Constitutive Expression of LIGHT on T Cells Leads to Lymphocyte Activation, Inflammation, and Tissue Destruction^{1,2}

Raziya B. Shaikh,*[‡] Sybil Santee,[†] Steven W. Granger,[†] Kristine Butrovich,[†] Tim Cheung,^{3†} Mitchell Kronenberg,*[‡] Hilde Cheroutre,⁴* and Carl F. Ware^{4†‡}

LIGHT, a member of the TNF family of cytokines (homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for herpesvirus entry mediator, a receptor expressed on T cells), is induced on activated T cells and mediates costimulatory and antitumor activity in vitro. Relatively little information is available on the in vivo effects of LIGHT expression, particularly within the T cell compartment. In this work, we describe transgenic mice that express human LIGHT under the control of the CD2 promoter, resulting in constitutive transgene expression in cells of the T lymphocyte lineage. LIGHT-transgenic animals exhibit abnormalities in both lymphoid tissue architecture and the distribution of lymphocyte subsets. They also show signs of inflammation that are most severe in the intestine, along with tissue destruction of the reproductive organs. These LIGHT-mediated effects were recapitulated when immune-deficient mice were reconstituted with bone marrow from LIGHT-transgenic donor mice. T cells in the LIGHT-transgenic mice have an activated phenotype and mucosal T cells exhibit enhanced Th1 cytokine activity. The results indicate that LIGHT may function as an important regulator of T cell activation, and implicate LIGHT signaling pathways in inflammation focused on mucosal tissues. *The Journal of Immunology*, 2001, 167: 6330–6337.

S everal members of the TNF superfamily of cytokines play important roles in lymphocyte activation, immune regulation, and the development of lymphoid tissues (1–3). The production of these cytokines is highly regulated and overproduction of some of them can lead to severe chronic inflammation and autoimmune diseases (4–5). LIGHT⁵ (homologous to lymphotoxin (LT), exhibits inducible expression and competes with HSV glycoprotein D for herpesvirus entry mediator (HVEM), a receptor expressed on T cells) (TNFSF14), is transiently expressed on the cell surface during activation of T cells (6). LIGHT is a ligand for two differentially expressed TNF family receptors, the herpesvirus entry mediator, expressed predominately on T lymphoid cells, and the LT β R, found on epithelial and stromal cells but conspicuously absent on lymphocytes (6–8). Recent evidence suggests the existence of a soluble binding protein for LIGHT, decoy receptor 3,

¹ This work was supported by National Institutes of Health Grants RO1 AI33068 and PO1 CA69381 (to C.F.W.), R29 DK54451 (to H.C.) and PO1 DK46763 (to M.K.); American Cancer Society Grant IM663 (to C.F.W.); and Universitywide AIDS Research Program of the State of California Postdoctoral Fellowship F98-LJIAI-11 (to S.S.).

² This publication is number 422 from the La Jolla Institute for Allergy and Immunology.

³ Current address: Research School of Biological Sciences, Australian National University, Canberra, Australian Capital Territory, Australia.

which can bind Fas ligand as well as LIGHT (9). These findings underscore the multiple regulatory mechanisms that control the function of TNF family molecules (9, 10).

Understanding the biological functions of LIGHT is further complicated by the fact that each of its receptors also binds to other TNF family ligands. For example, in addition to LIGHT, the LT β R binds the LT α 1 β 2 heterotrimer, while HVEM can also bind the secreted homotrimeric form of LT, LT α 3 (6). Therefore, the presence of LIGHT might either synergize with or antagonize the signals delivered by these alternate ligands for the receptors that bind LIGHT.

The results from several studies indicate that LIGHT can trigger apoptosis as well as cell activation, depending in part on the expression of its receptors on the target cells (11-13). Expression of LIGHT by transplanted tumors led to increased lymphocytic infiltrates, tumor necrosis, and enhanced T cell cytotoxic activity. Consistent with a role for LIGHT in T cell activation, inhibition of LIGHT binding with a soluble HVEM-Fc fusion protein reduced the severity of graft-vs-host disease (13). Reduced growth of LIGHT-expressing transplanted tumors in vivo also was observed, even in athymic and immune-deficient recipient mice, suggesting a LIGHT-mediated increase in apoptosis of the cancer cells in the absence of an adaptive immune response (12). It should be noted that LIGHT also may act as a deterrent to infection of dendritic cells and T cells by HSV through its ability to interfere with virus entry (6, 14). Both LIGHT-binding receptors participate in these diverse biologic effects of LIGHT. There are data suggesting that the enhanced activation of T cells by LIGHT occurs through its interaction with HVEM, which is also expressed on T lymphocytes (15). Interestingly, the activation-induced expression of LIGHT on responding T cells has been reported to negatively regulate HVEM expression in vitro on all activated T cell subsets, suggesting a regulatory feedback loop for controlling this potentially immune stimulatory pathway (16). The LTBR also is involved in mediating the effects of LIGHT, as LIGHT expression by tumor cells could induce cell death exclusively via the LTBR signaling pathway,

Divisions of *Developmental Immunology and [†]Molecular Immunology, La Jolla Institute for Allergy and Immunology, San Diego, CA 92121; and [‡]Department of Biology, University of California at San Diego, La Jolla, CA 92037

Received for publication July 9, 2001. Accepted for publication October 2, 2001.

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.

⁴ Address correspondence and reprint requests to Dr. Carl F. Ware or Dr. Hilde Cheroutre, La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, CA 92121. E-mail addresses: carl_ware@liai.org or hilde@liai.org

⁵ Abbreviations used in this paper: LIGHT, homologous to lymphotoxin, exhibits inducible expression, and competes with HSV glycoprotein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes; LT, lymphotoxin; HVEM, herpesvirus entry mediator; IEL, intraepithelial lymphocyte; LPL, lamina propria lymphocyte; MFI, mean fluorescence intensity.

although the tumor cells in this case expressed both the $LT\beta R$ and HVEM (11).

