LIGHT Regulates Inflamed Draining Lymph Node Hypertrophy

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Lymph node (LN) hypertrophy, the increased cellularity of LNs, is the major indication of the initiation and expansion of the immune response against infection, vaccination, cancer, or autoimmunity. The mechanisms underlying LN hypertrophy remain poorly defined. In this article, we demonstrate that LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by lymphocytes) (TNFSF14) is a novel factor essential for LN hypertrophy after CFA immunization. Mechanistically, LIGHT is required for the influx of lymphocytes into but not egress out of LNs. In addition, LIGHT is required for dendritic cell migration from the skin to draining LNs. Compared with wild type mice, LIGHT$^{-/-}$ mice express lower levels of chemokines in skin and addressins in LN vascular endothelial cells after CFA immunization. We unexpectedly observed that LIGHT from radioresistant rather than radiosensitive cells, likely Langerhans cells, is required for LN hypertrophy. Importantly, Ag-specific T cell responses were impaired in draining LNs of LIGHT$^{-/-}$ mice, suggesting the importance of LIGHT regulation of LN hypertrophy in the generation of an adaptive immune response. Collectively, our data reveal a novel cellular and molecular mechanism for the regulation of LN hypertrophy and its potential impact on the generation of an optimal adaptive immune response. ***The Journal of Immunology*****, 2011, 186: 7156–7163.**

Lymph node (LN) hypertrophy is one of the most basic clinical signs related to acute infection, inflammation, and tumor metastasis; it is thought to be the major indication for initiation and expansion of adaptive immune responses. During LN hypertrophy, dramatic changes in leukocyte trafficking take place: dendritic cells (DCs) carrying Ags from peripheral tissue are mobilized and migrate to draining LN (DLN); lymphocyte entry into DLN is increased; and lymphocyte egress is immediately shut down as early as within 1 h (1, 2). These steps in LN hypertrophy are considered important to favor encounters between APCs and rare Ag-specific T and B cells. In addition, dramatic changes in stromal cell function and number also accompany LN hypertrophy, steps that might play pivotal roles in regulating leukocyte trafficking (3–5). The cellular and molecular mechanisms of the dynamic LN hypertrophy process, however, are not well defined.

The understanding of the role of lymphotoxin β receptor (LTβR) signaling in LN hypertrophy regulation is emerging. LTβR was reported to be required for both high endothelial venue (HEV) homeostasis/function and lymphangiogenesis (6–8). Lymphotoxin (LT) has been thought to be the sole ligand for the actions described earlier. However, the lack of LN in LT$^{-/-}$ mice makes it difficult to study the mechanism of LT-mediated LN hypertrophy. LTβR can also bind to other molecules, such as LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by lymphocytes) (9). Most studies on LIGHT focus on its role as a T cell costimulator via its other receptor, herpes virus entry mediator (HVEM) (10). Unlike LT$^{-/-}$ mice that demonstrate a major defect in the development of lymphoid tissues, LIGHT$^{-/-}$ mice show no detectable defect in the development of lymphoid tissues (11). This raised doubt that LIGHT could be essential for LN hypertrophy. Unexpectedly, we observed that LIGHT$^{-/-}$ mice showed a remarkable defect in LN hypertrophy after immunization with a strong adjuvant, such as CFA, and now reveal a function for LIGHT in LN hypertrophy.

Materials and Methods

Mice

C57BL/6, CD45.1, OT-II, and Rag-1–deficient mice were purchased from The Jackson Laboratory. LIGHT knockout (KO) mice (12) and HVEM KO mice (13) were generated as previously described and backcrossed to the B6 background for >10 generations. Animal care and experiments were performed in accordance with the institution and National Institutes of Health guidelines, and approved by the animal use committee at The University of Chicago.

Immunization

IFA and *Mycobacterium tuberculosis* H37 RA were purchased from BD Diagnostic. CFA was made by mixing IFA and H37 RA of 10 mg/ml. Equal volume of CFA and sterile PBS was mixed, then emulsified before immunization. Mice were immunized with 100 μl emulsified CFA/PBS or CFA with Ag as indicated in *Results* and injected intradermally (i.d.) at the tail base. For Ag/CFA immunization, OVA (10 μg/mouse) or myelin oligodendrocyte glycoprotein (MOG) 35–55 peptides (10 μg/

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Abbreviations used in this article: Ad, adenovirus; CP, clobetasol propionate; DC, dendritic cell; DLN, draining lymph node; DTR, diphtheria toxin receptor; ELC, Epstein–Barr virus-induced molecule-1 ligand chemokine; HEV, high endothelial venue; HVEM, herpes virus entry mediator; i.d., intradermally; iLN, inguinal lymph node; KO, knockout; LC, Langerhans cell; LIGHT, homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by lymphocytes; LN, lymph node; LT, lymphotoxin; LTβR, lymphotoxin β receptor; mIg, mouse Ig; MOG, myelin oligodendrocyte glycoprotein; SLC, secondary lymphoid organ chemokine; VP, virus particles; WT, wild type.

mouse) were emulsified with CFA before immunization. The draining inguinal LNs were isolated at indicated time point for further analysis.

Fusion protein or adenovirus treatment

HVEM-mouse Ig (mIg) (14) was described previously. For in vivo blocking purpose, 200 μg HVEM-mIg, with mIg as control, was administrated s.c. on the back on days 0, 1, or 2 of CFA immunization. A total of 5×10^{10} virus particles (VP) of adenovirus (Ad)-null and Ad-LIGHT was administrated i.d. on the tail base.

