CD4+ T cells support cytotoxic T lymphocyte priming by controlling lymph node input

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Rapid induction of CD8+ cytotoxic T lymphocyte (CTL) responses is critical to combat acute infection with intracellular pathogens. CD4+ T cells help prime antigen-specific CTLs in secondary lymphoid organs after infection in the periphery. Although the frequency of naive precursors is very low, the immune system is able to efficiently screen for cognate CTLs through mechanisms that are not well understood. Here we examine the role of CD4+ T cells in early phases of the immune response. We show that CD4+ T cells help optimal CTL expansion by facilitating entry of naive polyclonal CD8+ T cells into the draining lymph node (dLN) early after infection or immunization. CD4+ T cells also facilitate input of naive B cells into reactive LNs. Such "help" involves expansion of the arteriole feeding the dLN and enlargement of the dLN through activation of dendritic cells. In an antigen- and CD40-dependent manner, CD4+ T cells activate dendritic cells to support naïve lymphocyte recruitment to the dLN. Our results reveal a previously unappreciated mode of CD4+ T-cell help, whereby they increase the input of naive lymphocytes to the relevant LN for efficient screening of cognate CD8+ T cells.

cell trafficking | herpes simplex virus | innate immunity | vascular biology | antiviral immunity

Antigen-specific cytotoxic T lymphocytes (CTLs) provide crucial protection against infection by viruses and intracellular bacteria. Previous studies have indicated that CD4+ T-cell help is important for the development and maintenance of CTLs. CD4+ T cells help the CD8+ T-cell response by at least two mechanisms (1–4): first, through direct contact between CD4+ and CD8+ T cells and, second, through licensing of dendritic cells (DCs) for antigen presentation to CD8+ T cells (5). DCs that have been activated by CD4+ T cells enable efficient primary and secondary CD8+ T-cell responses by inducing expression of CD25 and CD127 on antigen-specific CD8+ T cells. In addition, when DCs encounter activated antigen-specific CD8+ T cells, they attract polyclonal CD8+ T-cell precursors through secretion of CCL3 and CCL4, resulting in more efficient memory CTL generation (3).

Lymphocyte priming takes place within secondary lymphoid tissues, such as the lymph node (LN) and spleen. Individual naive C57BL/6 mice (B6) contain a total of only ~20–200 peptide-specific CD4+ T cells (6) and ~20–1,200 peptide-specific CD8+ T cells (7) in their spleen and LNs. Despite this rare frequency, the process of T-cell priming is remarkably efficient because of both macroscopic and microscopic mechanisms (8). At the level of the whole organism, inflammation induces a dramatic increase in the number of naive cells recruited to the reactive LN early after infection or immunization, resulting in LN swelling, a long-recognized sign of infection. LN hyperplasia is preceded by an increase in LN vasculature (9). The expansion of the arteriole feeding the LN enables more efficient screening for cognate lymphocytes, which promotes an effective adaptive immune response (10). However, the role of specific cell types involved in this process remains unclear.

In the present study, we use a variety of approaches to address the role of CD4+ T cells in the induction of primary CD8+ T-cell responses. We use a herpes simplex virus (HSV) infection model because immunity to HSV is characterized by a strong CTL response (11), and, in contrast to most infectious agents in which CD4+ T-cell help is only needed to generate memory CTLs (12–15), CD4+ T cells are needed to generate the primary CTL response to HSV (5, 16–21). Here, we demonstrate that CD4+ T cells are required for increased cellular input to the draining LN (dLN) after HSV2 infection. CD4+ T cells were required for remodeling of the LN feed arteriole to a larger diameter. Similar requirements for CD4+ T cells in LN hyperplasia were found after immunization with DCs. Using these systems, we demonstrate a critical role of DC–CD4+ T-cell interaction through major histocompatibility complex class II (MHCII)–T-cell receptor (TCR) and CD40–CD40 ligand (CD40L) in mediating naive lymphocyte input to the reactive LN. Ultimately, enhanced recruitment of naive lymphocytes results in the rapid onset of CTL responses caused by the increased screening capacity of the reactive LN. These results reveal a CD8+ T-cell–extrinsic role of CD4+ T-cell help in the primary immune response through increasing naive lymphocyte input to the sites of lymphocyte priming.

