MAdCAM-1 Expressing Sacral Lymph Node in the Lymphotoxin β-Deficient Mouse Provides a Site for Immune Generation Following Vaginal Herpes Simplex Virus-2 Infection[1]

Kelly A. Soderberg, Melissa M. Linehan, Nancy H. Ruddle, and Akiko Iwasaki[2]

The members of the lymphotoxin (LT) family of molecules play a critical role in lymphoid organogenesis. Whereas LTα-deficient mice lack all lymph nodes and Peyer's patches, mice deficient in LTβ retain mesenteric lymph nodes and cervical lymph nodes, suggesting that an LTβ-independent pathway exists for the generation of mucosal lymph nodes. In this study, we describe the presence of a lymph node in LTβ-deficient mice responsible for draining the genital mucosa. In the majority of LTβ-deficient mice, a lymph node was found near the iliac artery, slightly misplaced from the site of the sacral lymph node in wild-type mice. The sacral lymph node of the LTβ-deficient mice, as well as that of the wild-type mice, expressed the mucosal addressin cell adhesion molecule-1 similar to the mesenteric lymph node. Following intravaginal infection with HSV type 2, activated dendritic cells capable of stimulating a Th1 response were found in this sacral lymph node. Furthermore, normal HSV-2-specific IgG responses were generated in the LTβ-deficient mice following intravaginal HSV-2 infection even in the absence of the spleen. Therefore, an LTβ-independent pathway exists for the development of a lymph node associated with the genital mucosa, and such a lymph node serves to generate potent immune responses against viral challenge. ***The Journal of Immunology*****, 2004, 173: 1908–1913.**

Lymphotoxins (LT)[3] are cytokines structurally related to the TNF-mediating activities, including tumor cytotoxicity, cell proliferation, differentiation, and antiviral responses (1–3). The genes for LTα and β cluster along with that for TNF-α in the MHC locus. The LT are produced by a variety of cells, including activated lymphocytes and NK cells, and exist as either homotrimers (LTα_3) or heterotrimers (LT$\alpha_1\beta_2$ or the less prevalent LT$\alpha_2\beta_1$). Although the LTα_3 homotrimer is soluble, lacking a transmembrane domain, the heterotrimeric complexes can be retained on the cell surface because LTβ is a type II membrane protein. The LTα homotrimer has been shown to bind to p55 TNFR (TNFR-1 and TNFR-II), whereas the LT$\alpha_1\beta_2$ heterotrimer binds to the LTβR (4, 5).

Knockout mice have been generated for both LTα and LTβ, demonstrating the importance of LT in lymphoid organogenesis (4, 6–8). These mice exhibit both the absence of peripheral lymph nodes (PLNs) and Peyer's patches as well as disorganized splenic architecture. LTβ-deficient mice, however, maintain both the cervical and mesenteric lymph nodes (MLNs) (4, 8), indicating an LTβ-independent pathway for the generation of mucosal lymph nodes. Nevertheless, the mucosal lymph nodes that develop in the LTβ-deficient mice have disorganized T cell and B cell distribution and have altered expression of addressins on the high endothelial venules (HEVs) (9). Furthermore, LTα is sufficient to induce the expression of mucosal addressin cell adhesion molecule (MAdCAM)-1, specifically expressed by the mucosal lymph node venules in vivo (10). Early during embryonic development, all lymph node HEVs of wild-type mice express MAdCAM-1. However, a developmental switch occurs soon after birth, allowing PLN-HEVs to instead express peripheral node addressin (PNAd), while mucosal lymph node HEVs continue to express MAdCAM-1 in addition to PNAd (11).

In this study, using a mouse model of genital herpes infection, we dissect the mechanism of immune induction in the LTβ-deficient mouse. Intravaginal (ivag) inoculation of HSV-2 in progesterone-treated wild-type (WT) mice results in the generation of T cell responses in the iliac (consisting of 2–3 lumbar and 1 sacral lymph nodes) and inguinal lymph nodes that drain the vaginal mucosa (12). These lymph nodes were reported to be absent in the LTβ-deficient animals (4, 8). However, because MLNs and cervical lymph nodes, which drain the gut and oral/nasal mucosa, respectively, are present in these mice, we explored whether lymph nodes draining the genital mucosa are also retained in the LTβ-deficient mice. In this study, we report the presence of a lymph node adjacent to the iliac artery in the LTβ-deficient mice, situated at a location slightly misplaced from that of the sacral lymph node of the WT mice. This displaced sacral lymph node in the LTβ-deficient mice expresses MAdCAM-1 in addition to PNAd in a pattern similar to the MLN. Furthermore, we show that the sacral lymph node in WT mice also expresses MAdCAM-1, a characteristic not shared by the lumbar and other PLNs. Finally, we demonstrate that this sacral lymph node plays an important role in the generation of immunity

Department of Epidemiology and Public Health and Section of Immunobiology, Yale University School of Medicine, New Haven, CT 06520

Received for publication January 7, 2004. Accepted for publication May 28, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the Burroughs Wellcome Career Award in Biomedical Sciences (to A.I.), National Institutes of Health Grant NCI R01 CA 16885 (to N.H.R.), and a Minority Supplement to CA16885 (to K.A.S.).

