}^{\text{Cre}}$  mice (Figure 5a). This allowed for the development of mice that maintain a population of fully functional Tregs (from  $\text{Foxp3}^{\text{DTR}}$  host and donor cells) that

could be depleted upon administration of DT, leaving behind only Tregs that either lack CTLA-4 expression or act as  $\text{Foxp3}^{\text{Cre}}$  control cells (Figure 5b,c). When these chimeric mice were used to isolate DCs for our *ex vivo* T-cell antigen presentation assay (Figure 5d), we found that similar to Treg-depleted mice, DCs from HSV-2-infected mice with CTLA-4-deficient Tregs were less able than DCs from CTLA-4 WT mice to induce  $\text{IFN}\gamma$  production from T cells without addition of exogenous HSV-2 (Figure 5e). Thus it appears that Tregs mediate their impact on DC migration through a CTLA-4-dependent mechanism.

In sum, the data point to a previously unrecognized role for Tregs in maintaining the immune system in a state where it is capable of initiating an appropriate antigen-specific CD4 T-cell response upon microbial encounter (Figure 6).

#### DISCUSSION

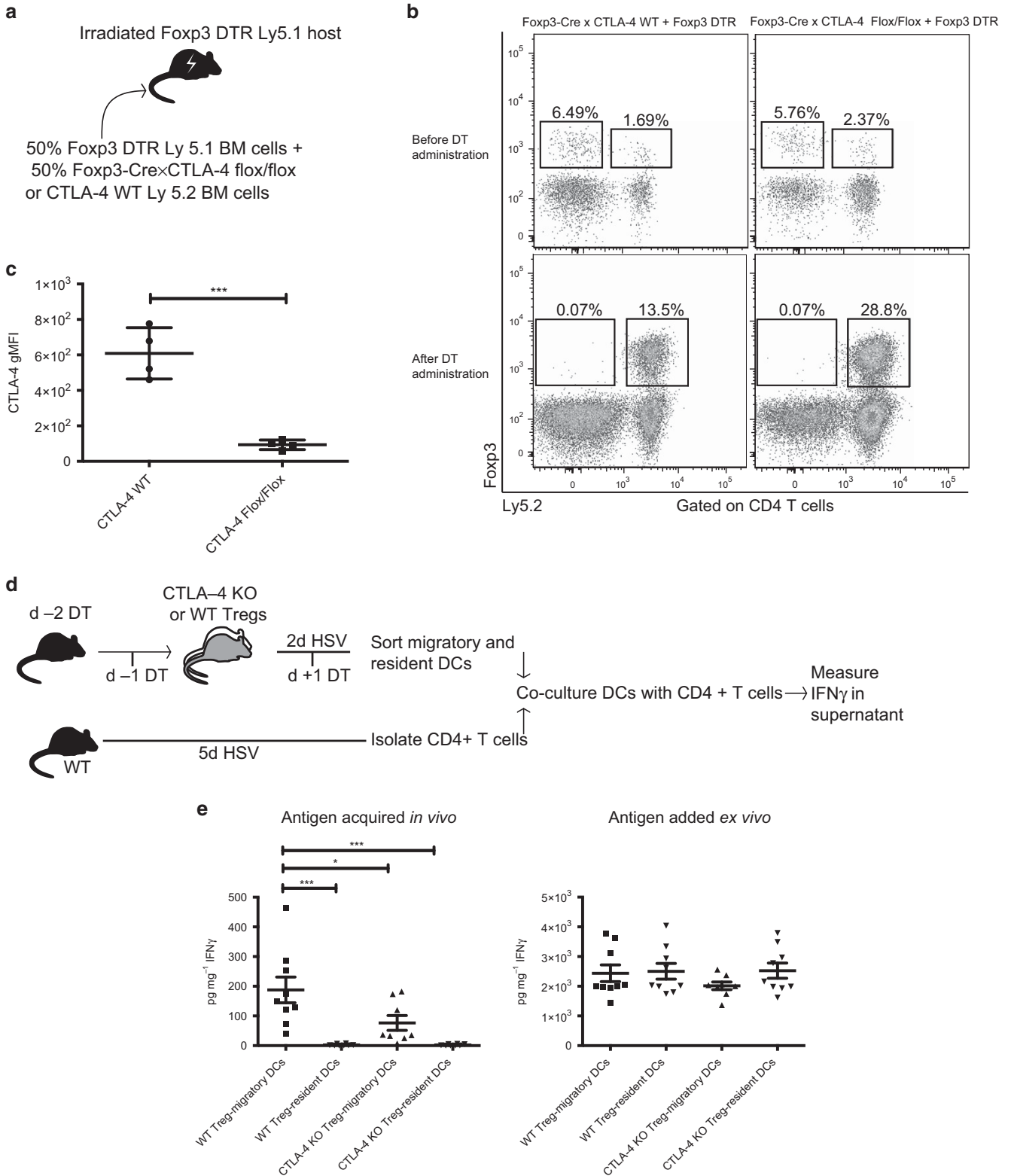
Current dogma places Tregs at the forefront of maintaining a well-regulated immune system. Not only do Tregs prevent autoimmunity and limit immunopathology<sup>1,36</sup> but we and others have also demonstrated that in some cases Tregs are required for the development of an appropriate immune response.<sup>11–14</sup> Here our data provide further insight into why Tregs are essential in the context of a vaginal HSV-2 infection and reveal that, contrary to expectations, Tregs are necessary for successful antigen-specific CD4 T-cell priming. When Tregs are depleted prior to HSV-2 infection, antigen entry into the dLN is limited because of decreased trafficking of migratory DCs into the dLNs where they can present cognate antigen to CD4 T cells, thereby initiating T-cell activation and clonal expansion. This change has the downstream effect of limiting T-cell priming and migration to the tissue, steps that are necessary for timely viral control.