Results

LN Expansion Is Impaired After Infection and Immunization in CD4+ T-Cell-Deficient Mice. To examine the role of CD4+ T-cell help during an immune response, WT, MHCII-deficient, or CD4-deficient mice transplanted with naive gBT-I cells were infected vaginally with thymidine kinase–defective HSV2 (TK HSV2). TK HSV2 infects and triggers robust immune responses without causing neuropathogenesis (22). The gBT-I cells, which express a transgenic TCR specific for HSV glycoprotein B, were used to track antigen-specific CTLs (5, 23). Although draining iliac LNs underwent robust expansion in WT recipients, the degree of expansion was significantly reduced in MHCII-deficient or CD4-deficient mice after infection (Fig. S1). Thus, these data suggest that CD4+ T cells contribute to inducible LN enlargement because the non-CD4+ T-cell compartment, including CD8+ T cells, plays a crucial role in LN hyperplasia in naive hosts, both of which lack a normal CD4+ T-cell compartment (ΔCD4T) (Fig. L4). A similar observation was reported by a previous study (21). The absence of maximal LN hyperplasia in ΔCD4T mice was not explained solely by the absence of CD4+ T cells in ΔCD4T mice because the non-CD4+ T-cell compartment, including CD8+ T cells and B cells, was also significantly reduced in these hosts (Fig. S1).

Although LN size in ΔCD4T mice was slightly reduced compared with WT mice at steady state, this reduction was accounted for by the lack of CD4+ T cells in ΔCD4T mice because the non-CD4+ T-cell compartment, including CD8+ T cells and B cells, was also significantly reduced in these hosts (Fig. S1). Thus, these data suggest that CD4+ T cells contribute to inducible LN enlargement but not to the steady-state function of a LN. In addition, the difference in LN hyperplasia was significantly reduced in ΔCD4T mice after footpad injection of TK HSV2 or bone marrow–derived DCs (BMDCs) (Fig. L4).


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dependent LN enlargement in CD8+ T-cell immunity. Here, we focus on the role and mechanism of the CD4+ T-cell activation. Infection of WT mice with TK HSV2 led to a significant increase in cellularity of the non-CD4 compartment in the dLNs (ΔΔCD4T mice showed a significantly reduced number of gBT-I cells was significantly reduced in ΔCD4T hosts (Fig. 1A), indicating that the requirement of CD4+ T cells for LN expansion is not restricted to the vaginal HSV infection model. Of note, LNs in ΔCD4T mice showed a significant increase in size and cellularity compared with the infected controls (Fig. L4), albeit to a lesser extent than WT mice, suggesting the presence of CD4+ T-cell–dependent and –independent mechanisms of LN enlargement in WT mice. Interestingly, injection of ΔCD4T mice with CpG in the absence of antigen led to similar reduction of CD25 expression on gBT-I cells without recombination (21) and CD25 up-regulation (5, 6, 21), depend on CD4

CD4+ T Cells Help Primary CD8+ T-Cell Responses Through Increasing Cellular Input. After intravaginal infection with TK HSV2, the number of gBT-I cells was significantly reduced in ΔCD4T recipients mice compared with WT hosts (Fig. 1B). Surprisingly, however, the percentage of gBT-I cells within the dLN was comparable among the hosts at all time points tested (Fig. 1C). DCs are unable to induce CD25 expression on CD8 T cells without receiving help from antigen-specific CD4+ T cells ("helpless") (5). CD25 controls late-phase expansion and differentiation of antigen-specific effector CD8+ T cells (6). However, this mode of DC activation cannot account for the reduced gBT-I cell number in HSV2-infected ΔCD4T hosts at 2 d postinfection (dpi) (Fig. 1D) because the up-regulation of CD25 occurred only after multiple cell divisions (Fig. S3) and no detectable division was observed at this time (Fig. S4). These data suggest that the reduced magnitude of primary CD8+ T-cell accumulation in the dLN of ΔCD4T hosts is largely dictated by the minimal increase in dLN cellularity in ΔCD4T hosts, and not by a cell-intrinsic defect of gBT-I cells.

To address this possibility, we assessed the activation and differentiation status of gBT-I cells. At 2 dpi, the numbers of gBT-I cells expressing high levels of an activation marker, CD69, were reduced in ΔCD4T hosts (Fig. 2A). This finding was not attributable to reduced efficiency of antigen presentation to gBT-I cells because the proportion of CD69+ cells within the transferred gBT-I population was comparable between WT and ΔCD4T hosts (Fig. 2B). Expression levels of CD69 in gBT-I cells and endogenous polyclonal CD8T cells were also intact in the absence of CD4+ T cells (Fig. 2C). Because the majority of the transferred gBT-I cells remained undivided at this time point (Fig. S4), any putative differences in cell division could not account for the difference in gBT-I cell numbers. In addition, effector CTL differentiation, as measured by granzyme B levels (Fig. 2D), was comparable at later time points in the LN and the spleen of ΔCD4T mice. Examination of the cell division rate of transferred gBT-I cells revealed no differences at 4 d after TK HSV2 infection (Fig. 2E). Together, these data indicate that, although late-stage CTL effector functions, including IFNγ and TNFα secretion (21) and CD25 up-regulation (5, 6, 21), depend on CD4

Fig. 1. CD4+ T cells are required for optimal dLN enlargement and CTL development after vaginal HSV2 infection. WT, MHCIIKO, or CD4KO mice were infected with 1 × 106 pfu of TK HSV2. Numbers of total cells (A), gBT-I CD8+ T cells (B), and percentages of gBT-I cells (C) in the dLN are shown. Each symbol represents one mouse. Data are pooled from two or more independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA (naïve, 2 and 4 dpi) and by two-tailed Student’s t test (7 dpi).