[2] Address correspondence and reprint requests to Dr. Akiko Iwasaki, Department of Epidemiology and Public Health and Section of Immunobiology, Yale University School of Medicine, 60 College Street, New Haven, CT 06520. E-mail address: akiko.iwasaki@yale.edu

[3] Abbreviations used in this paper: LT, lymphotoxin; DC, dendritic cell; FDC, follicular DC; HEV, high endothelial venule; ivag, intravaginal; LIGHT, LT homolog, which exhibits inducible expression, competes with HSV glycoprotein D for HveA and is expressed on T lymphocytes; MAdCAM, mucosal addressin cell adhesion molecule; MLN, mesenteric lymph node; p.i., postinfection; PLN, peripheral lymph node; PNAd, peripheral node addressin; RT, room temperature; TK, thymidine kinase; WT, wild type.

0022-1767/04/$02.00

within the LTβ-deficient mice following ivag infection with HSV-2. These results provide both phenotypic and functional evidence for the sacral lymph node that develops in the absence of LTβ as a site for immune induction against a sexually transmitted viral agent.

Materials and Methods

Virus

HSV-2 strain thymidine kinase (TK)-deficient 186TKΔKpn was a generous gift from D. Knipe (Harvard Medical School, Boston, MA), and was constructed, as described previously (13). All stocks were propagated and titered on the Vero cell line before use in ivag infections.

Animals and HSV-2 infection

Six- to 8-wk-old female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The generation of LTβ-deficient mice was described previously (4). For virus infection, mice were injected s.c. in the neck ruff with Depo-Provera (Pharmacia & Upjohn, Kalamazoo, MI) at 2 mg/mouse in 100 μl vol 5 days before infection, swabbed with calcium alginate, and inoculated with 1×10^6 PFU of HSV-2 strain 186TKΔKpn, or inoculated with noninfected Vero control lysate (mock infection) in 10 μl vol using a blunt-ended micropipette tip, as previously described (14). In some experiments, mice were splenectomized and, upon complete recovery, infected ivag with186TKΔKpn, as described above. All procedures complied with federal guidelines and institutional policies by the Yale animal care and use committee.

Visualization of lymph nodes

One percent of Evans blue was injected s.c. into the footpads of mice, and the lymph nodes were analyzed the following day.

ELISA

Immulon flat-bottom plates (Fisher Scientific, Hanover, IL) were coated with anti-HSV-2 Ab (DakoCytomation, San Diego, CA) in carbonate buffer overnight at 4°C. Plates were washed and blocked with 10% FCS in PBS for 2 h at room temperature (RT). HSV-2 lysate (Applied Biosystems, Columbia, MD) at a protein concentration of ~5.8 μg/ml was added for 3 h at RT; plates were washed; and dilutions of serum were added to the wells in triplicate. Known amounts of mouse mAb specific to HSV-2 gB (SS10 mouse IgG), kindly provided by G. Cohen and R. Eisenberg (University of Pennsylvania, Philadelphia, PA), were added in parallel as an internal reference. Plates were washed; HRP-conjugated anti-mouse IgG at 0.8 μg/ml (Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 1 h at RT; and the enzymatic reaction was measured using tetramethylbenzidine substrate (eBioscience, San Diego, CA). Upon completion, the reaction was stopped with 2 N H_2SO_4, and the OD was measured at 450 nm.

Antibodies

Abs used for the identification of specific cell populations and surface molecules were purchased from eBioscience: CD11c (N418), CD86 (GL-1), and CD8α (53-6.7). Abs specific for MAdCAM-1 (MECA 367) and PNAd (MECA 79) were purchased from BD Pharmingen (San Diego, CA). For localization of HSV-2-infected cells, FITC-conjugated HSV-2 (Virostat, Portland, ME) was used.

Immunofluorescence staining of tissues

To examine the expression of addressins, frozen sections of lymph nodes were stained with a combination of Abs to MAdCAM-1 and PNAd in a procedure similar to that described previously (14). The stained slides were analyzed by confocal microscopy (Zeiss LSM510; Oberkochen, Germany) using a ×63 objective lens with water.

Preparation of dendritic cells (DCs)

DCs were prepared from lymphoid organs of mice, as previously described (14). Briefly, the inguinal, iliac lymph nodes and MLNs from B6 control mice, as well as mesenteric and sacral lymph nodes from the LTβ-deficient mice were collected at the indicated time points following HSV-2 ivag infection. The tissues were digested with collagenase D and DNase I, followed by an incubation in 5 mM EDTA. Single cell suspensions were prepared and incubated with anti-mouse CD11c-coated magnetic beads (Miltenyi Biotec, Auburn, CA) and selected on MACS separation columns to obtain DCs, as previously described (14).

Stimulation of HSV-2-specific $CD4^+$ T cells

To analyze $CD4^+$ T cell responses in the lymphoid tissues following ivag HSV-2 infection, $CD4^+$ T cells isolated from the indicated lymph nodes of mice at 4 days postinfection (p.i.) using anti-CD4-conjugated magnetic beads (Miltenyi Biotec) were incubated with syngeneic splenocytes that had been irradiated at 2000 rad and pulsed with viral Ags or control lysate, as described previously (14). To determine the ability of DCs to stimulate HSV-2-specific T cells, 10^5 $CD4^+$ T cells isolated from the draining lymph nodes of WT mice infected ivag with HSV-2 4 days prior were cocultured with 10^5 DCs in the presence or absence of exogenously added viral Ags, and cytokine secretion from $CD4^+$ T cells was assayed by ELISA, as previously described (14).