DC migration, lymphocyte LN entry blockade, and lymphocyte egress blockade

To determine DC migration from skin to DLN, 100 μl 2% FITC (Sigma-Aldrich) in 1:1 (v/v) acetone/dibutylphthalate mixture was applied on shaved back skin. After 24 h, draining inguinal LN was collagenase digested and made into single-cell suspension as described previously (15). Migratory DC was determined as $CD11c^+FITC^+$ by FACS. To block lymphocyte entry into LN, we administered 100 μg anti-CD62L (clone MEL-14; BioLegend) i.v. as described previously (3). To block lymphocyte egress from LN, we pretreated lymphocytes with 0.5 μg/ml FTY720 for 1 h at 37°C before adoptive transfer as described previously (16).

Bone marrow reconstitution, lymphocyte adoptive transfer, and Langerhans cell depletion

Mice were lethally irradiated with 1050 rad and adoptively transferred i.v. with 2×10^6 bone marrow cells the next day. Bactrim was added to the drinking water for 4 wk starting 1 d before irradiation. Mice were used after 8 wk. For lymphocyte adoptive transfer experiments, splenocytes were harvested, CSFE-labeled, counted, and transferred to recipient mice at doses described in *Results*. For skin Langerhans cell (LC) depletion, 0.1% clobetasol propionate (CP; Sigma) in DMSO was topically applied on the skin for 4 consecutive days. Mice were rested for 2 wk before immunization.

Flow cytometry analysis and cell sorting

Single-cell suspensions from the LNs were stained with anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD19 (1D3), anti-CD11c (N418), anti-CD45.1 (A20), anti-CD45 (30-F11), and anti-CD62L (MEL-14) mAbs (BD Biosciences), and anti-CD31 (MEC13.3) anti-CD103 (2E7), anti-epithelial cell adhesion molecule (G8.8) (BioLegend) in PBS containing 0.2% BSA and 0.09% sodium azide. Before staining, the cells were preincubated with anti-FcγRIII/II (2.4G2; BD Biosciences). Anti-Ki67 was purchased from Santa Cruz Biotechnology, and intracellular staining was conducted as per the Mouse Regulatory T Cell Staining Kit (eBioscience) protocol. Stained cells were analyzed on FACSCanto (BD Biosciences). Blood vascular endothelial cells were determined as $CD45^-CD31^+$ by FACS and sorted on FACSAria. LCs were determined as $CD45^-CD11c^+CD103^-$epithelial cell adhesion $molecule^+$. The purity was routinely >90%.

Real-time PCR

cDNA was prepared from DNase I-treated RNA extracted from skin, LN, or purified cells. PCR was conducted using Power SYBR Green PCR Master Mix (Applied Biosystems) on ABI 7300. The primers used are as follows: secondary lymphoid organ chemokine (SLC)—forward, 5′-AGACTC-AGGAGCCCAAAGCA-3′; reverse, 5′-GTTGAAGCAGGGCAAGGGT-3′; Epstein–Barr virus-induced molecule-1 ligand chemokine (ELC)—forward, 5′-ATGCGGAAGACTGCTGCCT-3′; reverse, 5′-GGCTTTCA-CGATGTTCCCAG-3′; VCAM-1—forward, 5′-AATCTCTTGTTTGCCT-CGCT-3′; reverse, 5′-GGGCAACGTTGACATAAAGA-3′; LIGHT—forward, 5′-CCTGAGACTGCATCAACGTC-3′; reverse, 5′-TTGGCTCCT-GTAAGATGTGC-3′; and β-actin—forward, 5′-ACACCCGCCACCAG-TTCGC-3′; reverse, 5′-ATGGGGTACTTCAGGGTCAGGATA-3′.

Statistical analysis

Statistical analysis was performed with GraphPad Prism statistical software (GraphPad Software). The nonparametric two-tailed *t* test was used to compare mean values between groups.

Results

LIGHT is required for LN hypertrophy on CFA immunization

$LIGHT^{-/-}$ mice develop a complete set of primary and secondary lymphoid organs including peripheral and mesenteric LNs and Peyer's patches (PP) with unaltered microarchitecture (11). To determine whether LIGHT plays a role in immune responses, we i.d. immunized $LIGHT^{-/-}$ or wild type (WT) mice with CFA. $LIGHT^{-/-}$ mice demonstrated smaller DLNs within days of immunization. To more carefully determine the role of LIGHT, we compared the total cellularity of draining inguinal LN, which reflects the extent of LN hypertrophy, between WT and $LIGHT^{-/-}$ mice on days 0, 3, and 7 postimmunization. A significantly lower total DLN cellularity in $LIGHT^{-/-}$ mice was found at days 3 and 7 compared with DLN of WT mice (Fig. 1*A*). Further analysis revealed a general reduction of several major subsets of cells including $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells, and DC (Fig. 1*B* and data not shown). Thus, $LIGHT^{-/-}$ mice have a defect that leads to significantly impaired LN hypertrophy, indicating that LIGHT is required for normal LN hypertrophy on immunization.

Early LIGHT signaling is not only essential but can also enhance LN hypertrophy

LIGHT engages two receptors, LTβR and HVEM, where LTβR signaling has been well documented in lymphoid organogenesis (17, 18). To determine whether the difference in LN hypertrophy was due to developmental defects in $LIGHT^{-/-}$ mice, we used HVEM-Ig to block LIGHT signaling in WT mice, together with CFA immunization. HVEM-Ig treatment alone has no impact on LN cellularity (data not shown). Three days after CFA immunization, LN hypertrophy, as assessed by DLN cellularity, was significantly reduced after HVEM-Ig blockade and CFA immunization (Fig. 2*A*), indicating that LIGHT signaling at the time of immunization regulates LN hypertrophy. To further examine the kinetics of LIGHT signaling required for LN hypertrophy, we administered HVEM-Ig at different time points after CFA immunization. It was found that only simultaneous HVEM-Ig treatment with CFA inhibits LN hypertrophy, whereas HVEM-Ig treatment 1 d after CFA has no impact on LN hypertrophy (Fig. 2*B*). This result suggests that early LIGHT signaling is essential for CFA-induced LN hypertrophy.