Fig. 2. Primary CTL responses in ΔCD4T hosts are reduced in magnitude but are phenotypically intact. WT, MHCIIKO, or CD4KO mice were infected with carboxyfluorescin diacetate succinimidyl ester (CFSE)–labeled naïve gBT-I cells and infected as in Fig. 1. (A–C) Expression of CD69 2 d after infection. Numbers of CD69+ gBT-I cells per dLN (A) and percentages of CD69+ cells among total gBT-I cells in the dLN (B) are shown. Data are pooled from three independent experiments. *P < 0.05, **P < 0.01 by one-way ANOVA. In C, CD69 expression on gBT-I cells (thick line), endogenous CD8+ T cells (dashed line), and CD8+ T cells in naïve WT mouse (gray histogram) are shown. (D and E) Intracellular granzyme B expression on 4 and 7 dpi (D) and CFSE dilution on 4 dpi (E) in the donor gBT-I cells in indicated organs. Gray histograms indicate isotype staining (D) or uninfected controls (E).
help, the reduced ability of helpless gBT-I cells to accumulate in ΔCD4T hosts at early stages is not attributable to a cell-intrinsic deficiency. Rather, it appears to be linked to the defect in LN expansion.

**CD4$^+$ T Cells Support Primary CD8$^+$ T-Cell Responses Through Interaction with DCs.** To understand the mechanism by which CD4$^+$ T cells support LN expansion, we asked whether this process requires the interaction of CD4$^+$ T cells with MHCII$^+$ antigen-presenting cells (APCs). First, we examined whether the APCs in ΔCD4T hosts are intrinsically defective in inducing accumulation of gBT-I cells within the dLN upon HSV infection. To this end, we partially reconstituted ΔCD4T hosts with WT polyclonal CD4$^+$ T cells before gBT-I transfer. Consistent with previous reports (5, 6, 21), CD25 expression was impaired in gBT-I cells in ΔCD4T hosts (Fig. S3). However, ~10% reconstitution of the CD4$^+$ T-cell compartment in CD4-deficient, but not MHCII-deficient, hosts partially restored the expression of CD25 on activated gBT-I cells (Fig. S3) and dLN enlargement after footpad infection with HSV2 (Figs. S5A and B and S6C). In addition, DCs from Cd4$^{-/-}$ mice were able to induce LN expansion in WT, but not in Cd4$^{-/-}$ hosts (Fig. S2C). These data indicate that help is mediated via the ability of CD4$^+$ T cells to interact with APCs through MHCII and that such help can be provided transiently in situ.

Next, we examined the type of MHCII$^+$ APC responsible for supporting CD4$^+$ T-cell–dependent LN enlargement. We found that injection of DCs, but not B cells, induced LN enlargement (Fig. S5C). In addition, depletion of DCs before HSV2 infection abolished LN enlargement (Fig. S5D), indicating that DCs are both required and sufficient to induce dLN hyperplasia, consistent with previous studies (24, 25). Although B-cell injection was not sufficient to induce LN enlargement in helpless mice, B cells are known to mediate inflammation-induced lymphangiogenesis and LN remodeling (26, 27). To examine the requirement of B cells in HSV-induced LN hyperplasia, we infected WT or μMT mice, which lack B cells, in the footpad with TK HSV2. Although total cellularity of the dLN was smaller in μMT mice compared with WT mice (Fig. S5E), the size of the non–B-cell compartment was comparable (Fig. S5F), indicating that B cells are not required to increase cell input into the dLN. Thus, DCs, but not B cells, are causally involved in CD4$^+$ T-cell–dependent LN enlargement.