Results

Identification of a genital lymph node in the LTβ-deficient mice

LTβ-deficient mice are reported to lack PLNs, yet retain the lymph nodes draining the gut and oral/nasal mucosa. Therefore, we wished to examine whether lymph nodes draining the genital mucosa are also retained in these mice. Both naive (Fig. 1*A*) and HSV-2-infected (Fig. 1*B*) WT mice possess a collection of lymph nodes known as the iliac lymph nodes that consist of two to three lumbar (yellow arrows) and one sacral (green arrow) nodes. All of the iliac lymph nodes of the infected WT mice became enlarged as a result of HSV-2 infection (Fig. 1*B*). Upon careful examination of the LTβ-deficient mice, we discovered the presence of a lymph node located adjacent to the iliac artery (Fig. 1, *C* and *D*; red arrow), slightly misplaced from the location of the WT sacral lymph node (Fig. 1, *A* and *B*; green arrow). This sacral lymph node was also found in majority of the naive LTβ-deficient mice, although much smaller in size (Fig. 1*C*). In a minority of LTβ-deficient mice, the sacral lymph node was not visible (Table I). The diminutive size of this lymph node may explain why it was not generally noted in previous publications, in which either naive mice or those immunized by other routes were evaluated (4, 8).

The sacral lymph node was present in both the naive (70%) and the HSV-2-infected (76%) LTβ-deficient animals at a frequency similar to what had been reported for MLNs present in these mice

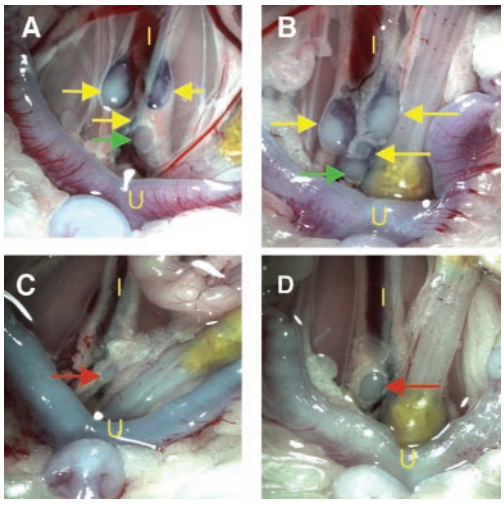

FIGURE 1. A lymph node is present near the iliac artery in LTβ-deficient mice. Lumbar and sacral lymph nodes in WT mice either naive (*A*) or infected ivag with HSV-2 for 4 days (*B*) are depicted. A lymph node is present near the iliac artery in a majority of the LTβ-deficient mice either naive (*C*) or infected with HSV-2 for 4 days (*D*). Lumbar lymph nodes (yellow arrows), sacral lymph nodes (green arrows), and the displaced sacral lymph nodes of the LTβ-deficient mice (red arrows) are indicated. U, uterus; I, iliac artery. A total of 83 mice was examined in a similar manner as described in Table I.

Table I. *Presence of sacral lymph node in LTβ$^{-/-}$ mice*

Infection Status	Total No. Mice	Sacral Lymph Node		% Total
		Yes	No	
Mock	34	24	10	70
HSV-2 infected	49	37	12	76

(8) (Table I). Furthermore, this sacral lymph node was present in both female and male LTβ-deficient mice.

Sacral lymph node displays MAdCAM-1

Naive T and B lymphocytes enter lymph nodes and Peyer's patches via the HEVs following a series of lymphocyte-endothelial cell interactions initiated by receptor binding to the HEV addressins (15, 16). Although naive lymphocytes bind via L-selectin to the addressin PNAd expressed by the HEVs of the PLNs, the mucosal vascular addressin, MAdCAM-1, is specifically displayed by the HEV of lymph nodes draining the mucosal surfaces (17, 18). MAdCAM-1 expressed on the endothelial cells of mucosal lymphoid tissues can mediate entry of lymphocytes via L-selectin or $\alpha_4\beta_7$. Because the sacral lymph node was retained in the LTβ-deficient mice, we examined whether this lymph node also possessed characteristics of a mucosal lymph node.

Consistent with the previously reported addressin phenotypes of the PLNs (11), MAdCAM-1 staining was absent from HEVs of the lumbar lymph nodes of the WT mice (Fig. 2*A*). Interestingly, the sacral lymph node of WT mice displayed both PNAd and MAdCAM-1 on their HEVs (Fig. 2*B*). This pattern of expression was similar to the MLN (Fig. 2*C*), but not to the PLN (Fig. 2*A*). The sacral lymph node present in the LTβ-deficient mice also co-expressed PNAd and MAdCAM-1 (Fig. 2*D*), similar to expression patterns found on the MLN HEVs (Fig. 2*E*). The MLNs from both the LTβ-deficient mice (Fig. 2*E*) and the WT control mice (Fig. 2*C*) contained HEVs expressing MAdCAM-1 and PNAd, as expected. The expression pattern of PNAd within the LTβ knockout sacral lymph node was similar to what has been previously reported in the MLN of these mice (9), namely, that some of the HEVs expressed only abluminal PNAd (Fig. 2*D*). Furthermore, the sacral lymph node appeared to have disrupted T/B organization similar to what has been previously reported (4, 8) for the MLN of LTβ-deficient mice (data not shown).