Knowing that active signaling by LIGHT regulates LN hypertrophy, we next studied which receptor is engaged by LIGHT for LN hypertrophy control. We used $HVEM^{-/-}$ mice to study LN

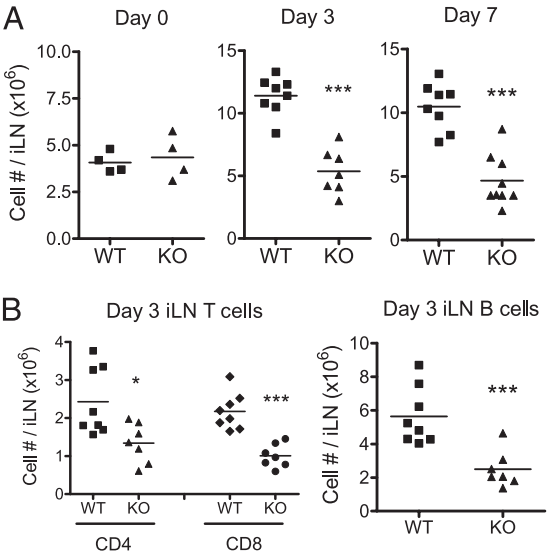

FIGURE 1. Impaired hypertrophy of DLN in $LIGHT^{-/-}$ mice. *A*, WT and $LIGHT^{-/-}$ mice were immunized with CFA/PBS (v/v = 1:1) i.d. at tail base. At days 0 (resting state), 3, and 7, iLNs were collected and total cell numbers were counted. Representative of at least three experiments. *B*, Different subsets of lymphocytes were analyzed by flow cytometry. Representative of at least three experiments. $*p < 0.05$, $***p < 0.001$.

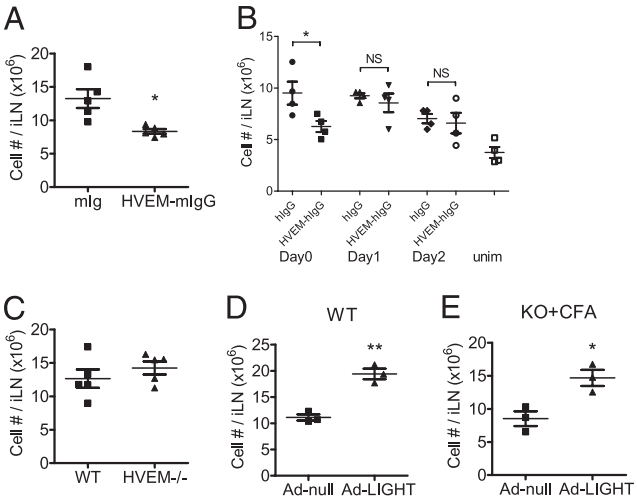

FIGURE 2. LIGHT signaling is not only essential but can also enhance LN hypertrophy. *A*, WT mice were treated i.d. with HVEM-IgG and control IgG, immediately followed by CFA immunization i.d. at tail base. iLN cellularity was determined at day 3. Representative of two experiments. *B*, WT mice were treated i.d. with HVEM-IgG or control IgG, at days 0, 1, and 2 of CFA immunization. iLN cellularity was determined at day 3. $*p < 0.05$, NS. Representative of two experiments. *C*, WT and HVEM$^{-/-}$ mice were immunized with CFA i.d. at tail base, and iLN cellularity was determined at day 3. $p > 0.05$. Representative of two experiments. *D*, WT mice were treated with Ad-null and Ad-LIGHT (5×10^{10} VP) i.d. at the tail base, and iLN cellularity was determined at day 3. Representative of two experiments. *E*, LIGHT$^{-/-}$ mice were treated with Ad-null and Ad-LIGHT (5×10^{10} VP) i.d. at the tail base followed by CFA immunization. iLN cellularity was determined at day 3. Representative of two experiments. $*p < 0.05$, $**p < 0.01$.

hypertrophy because LTβR$^{-/-}$ mice lack LNs. HVEM$^{-/-}$ mice have no apparent lymphoid organ developmental defect (data not shown). HVEM$^{-/-}$ and WT mice were immunized with CFA, and HVEM$^{-/-}$ mice showed comparable LN hypertrophy to WT mice at day 3 postimmunization (Fig. 2*C*). Thus, LIGHT signaling likely acts on LTβR, the other LIGHT-binding molecule, to control LN hypertrophy.

To further test whether active LIGHT signaling can promote LN hypertrophy induction during inflammation, we delivered LIGHT signaling to WT mice i.d. via Ad vector (Ad-LIGHT). Although Ad infection itself (Ad-null) was able to induce LN hypertrophy, Ad-LIGHT delivery induced significantly more LN hypertrophy than Ad-null (Fig. 2*D*), suggesting an important role of LIGHT signaling in promoting LN hypertrophy. To further test whether LIGHT signaling could rescue impaired CFA-induced LN hypertrophy in LIGHT$^{-/-}$ mice, we immunized LIGHT$^{-/-}$ mice with CFA followed by local treatment of either Ad-null or Ad-LIGHT. Three days later, Ad-LIGHT was found to induce more LN hypertrophy than Ad-null in LIGHT$^{-/-}$ mice (Fig. 2*E*), demonstrating that exogenous LIGHT signaling is sufficient to restore LN hypertrophy defect in LIGHT$^{-/-}$ mice after CFA immunization. These experiments suggest that exogenous LIGHT signaling is able to enhance LN hypertrophy regardless of the presence of endogenous LIGHT. Thus, active LIGHT signaling is essential for LN hypertrophy induction during the inflammation condition; in addition, exogenous LIGHT signaling can also enhance LN hypertrophy.