We propose a model whereby depletion of Tregs or elimination of CTLA-4-expressing Tregs leads to decreased entry of antigen-bearing DCs into the dLNs (Figure 6). In the steady state, Tregs bind to DCs in the LN via high affinity CTLA-4-B7 molecule interactions, thereby outcompeting CD28-expressing effector T cells and preventing T cells from forming a synapse with DCs. This maintains the LN in a homeostatic state without excessive inflammatory cytokines produced, as well as helping to prevent expansion of T cells when there is no pathogenic threat present, including

**Figure 4** Dendritic cell (DC) trafficking from the vaginal mucosa is impaired in the absence of regulatory T cells (Tregs).  $\text{Foxp3}^{\text{DTR}}$  or control mice were injected with diphtheria toxin (DT) and infected intravaginally with herpes simplex virus-2 (HSV-2) 186ΔKpn as above. (a) Expression of C-C motif chemokine ligand 21 (CCL21) in the draining lymph nodes (dLNs) of Treg-sufficient or Treg-depleted mice 2 days after infection determined by quantitative PCR analysis (relative to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT)). (b) Fluorescein isothiocyanate (FITC) painting experimental layout. (c) Representative flow plots showing the fraction of  $\text{CD11b}^+/\text{CD8}^-$  DCs in the dLNs that stained positive for FITC 2 days after infection. Cells were first gated on major histocompatibility complex (MHC) II/ $\text{CD11c}^+/\text{CD11b}^+$  and  $\text{CD8}^-$  cells. (d) Representative data showing the number of FITC<sup>+</sup> DCs that had entered the dLNs from the infected vaginal tract 2 days after the mice were infected. (e) *Ex vivo* antigen presentation experimental layout. (f) The amount of interferon- $\gamma$  ( $\text{IFN}\gamma$ ) produced by anti-HSV-2 CD4 T cells after 60 h of co-culture with DCs sorted from Treg-sufficient or Treg-depleted mice 2 days after infection. On the left, cells were cultured without addition of exogenous antigen, whereas, on the right, cells were provided HSV-2 *ex vivo* during culture. All data come from or are representative of at least two similar, independent experiments with 3–5 mice in each group that showed similar results. Each data set was first screened for outliers using the ROUT test with a *Q* value of 0.2%. Statistical significance was then determined using an ordinary one-way analysis of variance followed by Tukey's test to compare the mean of each group with the mean of all other groups. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ . Error bars show s.d. WT, wild type.

restraining activation of auto-reactive T cells. However, in the absence of Tregs, or of CTLA-4 expression on Tregs, activation and expansion of polyclonal T cells is unleashed, leading to an inflamed LN full of expanding T cells producing cytokines, as well as decreased expression of CCL21.