Despite these previous studies, there remains relatively little information on the in vivo effects of LIGHT expression, particularly within the T cell compartment, where LIGHT normally is expressed. This question is of special interest because of the proposed role of LIGHT as a highly regulated costimulatory molecule for T cells and its potential role in virus defense.

In the current study, we generated transgenic mice with constitutive expression of human LIGHT in T cells, under the control of the CD2 promoter. The results demonstrate an important role for LIGHT in driving the sustained activation of T cells, indicating that the regulation of expression of this TNF ligand on T lymphocytes plays a major role in orchestrating inflammatory responses. Interruption of this regulated expression on T cells leads to chronic inflammation and the destruction of selected tissues.

Materials and Methods

LIGHT-transgenic mice

A human LIGHT cDNA containing 5' EcoRI and 3' SmaI sites was generated by PCR, using a LIGHT cDNA isolated from the human T cell hybridoma line II23.D7 as a template. Forward primer 5'-cccagggaattcagc cctgctccagagacctt-3' and reverse primer 5'-gggtgtcagacccatgtccaat-3' were used with the following PCR parameters: 94°C for 4 min, 30 cycles at 94°C for 30 s, 53°C for 30 s, 72°C for 45 s, followed by a 10-min extension at 72°C (11). This PCR fragment was subcloned into the EcoRI and SmaI sites of a vector containing the human CD2 promoter (kindly provided by Dr. S. Hedrick, University of California at San Diego, La Jolla, CA) (17). The final DNA fragment for microinjection was generated by SalI and XbaI digestion. It contains the human CD2 promoter and human growth hormone gene 3' untranslated region, and the human LIGHT cDNA and sequence for polyadenylation. PCR analysis of tail biopsy DNA was used to screen for transgenic animals with the forward primer 5'-ctaggagagatggt cacc-3' and the reverse primer 5'-cttccttcacaccatgaaagc-3'. The same parameters were used as described above for 40 cycles to generate a 534-bp product.

Necropsy of transgenic mice

Two LIGHT-transgenic founder mice were generated by injecting (C3H \times C57BL/6) F₁ eggs that were fertilized by C57BL/6 males. Fertility of the mice was limited, but one female LIGHT-transgenic offspring was generated by mating a founder to a C57BL/6 male. Mice were necropsied when they appeared ill, at 5 and 8 mo of age for the founders and at 4 mo of age for the offspring. Transgenic mice and littermate control animals were sacrificed and dissected, and portions of the salivary gland, lungs, liver, spleen, mesenteric lymph nodes, peripheral lymph nodes, uterus, and small and large intestine were placed in either formalin and/or OCT compound for histology analysis. Tissue was also flash frozen using liquid nitrogen for RNA preparation. Sera were prepared using serum separator tubes (Fisher Scientific, Pittsburgh, PA) and stored at -80°C. Following mechanical disruption, portions of the tissues (liver, spleen, mesenteric lymph nodes, peripheral lymph nodes, small and large intestine, and bone marrow) were used to prepare cell suspensions for flow cytometry analysis. Mononuclear cell preparations were prepared from liver as previously described (18). Intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) from the small and large intestine were prepared essentially as described previously (19).

Flow cytometry

Cell preparations were used for flow cytometry analysis with the following mAbs: rat anti-mouse CD4, CD8, CD122, B220, CD2, CD44, CD45RB, and CD19, and hamster anti-mouse CD3 ϵ , TCR β and TCR δ . Monoclonal Abs were directly conjugated to either FITC, CyChrome, or PE (BD Biosciences, San Diego, CA). Mouse HVEM was detected with rat anti-mouse HVEM mAb 4CG4 (IgM) derived from a rat immunized with mouse HVEM-Fc and secondary mouse anti rat-IgM-FITC (clone G53–238; BD Biosciences). The presence of the LIGHT transgene was detected by flow cytometry using a human HVEM-Fc fusion protein (6) (10 μ g/ml) followed by an anti-human IgG secondary Ab conjugated to PE (Southern Biotechnology Associates, Birmingham, AL). Human IgG1 (Sigma-Aldrich, St. Louis, MO) was used as a control for staining with receptor-Fc fusion proteins, and the analyzed cells were pretreated with the 2.4G2 anti-Fc γ R mAb to prevent nonspecific secondary Ig binding. Analyses

were performed on a FACSCalibur (BD Biosciences) flow cytometer. Profiles consist of lymphocytes that were gated by analysis of forward and side angle light scatter, or in some cases by gating on expression of CD2.

Human LIGHT receptor binding assay

HEK293 cells expressing either human or mouse LIGHT were generated as described previously (6). Mouse LT β R and HVEM-Fc fusion proteins were produced in a baculovirus-mediated protein production system (6, 20) and purified by protein G affinity chromatography (11). Binding assays for LT β R-Fc or HVEM-Fc to human or mouse LIGHT were accomplished by staining stably transfected HEK293 cells (10⁵ cells in 100 μ l of FACS binding buffer) with graded concentrations of Fc proteins for 60 min at 0°C followed by addition of 5 μ g/ml goat Fab anti-human IgG conjugated to R-PE. The specific mean fluorescence intensity (MFI) was calculated by subtracting the background MFI obtained with the equivalent amount of human IgG from the MFI of LT β R-Fc or HVEM-Fc. EC₅₀ values were determined by nonlinear regression analysis of the fusion protein binding (Prism GraphPad software; San Diego, CA).

Bone marrow chimeras

129SV Rag $2^{-/-}$ mice aged 6–8 wk (Jackson ImmunoResearch Laboratories, West Grove, PA) were lethally irradiated (1100 rad) before injection via the tail vein of $3-5 \times 10^6$ bone marrow cells. Full necropsies were performed as described above, 8–10 wk after cell transfer.