**CD4$^+$ T-Cell–Mediated LN Expansion Requires MHCII and CD40 Expression by DCs.** Next, we examined the molecules involved in LN expansion after CD4$^+$ T–DC interaction. Injection of BMDCs alone is known to induce LN enlargement without the need for antigen pulsing (Fig. S2B and C) (24, 25). Such LN hyperplasia is unlikely to be caused by LPS contamination of the BMDCs because we did not observe any signs of activation in the WT BMDC preparation (Fig. S2D). Furthermore, BMDC-intrinsic TLR signaling is not responsible for DC-induced LN hyperplasia because cellularity was comparable in the dLNs of mice injected with WT or MyD88/Toll/interleukin-1 receptor-domain-containing adapter inducing interferon β (TRIF) double-deficient BMDCs (Fig. S2E). In contrast, we observed a significant defect in the ability of MHCIIKO BMDCs to induce expansion of the dLNs (Fig. 3A). These data indicate that BMDCs must interact with CD4$^+$ T cells through MHCII to mediate LN expansion. Activation of DCs by CD4$^+$ T cells through CD40 and CD40L interaction represents one important mechanism of CD4$^+$ T-cell help (17–19). Thus, we assessed the role of CD40–CD40L interaction in LN enlargement. A significant reduction in the degree of LN expansion upon injection of CD40-deficient BMDCs was observed compared with WT BMDCs (Fig. 3B), indicating that CD40 signaling in DCs is required to mediate LN enlargement. The incomplete LN enlargement induced by MHCII- and CD40-deficient BMDCs is not explained by impaired migration of these DCs because they reached the dLN at levels comparable to WT BMDCs (Fig. 3C). Together, these data indicate that CD4$^+$ T cells provide help through the engagement of both MHCII and CD40 on DCs.

**CD4$^+$ T Cells Promote Increased Recruitment of Naïve Lymphocytes to the Draining LN.** We hypothesized that the CD4$^+$ T-cell–dependent increase in dLN cellularity results in optimal CD8$^+$ T-cell expansion by increasing naïve polyclonal CD8$^+$ T-cell input into the reactive LN. To avoid complications from putative developmental bias in CD8$^+$ T-cell population in ΔCD4T mice, first we monitored accumulation of adoptively transferred WT naïve polyclonal CD8$^+$ T cells in the dLN after footpad infection of TK HSV2. To this end, polyclonal CD8$^+$ T cells were purified from the spleen and LNs of naïve WT mice and transferred into WT or ΔCD4T recipient mice that had been infected in the footpad for 12 h with TK HSV2. At 12 h after the transfer of the CD8$^+$ T cells, the draining ipsilateral and nondraining contralateral popliteal LNs were removed, and the donor CD8$^+$ T cells were counted. At this time point, the total cellularity in the dLN was not significantly different between WT and ΔCD4T mice, although the dLN enlargement was evident in all mice compared with the nondraining LN counterpart (Fig. 4A), suggesting that the CD4$^+$ T-cell–independent mode of LN enlargement precedes the CD4$^+$ T-cell–dependent mode of hyperplasia. In contrast, the number of donor CD8$^+$ T cells in the dLN was significantly reduced in the ΔCD4T hosts, suggesting that CD4$^+$ T “help” accelerates the accumulation of polyclonal naïve CD8$^+$ T cells within the dLN starting from very early time points (Fig. 4A).

Next, we asked whether such CD4$^+$ T-cell–dependent accumulation of CD8$^+$ T cells in the dLN is (i) explained by enhanced entry of lymphocytes and (ii) applicable to other routes of infection because different sets of DCs are involved in T-cell priming after footpad vs. vaginal HSV infection (28). WT and CD4-deficient mice were infected with TK HSV2 intravaginally and then injected with spleen and LN cells from naïve congenic CD45.1$^+$ WT mice. Two hours after the transfer, the draining iliac LNs and the nondraining brachial LNs were harvested, and donor cell numbers were compared. The number of donor cells was significantly reduced in the draining iliac LNs, but not the non-draining brachial LNs, in ΔCD4T hosts (Fig. 4B Left). These
results suggest that the impaired accumulation of naïve CD8+ T cells in CD4-T mice is accounted for, at least in part, by the reduced lymphocyte entry into the dLN in these hosts. Of note, we observed a similar requirement for CD4+ T cells for the recruitment CD8+ T cells and B cells (Fig. AB Center and Right), suggesting that this mode of CD4+ T-cell help is not specific to CD8+ T cells and that the CD4+ T cells may also help B-cell responses through increased input.