It has been documented that, following the initiation of an immune response after antigen exposure through a variety of routes, production of CCL21 in the lymphoid tissues decreases. This decrease affects CD8 T-cell priming following a subsequent infection, similar to our observation with decreased

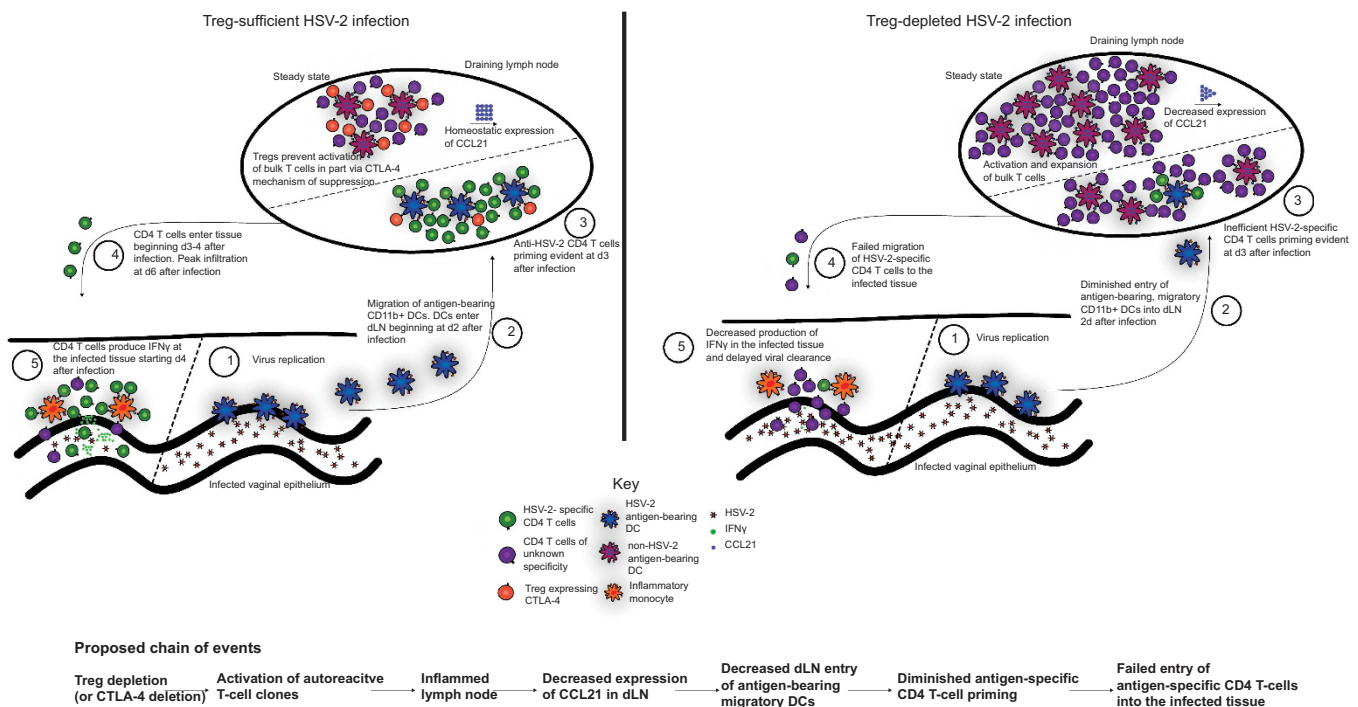




CD4 T-cell priming. In the context of lymphocytic choriomeningitis virus infection, the drop in LN production of CCL21 was found to be dependent on CD4 T-cell production of IFN $\gamma$ .<sup>32</sup> As Treg depletion causes increased production of IFN $\gamma$  from CD4 T cells within the LNs,<sup>12</sup> we initially hypothesized that, if we prevented IFN $\gamma$  production, we may be able to restore CCL21 to homeostatic levels, even in Treg-depleted mice, and thus restore migratory DC entry into the dLNs. However, after crossing IFN $\gamma$  knockout mice with Foxp3<sup>DTR</sup> mice, CCL21 production was still reduced in Treg-depleted, IFN $\gamma$  knockout mice to a similar degree as in Treg-depleted IFN $\gamma$ -WT mice (data not shown). However, as we previously demonstrated that Treg depletion leads to increased cytokine production by CD4 T cells even in the absence of infection, likely owing to

activation of auto-reactive cells,<sup>12</sup> it remains possible that Treg depletion results in the production of multiple inflammatory cytokines that are able to compensate for a loss of IFN $\gamma$ , thereby still leading to diminished CCL21. Indeed, interleukin (IL)-1 and IL-12 were also implicated in leading to decreased CCL21 levels,<sup>32</sup> so it may be that eliminating the effects of multiple cytokines would be necessary to restore homeostatic CCL21 expression in Treg-depleted mice.