Intracellular cytokine staining

Suspensions containing freshly isolated spleen, small intestine IEL, or large intestine IEL were seeded at 5×10^5 – 1×10^6 cells/ml in 24-well plates coated with 10 μ g/ml coated anti-CD3 ϵ (2C11) mAb. These cells were incubated at 37°C for 5–7 h in the presence of GolgiStop (BD Biosciences). A kit was then used to permeabilize and stain the cells according to the manufacturer's protocol (BD Biosciences). Rat anti-mouse IFN- γ , IL-4, TNF, and rat IgG isotype control PE Abs were used to stain for intracellular cytokines, and TCR β CyChrome Abs were used to identify T cells.

Ig isotype measurements

Ig isotype levels in sera were detected using standard capture ELISAs according to the manufacturer's protocol (Southern Biotechnology Associates), with biotinylated anti-Ig Abs and streptavidin-labeled HRP for detection.

Results

Human LIGHT binds to both mouse HVEM and $LT\beta R$

Human LIGHT was investigated for its ability to bind to mouse receptors as measured by the binding of surrogate receptor Fc fusion proteins to human or mouse LIGHT-transfected cells. Human LIGHT binds to mouse LT β R-Fc and mouse HVEM-Fc nearly as efficiently as these receptors bind mouse LIGHT (Fig. 1*A*). Thus, the in vivo effects of human LIGHT are likely to be due to its interaction with either one or both of these receptors. It should be noted that mixed heterotrimers of human and mouse LIGHT can be formed in cotransfected cell lines (data not shown), suggesting that human LIGHT is unlikely to interfere with the cell surface expression of endogenous mouse LIGHT. However, as endogenous mouse LIGHT is normally only transiently expressed by T cells, it is likely that human LIGHT homotrimers are the predominant species in transgenic mice with constitutive human LIGHT expression.

The human HVEM-Fc binds human LIGHT with significantly higher affinity than mouse LIGHT (Fig. 1*B*). Saturation binding was reached with both LIGHT homologs, but the midpoint (EC₅₀) is consistent with a nearly 10-fold greater affinity of human HVEM-Fc for human LIGHT. This preferential binding allowed the use of human HVEM-Fc (at 10 μ g/ml) to specifically detect human LIGHT on cells from the transgenic mice.

Expression of human LIGHT in transgenic mice

Cell suspensions from the LIGHT-transgenic mice were analyzed for surface expression of human LIGHT. Although the expression



FIGURE 1. Human LIGHT binds to both mouse HVEM and LT β receptor. *A*, Human HEK293 cells stably transfected with human LIGHT (*upper panel*) or mouse LIGHT (*lower panel*) were incubated with either mouse HVEM-Fc or mouse LT β R-Fc and stained with a goat Fab antihuman IgG conjugated to R-PE. Incubation with human IgG followed by goat Fab anti-human IgG was used to measure nonspecific staining. *B*, Titration of human HVEM-Fc binding to mouse or human LIGHT. HEK293 cells expressing human (\blacktriangle) or mouse (\blacksquare) LIGHT were stained with human HVEM-Fc. HVEM-Fc binding was detected by incubation with a goat anti-human R-PE-conjugated secondary Ab and analyzed by flow cytometry.

of the CD2 promoter-driven transgene correlates with the expression of endogenous mouse CD2, the correlation is only partial (data not shown). This could be due to several factors, including the restriction in humans of CD2 expression to the T cell lineage, while in mouse CD2 has a broader expression pattern, including B cells as well as T cells. As expected, in the absence of activation, CD2⁺ mononuclear cells isolated from nontransgenic control animals did not express high levels of LIGHT when detected with either a human HVEM-Fc (Fig. 2) or a mouse HVEM-Fc (data not shown). Because many IEL are CD2⁻ (21), we analyzed total lymphocytes in IEL preparations that were gated by light scatter. In control mice, IEL did not express high levels of LIGHT.

By contrast with these results indicating a limited amount of LIGHT expression in the control animals, thymocytes from the transgenic mice had a higher MFI for LIGHT staining (Fig. 2). Additionally, a fraction of the cells from peripheral lymph node (Fig. 2) and mesenteric lymph node (data not shown) were highly positive for LIGHT expression. The percentage of lymphocytes present in the spleen of transgenic mice was greatly reduced (see below), and the level of HVEM-Fc staining on these splenic lymphocytes was not significantly different from the endogenous

LIGHT expressed by control splenocytes. Similar to the spleen, the CD2⁺ population in the liver was reduced in the LIGHT-transgenic animals and expression of the LIGHT transgene was limited (Fig. 2). The greatest percentage of cells expressing high levels of human LIGHT in the transgenic mice was observed on IEL (Fig. 2) and LPL (data not shown) from the small and large intestine.

Reduced viability and infertility in LIGHT-transgenic mice

Mice expressing the human LIGHT transgene were small compared with their nontransgenic littermates, and they appeared sick by several months of age. Necropsies of the mice showed visible changes in the gross anatomy of these transgenic animals (Table I). They were anemic, and the small intestine, cecum, mesenteric and peripheral lymph nodes, and spleen were all enlarged, while the thymus was drastically reduced in size when compared with an age-matched nontransgenic littermate (Fig. 3A). However, the large intestine was not increased in size and the mice did not have diarrhea. As noted in Materials and Methods, there were two LIGHT-transgenic founder mice, which were both females. One of these mice produced one litter with one transgenic offspring, and the other failed to reproduce. Gross morphology of these mice indicated that their inability to produce litters correlated with severe atrophy of the reproductive organs (Fig. 3B). However, the colon and liver appeared not to be inflamed upon necropsy.