Activation of CD4$^+$ T cells within the sacral lymph node

Previously, we demonstrated that HSV-2-specific CD4$^+$ T cells are present within the vaginal draining lymph nodes of WT mice as early as 3 days following ivag infection with HSV-2 (14). To examine the functional relevance of the sacral lymph node in the LTβ-deficient mice, we analyzed the presence of HSV-2-specific CD4$^+$ T cells in this lymph node following ivag infection. CD4$^+$ T cells were isolated 4 days following ivag HSV-2 infection from the sacral lymph node of the LTβ-deficient mice, and their ability to secrete IFN-γ following incubation with APCs presenting viral Ags was assessed. To specifically compare CD4$^+$ T cell responses generated in the sacral lymph nodes, we separately isolated CD4$^+$ T cells from the genital draining lymph nodes of WT mice. Thus, CD4$^+$ T cells from inguinal, lumbar, and sacral lymph nodes of WT mice, or sacral lymph nodes of LTβ-deficient mice were collected. This analysis revealed that Ag-specific CD4$^+$ T cells capable of secreting high levels of IFN-γ were present in the sacral lymph node of the LTβ-deficient mice at 4 days p.i., albeit at slightly reduced levels compared with those of WT mice (Fig. 3). WT mice also contained HSV-2-specific CD4$^+$ T cells in the lumbar, sacral, and inguinal lymph nodes (Fig. 3). Moreover, nondraining lymph nodes such as the MLN did not contain Ag-specific T cells in either the LTβ-deficient or WT mice (data not shown). These results suggested that the sacral lymph node that develops in the LTβ-deficient mice provides a site for CD4$^+$ T cell priming following genital viral infection.

DCs present MHC class II-associated HSV-2 peptides in the sacral lymph node of LTβ-deficient mice

Because activated HSV-2-specific CD4$^+$ T cells were discovered within the sacral lymph node, but not in the MLN of LTβ-deficient mice at 4 days p.i., we tested whether this lymph node provides a site for CD4$^+$ T cell priming, and whether DCs are involved in this process. Because DC migration into lymphoid tissues has been

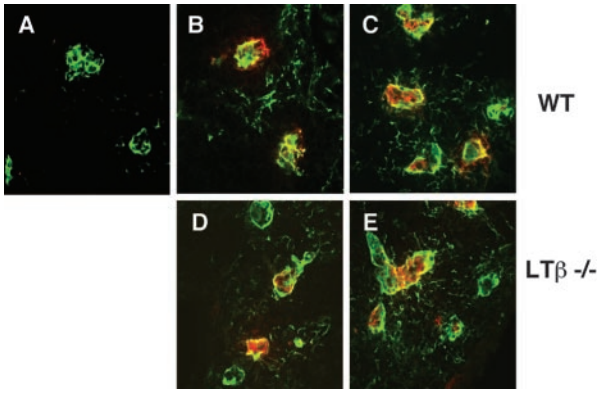

FIGURE 2. MAdCAM-1 and PNAd are expressed in both the sacral lymph node of the LTβ-deficient mice and the WT mice. Cryosections of the lumbar (*A*), sacral (*B*), and the MLN (*C*) of WT mice or the sacral lymph node (*D*) and the MLN (*E*) of the LTβ-deficient were stained with Abs to PNAd (green) and MAdCAM-1 (red) and analyzed by confocal microscopy. These figures are representative of lymph nodes obtained from 8–10 mice in each group.

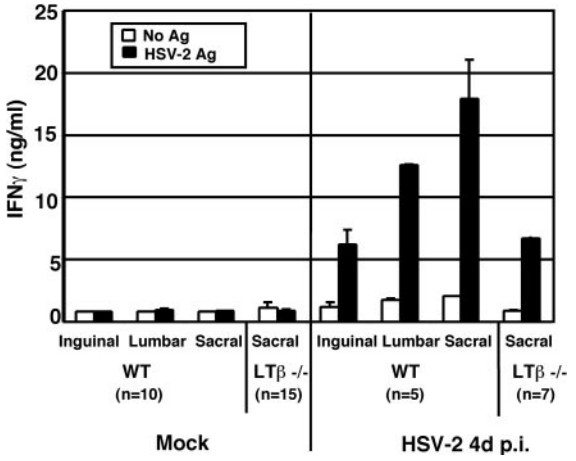

FIGURE 3. Cytokine secretion from CD4$^+$ T cells in the sacral lymph node of ivag HSV-2-infected LTβ-deficient mice. CD4$^+$ T cells were isolated from the sacral lymph node of LTβ-deficient mice, or from the individual vaginal draining lymph nodes (inguinal, lumbar, or sacral) of WT mice that have been either infected with HSV-2 ivag for 4 days or mock infected. The lymph node CD4$^+$ T cells were cocultured with irradiated syngeneic splenocytes in the presence (■) or absence (□) of viral Ags for 3 days, and supernatants were analyzed for IFN-γ. The numbers in brackets indicate the number of mice used in each group in a representative experiment. These data are representative of five similar experiments.

shown to be severely disrupted in the absence of the LT$\alpha\beta$ complex (19), we examined DC migration to the sites of infection and their subsequent migration into secondary lymphoid tissues following ivag HSV-2 infection. In WT mice, upon vaginal infection with HSV-2, DCs are recruited to the submucosa of the infected epithelium by 24 h p.i. (14). There were no significant differences in the influx of DCs near the HSV-2-infected vaginal epithelium in LTβ-deficient mice compared with WT control mice (data not shown). Thus, the migration of DCs into the peripheral tissues in response to HSV-2 infection was intact in the LTβ-deficient mice.