Thus, following this drop in CCL21 production in the absence of Tregs, there is diminished entry of antigen-bearing, migratory DCs into the dLNs following HSV-2 infection, therefore resulting in inefficient HSV-2-specific CD4 T-cell priming and, consequently, failed migration of these cells to the infected tissue. Thus we have demonstrated for the first time



**Figure 6** Visual summary of conclusions. In the absence of regulatory T cells (Tregs), homeostasis is disrupted and, among other changes, C-C motif chemokine ligand 21 (CCL21) expression is reduced. Following intravaginal herpes simplex virus-2 (HSV-2) infection in Treg-depleted mice, the antigen-specific CD4 T-cell response fails to develop properly. Fewer antigen-bearing migratory dendritic cells (DCs) enter the draining lymph nodes (dLNs) from the infected tissue, antigen-specific CD4 T-cell priming is inefficient, and antigen-specific CD4 T cells ultimately fail to migrate to the infected tissue and mediate viral control. IFN $\gamma$ , interferon- $\gamma$ .

**Figure 5** CTLA-4 expression on regulatory T cells (Tregs) is needed to promote proper migration of antigen-bearing dendritic cells (DCs) from the infected tissue to the draining lymph nodes (dLNs). (a) Mixed bone marrow (BM) chimera setup. (b) Representative flow plots from blood (before diphtheria toxin (DT) administration) or lymphoid tissue (after DT administration) showing two congenically distinct Treg populations in mature BM chimeras. Mixed BM chimera mice were injected with DT on days -2, -1, and 1 relative to the day of infection to deplete the fully functional Foxp3<sup>DTR</sup> Tregs in each mouse. Following DT administration, Ly 5.1 Foxp3<sup>DTR</sup> Tregs are no longer present and the populations of Foxp3<sup>Cre</sup> x CTLA-4<sup>WT/WT</sup> or CTLA-4<sup>flox/flox</sup> Ly 5.2 Tregs have expanded. (c) Expression of CTLA-4 on Tregs measured on the day of harvest, when all fully competent, Foxp3<sup>DTR</sup> Ly 5.1 cells have been eliminated. (d) Experimental layout. (e) Amount of interferon- $\gamma$  (IFN $\gamma$ ) produced by anti-herpes simplex virus-2 (anti-HSV-2) CD4 T cells following 60 h of co-culture with DCs sorted from mice with either wild-type (WT) or CTLA-4 conditional knockout (KO) Tregs 2 days after infection. On the left, cells were cultured without addition of exogenous antigen, whereas, on the right, cells were provided HSV-2 *ex vivo* during culture. All data come from or are representative of at least two similar, independent experiments with 4–5 mice in each group that showed similar results. Each data set was first screened for outliers using the ROUT test with a *Q* value of 0.2%. Statistical significance was then determined using an unpaired *t*-test (c) or a one-way analysis of variance followed by Tukey's test to compare the mean of each group with the mean of all other groups (f) \**P* ≤ 0.05, \*\*\**P* ≤ 0.001. Error bars show s.d.

that Tregs assist in focusing the CD4 T-cell response to be directed against an invading microbe upon infection.