Histopathology of LIGHT-transgenic mice

Analysis of fixed tissues of transgenic mice consistently showed pathologic changes that were relatively selective with respect to different organs. Spleen and lymph nodes were enlarged. H&E staining of spleen tissue demonstrated a marked increase in extramedullary hematopoiesis (primarily cells in the erythroid series were evident) and an absence of a well-defined red and white pulp separation and reactive lymphoid follicles (Fig. 3*C*). The enlarged small intestine showed signs of chronic inflammation with substantial infiltration of mononuclear cells, loss of goblet cells, distortion and hyperplasia of crypts, and villus atrophy (Fig. 3*D*). Liver sections stained with H&E showed cellular infiltrates into the portal areas, characterized by marked bile duct hyperplasia and



FIGURE 2. LIGHT expression on lymphocytes. LIGHT was detected by incubating cell preparations from either LIGHT-transgenic mice (filled histograms) or littermate control mice (open histograms) with a human HVEM-Fc followed by a goat Fab anti-human-IgG conjugated to R-PE (as in Fig. 1). CD2-expressing cells were gated on to detect LIGHT expression in the thymus, spleen, lymph nodes, and liver. Lymphocytes were gated on by forward angle and side angle light scatter profile to detect LIGHT expression on IEL of the small and large intestine. Fluorescence intensity was measured on a log scale. Data shown are representative of three analyses.

| Phenotype | LIGHT-Transgenic Mice ^a | | LIGHT-Transgenic Bone Marrow-Chimeric Mice ^b | |
|------------------------------|------------------------------------|-----------|--|-------|
| Appearance ^c | Not healthy | $(3/3)^d$ | Not healthy | (3/3) |
| Weight ^e | Reduced | (3/3) | Reduced | (3/3) |
| Thymus ^f | Atrophied | (3/3) | Atrophied | (2/3) |
| Spleeng | Splenomegaly | (2/3) | Splenomegaly | (1/3) |
| Peripheral LN ^h | Enlarged | (2/3) | Enlarged | (2/3) |
| Mesenteric LN ^h | Enlarged | (2/3) | Enlarged | (2/3) |
| Liver ⁱ | Normal | (3/3) | Normal | (3/3) |
| Small intestine ^j | Severe inflammation | (3/3) | Inflammation | (2/3) |
| Caecum ^k | Enlarged | (2/3) | Enlarged | (2/3) |
| Large intestine ^k | Normal | (3/3) | Normal | (3/3) |
| Uterine horns | Atrophied | (3/3) | Atrophied | (3/3) |

Table I. Gross anatomical characteristics of LIGHT-transgenic (tg) mice and LIGHT-transgenic bone marrow-chimeric mice

^{*a*} LIGHT transgene is expressed under the control of the CD2 promoter. Three LIGHT-transgenic mice were analyzed. ^{*b*} Bone marrow chimeras were made by reconstituting lethally irradiated RAG^{-/-} mice with $3-5 \times 10^6$ bone marrow cells. LIGHT-transgenic bone marrow-chimeric mice were analyzed.

^c Mice had a hunched appearance, showed piloerection, and had slower movement compared to control mice.

^d Number of mice with phenotype/number of mice analyzed.

^e LIGHT-transgenic mice had 27–35% reduced weight compared to control mice. Similarly, LIGHT-transgenic bone marrow chimeras had 22–25% reduced weight compared to the respective control mice.

^f The total cellularity of LIGHT-transgenic and LIGHT-transgenic bone marrow-chimeric mice thymuses was 10-20% of respective control mice.

^g Spleens of LIGHT-transgenic and LIGHT-transgenic bone marrow-chimeric mice with enlarged spleens were six to eight times the size of corresponding control spleens based on total cellularity.

^h Visually, all lymph nodes were grossly enlarged in two of three of each LIGHT-transgenic and LIGHT-transgenic bone marrow-chimeric mice. LN, lymph nodes.

ⁱ No gross abnormalities were seen in the liver.

^{*j*} LIGHT-transgenic and LIGHT-transgenic bone marrow-chimeric mice showing small intestinal abnormalities visually had patchy to complete inflammation. IEL preparations showed a 2- to 50-fold increase in cell numbers and LPL preparations showed a 12- to 33-fold increase in cell numbers. LPL were counted only in LIGHT-transgenic mice.

^k While some LIGHT-transgenic and LIGHT-transgenic bone marrow-chimeric mice had visually enlarged cecums, no gross abnormalities were seen in the large bowel.

accompanied by a moderate, mixed inflammation of neutrophils and lymphocytes (Fig. 3*E*). The reproductive organs showed extensive changes, although a massive mononuclear infiltrate was not observed (data not shown). The effect of constitutive LIGHT expression in lung tissue was less pronounced. There was a slight increase in polymorphonuclear cells in the septi in some mice (data not shown).

Altered composition of lymphoid tissues in LIGHT-transgenic mice

Dramatic alterations in the number and composition of lymphocyte populations were observed in both peripheral and central lymphoid organs of the LIGHT-transgenic mice. The constitutive expression of human LIGHT on thymocytes greatly reduced the cellularity as well as the size of the thymus. The double positive thymocytes showed the greatest reduction in percentage, while the percentage of single positive CD8 $\alpha\beta$ and CD4 thymocytes increased (Fig. 4A). A subset of these mature, single positive thymocytes had an activated phenotype, as determined by high levels of CD44 as well as CD25 expression (Fig. 4A). The spleen, although grossly enlarged, had a significant decrease in the percent of CD3⁺ T cells as well as $B220^+CD19^+$ B cells (Fig. 4B). In contrast, the enlarged lymph nodes of the LIGHT-transgenic mice did contain a significant population of T cells, although the percentage of B cells was drastically reduced (Fig. 4C). Similar to the mature thymocytes of these LIGHT-transgenic animals, the T cells in the spleen and lymph nodes also expressed high levels of CD44, suggesting an activated phenotype (data not shown). IEL in the LIGHT-transgenic mice also were increased, due to a strong and selective accumulation of activated T cells with a conventional TCR and coreceptor phenotype, namely $TCR\alpha\beta^+$ cells that are either $CD8\alpha\beta^+$ or $CD4^+$ (Fig. 4D). By contrast, the $TCR\gamma\delta^+$ and TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$ IEL populations, which are characteristic of the intestine, were greatly decreased in percentage, reflecting a lack of increase in these populations (Fig. 4*D*). Surprisingly, the population of LPL of the intestine did not show a decrease in the percentage of B cells ($B220^+CD19^+$) in both the small (Fig. 4*E*) and large intestine (data not shown), and in fact a slight increase was observed. This was in sharp contrast to the reduced B cell populations observed in the other tissues analyzed.