Cognate CD4+ T Cells Enable LN Expansion. Thus far, our data do not distinguish whether CD4+ T cells exert help in an antigen-specific manner. Although injection of antigen-unpulsed DCs or CpG was sufficient to drive LN hyperplasia (Fig. S2), it is impossible to rule out the contributions from unintended antigens. For instance, unpulsed BMDC injection alone is known to induce an antigen-specific antibody response against FBS contained in the culture media (29). To determine whether antigen specificity of CD4+ T cells is required to increase dLN cellularity, we assessed naïve CD8+ T-cell accumulation in OT-II TCR transgenic mice on recombination activating gene (RAG)-deficient background. In these mice, the only CD4+ T cells available for help are specific to ovalbumin and not to HSV-2. Footpad infection of OT-II/Rag-1−/− mice with TK HSV2 resulted in a significant increase in LN cellularity compared with the Rag-1−/− counterpart, mainly accounted for by the influx of OT-II TCR transgenic T cells (Fig. S6A). However, accumulation of naïve polyclonal CD8+ T cells in these mice was comparable to the Rag-1−/− mice (helpless) (Fig. S6B). One caveat to this experimental approach is that Rag−/− mice lack all lymphocytes, making it difficult to compare cellular influx in the context of normal LN composition. To compare influx of endogenous lymphocyte populations in a more physiological setting in the presence or absence of cognate CD4+ T cells, ΔCD4 mice reconstituted with either WT polyclonal CD4+ T cells (containing a rare population of HSV-specific T cells) or with OT-II/Rag-1−/− CD4+ T cells (nonspecific to HSV-2) were infected with TK HSV2 in the footpad. Four days later, LN cellularity and numbers of endogenous B and CD8+ T cells were measured (Fig. S6C). These data clearly indicated that (i) antigen-nonspecific CD4+ T cells are not sufficient to support infection-induced cellular influx to the dLN and (ii) CD4+ T cells must interact with MHCII+ APCs to bring about this exchange because MHCII−/− recipients of polyclonal CD4+ T cells failed to undergo LN expansion. Together, these data indicate that CD4+ T cells promote accumulation of naïve lymphocytes to the dLN after antigen-specific interaction with MHCII+ APCs.

CD4+ T Cells Support Expansion of the Arteriole Feeding the Reactive LN. The increase in naïve lymphocyte entry into the reactive LN may be attributable to an increase in blood flow. We previously demonstrated that the dLN feed arteriole remodels to a larger diameter upon infection or immunization to increase supply of naïve lymphocytes into the dLN (10). To examine whether CD4+ T cells might mediate larger lymphocyte input by inducing arteriole remodeling, the vessel diameter of the primary arteriole feeding the inguinal dLN was measured in WT and CD4-deficient mice after vaginal infection with TK HSV2. Consistent with our previous report (10), both resting and maximal vessel diameter expanded after infection in WT mice (Fig. 4C), indicating that the arteriole had undergone remodeling. The increase in arteriolar diameter was observed as early as 1 d after infection. In contrast, the arteriole failed to expand in CD4-deficient mice at all time points tested (Fig. 4C), indicating that CD4+ T cells are required for infection-induced arteriole remodeling. Collectively, these data indicate that CD4+ T-cell help is required for immunization-induced increase in lymphocyte entry into reactive LN through arteriole remodeling and expansion.

Discussion
The immune system faces the daunting task of screening for cognate lymphocytes for a given antigen. Because the precursor frequencies of naïve T and B lymphocytes are extremely low, its capacity to rapidly find and induce expansion of cognate lymphocytes are critical in combating an acute infection. Our data establish a previously undescribed role of CD4+ T-cell help in the initiation of an immune response, namely increasing naïve lymphocyte input into the reactive LN. Our data are consistent with a model by which DCs migrating into the LN interact with CD4+ T cells through MHCII and receive help through CD40. This process is accompanied by an expansion of the LN feed arteriole, promoting the enhanced recruitment of polyclonal naïve CD8 T cells into the LN and resulting in more efficient screening for cognate CD8+ T cells (10). This mode of CD4+ T-cell help likely occurs before and/or in parallel to the other help pathways known to be mediated by CD4+ T cells, such as licensing of DCs for efficient formation of effector and memory cells (5) and...
guiding of CD8+ T cells to antigen-presenting DCs within the LNs (3). Although this mode of help depends on TCR engagement by MHCIi plus peptide complex, it occurs very rapidly (24 h) and is likely mediated by a small number of cognate CD4+ T cells engaging antigen-presenting DCs. In fact, naïve CD4+ T cells are known to constitutively express abundant CD40L and engage CD40 on APCs (30).

Our data indicate that there are two separate pathways that mediate LN enlargement. The first pathway is innate, in that it does not require CD4+ T cells or specific antigen. This type of enlargement is observed at very early time points after immunization (12 h) (Fig. 4 A), in the absence of adaptive immunity (Fig. 6A) (25), or after inoculation of CpG without antigen (Fig. 8A). We do not yet understand the mechanism that governs the innate stage of LN expansion. However, we start to see signs of CD4+ T-cell–dependent LN input even at this stage because donor CD8+ T-cell entry into dLN is enhanced in the presence of CD4+ T cells (Fig. 4 A Right). The second pathway is adaptive, in that it requires antigen-specific CD4+ T cells and DC interaction. This latter pathway is evident after 24 h of infection (Fig. 4 B and C) and is mediated by remodeling of feed arteriole to a larger diameter. During infection or immunization, LN enlargement likely reflects a combination of these two pathways because varying degrees of CD4-independent enlargement is observed under most conditions tested.