Next, we examined the migration and activation of DCs in the vaginal draining lymph nodes. DCs from the sacral lymph node of LTβ deficient or from the vaginal draining lymph nodes (inguinal, lumbar, and sacral) of WT mice were collected at 3 days p.i. Similar to DCs within the vaginal draining lymph nodes of WT mice, a small percentage (~3%) of the CD11c$^+$CD8α^- DCs within the LTβ-deficient sacral lymph node expressed increased levels of CD86 at 3 days p.i. (Fig. 4). The CD11c$^+$CD8α^-CD11b$^+$ DCs have been shown to mediate CD4$^+$ T cell priming following genital HSV-2 infection (14). These results suggested that the activation and migration of DCs from the site of infection to the draining lymph node occur in the LTβ-deficient mice.

Finally, the ability of DCs from each of these lymphoid organs to present in vivo derived viral Ags was examined. HSV-2-specific CD4$^+$ T cells from WT mice were incubated with purified DCs from the sacral lymph nodes of LT$\beta^{-/-}$ or total vaginal draining lymph nodes (inguinal, lumbar, and sacral) of WT mice at 3 days p.i. in the absence of exogenously added Ags (Fig. 5*A*). In the WT mice, Ag-presenting DCs were only present in the vaginal draining lymph nodes and not in the MLN (Fig. 5*A*). Similarly, IFN-γ secretion from HSV-2-specific CD4$^+$ T cells was induced only by the DCs isolated from the sacral lymph node, but not from the MLNs, of the LTβ-deficient mice. Upon addition of exogenous viral Ags, all DCs were found to stimulate IFN-γ secretion from CD4$^+$ T cells (Fig. 5*B*), indicating that Ag-presenting functions in general were intact in various DC isolates. Therefore, collectively, these data indicated that the sacral lymph node of the LTβ-deficient mice serves as a site for CD4$^+$ T cell activation following ivag HSV-2 infection.

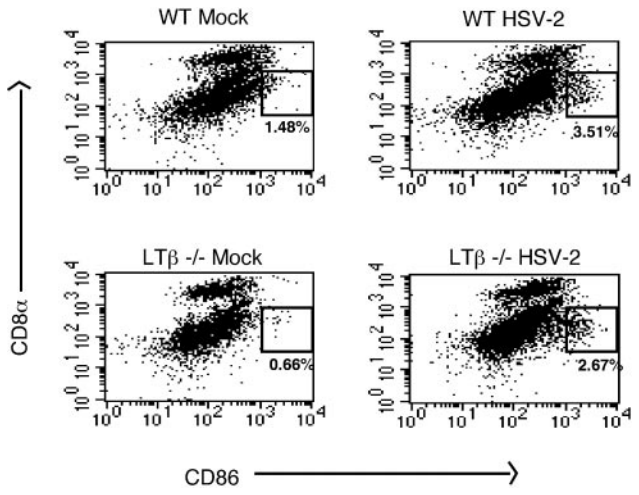

FIGURE 4. Activation of DCs in the LTβ-deficient sacral lymph node. Lymphocytes were isolated from the sacral lymph node of LTβ-deficient mice and vaginal draining lymph nodes of WT mice that were infected ivag 3 days with HSV-2 or inoculated with control lysate (mock). Costimulatory molecule CD86 expression levels were determined by flow cytometry. The square gate indicates a population of CD11c$^+$/CD8α^- DCs that expresses high levels of CD86. This experiment was repeated twice with similar results.

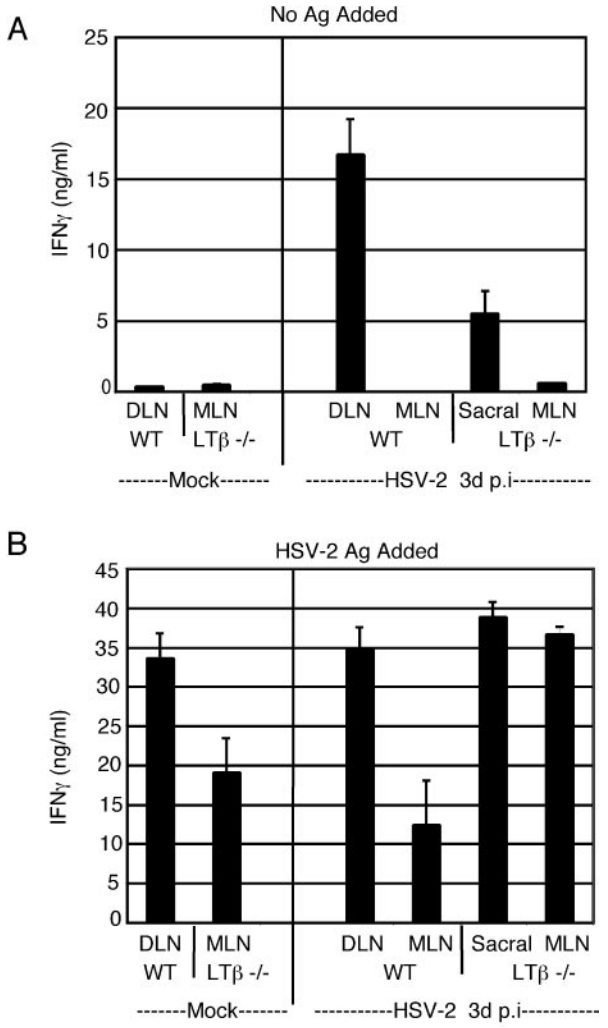

FIGURE 5. DCs present viral peptides to CD4$^+$ T cells in the sacral lymph node of LTβ-deficient mice following ivag HSV-2 infection. To demonstrate the Ag specificity of the T cell responses, DCs were isolated from the indicated lymph nodes of WT or LTβ-deficient mice either mock infected or at day 3 HSV-2 infection. Isolated DCs were coincubated with WT HSV-2-specific CD4$^+$ T cells in the absence (*A*) or presence (*B*) of exogenously added viral Ags. IFN-γ secretion from CD4$^+$ T cells was measured by ELISA. DLN, vaginal draining lymph nodes consisting of inguinal, lumbar, and sacral nodes. These results are representative of three similar experiments.