Hematopoietic cell expression of LIGHT determines the phenotype of the transgenic mice

To determine whether the in vivo effect of constitutively expressed LIGHT is due solely to transgene expression by bone marrowderived cells, we generated chimeric mice by reconstituting lethally irradiated immune-deficient RAG $2^{-/-}$ mice with bone marrow from the LIGHT-transgenic or littermate control mice. The rate of successful bone marrow reconstitution, defined as survival beyond 4 wk post-transfer, was relatively low when LIGHT-transgenic bone marrow was transferred. Only three of eight mice reconstituted with LIGHT bone marrow survived, whereas eight of nine recipients survived when reconstituted with bone marrow from control donor mice. The LIGHT bone marrow chimeras were similar to the LIGHT-transgenic mice with regard to phenotype and gross anatomy (Table I). The recipients of LIGHT-transgenic bone marrow had a hunched appearance, a reduction in body fat, and a >20% decrease in weight, and they were also anemic. The LIGHT bone marrow chimeras also had enlarged lymph nodes, both peripheral and mesenteric. Similar to the donor mice, the LIGHT bone marrow recipient mice showed signs of inflammation of the intestine with enlarged cecum, and two of three recipients had patchy to severe inflammation of the small intestine and ascites in their peritoneal cavity. The atrophy of the reproductive organs was recapitulated in all LIGHT bone marrow chimeras as well,



FIGURE 3. Gross anatomy and histology of tissues of LIGHT-transgenic mice. *A*, Decreased thymus size. A representative thymus lobe of a transgenic mouse is compared with a lobe from an age-matched (4 mo) control mouse. *B*, Atrophy of the reproductive organs. The uterine horns from a LIGHT-transgenic mouse are compared with those from an agematched control mouse. *C*–*E*, Tissues were obtained and processed for histological examination and stained with H&E. Representative tissue sections of the LIGHT-transgenic mice were compared with the relevant tissue (*C*, spleen; *D*, small intestine; and *E*, liver) isolated from age-matched control littermates. Shown are representative data from one of three separate analyses (×100 magnification).

indicating that the destruction of this tissue was mediated by LIGHTexpressing hematopoietic cells, as opposed to unanticipated LIGHT expression in the reproductive tract. Control chimeras generated by transfer of bone marrow from littermate control mice appeared healthy and did not show any of these abnormalities.

Although the histopathologic changes are consistent with those observed in the intact LIGHT-transgenic mice, they were less severe in the chimeric mice. This difference most likely reflects the low reconstitution efficiency of the lymphocyte populations in the LIGHT bone marrow-chimeric animals and the relatively short time post-transfer before analysis of the recipient mice. While the intact transgenic animals were analyzed between 16 and 32 wk of age, bone marrow-chimeric animals were aged 14–18 wk, but were analyzed between 8 and 10 wk post-transfer.

The phenotype of the lymphocytes reconstituted in the chimera that received transgenic bone marrow also showed similarities with those observed in the intact LIGHT-transgenic mice. A reduced percentage of double positive thymocytes together with an increase in the percentage of $CD8\alpha\beta$ and CD4 single thymocytes was observed (Fig. 5A). Similar to the LIGHT-transgenic animals, the percentage of lymphocytes among the splenocytes of the LIGHT bone marrow-chimeric mice was significantly reduced when compared with the splenocytes of the control chimeric mice. The reduction was seen among the T cells (Fig. 5B) as well as among the B cells (data not shown). The lymph nodes in the LIGHT chimeric animals contained an increased population of mature CD4 and CD8 $\alpha\beta$ single positive T cells (Fig. 5C), while as was the case in the LIGHT-transgenic donor animals, B cells were drastically reduced (data not shown). Additionally, the IEL in the small intestine of the LIGHT bone marrow chimeras showed a decreased percentage of the typical intestinal CD8 $\alpha \alpha^+$ T cells, including the TCR $\gamma\delta^+$ cells, while an increased percentage of conventional TCR $\alpha\beta$ T cells was observed (Fig. 5D). Consistent with the less severe alterations seen in the chimeras, there was no significant lymphocyte infiltration observed in either the liver or the lungs of the chimeric animals (data not shown).

Altered immune function in lymphocytes of LIGHT-transgenic mice

To assay for changes in the function of T cells from the LIGHTtransgenic mice, intracellular cytokine staining was done on splenocytes and IEL from LIGHT-transgenic bone marrow chimeras and control chimeras. Cytokines were measured after a brief in



FIGURE 4. LIGHT-transgenic mice have altered populations of T and B cells. Flow cytometric profiles are shown with cells gated on the lymphocyte population by forward and side angle light scatter. *A*, Thymus. Dot plot profile of LIGHT-transgenic thymocytes and control thymocytes stained with anti-CD4 and anti-CD8 β mAb (*left*), and histogram profile of LIGHT-transgenic thymocytes (filled histograms) and control thymocytes and stained with either anti-CD25 or anti-CD44 mAbs (*right*). Dot plot profiles of LIGHT-transgenic and control lymphocytes stained with either anti-CD25 or anti-CD44 mAbs (*right*). Dot plot profiles of LIGHT-transgenic and control lymphocytes stained with either anti-CD19 for B cells. Cells from the following organs were analyzed: spleen (*B*); lymph node (*C*); and small intestine IEL (*D*). Staining of small intestine IEL (*E*) and LPL (*F*) with anti-TCR β mAbs. Shown is one of three repeated experiments.