We found that CD4+ T cells are necessary to remodel the LN feed arteriole to a larger diameter. We observed a striking impairment of the primary feed arteriole to enlarge in the absence of CD4+ T cells after HSV-2 infection. Of note, the average diameter of the LN feed arteriole in naïve Cd4−/− mice was consistently larger than in the WT counterpart. These data suggest that CD4+ T cells also regulate vascular function at the steady state. The precise mechanism by which CD4+ T cells support arteriole remodeling remains unclear. It is known that antigen-bearing DCs migrating from inflamed tissues preferentially wrap around high endothelial venules, enabling the DCs to scan the arriving lymphocytes at their site of entry into the LN (31). It is possible that “helped” DCs transmit signals to the high endothelial venules, inducing remodeling of the arteriole upstream. Although the requirement of CD40 in BMDCs to induce full enlargement of the dLN (Fig. 3 A) suggests that licensing of DC by CD4+ T cells is involved in this process, it is also possible that antigen-specific CD4+ Th1 cells themselves, after interacting with DCs, directly enhance naïve lymphocyte entry. Indeed, IFN-γ, a major effector cytokine produced by antigen-specific Th1 cells, is known to activate high endothelial venules and enhance attachment and transendothelial migration of lymphocytes (32–35). However, this latter possibility is likely only relevant at later time points and would not explain LN enlargement at 24 h. Future studies are needed to determine the mechanism by which arteriole remodeling is induced by CD4+ T–DC interaction.

Our results demonstrated that naïve lymphocyte input is significantly impaired in ΔCD4T mice. However, we have not ruled out the possibility that CD4+ T cells also induce LN hyperplasia by increasing retention of lymphocytes within the reactive LNs. It is known that LN shutdown occurs rapidly after infection or immunization (36), and recent studies illustrate the underlying molecular mechanisms (37). It is possible that CD4+ T–DC interaction leads to a more efficient shutdown of lymphocyte egress, which would contribute to LN enlargement and more efficient activation of cognate CD8+ T cells and B cells.

We believe that there are multiple pathways that link CD4+ T cells and enhanced LN input, some of which might use molecules that are redundant with CD40. For instance, TLR stimulation or overt infection could induce signals that either override the requirement for CD4+ T-cell help altogether or induce CD40-independent signals in DCs and other cells to achieve LN enlargement. The immune system is set up to maximize the recruitment of naïve antigen-specific CD8+ T cells in response to infection in WT animals (38). Our data suggest that CD4+ T-cell help in maximizing naïve lymphocyte input to the reactive LN is critical in achieving such efficient screening for cognate antigen by naïve CD8+ T cells. In addition, this mode of CD4+ T-cell help is likely not restricted to CD8+ T cells because polyclonal B-cell entry into reactive LNs was also severely reduced in helpless mice. Finally, our current findings may in part explain the severe immune deficiencies in CD8+ T and B-cell compartments observed after depletion of CD4+ T cells during the AIDS stage of HIV-1 infection.

### Methods

**Mice.** B6, B6.SJL-PtprcPep3BoyJ (congenic CD45.1 mice on B6 background), B6.129S-H2M–/–, Cd40–/–, and Cd44– mice were purchased from the National Cancer Institute and Jackson Laboratories. The gB−1 TCR transgenic mice (5, 23), which are specific for the immunodominant HSV glycoprotein B peptide gB55–60, and CD11c-DTR mice (39), which express diphtheria toxin receptor under the CD11c promoter, were previously described. All procedures used in this study complied with federal guidelines and institutional policies of the Yale Animal Care and Use Committee and University of Northern British Columbia Animal Use Committee.

**Mouse Treatment.** For vaginal HSV infection, mice were treated with Depo-Provera at 4 d before infection and then inoculated with 106 pfu TK-HSV2 in the vagina (40). The draining iliac LNs were harvested at indicated time points. For footpad inoculation, TK-HSV2, CpG2216, splenic DCs, splenic B cells, or BMDCs were injected into the hind footpad in a 20-50 μl volume. The ipsilateral draining or contralateral nondraining popliteal LNs were harvested at indicated time points. For DC depletion, WT or CD11c-DTR mice were injected i.p. with diphtheria toxin (300 ng per mouse; Sigma) 1 d before infection with TK-HSV2 (106 pfu) in the footpad. Popliteal LNs were harvested at 2 d. DC depletion was confirmed by flow cytometry.

**Intravital Microscopy.** LN feed arteriole diameter was measured as previously described (10). Briefly, a midline incision was made along the ventral surface of the abdominal cavity of anesthetized mice. The skin was retracted and pinned onto a pedestal while continuously superfused with a bicarbonate-buffered physiological salt solution equilibrated with 5% CO2/95% N2 (pH 7.4, 34 °C). Surrounding adipose tissue was cleared, and the main arterial segment lying adjacent to the LN was visualized and evaluated. The preparation was secured on an intravital microscope, and the feed arteriole was observed at a total magnification of ×950 using video microscopy. Internal vessel diameter was determined from the width of the red blood cell column by using a video caliper. The same vessel segment was studied in each mouse per group. The resting internal diameter was measured after 60-min equilibration with physiological salt solution. The preparation was then equilibrated with sodium nitroprusside (100 mM; Sigma-Aldrich) to obtain maximal diameter in vivo.