HSV-2-specific Ig production in LTβ-deficient mice

Iliac and inguinal lymph nodes represent the only sites for CD4$^+$ T cell priming following ivag HSV-2 infection in WT mice (12, 14). Although LTβ knockout mice had been reported to lack both of these lymph nodes, a sacral lymph node was discovered (Fig. 1), and demonstrated to contain Ag-specific CD4$^+$ T cells (Fig. 3) and DCs presenting viral Ags (Fig. 5). These observations suggested that normal immune effector responses could be generated in the LTβ-deficient mice. Thus, we examined whether Th-dependent Ig responses are induced in the LTβ-deficient mice following ivag infection with HSV-2. Consistent with the results obtained with the individual lymph nodes, the HSV-2-infected LTβ-deficient mice produced Ag-specific IgG responses with kinetics and magnitude similar to that of WT mice (Fig. 6). Similar levels of Ag-specific

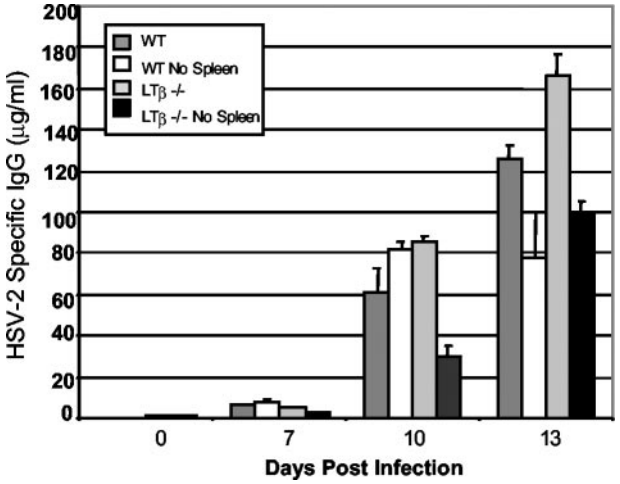

FIGURE 6. HSV-2-specific IgG responses occur in LTβ-deficient mice. Sera were collected from either the splenectomized or intact WT or LTβ-deficient mice at various time points following HSV-2 infection and tested for HSV-specific IgG by ELISA. These data are representative of three separate experiments.

IgG subtypes and IgA were found in the LT$\beta^{-/-}$ and WT mice (data not shown). Although the LTβ-deficient mice lack all PLNs, they retain a disorganized spleen. To more specifically illustrate the contribution of the sacral LN in the Ig responses to genital HSV-2 infection, LT$\beta^{-/-}$ mice and WT mice were splenectomized. HSV-2-specific IgG levels were measured in the infected splenectomized LT$\beta^{-/-}$ mice and WT mice at different time points post-HSV-2 infection, and were compared with those of intact mice (Fig. 6). This analysis showed that albeit at reduced levels, IgG responses occurred even in the absence of spleen in both the WT and the LT$\beta^{-/-}$ mice. Importantly, the reduction in the IgG levels occurred to a similar extent in both WT and LT$\beta^{-/-}$ mice as a result of splenectomy. Thus, these results revealed that the spleen is not necessary for the IgG responses following HSV-2 infection, and sacral lymph node in the LT$\beta^{-/-}$ mice serves as an important immune inductive organ in these mice.

Discussion

Studies using knockout animals for a variety of TNF/LT ligand-receptor members have illustrated the critical roles of these molecules in lymphoid organ development (4, 6, 8, 20–23). Unlike the LTα-deficient mice, the LTβ-deficient mice retain their MLNs and cervical lymph nodes, while LTβR gene-deficient mice are reported to be devoid of all lymph nodes (20). In this study, we demonstrated that the LTβ-deficient mice retain a sacral lymph node located adjacent to the iliac artery. We further provide the first demonstration that this sacral lymph node in the LTβ-deficient mice, as well as the sacral lymph node in WT mice, possessed mucosal characteristics, expressing high levels of MAdCAM-1 on their HEVs. These observations support the previously suggested concept that a unique pathway is present for mucosal lymph node development that is not shared by all other PLNs (1, 24). Our finding of a sacral lymph node in the LTβ-deficient mice is consistent with the role of other factors that can bind to the LTβR, such as LT homolog, which exhibits inducible expression, competes with HSV glycoprotein D for HveA and is expressed on T lymphocytes (LIGHT) in the development of mucosal lymph nodes. In fact, simultaneous disruption of LIGHT and LTβ also results in the ablation of the MLN (25). Because LTβ-deficient mice retain only their mucosal lymph nodes, it remains possible that factors such as LTα and LIGHT are sufficient for mucosal lymph node development. Future analysis of the sacral lymph node in the LTβR gene-deficient mice or the LIGHT-LTβ-disrupted mice will provide further mechanistic insights to the molecular requirements for the development of this lymph node.