LIGHT is required for lymphocyte and DC migration into LN

Reduced number of cells in the LIGHT$^{-/-}$ DLN could be attributed to reduced proliferation, increased apoptosis, or balance of migration in and out of LN. Because ~90% of the lymphocytes in DLN are naive nonproliferating cells at such an early stage (day 3) after CFA immunization (Supplemental Fig. 1) (19), the hypertrophy defect is unlikely due to impaired proliferation. There is also no difference in the number of apoptotic cells in the LN between WT and LIGHT$^{-/-}$ mice (data not shown). Actually, few lymphocytes undergo apoptosis during LN hypertrophy. We therefore explored whether LIGHT regulates lymphocyte trafficking to and accumulation in the LN. To directly address the lymphocyte trafficking issue after immunization, we immunized WT and LIGHT$^{-/-}$ mice with CFA as described earlier. All mice were immediately adoptively transferred (i.v.) with Ly5.1 splenocytes. Twenty-four hours later, accumulation of Ly5.1$^+$ lymphocytes in inguinal LNs (iLNs) was counted and analyzed. A significantly higher number of Ly5.1$^+$ B and T cells were found accumulated in WT LN compared with LIGHT$^{-/-}$ LN (Fig. 3*A*). This suggested that LIGHT dictates the accumulation of adoptively transferred circulating lymphocytes into the DLN.

To directly address whether LIGHT has a role in lymphocyte influx after immunization, we treated Ly5.1$^+$ splenocytes with FTY720 as described previously (16) before adoptive transfer and CFA immunization in WT versus LIGHT$^{-/-}$ mice. FTY720 treatment downregulates sphingosine 1-phosphate receptor and therefore inhibits lymphocyte egress from LN (16, 20). Thus, the accumulation of FTY720-treated lymphocytes reflects lymphocyte influx into, but not egress out of, DLN within 24 h. We found dramatically more accumulation of FTY720-treated Ly5.1 B and T cells in DLN of WT mice than that in LIGHT$^{-/-}$ mice (Fig. 3*B*). This strongly suggests that LIGHT regulates the influx of lymphocytes in LNs after CFA immunization.

Lymphocyte accumulation in LNs is a balance between influx and egress. Under inflammation conditions, newly immigrated lymphocytes emigrate as early as 5 h after entering LN and emigration peaks at 12 h (21). We found no significant change of the lymphocyte retention rate between WT and LIGHT$^{-/-}$ mice (Fig. 3*C*), suggesting that LIGHT does not control lymphocyte egress in the LN after immunization. Thus, reduced lymphocyte accumulation in LIGHT$^{-/-}$ LN is likely due to impaired lymphocyte influx.

DCs migrate rapidly to DLN after CFA immunization; this has been shown play a critical role for LN hypertrophy. To test whether LIGHT regulates DC migration, we traced migratory DC in WT and LIGHT$^{-/-}$ mice using the FITC skin painting method. FITC was applied immediately after CFA immunization to the area around the CFA immunization site. Six and 24 h later, the numbers of FITC$^+$ DCs from collagenase-digested DLN were analyzed by FACS. Significantly fewer migratory DC (FITC$^+$) numbers were found in the DLN of LIGHT$^{-/-}$ mice compared with WT mice at both time points (Fig. 3*D*). The reduced number of migratory DCs is unlikely due to more migratory DC death in LIGHT$^{-/-}$ LN, because the reduced number is already apparent in LIGHT$^{-/-}$ LN as early as 6 h after CFA immunization. Therefore, the data suggest that LIGHT is required for DC migration from skin to DLN after CFA immunization.

LIGHT is required for chemokine and adhesion molecule expression

In studying the downstream molecular mechanism how LIGHT might regulate DC and lymphocyte migration to LN, we hypothesized that LIGHT might regulate vascular activation, that is, upregulation of chemokines and adhesion molecules, which has been well documented downstream of LTβR signaling, and might play important roles in leukocyte trafficking. SLC and ELC expression in the skin are critical for DC migration to DLN (5, 22). We tested whether production of these chemokines is impaired