**Statistical Analysis.** Statistical significance was tested either by two-tailed Student’s t test or by one-way ANOVA followed by Tukey’s post test using GraphPad PRISM software (Version 4.0b; GraphPad Software). Data are presented as mean ± SEM.

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Supporting Information

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SI Methods

Adoptive Transfer of Lymphocytes and Dendritic Cells (DCs). CD4+ or CD8+ T cells were negatively isolated from the spleen and lymph nodes (LNs) of naïve WT C57BL/6 (B6) or gBT-I T-cell receptor (TCR) transgenic (on B6CD45.1 background) mice by using CD4+ T Cell Isolation Kit II and CD8+ T Cell Isolation Kit (Miltenyi Biotec) according to manufacturer’s protocol. In general, the purity of isolated cells was ~90--95%. Cells were labeled with 0.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen), then transferred retro-orbitally. For homing experiments in ΔCD4T hosts, mice were infected with thymidine kinase–defective herpes simplex virus 2 (TK−HSV2) in the footpad for 12 h, and then 1.5 × 10⁶ CFSE-labeled naïve WT CD8+ T cells were transferred retro-orbitally. The popliteal LNs were harvested for analysis 12 h after the transfer of CD8+ T cells. For homing experiments in Rag-1−/− hosts, Rag-1−/− or OT-II/Rag-1−/− mice were infected with TK−HSV2 in the footpad for 7 h, and then 1.25 × 10⁶ CFSE-labeled naïve WT CD8+ T cells were transferred retro-orbitally. The popliteal LNs were harvested for analysis 23 h after the transfer of CD8+ T cells. For short-term homing experiments in the iliac LNs, mice were infected intravaginally with TK−HSV2 for 24 or 43 h, and then 5 × 10⁷ total spleen and LN cells prepared from congenic CD45.1+ naïve WT mice were injected retro-orbitally. The draining iliac and nondraining brachial (both sides) LNs were harvested at 2 h after the donor cell transfer. For splenic B-cell and DC isolation, cells were positively selected from total splenocytes by using anti-B220 or anti-CD11c microbeads (Miltenyi Biotec), respectively, according to manufacturer’s protocol. One million cells were injected into the hind footpad, and LNs were harvested at 4 d postinfection (dpi). For DC migration assay, bone marrow–derived DCs (BMDCs) were labeled with 0.5 μM CFSE and injected into the hind footpad, and the draining LNs (dLNs) were harvested at 2 d after injection.

Preparation of BMDCs. Bone marrow cells from mice of indicated genotype were culture for 5--7 d in RPMI medium 1640 supplemented with 10% FCS and GM-CSF (1). DCs were then purified with anti-CD11c microbeads (Miltenyi Biotec), and 5--10 × 10⁵ cells were injected into the hind footpad of the indicated host mice.

Flow Cytometric Analysis. For determining LN cellularity and phenotyping gBT-I cells, LNs and spleens were minced with fine scissors, mashed with a plunger of a 3-mL syringe, and digested in 2 mg/mL collagenase D (Roche) and 0.1 mg/mL DNase I (Roche) in RPMI medium 1640 supplemented with 10% FCS for 30 min at 37 °C. Total cells were counted with a hemocytometer. For detecting donor gBT-I cells, cells were stained with anti-CD4 (GK1.5; BioLegend), anti-CD8a (53-6.7; BioLegend), anti-B220 (RA3-6B2; BD Pharmingen), anti-CD45.1 (A20; BioLegend), anti-TCRVα2 (B20.1; BD Pharmingen), anti-CD69 (H1.2F3; eBioscience), and/or anti-CD25 (PC61.5; eBioscience) along with 7-amino-actinomycin D (eBioscience) staining for viability. For intracellular staining of granzyme B, after staining for the cell surface antigens, cells were fixed and permeabilized with Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience) according to the manufacturer’s protocol and stained with anti-granzyme B (16G6; eBioscience). Cells were analyzed on an LSRII (BD Bioscience).


Fig. S1. The number of non-CD4+ T cells in the dLN is reduced in ΔCD4T mice compared with WT mice after vaginal HSV2 infection. Mice were infected as in Fig. 1. Numbers of non-CD4+ T cells were calculated by subtracting the number of CD3ε+ CD4+ cells from the total cellularity. Data are pooled from two or more independent experiments. *P < 0.05, **P < 0.01 by one-way ANOVA.