During early embryonic development, HEVs of all lymph nodes have been shown to express MAdCAM-1. Soon after birth, all HEVs begin to express PNAd. Whereas PLN HEVs later lose MAdCAM-1 expression, it is maintained within the MLN (11). The striking correlation between the MAdCAM-1 expression and the development of the mucosal lymph nodes suggests that the factors required to maintain MAdCAM-1 expression on the HEV are coregulated with those required for lymphoid organogenesis.

Although the aspects of lymphoid organogenesis have been well characterized in mice genetically lacking a member of the TNF/LT families, the role of the remaining lymphoid structures in the generation and maintenance of immunity in these mice is unclear. In this study, we provide the first demonstration that a lymph node present in the LT$\beta^{-/-}$ mice can serve as a site for CD4$^+$ T cell activation following a genital viral infection. Significant levels of IFN-γ were secreted by the CD4$^+$ T cells isolated from the sacral lymph node of the HSV-2-infected LT$\beta^{-/-}$ mice (Fig. 3), indicating that strong Th1 responses are generated in these mice against a viral pathogen. This level of IFN-γ secretion was consistent with the Ag-presenting activities of the DCs found in the LT$\beta^{-/-}$ sacral lymph nodes (Fig. 5).

Conflicting data exist for the ability of LTβ-deficient mice to elicit Ag-specific Ig. The LTβ-deficient mice lack follicular DCs (FDCs) that are thought to play an important role in Ig responses by retaining Ag-Ig complexes to aid in affinity maturation and B cell memory (4, 8). In this study, we demonstrated the ability of the LTβ-deficient mice to mount HSV-2-specific IgG responses that are similar in kinetics, isotypes, and magnitude to WT mice following ivag HSV-2 infection. Furthermore, significant IgG responses were generated in the absence of the spleen. Previous studies have shown that LTβ-deficient mice have reduced or negligible isotype switching following i.p. administration of sheep RBC, yet showed near normal isotype switching following i.p. nitrophenol-haptenated chicken γ globulin absorbed in alum (4). Thus, the ability of the LTβ-deficient mice to produce Ag-specific IgG may depend on several factors, including the route of administration, the type of Ag, and the presence of adjuvants. It is possible that Ag persistence or repeated immunizations overcome the requirement for the germinal center and FDCs to generate an effective humoral response (26). Besides simple Ag persistence, the presence of microbial signals present either in the adjuvant or in the infectious agents might induce activation of DCs and Th responses that are adequately generated in the LT$\beta^{-/-}$ mice. This theory would account for the differences observed in the Ig responses in the LT$\beta^{-/-}$ mice when using protein Ags (4) vs infectious agents, such as in this study. Thus, the germinal center/FDC complex might be important to support affinity maturation when the Ag is present transiently without microbial stimuli (protein Ag), although it is not required for Ags that are long-lived and/or infectious (presence of adjuvant or infectious agents).

Our study characterized the phenotype and function of a sacral lymph node in the LTβ-deficient mice. We demonstrated that this lymph node displayed MAdCAM-1 on the HEVs and played a role in immune induction following ivag HSV-2 infection. Furthermore, we show that the sacral lymph node in WT mice expresses MAdCAM-1, suggesting that it may serve a unique function in the generation of mucosal immunity compared with the lumbar lymph nodes, which exhibit a PLN phenotype. Future studies will be needed to address the differences in the behavior of lymphocytes

generated among the lymph nodes draining the vaginal mucosa in providing protective immunity to sexually transmitted pathogens. In conclusion, while LT and TNF may provide critical signals required in organization and maintenance of secondary lymphoid organs, neither proper T/B organization nor FDCs were required within the LTβ-deficient mice for the generation of Th1 and IgG responses following genital HSV-2 infection.

Acknowledgments

We thank Myriam Hill for technical assistance and Dr. Fadi Lakkis for critical discussions.