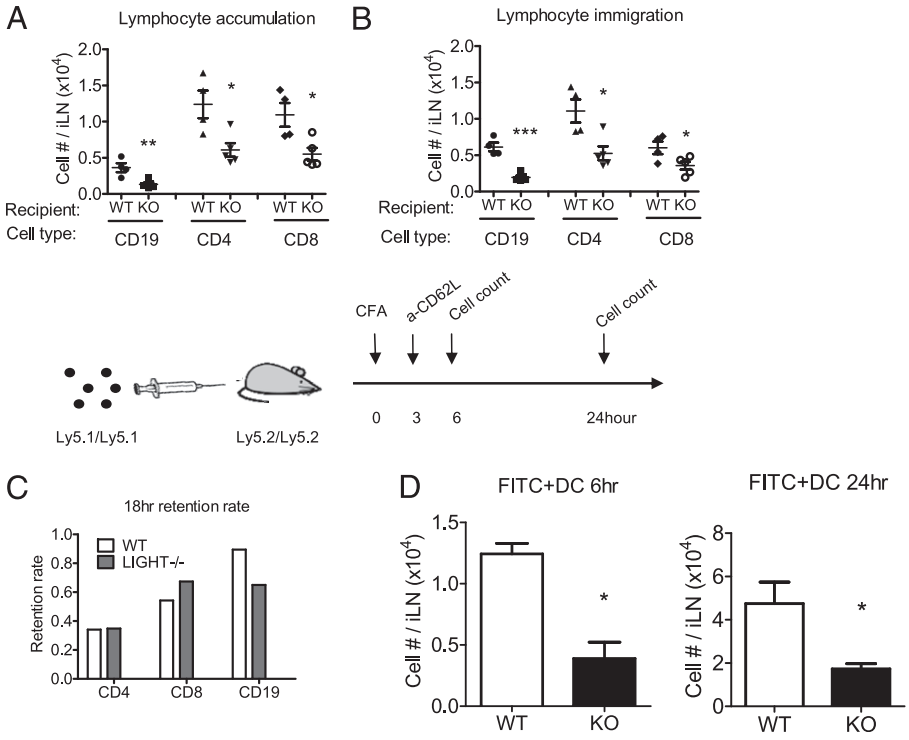

FIGURE 3. LIGHT is required for lymphocytes and DC migration to inflamed LN. WT and LIGHT$^{-/-}$ mice were immunized with CFA. *A*, Immediately after CFA immunization, Ly5.1 WT splenocytes were adoptively transferred to mice. Accumulation of Ly5.1$^+$ cells in iLN was analyzed 24 h after immunization. Representative of two experiments. *B*, Ly5.1 splenocytes were treated with FTY720 0.5 μg/ml for 1 h before adoptive transfer and CFA immunization in WT versus LIGHT$^{-/-}$ mice. Twenty-four hours after CFA immunization, accumulation of Ly5.1 T and B cells in DLN were counted by flow cytometry. Representative of two experiments. *C*, Immediately after CFA immunization, Ly5.1 WT splenocytes were adoptively transferred i.v. to immunized mice. Three hours later, further immigration to LN was blocked with 100 μg anti-CD62L. Six and 24 h after CFA, DLNs were collected, and Ly5.1$^+$ lymphocytes were counted by FACS. Retention rate was calculated as the ratio of cell numbers between 24 and 6 h. *D*, WT and LIGHT$^{-/-}$ mice were immunized with CFA. We applied 2% FITC in acetone and dibutyl phthalate (v/v = 1:1) near the CFA site in 100 μl. Six or 24 h later, iLN was collagenase digested and migratory DC (FITC$^+$CD11c$^+$) was determined by flow cytometry. Representative of two experiments. $*p < 0.05$, $**p < 0.01$. $***p < 0.001$.

compared with WT in the skin of LIGHT$^{-/-}$ mice on CFA immunization. Skin tissue at CFA immunization sites was collected 22–24 h after CFA immunization. Quantitative PCR was performed to determine the expression of several chemokines and adhesion molecules related to DC migration. We found that both SLC and ELC are dramatically lower in the skin of LIGHT$^{-/-}$ as compared with WT (Fig. 4*A*). This suggests that LIGHT regulates the production of SLC and ELC from cells resident in skin tissues.

Lymphocyte migration to LN is regulated by LN vascular endothelial cells, especially HEV (1, 23). To test whether LIGHT is required for LN vascular endothelial cell activation, we sorted CD45$^-$CD31$^+$ cells from DLN at 22–24 h after CFA immunization and performed quantitative PCR to check their activation status. Among several key molecules involved in vascular endothelial cell activation and function, we found that the expression level of VCAM-1, but not GlyCAM-1, CD34, or FucTVII, is significantly lower in LN vascular endothelial cells from LIGHT$^{-/-}$ mice after CFA immunization (Fig. 4*B*). This difference is seen only under immunization conditions, because VCAM-1 expression levels are comparable between WT and LIGHT$^{-/-}$ in unimmunized mice (Fig. 4*B*). In accordance with this, previous studies have demonstrated an important role of VCAM-1 in lymphocyte transmigration and entry to mesenteric LN and PP (24, 25).

Other proinflammatory cytokines have also been reported to regulate vascular endothelial cell activation. To further determine the relation between LIGHT and other proinflammatory cytokines, we examined some typical proinflammatory cytokine expression in skin by CBA 22–24 h after CFA immunization. We found dramatic impairment of TNF production from skin of LIGHT$^{-/-}$ mice compared with WT mice (Fig. 4*C*), whereas other proinflammatory cytokines tested, including IL-6, IFN-γ, and MCP-1, were normal. Furthermore, we found that local TNF signaling blockade by TNFR-Ig also significantly inhibits LN hypertrophy induced by CFA (Fig. 4*D*).

Radioresistant cell-derived LIGHT is unexpectedly required for LN hypertrophy

LIGHT is expressed on immature DCs and activated T cells, both of which are bone marrow-derived radiosensitive cells. To explore what cell delivers LIGHT for LN hypertrophy, we generated bone marrow chimeric mice as noted in Fig. 5*A*. Six to 8 wk later, when the hematopoietic compartment had completely reconstituted, mice were immunized with CFA as described earlier and LN hypertrophy was determined. Interestingly, radiosensitive bone marrow-derived, LIGHT-expressing cells are not essential for LN hypertrophy, because LIGHT$^{-/-}$ bone marrow does not decrease LN size in WT mice (Fig. 5*A*). In contrast, we found that radioresistant cells are the essential LIGHT-expressing cells contributing to LN hypertrophy after CFA immunization, because WT bone marrow into LIGHT$^{-/-}$ recipient mice phenocopied the decrease in LN hypertrophy observed in LIGHT$^{-/-}$ mice (Fig. 5*A*). This unexpected result led us to hypothesize that some nonconventional DC or T cell might deliver LIGHT signaling for LN hypertrophy.