FIGURE 5. Flow cytometry analysis of the T cells isolated from chimeric RAG $2^{-/-}$ recipients of LIGHT-transgenic or control bone marrow. The populations were gated on lymphocytes by forward and side angle light scatter profile. Dot plot profiles of T cells stained with anti-CD4 and anti-CD8 β in the following organs: thymocytes (*A*); splenocytes (*B*); and lymph node cells (*C*). *D*, A dot plot profile of small intestine IEL stained with anti-TCR δ and anti-TCR β mAbs is shown. The data shown are representative of one of three repeated analyses.

vitro activation with plate-bound CD3 ϵ Ab. These experiments showed a significant increase in the percentage of cells with intracellular IFN- γ in small intestine IEL of LIGHT-transgenic bone marrow-chimeric mice (Fig. 6A), while the percentage of cytokine producing TCR β^+ cells in the spleen of these animals was not changed when compared with control chimeric animals (data not



shown). IEL from the large intestine also showed increased levels of intracellular IFN- γ , although less pronounced (data not shown). In no case did cells from the chimeric mice show production of intracellular IL-4.

The effect of constitutive LIGHT expression on serum Ig levels showed that IgA levels in the LIGHT-transgenic mice were significantly higher than in the control animals (p < 0.05), while IgG1, IgG2a, IgG2b, IgG3, and IgM levels were not considerably different from control mice (Fig. 6*B*). This finding of increased production of IgA is consistent with the singular increase in the B cell population observed in the lamina propria of the LIGHT-transgenic animals.

Constitutive expression of LIGHT does not down-regulate HVEM

The induction of LIGHT expression on activated T cells has been reported to correlate with a reciprocal in vitro down-regulation of HVEM on those cells (16). Surprisingly, when analyzed for HVEM expression using a specific Ab, lymph node cells of chimeric mice that received LIGHT-transgenic bone marrow, which express significant levels of the LIGHT transgene, also expressed equivalent or even higher levels of HVEM compared with the control mice (Fig. 7). This suggests that this ligand-receptor interaction does not initiate sustained down-regulation of HVEM in vivo. Alternatively, the level of transgene-derived LIGHT expressed, or differences in binding HVEM between mouse and human LIGHT, could account for the difference in receptor down-modulation.

Discussion

The role of LIGHT expression on T lymphocytes in vivo was investigated in mice with a human LIGHT transgene expressed under the control of the CD2 promoter. These mice constitutively express the transgene-derived LIGHT on T lineage cells, and as a result they developed inflammation and tissue destruction. Because of atrophy of the reproductive organs, these mice cannot breed; however, the analysis of different founders confirmed that the effects observed were not due to insertional mutagenesis.

The inflammation observed was particularly severe in the intestine, and this correlated with other changes in the mucosal immune system of these transgenic mice, including selective increases in lamina propria B cells along with increases in serum IgA, despite decreases in B lymphocytes in other sites. The accumulation of mature IgA-producing plasma cells in the lamina propria of the intestine and the terminal differentiation of plasma cells to IgA secretion are T cell-mediated phenomena that are up-regulated during inflammation. Additionally, increased production of IFN- γ was obtained from the IEL of LIGHT-transgenic mice. This is



FIGURE 6. Increased IFN- γ production by IEL from LIGHT-transgenic mice. IEL were stimulated in vitro with an anti-CD3 ϵ mAb and then stained for intracellular IFN- γ . *A*, The profile depicts intracellular cytokine staining in gated TCR β^+ IEL. Filled histograms show the cytokine profile of a chimeric recipient of LIGHT-transgenic bone marrow, and the shaded histogram shows the IFN- γ production by control TCR β^+ IEL. *B*, Ig isotype levels measured from the sera of recipients of LIGHT-transgenic or control bone marrow. *, Using the Student *t* test, *p* < 0.05 for IgA levels.

FIGURE 7. Expression of LIGHT and HVEM on lymph node cells isolated from chimeric RAG $2^{-/-}$ recipients of LIGHT-transgenic and control bone marrow. Lymph node cells were stained for LIGHT transgene expression using a human HVEM-Fc detected with an anti-human IgG secondary Ab conjugated to PE. Endogenous mouse HVEM expression was detected using rat anti-HVEM (4CG4). Data shown are representative of one of two experiments.

consistent with previous data indicating that LIGHT is a costimulatory molecule that induces a Th1-type proinflammatory cytokine response in vitro (13). Interestingly, the typical intestinal intraepithe lial CD8 $\alpha \alpha^+$ T cells, including the TCR $\gamma \delta^+$ T cells, were not increased in these mice, and few cells could be detected. Such alterations in IEL subpopulations are a characteristic feature of several colitis models in mice (22, 23). Evidence consistent with an important role for LIGHT in the induction of inflammation in the intestine also comes from the analysis of immune-deficient mice in which colitis is induced by transfer of CD4⁺CD45RB^{high} T cells. In these recipient mice, blocking of both LIGHT and $LT\alpha\beta$ with soluble $LT\beta$ R-Fc decoy receptor prevented colitis (24), but there is other evidence suggesting that expression of $LT\alpha\beta$ in recipient mice is not required for pathogenesis (25), implicating LIGHT as the relevant ligand neutralized by the $LT\beta R$ -Fc and responsible for the beneficial effects of this treatment. Overall, these results support the hypothesis that increased or sustained expression of LIGHT on activated T cells is one factor causing the induction and/or persistence of inflammation in the intestine.