References

1. Mebius, R. E. 2003. Organogenesis of lymphoid tissues. *Nat. Rev. Immunol. 3:292*.
2. Gommerman, J. L., and J. L. Browning. 2003. Lymphotoxin/LIGHT, lymphoid microenvironments and autoimmune disease. *Nat. Rev. Immunol. 3:642*.
3. Sacca, R., A. Kratz, A. Campos-Neto, M. S. Hanson, and N. H. Ruddle. 1995. Lymphotoxin: from chronic inflammation to lymphoid organs. *J. Inflamm. 47:81*.
4. Koni, P. A., R. Sacca, P. Lawton, J. L. Browning, N. H. Ruddle, and R. A. Flavell. 1997. Distinct roles in lymphoid organogenesis for lymphotoxins α and β revealed in lymphotoxin β-deficient mice. *Immunity 6:491*.
5. Fu, Y. X., G. Huang, M. Matsumoto, H. Molina, and D. D. Chaplin. 1997. Independent signals regulate development of primary and secondary follicle structure in spleen and mesenteric lymph node. *Proc. Natl. Acad. Sci. USA 94:5739*.
6. Banks, T. A., B. T. Rouse, M. K. Kerley, P. J. Blair, V. L. Godfrey, N. A. Kuklin, D. M. Bouley, J. Thomas, S. Kanangat, and M. L. Mucenski. 1995. Lymphotoxin-α-deficient mice: effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol. 155:1685*.
7. De Togni, P., J. Goellner, N. H. Ruddle, P. R. Streeter, A. Fick, S. Mariathasan, S. C. Smith, R. Carlson, L. P. Shornick, J. Strauss-Schoenberger, et al. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science 264:703*.
8. Alimzhanov, M. B., D. V. Kuprash, M. H. Kosco-Vilbois, A. Luz, R. L. Turetskaya, A. Tarakhovsky, K. Rajewsky, S. A. Nedospasov, and K. Pfeffer. 1997. Abnormal development of secondary lymphoid tissues in lymphotoxin β-deficient mice. *Proc. Natl. Acad. Sci. USA 94:9302*.
9. Drayton, D. L., X. Ying, J. Lee, W. Lesslauer, and N. H. Ruddle. 2003. Ectopic LT$\alpha\beta$ directs lymphoid organ neogenesis with concomitant expression of peripheral node addressin and a HEV-restricted sulfotransferase. *J. Exp. Med. 197:1153*.
10. Cuff, C. A., J. Schwartz, C. M. Bergman, K. S. Russell, J. R. Bender, and N. H. Ruddle. 1998. Lymphotoxin α3 induces chemokines and adhesion molecules: insight into the role of LTα in inflammation and lymphoid organ development. *J. Immunol. 161:6853*.
11. Mebius, R. E., P. R. Streeter, S. Michie, E. C. Butcher, and I. L. Weissman. 1996. A developmental switch in lymphocyte homing receptor and endothelial vascular addressin expression regulates lymphocyte homing and permits $CD4^+$ $CD3^-$ cells to colonize lymph nodes. *Proc. Natl. Acad. Sci. USA 93:11019*.
12. McDermott, M. R., C. H. Goldsmith, K. L. Rosenthal, and L. J. Brais. 1989. T lymphocytes in genital lymph nodes protect mice from intravaginal infection with herpes simplex virus type 2. *J. Infect. Dis. 159:460*.
13. Jones, C. A., T. J. Taylor, and D. M. Knipe. 2000. Biological properties of herpes simplex virus 2 replication-defective mutant strains in a murine nasal infection model. *Virology 278:137*.
14. Zhao, X., E. Deak, K. Soderberg, M. Linehan, D. Spezzano, J. Zhu, D. M. Knipe, and A. Iwasaki. 2003. Vaginal submucosal dendritic cells, but not Langerhans cells, induce protective Th1 responses to herpes simplex virus-2. *J. Exp. Med. 197:153*.
15. Warnock, R. A., S. Askari, E. C. Butcher, and U. H. von Andrian. 1998. Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. *J. Exp. Med. 187:205*.
16. Kraal, G., and R. E. Mebius. 1997. High endothelial venules: lymphocyte traffic control and controlled traffic. *Adv. Immunol. 65:347*.
17. Bargatze, R. F., M. A. Jutila, and E. C. Butcher. 1995. Distinct roles of L-selectin and integrins $\alpha_4\beta_7$ and LFA-1 in lymphocyte homing to Peyer's patch-HEV in situ: the multistep model confirmed and refined. *Immunity 3:99*.
18. Girard, J. P., and T. A. Springer. 1995. High endothelial venules (HEVs): specialized endothelium for lymphocyte migration. *Immunol. Today 16:449*.
19. Abe, K., F. O. Yarovinsky, T. Murakami, A. N. Shakhov, A. V. Tumanov, D. Ito, L. N. Drutskaya, K. Pfeffer, D. V. Kuprash, K. L. Komschlies, and S. A. Nedospasov. 2003. Distinct contributions of TNF and LT cytokines to the development of dendritic cells in vitro and their recruitment in vivo. *Blood 101:1477*.
20. Futterer, A., K. Mink, A. Luz, M. H. Kosco-Vilbois, and K. Pfeffer. 1998. The lymphotoxin β receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity 9:59*.
21. Koni, P. A., and R. A. Flavell. 1998. A role for tumor necrosis factor receptor type 1 in gut-associated lymphoid tissue development: genetic evidence of synergism with lymphotoxin β. *J. Exp. Med. 187:1977*.
22. Kuprash, D. V., M. B. Alimzhanov, A. V. Tumanov, A. O. Anderson, K. Pfeffer, and S. A. Nedospasov. 1999. TNF and lymphotoxin β cooperate in the maintenance of secondary lymphoid tissue microarchitecture but not in the development of lymph nodes. *J. Immunol. 163:6575*.
23. Kuprash, D. V., M. B. Alimzhanov, A. V. Tumanov, S. I. Grivennikov, A. N. Shakhov, L. N. Drutskaya, M. W. Marino, R. L. Turetskaya, A. O. Anderson, K. Rajewsky, et al. 2002. Redundancy in tumor necrosis factor (TNF) and lymphotoxin (LT) signaling in vivo: mice with inactivation of the entire TNF/LT locus versus single-knockout mice. *Mol. Cell. Biol. 22:8626*.
24. Drayton, D. L., K. Chan, W. Lesslauer, J. Lee, X. Y. Ying, and N. H. Ruddle. 2002. Lymphocyte traffic in lymphoid organ neogenesis: differential roles of Ltα and LT$\alpha\beta$. *Adv. Exp. Med. Biol. 512:43*.
25. Scheu, S., J. Alferink, T. Potzel, W. Barchet, U. Kalinke, and K. Pfeffer. 2002. Targeted disruption of LIGHT causes defects in costimulatory T cell activation and reveals cooperation with lymphotoxin β in mesenteric lymph node genesis. *J. Exp. Med. 195:1613*.
26. Wang, Y., G. Huang, J. Wang, H. Molina, D. D. Chaplin, and Y. X. Fu. 2000. Antigen persistence is required for somatic mutation and affinity maturation of immunoglobulin. *Eur. J. Immunol. 30:2226*.