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**Fig. S2.** CD4+ T cells are required for inflammation-induced dLN enlargement. (A and B) CpG 2216 oligo DNA (10 μg), TK− HSV2 (1 × 10^6 pfu) (A), or BMDC (1 × 10^6 cells) from WT mice (B) were injected in the footpad of the indicated host in separate groups. The draining popliteal LNs were collected at 4 dpi. Data are pooled from two or more independent experiments. *P < 0.05, ***P < 0.001 by two-tailed t test. (C) WT or CD4KO mice were injected with BMDCs (1 × 10^6) from WT or CD4KO mice. Cell numbers in the popliteal dLNs (4 d after injection) are shown. **P < 0.01 by one-way ANOVA. (D) WT or Myd88−/− Ticam1−/− BMDCs purified by magnetic beads were cultured overnight with or without LPS. Expression of major histocompatibility complex class II (MHCII), CD80, and CD86 were examined by flow cytometry. Note that the expression levels of these molecules in unstimulated WT BMDCs are virtually identical to that observed in Myd88−/− Ticam1−/− BMDCs, suggesting no contamination of LPS in the culture media. Therefore, BMDC-induced LN hyperplasia shown in this study is not caused by LPS contamination. (E) Unstimulated WT or Myd88−/− Ticam1−/− BMDCs (1 × 10^6 cells) were injected in the right footpad, and PBS was injected in the left footpad. Total cellularity in the dLN at 4 dpi is shown.

**Fig. S3.** Reconstitution of CD4+ T cells in CD4KO mouse recovers CD25 induction in cytotoxic T lymphocytes (CTLs). WT naïve polyclonal CD4+ T cells (2.5 × 10^7 cells) were transferred retro-orbitally into MHCIIKO or CD4KO hosts. Then 1 d later, 1 × 10^6 CFSE-labeled naïve gBT-I cells were transferred retro-orbitally into these hosts as well as into untreated WT recipients. At 1 d after the gBT-I transfer, mice were infected vaginally with TK− HSV2. dLNs were collected at 4 dpi. Note that cells in the CFSE–CD25+ gate (percentage shown in bold) increased in CD4KO hosts with CD4+ T reconstitution compared with MHCIIKO hosts.
Fig. S4. CD69 expression occurs before division of gBT-I cells after intravaginal HSV infection. CFSE-labeled gBT-I cells were transferred, and the host mice were infected as in Fig. 1. CFSE levels in gBT-I cells (CD8α+ TCRα2+ CD45.1+) at 2 dpi are shown.

Fig. S5. Cellular and molecular requirement for HSV-induced LN hypertrophy. (A and B) Mice of indicated genotypes were adoptively transferred with ∼5 × 10⁶ WT polyclonal CD4+ T cells and then infected with 10⁶ pfu TK− HSV2 in the footpad. Numbers of reconstituted CD4+ T cells (A) and total draining popliteal LN cells (B) at day 4 are shown. Data are pooled from two independent experiments. (C) DCs or B cells (1 × 10⁶ cells each) isolated from the spleens of WT mice were injected into the footpad of WT mice. LN cellularity was measured on day 4. (D) DCs were depleted in CD11c-DTR mice 1 d before injection of 10⁶ pfu TK− HSV2 in the footpad, and LN cellularity was analyzed at 2 dpi. Data are representative of two independent experiments. (E and F) WT or B-cell-deficient μMT mice were infected with 10⁶ pfu TK− HSV2 in the footpad. Numbers of total popliteal LN cells (E) or B220− non-B cells (F) are shown. Data are representative of three independent experiments. *P < 0.05, ***P < 0.001 by two-tailed t test (B, D, and E) or one-way ANOVA (C). n.s., not significant.
Fig. S6. Antigen nonspecific CD4+ T cells are insufficient to induce CD8+ T-cell–dependent naïve CD8+ T-cell entry into the dLNs. (A and B) OT-II/rag-1−/− or rag-1−/− mice were infected in the footpad with TK−HSV2 (1 × 10^6 pfu) for 7 h, and then 1.25 × 10^6 CFSE-labeled WT polyclonal CD8+ T cells were transferred retro-orbitally. Total LN cells (A) and donor CD8+ T cells (B) were enumerated at 30 h postinfection. (C) WT naïve polyclonal or OT-II/rag-1−/− CD4+ T cells (2.5 × 10^7 cells) were transferred retro-orbitally into MHCIIKO or CD4KO hosts. Then 1 d later, the recipients were infected with 1 × 10^6 pfu TK−HSV2 in the footpad. The dLNs were harvested at 4 dpi, and the cellularity was calculated. *P < 0.05, **P < 0.01, ***P < 0.001 by two-tailed t test (A, B, and C, MHCIIKO) or one-way ANOVA (C, CD4KO). n.s., not significant.