Mice expressing the LIGHT transgene also presented with altered lymphoid tissues, including enlarged lymph nodes and decreased thymic cellularity. The spleen, although enlarged, was lymphopenic, and the normal splenic architecture was not present with the loss of clearly defined marginal zones and an altered cellular composition. Histology showed that the splenomegaly was probably due to dividing erythrocyte precursors. In contrast, the thymus showed a significant reduction in CD4/CD8 double positive cells. It cannot be determined whether this reduction in thymocytes and the presence of single positive cells with an activated phenotype is purely a developmental effect, or whether it is secondary to the inflammatory phenotype exhibited by these LIGHTtransgenic animals. The presence of increased T cell populations in several peripheral tissues of these mice, together with the observation that some of these activated T cells might display autoreactivity resulting in tissue destruction, indicate that a general enhancement of negative selection, or failure of positive selection, are most likely not the sole causes of the reduced double positive thymocyte population.

Chimeric recipients of LIGHT-transgenic bone marrow exhibited a similar pathology as the transgenic donor mice. Although the effects were less severe, changes in lymphoid organs, the intestine, and the reproductive tract were observed, indicating that the effects of constitutive LIGHT expression are due to bone marrow-derived cells. Because the LIGHT transgene is expressed under the control of the CD2 promoter, it is likely that the constitutive expression of LIGHT by activated T lymphocytes is responsible for the inflammatory and autoimmune-like phenotype observed in both the transgenic mice and chimeric recipients of transgenic bone marrow.

The potent inflammatory effect of LIGHT expression in vivo may reveal its importance for viral immunity and evasion. In particular, the use of the LIGHT receptor HVEM by HSV as a point of access to the immune system is not likely to be fortuitous. Rather, this pathway may have been specifically targeted to suppress immune function by HSV (14, 26). The ability of envelope glycoprotein D of HSV to compete with LIGHT-HVEM binding supports this notion (6). In an opposite strategy, as a consequence of the action of the *nef* gene product of HIV, LIGHT expression at the cell surface is significantly sustained on activated T cells (27), possibly contributing to nonspecific T cell-mediated pathogenesis, similar to that observed in the LIGHT-transgenic animals.

The effects of LIGHT in the transgenic mice could be the result of its interaction with the LT β R, HVEM, or both (6, 12). Signaling through the LT β R by binding of its other ligand, LT $\alpha\beta$, is critical for the normal morphogenesis of the spleen (28-29). Although both LIGHT and $LT\alpha\beta$ can effectively interact with the $LT\beta R$, the downstream signaling pattern may differ. It is therefore possible that LIGHT competes with $LT\alpha\beta$ when it is expressed constitutively, thus interrupting the formation of normal splenic architecture in these transgenic animals. HVEM is the only known receptor for LIGHT expressed on T cells, and in vitro studies suggest that LIGHT-induced increases in proinflammatory Th1 cytokine production are most likely due to its interaction with HVEM expressed on the activated effector T cells (13). However, it remains to be determined if HVEM is the only receptor for the induction of LIGHT-mediated inflammatory changes seen in the mucosal tissues and elsewhere in these transgenic mice. Interestingly, increased expression of HVEM was observed in T cells from the LIGHT-transgenic mice, indicating that the balanced expression of LIGHT and its receptor on T cells may be disrupted. We speculate that dysregulation of the LIGHT-HVEM system (e.g., by sustained expression) could result in an exaggerated T cell-mediated immune response, which consequently could lead to chronic inflammation and autoimmune disease. The distribution of the activated effector cells in the LIGHT-transgenic and bone marrow-chimeric animals indicates that mucosal tissues are a prominent target for this receptor-ligand deregulation. These results suggest that targeting the LIGHT-HVEM ligand-receptor pair may provide new opportunities for intervention in inflammatory and autoimmunebased diseases, in particular those involving mucosal tissues.

Acknowledgments

We thank Drs. Scott Binder (University of California, Los Angeles, CA) and Kent Osborne (The Scripps Research Institute, La Jolla, CA) for analysis of tissue sections.

References

- Locksley, R. M., N. Killeen, and M. J. Lenardo. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 104:487.
- Matsumoto, M. 1999. Role of TNF ligand and receptor family in the lymphoid organogenesis defined by gene targeting. J. Med. Invest. 46:141.
- Ruddle, N. H. 1999. Lymphoid neo-organogenesis: lymphotoxin's role in inflammation and development. *Immunol. Res.* 19:119.
- Probert, L., J. Keffer, P. Corbella, H. Cazlaris, E. Patsavoudi, S. Stephens, E. Kaslaris, D. Kioussis, and G. Kollias. 1993. Wasting, ischemia, and lymphoid abnormalities in mice expressing T cell-targeted human TNF transgenes. J. Immunology 151:1894.
- Kontoyiannis, D., M. Pasparakis, T. T. Pizarro, F. Cominelli, and G. Kollias. 1999. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AUrich elements: implications for joint and gut associated immunopathologies. *Immunity* 10:387.
- Mauri, D., R. Ebner, R. I. Montgomery, K. D. Kochel, T. C. Cheung, G. L. Yu, S. Ruben, M. Murphy, R. J. Eisenberg, G. H. Cohen, et al. 1998. LIGHT, a new member of the TNF superfamily, and lymphotoxin α are ligands for herpesvirus entry mediator. *Immunity* 8:21.
- Browning, J. L., K. Miatkowski, I. Sizing, D. Griffiths, M. Zafari, C. D. Benjamin, W. Meier, and F. Mackay. 1996. Signaling through the lymphotoxin β receptor induces the death of some adenocarcinoma tumor lines. J. Exp. Med. 183:867.
- Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear. 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* 87:427.
- Yu, K. Y., B. Kwon, J. Ni, Y. Zhai, R. Ebner, and B. S. Kwon. 1999. A newly identified member of tumor necrosis factor superfamily (TR6) suppresses LIGHT mediated apoptosis. J. Biol. Chem. 274:13711.
- Pitti, R., S. A. Marsters, D. A. Lawrence, M. Roy, F. C. Kischkel, P. Dowd, A. Huang, C. J. Donahue, S. W. Sherwood, D. T. Baldwin, et al. 1998. Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* 396:699.
- Rooney, I. A., K. D. Butrovich, A. A. Glass, S. Borboroglu, C. A. Benedict, J. C. Whitbeck, G. H. Cohen, R. J. Eisenberg, and C. F. Ware. 2000. The lymphotoxin-β receptor is necessary and sufficient for LIGHT mediated apoptosis of tumor cells. J. Biol. Chem. 275:14307.
- 12. Zhai, Y., R. Guo, T. L. Hsu, G. L. Yu, J. Ni, B. S. Kwon, G. W. Jiang, J. Lu, J. Tan, M. Ugustus, et al. 1998. LIGHT, a novel ligand for lymphotoxin β receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. J. Clin. Invest. 102:1142.
- Tamada, K., K. Shimozaki, A. I. Chapoval, G. Zhu, G. Sica, D. Flies, T. Boone, H. Hsu, Y. X. Fu, S. Nagta, et al. 2000. Modulation of T-cell mediated immunity