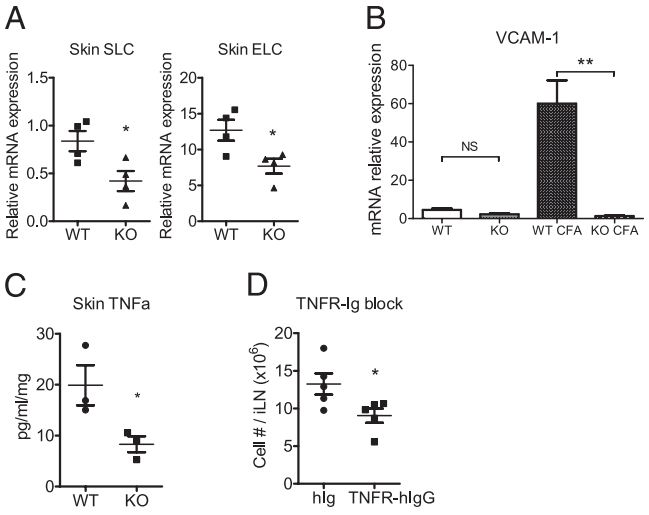

FIGURE 4. Impaired vascular activation and local TNF production in LIGHT$^{-/-}$ mice after CFA immunization. *A*, WT or LIGHT$^{-/-}$ mice were immunized with CFA; 22–24 h later, skin on top of the CFA site was collected and RNA was isolated. SLC and ELC expression levels were determined by quantitative PCR. Representative of two experiments. *B*, WT or LIGHT$^{-/-}$ mice were immunized with CFA or left untreated; 22–24 h later, LNs from each group were pooled and collagenase digested. CD45^{-}CD31^{+} cells were sorted by flow cytometry, and RNA was isolated. VCAM-1 expression level was determined by quantitative PCR. Data were representative from two experiments. *C*, Skin homogenate was made 22 h after CFA immunization from either WT or LIGHT$^{-/-}$ mice; TNF production was measured by CBA. Representative of two experiments. *D*, TNFR-hIgG or control IgG was injected i.d. locally at the tail base, followed immediately by CFA immunization. iLN cellularity was determined at day 3. Representative of two experiments. $*p < 0.05$, $**p < 0.01$.

We therefore asked whether skin LC could be the LIGHT-expressing radioresistant cell involved in LN hypertrophy regulation. Skin LC is a specialized radioresistant DC subset whose role in LN hypertrophy is currently unknown. To test whether skin LC could be important for LN hypertrophy, we depleted skin LCs by the pharmacological drug CP (Supplemental Fig. 2) (26). Given the slow homeostatic rate of skin LCs, they do not repopulate in epidermis within 2 wk of CP treatment and only partially repopulate in dermis, whereas conventional dermal DC is largely recovered (27–29). Therefore, mice were rested for 2 wk before CFA immunization. We found that CP-treated mice showed significantly lower LN hypertrophy than vehicle-treated mice (Fig. 5*B*). Furthermore, LIGHT expression was significantly upregulated on skin LCs after CFA immunization, supporting the role of LC-derived LIGHT on LN hypertrophy (Fig. 5*C*).

LIGHT promotes adaptive Ag-specific T cell response to immunization

Because LIGHT$^{-/-}$ fail to enlarge their LN after immunization, we next examined whether the subsequent immune response is also impaired in LIGHT$^{-/-}$ mice. To do this, we performed CFA/MOG immunization to examine MOG-specific T cell response. Seven days after CFA/MOG immunization, DLN cells were collected and restimulated with MOG peptide for 5 d and cytokine production was measured. Dramatically reduced IL-17 and IFN-γ production was found from cells from LIGHT$^{-/-}$ mice (Fig. 6*A*, 6*B*).

LIGHT expression on T cells has been well accepted as a T cell costimulatory molecule, and the impaired T cell immune response found earlier could be caused by a T cell costimulatory defect in LIGHT$^{-/-}$ mice. To exclude this possibility, we used a T cell adoptive transfer model, in which T cells are WT, whereas the hosts are WT or LIGHT$^{-/-}$. In this experiment, mice were adoptively transferred i.v. with 1×10^6 CFSE labeled OVA-specific CD4^{+} OT-II cells, followed immediately by i.d. injection of 10 μg OVA/CFA at the tail base. At day 3 after immunization, iLN cells were harvested for CFSE dilution and IFN-γ production analysis. For IFN-γ assay, iLN cells were restimulated with OT-II peptide for 14 h, and IFN-γ production was measured using intracellular staining. We found that LIGHT$^{-/-}$ iLNs have dramatically reduced OT-II cell proliferation and reduced production of IFN-γ compared with that in WT mice (Fig. 6*C*, 6*D*). These data suggest that a costimulation-independent function of LIGHT is required for T cell proliferation and differentiation. Future study is required to determine whether LIGHT regulates T cell response through LN hypertrophy per se or other mechanisms.

Discussion

LTβR signaling is transmitted by two TNF superfamily members, membrane LT and LIGHT. LT, but not LIGHT, is essential for normal lymphoid tissue organogenesis (17, 18, 30, 31). Although LTβ-deficient mice lack peripheral lymphoid organs and organized splenic structure, LIGHT-deficient mice have normal lymphoid development and organization in LN (11). Therefore, most studies focus on the role of LT for the development of lymphoid tissues and on the role of LIGHT on T cell costimulation. The role of LT and LTβR in LN hypertrophy has been difficult to study because both LT KO and LTβR KO mice lack peripheral LN