The degree to which these findings can be extended to other infections requires further investigation. One aspect of HSV-2 infection biology that may be crucial for this observed phenotype is the relative scarcity of antigen that enters the dLNs following the natural route of exposure. When other methods of exposure are used, antigen can enter the LN without the help of migratory DCs by traveling in the lymph. In one study, HSV-1 administered into the vaginal tract tissue through needle injection moved into the dLN very quickly and was presented by lymphoid organ-resident DCs, while the same virus delivered to the mucosal surface of the vaginal canal resulted in antigen movement to the dLN that was dependent on migratory DCs.<sup>33</sup> Thus, during systemic infections, or infections where antigen itself drains into secondary lymphoid organs, there may be sufficient antigen presentation to prime a robust antigen-specific T-cell response in the absence of Tregs. However, following ivag HSV-2 infection in mice, antigen only moves to the dLNs when carried by migratory DCs<sup>23</sup> and is thus subject to changes in the chemotactic gradients that facilitate migratory DC entry into the dLNs. Furthermore, regardless of applicability to other infections, our study points to the crucial role that Tregs have in both restraining, as well as coordinating, immune responses. Although it is well established that Tregs restrain T-cell responses in order to maintain peripheral tolerance, we demonstrate here that Tregs also have a pivotal role in promoting an appropriate immune response upon infection.

The data also highlight the important role that Tregs have in focusing an immune response against the invading pathogen. Although some antigen did still enter the dLNs carried by migratory DCs in Treg-depleted mice (Figure 4), there was a near complete failure to target the antigen-specific gDT-II cells to the infected tissue (Figure 1). Consequently, the immune response in the vaginal tract of Treg-sufficient mice was dominated by the antigen-specific CD4 T cells that were transferred into the mice prior to infection, though these same cells were scarcely present in the vaginal tracts of Treg-depleted mice.

Tregs are known to affect both the priming of antigen-specific CD8 T cells by preventing activation of low-affinity CD8 T-cell clones<sup>19</sup> and the development of CD8 T-cell memory through the production of IL-10.<sup>37</sup> Our data may be related to these observations; however, we focused on the priming of a specific CD4 T-cell clone, and to our knowledge, the impact of Tregs on CD4 T-cell priming has not been explored in detail. Furthermore, we did not assess how Treg depletion in our model affected the development of T-cell memory. In our model, the combination of decreased entry of antigen into the dLNs (Figure 4) and possible increased ability for low-affinity clones to engage with antigen-bearing DCs<sup>19,38</sup> is a potential explanation for the decrease in priming of TCR transgenic CD4 T cells in Treg-depleted mice.

In situations where a more robust immune response is desired, such as during chronic viral infections or cancer, it has

been proposed that therapies targeted toward Treg function may be helpful. The findings presented here could have relevance when considering possible unintended consequences from such therapies. Our data suggest that very quickly after inhibition of Treg function, well before any overt signs of autoimmunity would be expected, individuals with lessened Treg function could be more susceptible to some forms of infection because of a diminished ability to mount a proper immune response.

In summary, our findings highlight an underappreciated role for Tregs in allowing the immune system to prime and target an immune response against a pathogen that is localized to a peripheral tissue and provide further insight into the importance of Tregs in not only curtailing immunity but also in promoting appropriate immune responses against infections.

## METHODS

**Mice.** Six-to-eight-week-old female mice were used for experimental groups. All animal experiments were approved by the Fred Hutch IACUC (NIH OLAW approval no. A3226-01), and this study was carried out in strict compliance with the PHS Policy on Humane Care and Use of Laboratory Animals. For more information on strains of mice used and generation of bone marrow chimeras, see **Supplementary Experimental Procedures** online.

**Infections and vaginal washes.** Mice were injected subcutaneously with 2 mg of Depo Provera (Greenstone, Peapack, NJ) 5–7 days prior to being infected with  $1.88 \times 10^5$ – $1.0 \times 10^6$  plaque-forming units of HSV-2 by application of virus to the vaginal canal. HSV-2 was propagated on and titered by plaque assay on Vero cells. Vaginal washes were collected from mice by swabbing vaginal canals and washing the vaginal canals with 50  $\mu$ L of titration buffer. Viral titer of vaginal washes was determined by plaque assay on Vero cells. IFN $\gamma$  concentration in vaginal washes was determined using the Ready-Set-Go ELISA Kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. For more details, see **Supplementary Experimental Procedures**.

**Treg depletion.** In all experiments, *Foxp3<sup>DTR</sup>* mice were injected intraperitoneally with 30  $\mu$ g kg<sup>-1</sup> of DT (EMD Millipore, Darmstadt, Germany) dissolved in phosphate-buffered saline upon first injection and 10  $\mu$ g kg<sup>-1</sup> of DT for all subsequent injections. In experiments where Tregs were depleted prior to infection, DT injections were carried out on days -2, -1, and +1 relative to the day of infection. In experiments where Tregs were depleted after T-cell priming, DT injections were done on days +3 and +4 relative to the day of infection. In some experiments, Treg-sufficient mice were a non-DTR-expressing strain injected with DT, and no differences were noted upon using either Treg-sufficient control setup.