- Raftery, M. J., C. K. Behrens, A. Muller, P. H. Krammer, H. Walczak, and G. Schonrich. 1999. Herpes simplex virus type 1 infection of activated cytotoxic T cells: induction of fratricide as a mechanism of viral immune evasion. *J. Exp. Med.* 190:1103.
- Harrop, J. A., P.C. McDonnell, M. Brigham-Burke, S. D. Lyn, J. Minton, K. B. Tan, K. Dede, J. Spampanato, C. Silverman, P. Hensley, et al. 1998. Herpesvirus entry mediator ligand (HVEM-L), a novel ligand for HVEM/TR2, stimulates proliferation of T cells and inhibits HT29 cell growth. J. Biol. Chem. 273: 27548.
- Morel, Y., J. M. Schiano de Colella, J. Harrop, K. C. Deen, S. D. Holmes, T. A. Wattam, S. S. Khandekar, A. Truneh, R. W. Sweet, J. A. Gastaut, et al. 2000. Reciprocal expression of the TNF family receptor herpesvirus entry mediator and its ligand LIGHT on activated T cells: LIGHT down-regulates its own receptor. J. Immunol. 165:4397.
- Zhumabekov, T., P. Corbella, M. Tolaini, and D. Kioussis. 1995. Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice. J. Immunol. Methods 185:133.
- Watanabe, H., K. Ohtsuka, M. Kimura, Y. Ikarashi, K. Ohmori, A. Kusumi, T. Ohteki, S. Seki, and T. Abo. 1992. Details of an isolation method for hepatic lymphocytes in mice. *J. Immunol. Methods* 146:145.
- Aranda, R., B. C. Sydora, P. L. McAllister, S. W. Binder, H. Y. Yang, S. R. Targan, and M. Kronenberg. 1997. Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4⁺, CD45RB^{high} T cells to SCID recipients. J. Immunol. 158:3464.
- Force, W. R., B. N. Walter, C. Hession, R. Tizard, C. A. Kozak, J. L. Browning, and C. F. Ware. Mouse lymphotoxin-β receptor: molecular genetics, ligand binding, and expression. J. Immunol. 155:5280.

- Van Houten, N., P. M. Mixter, J. Wolfe, and R. C. Budd. 1993. CD2 expression on murine intestinal intraepithelial lymphocytes is bimodal and defines proliferative capacity. *Int. Immunol.* 5:665.
- Poussier, P., T. Ning, J. Chen, D. Banerjee, and M. Julius. 2000. Intestinal inflammation observed in IL-2R/IL-2 mutant mice is associated with impaired intestinal T lymphopoiesis. *Gastroenterology* 118:880.
- Kronenberg, M., and H. Cheroutre. 2000. Do mucosal T cells prevent intestinal inflammation? *Gastroenterology* 118:974.
- Mackay, F., S. A. Woodcock, P. Lawton, C. Ambrose, M. Baetscher, P. Schneider, J. Tschopp, and J. L. Browning. 1999. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J. Exp. Med.* 190:1697.
- Corazza, N., S. Eichenberger, H. P. Eugster, and C. Mueller. 1999. Nonlymphocyte derived tumor necrosis factor is required for induction of colitis in recombination activating gene (RAG)2^{-/-} mice upon transfer of CD4⁺CD45RB^{high} T cells. J. Exp. Med. 190:1479.
- Salio, M., M. Cella, M. Suter, and A. Lanzavecchia. 1999. Inhibition of dendritic cell maturation by herpes simplex virus. *Eur. J. Immunol.* 29:3245.
- Lama, J., and C. F. Ware. 2000. Human immunodeficiency virus type 1 Nef mediates sustained membrane expression of tumor necrosis factor and the related cytokine LIGHT on activated T cells. J. Virol. 74:9396.
- Ettinger, R., J. L. Browning, S. A. Michie, W. van Ewijk, and H. O. McDevitt. 1996. Disrupted splenic architecture, but normal lymph node development in mice expressing a soluble lymphotoxin-β receptor IgG1 fusion protein. *Proc. Natl. Acad. Sci. USA* 93:13102.
- Rennert, P., J. L. Browning, R. Mebius, F. Mackay, and P. S. Hochman. 1996. Surface lymphotoxin α/β complex is required for the development of peripheral lymphoid organs. J. Exp. Med. 184:1999.