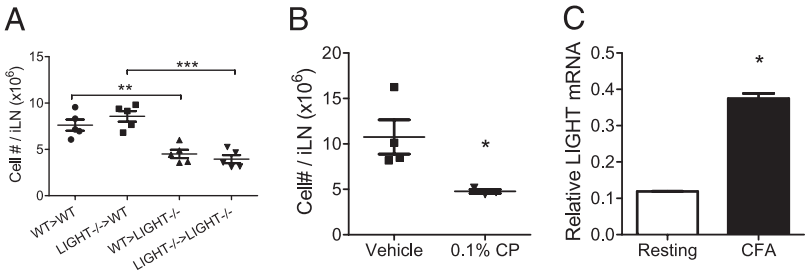

FIGURE 5. Radioresistant cell-derived LIGHT is required for LN hypertrophy. *A*, Bone marrow chimeric mice are generated with 2×10^6 bone marrow cells in 1050 rad lethally irradiated recipient mice. Six to 8 wk after bone marrow transplant, mice were immunized with CFA in the skin and DLN hypertrophy was analyzed at day 3 after immunization. Representative of two experiments. *B*, Shaved tail base skin was topically treated with DMSO or 0.1% CP in DMSO for 4 consecutive days. Mice were rested for 2 wk before CFA immunization. LN hypertrophy was checked at day 3 after immunization. $p < 0.05$. Representative of two experiments. *C*, WT mice were immunized with CFA; 24 h later, skin on top of the CFA site was collected and digested. LCs were isolated by FACS, and the expression level of LIGHT was determined by quantitative PCR. Representative of two experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

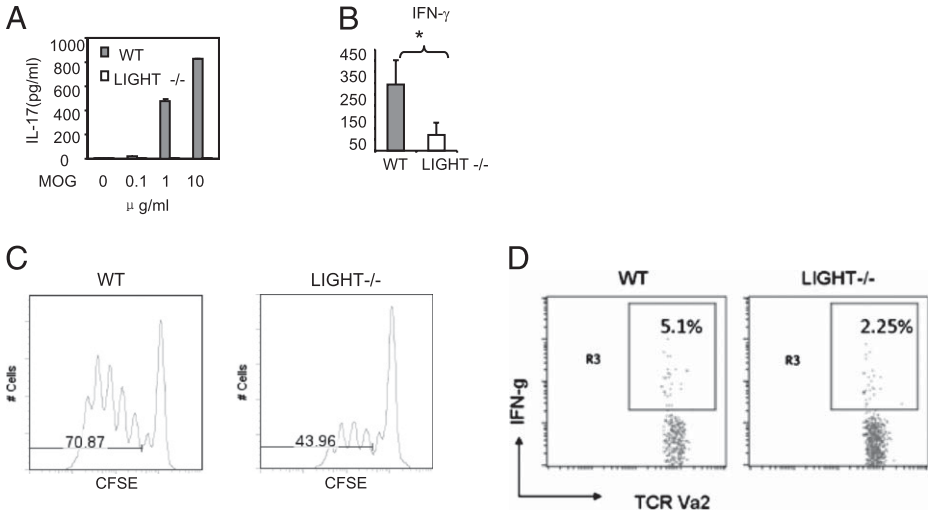

FIGURE 6. LIGHT expression on non-T cells is essential for T cell response. *A* and *B*, WT and LIGHT$^{-/-}$ mice were immunized with 10 μg MOG/CFA i.d. in the tail base. Seven days postimmunization, inguinal DLN cells were restimulated with MOG peptide at indicated concentration for 5 d in vitro. The production of IL-17 in the culture supernatants was determined by ELISA (*A*); IFN-γ production was measured by CBA (*B*). Representative of two experiments. *C*, CFSE-labeled OT-II cells (1×10^6) were i.v. transferred to WT and LIGHT$^{-/-}$ mice followed by immediate CFA/OVA 10 μg immunization i.d. at the tail base. Three days later, iLN cells were harvested for CFSE dilution analysis by FACS. *D*, After the iLNs cells were harvested as described in *C*, the cells were further restimulated with OT-II peptide (10 μg/ml) for 14 h. IFN-γ production was determined by intracellular staining. Representative of three experiments. $*p < 0.05$.

because of the developmental defect. However, rescued LN from LT KO mice treated at embryonic stage with LTβR agonistic Ab show HEV dysfunction similar to that in WT mice treated with LTβR-Ig to block LTβR signaling (6, 8). Thus, LT was attributed to play an essential role in regulating LN hypertrophy, whereas the role of LIGHT has been thought to be insignificant. Surprisingly, we found that LIGHT plays a critical role for CFA-mediated LN hypertrophy. Although expression of LIGHT only on T cells and bone marrow-derived DCs has been reported, LIGHT-expressing T cells or bone marrow-derived DCs are not essential. Unexpectedly, a radioresistant cell (likely LC) expressing LIGHT controls LN hypertrophy on CFA immunization. Importantly, LIGHT$^{-/-}$ mice have also exhibited a defect in T cell response after immunization.

Peripheral DC has been demonstrated to play a critical role for LN hypertrophy on CFA immunization using the CD11c$^-$diphtheria toxin receptor (DTR) DC depletion system (3). Several DC subsets exist in the skin, LCs in the epidermis, and Langerin$^+$ DCs and Langerin$^-$ DCs in the dermis. Although LCs are radioresistant, both Langerin$^+$ dermal DCs and Langerin$^-$ dermal DCs are radiosensitive (27, 32). In the CD11c$^-$DTR system, the major DC depleted by DT treatment is dermal DC, whereas LCs are preserved because of low CD11c expression (33). These pieces of information together led us to suspect that radioresistant LCs might also play an essential role for LIGHT-mediated LN hypertrophy, at least at the early phase of response. Our kinetic study using HVEM-Ig blockade showed that blocking LIGHT at the time of CFA immunization (day 0), but not after (day 1), inhibits LN hypertrophy. This suggests an early requirement of LIGHT likely at the dermis, the primary site of immunization. To test whether LCs in the skin are required, we used a topical treatment of CP to deplete LCs (26) and found that LN hypertrophy was dramatically reduced. Furthermore, LCs actually increased LIGHT expression after CFA immunization, in stark contrast with conventional DCs that reduce LIGHT expression after activation (34), further implicating LIGHT as a signal delivered by LCs to control LN hypertrophy. Although these data are all in line with our hypothesis that LC-derived LIGHT plays an important role at the early phase of LN hypertrophy, alternative explanations exist. For instance, topical CP treatment could lead to skin damage or have other effects on LN lymphatic vessels or other stromal cells, which may be critical for DC or lymphocyte migration. Therefore, a more conclusive answer about the role of LC-derived LIGHT in LN hypertrophy requires more specific tools, such as Langerin$^-$DTR mice and LC-specific LIGHT KO mice.