**Cell sorting and flow cytometry.** For *ex vivo* co-culture experiments, DCs were sorted from iliac LNs based on the expression of CD11c and MHC II. Cells were blocked for Fc binding and then stained with anti-MHC II antibody and anti-CD11c antibody (eBioscience). Cells from dLNs were sorted into two groups: an MHC II high/CD11c mid population of migratory DCs and MHC II mid/CD11c + population of non-migratory DCs. All sorting was carried out on a FACSAria (BD Biosciences, San Jose, CA). CD4 T cells that were already enriched using the CD4+ T-cell Negative Selection Kit (Stem Cell Technologies, Vancouver, Canada) were also stained using anti-CD4 and anti-MHC II antibodies (eBioscience) and sorted into a population of CD4+ and MHC II- cells to ensure that the CD4 T cells were not contaminated with antigen-presenting cells.

For flow cytometry, cells were incubated in fixable viability dye (eBioscience), blocked for Fc binding, and then stained for surface proteins. Data were analyzed using the FlowJo software (Treestar, Ashland, OR). For more details, including antibodies used, see **Supplementary Experimental Procedures**.

**Ex vivo co-culture.** DCs and CD4 T cells were sorted as described above. A total of 2500–3000 DCs were then co-cultured with 100 000 CD4 T cells in 120  $\mu$ l of volume in a 384-well culture plate. After 60 h of co-culture at 37 °C, supernatant was assessed for IFN $\gamma$  using the Ready-Set-Go ELISA Kit (eBioscience) according to the manufacturer's instructions. Where noted, *ex vivo* cultures were supplemented with  $1 \times 10^5$  plaque-forming units of inactivated HSV-2.

**FITC painting.** Four hours prior to infection, mouse vaginal tracts were swabbed with calcium-alginate-tipped swabs before having 10  $\mu$ l of 1% FITC (Sigma-Aldrich, St Louis, MO) in dimethyl sulfoxide applied using a pipette. FITC + DCs were enumerated in the dLNs by gating on FITC + /CD11b + /CD8 – DCs and multiplying the gate percentages by the number of lymphocytes in the sample. The FITC + gate was set using cells from a mouse that had dimethyl sulfoxide applied to the vaginal canal.

**Quantitative PCR.** dLNs were harvested directly into RNeasy Lysis Buffer (Thermo Fisher Scientific, Waltham, MA), and then RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Any residual genomic DNA was then eliminated using the DNase Away Kit (Thermo Fisher Scientific). A cDNA library was then prepared using the RT Kit (Thermo Fisher Scientific). Quantitative PCR was performed on CCL21 and hypoxanthine-guanine phosphoribosyl-transferase (HPRT) using TaqMan primers (Applied Biosystems) and 2  $\times$  quantitative PCR master mix (Thermo Fisher Scientific) diluted with water and 250 ng of RNA from each sample. Relative expression of CCL21 in relation to HPRT was then calculated for each sample using the delta Ct method. All kits were used according to the manufacturer's instructions.

**Statistical analysis.** All statistical analysis was performed using the Prism software (GraphPad Software, San Diego, CA). All data sets were first screened for outliers using the ROUT test with a Q value of 0.2%. When comparing groups in experiments with more than two experimental groups, an ordinary one-way analysis of variance followed by Tukey's test was conducted to determine statistical significance between groups. When comparing only two experimental groups, an unpaired, two-tailed, parametric *t*-test was conducted to determine statistical significance. When comparing multiple rows of data, multiple *t*-tests were performed with correction for multiple comparisons using the Holm–Sidak method. In all cases, a *P*-value < 0.05 was considered significant.

**SUPPLEMENTARY MATERIAL** is linked to the online version of the paper at <http://www.nature.com/mi>

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#### AUTHOR CONTRIBUTIONS

AGS and AC performed all experiments. AGS performed data and statistical analyses. AGS and JML designed the study, conceived the experiments and wrote the first draft of the manuscript, and all authors provided editorial contribution and approved the final draft.

#### DISCLOSURE

The authors declared no conflict of interest.

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