Our study also reveals a novel role of LIGHT in regulating vascular activation. Reduced chemokine and addressin expression was found in skin and LN vascular endothelial cells, respectively. It remains to be determined how LIGHT regulates this process. Our earlier published data demonstrate that LIGHT directly stimulates chemokine or adhesion molecule expression in stromal cells, suggesting a direct role of LIGHT (35). In addition, LIGHT could control expression of these genes indirectly. Supporting the latter, LIGHT can stimulate mast cells to produce various inflammatory cytokines, including TNF and IL-6 (36), where TNF is a cytokine well-known to activate endothelial cells (37, 38). Thus, TNF derived from LIGHT-activated mast cells could be a mediator for the effect of LIGHT on vascular endothelial cell activation. Indeed, mast cells are one of the major producers of proinflammatory cytokines, such as TNF and IL-1β, in the skin (39). As shown previously, TNF produced by mast cells in the skin can travel into DLN and is essential for LN hypertrophy (40, 41). Thus, LIGHT could have both a direct role and an indirect role on vascular endothelial cell activation. Conditional ablation of LIGHT-LTβR signaling on mast cells versus endothelial cells would help to clarify this issue in the future.

In addition to vascular endothelial cell activation, the growth of vascular endothelial cells might be also important for lymphocytes and DC recruitment, especially at later stages of LN hypertrophy. In fact, DC has been reported to be important for LN vascular endothelial cell growth (3). Because DC migration is reduced in LIGHT$^{-/-}$ mice, it would be interesting in the future to test whether this could lead to impaired vascular endothelial cell growth, thus affecting later lymphocyte migration.

The biological function of LIGHT in immune response generation is currently unclear. In one study, similar Ab response and

CTL response was found in LIGHT$^{-/-}$ and WT mice after vesicular stomatitis virus infection (11). In another study, only a minor role for LIGHT was found in CD8, but not CD4, T cell response generation after staphyloccoal enterotoxin B immunization (12). Ab response in LIGHT$^{-/-}$ mice after trinitrophenyl-keyhole limpet hemocyanin immunization was also found to be normal (12). However, in our study, a dramatically lower CD4 T cell response to immunization was found in LIGHT$^{-/-}$ mice compared with WT mice. This could be due to the difference of vaccination/infection protocol used in each study. In our study, a low dose of Ag was used for immunization s.c. in the skin, whereas mice were vaccinated/infected with high-dose i.v. in previous studies. The significance of LIGHT could therefore be more critical under suboptimal conditions. In fact, when a high dose of Ag (100 μg/mouse) was used in our study, OT-II T cell proliferation was comparable between WT and LIGHT$^{-/-}$ mice (data not shown). Considering that a natural infection usually presents with a low dose of Ag, the role of LIGHT could be more important than previously thought. This remains to be tested using an infection model. The more apparent role of LIGHT under low-dose Ag immunization indicates that LIGHT might regulate the immune response by indirect regulation of LN hypertrophy. Indeed, LN hypertrophy has been thought to be important for interaction between rare Ag-specific lymphocytes and APCs. Because pathogens can replicate exponentially early postinfection, a timely generation of a strong effector response would benefit the infected host. Therefore, fully developed LN hypertrophy might facilitate the generation of effector response. Further studies are needed to separate the role of LIGHT per se versus LN hypertrophy on immune response generation.

LN hypertrophy is a process involving multiple cell types and cytokines with finely regulated dynamics. In addition to DCs, B cells and mast cells have also been found to play important roles in LN hypertrophy induced by CFA or *Escherichia coli* infection (4, 40). On a molecular level, it is currently unclear how LIGHT, LT, and TNF mechanistically regulate LN hypertrophy. It is already known that LTβR signaling pathway and LT are critical for both HEV function and lymphangiogenesis (6–8), and that LTβR enhances mast cell activation and proinflammatory cytokine production (36). What remains to be discovered is how LIGHT and LT can both be required for LN hypertrophy given that they both the engage the same receptor. Do they have specified roles according to a temporal or spatial pattern during the response? Is TNF a downstream mediator of LIGHT or LT in controlling LN hypertrophy? How do these factors coordinate, and what is the unique role of each factor in LN hypertrophy? Our kinetic study showed that LIGHT is essential only for the first 24 h after immunization. This suggests that LIGHT might work at a very early stage after CFA immunization. Based on our data, it is possible that LIGHT expressed on a radioresistant cell from local skin tissue, likely LC, is an initiator of LN hypertrophy. Because LT controls LN hypertrophy and significant B cell migration at 2–3 d after CFA immunization, we hypothesize that B cell-derived LT works at a later stage of CFA immunization than LIGHT and works inside the LN. Further studies are needed to test this hypothesis in more detail. As mentioned earlier, it will be interesting to investigate whether LIGHT regulates hypertrophy of LNs under infection conditions.

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Disclosures

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