Published in final edited form as:
J Immunol. 2011 June 15; 186(12): 7156–7163. doi:10.4049/jimmunol.1002097.

LIGHT regulates inflamed draining lymph node hypertrophy

Mingzhao Zhu[*,†], **Yajun Yang**[*], **Yugang Wang**[†], **Zhongnan Wang**[*], and **Yang-Xin Fu**[†]

[*] Key Laboratory of Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China

[†] The Department of Pathology and Committee on Immunology, The University of Chicago, Chicago, Illinois, USA

Abstract

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. Here, we demonstrate that LIGHT (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 DC migration from the skin to draining LNs. Compared with WT 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, antigen-specific T cell responses were impaired in DLN 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.

Introduction

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 (DC) carrying antigens 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 one hour (1, 2). These steps in LN hypertrophy are considered important in order to favor encounters between antigen-presenting cells and rare antigen 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 LTβR signaling in LN hypertrophy regulation is emerging. LTβR was reported to be required for both high endothelial venue (HEV) homeostasis/

Address correspondence to: Mingzhao Zhu, Key Laboratory of Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Chaoyang District, Beijing 100101, China. Phone: 86-10-64888775; Fax: 86-10-64884618, zhumz@ibp.ac.cn; Yang-Xin Fu, Department of Pathology and Committee on Immunology, The University of Chicago, 5841 S. Maryland, Room J541, MC3083, Chicago, IL60637, USA. Phone: 773-702-0929; Fax: 773-834-8940;, yfu@uchicago.edu.

The authors declare no competing financial interests.

function and lymphangiogenesis (6–8). Lymphotoxin (LT) has been thought to be the sole ligand for the actions described above. 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 (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 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

Incomplete Freund's adjuvant (IFA) and M. Tuberculosis H37 RA were purchased from BD Diagnostic. Complete Freund's adjuvant (CFA) was made by mixing IFA and H37 RA of 10mg/ml. Equal volume of CFA and sterile PBS was mixed, emulsified before immunization. Mice were immunized with 100μl of emulsified CFA/PBS or CFA with antigen as indicated in the text and injected intradermally at tail base. For antigen/CFA immunization, ovalbumin (10μg/mouse) or MOG 35–55 peptide (10μg/mouse) were emulsified with CFA before immunization. The draining inguinal lymph nodes were isolated at indicated time point for further analysis.

Fusion protein or Adenovirus treatment

HVEM-mIg (14) was described as previously. For in vivo blocking purpose, 200μg HVEM-mIg, with mIg as control, were administrated subcutaneously at back on day0, 1 or 2 of CFA immunization. 5×10^{10} virus particles (VP) of adenovirus-null and adenovirus-LIGHT were administrated intradermally on the tail base.

DC migration, lymphocyte LN entry blockade, lymphocyte egress blockade

To determine DC migration from skin to draining LN, 100μl of 2% FITC (Sigma-Aldrich) in 1:1 (v/v) acetone/dibutylphthalate mixture was applied on shaved back skin. After 24 hours, 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 lymphocytes entry into LN, 100μg anti-CD62L (clone MEL-14, BioLegend) was administrated intravenously as described previously (3). To block lymphocytes egress from LN, lymphocytes were pretreated with 0.5μg/ml FTY720 for 1hr at 37°C before adoptive transfer as described previously (16).

Bone marrow reconstitution, lymphocyte adoptive transfer and LC depletion

Mice were lethally irradiated with 1050 rads 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 day before irradiation. Mice were used after 8wks. For lymphocyte adoptive transfer experiments, splenocytes were harvested, CSFE-labeled, counted and transferred to recipient

mice at doses described in the text. For skin LC depletion, 0.1% clobetasol propionate (Sigma) in DMSO was topically applied on the skin for four consecutive days. Mice were rested for 2wks before immunization.

Flow cytometry analysis and cell sorting

Single cell suspensions from the lymph nodes 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), anti-CD62L (MEL-14) mAbs, (BD Biosciences) and anti-CD31 (MEC13.3) anti-CD103 (2E7), anti-EpCAM (G8.8) (BioLegend) in PBS containing 0.2% BSA and 0.09% sodium azide. Before staining, the cells were preincubated with anti-Fc III/II receptor (2.4G2; BD Biosciences). Anti-Ki67 was purchased from Santa Cruz 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-EpCAM+. The purity was routinely higher than 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. For SLC, forward: AGACTCAGGAGCCCAAAGCA, reverse: GTTGAAGCAGGGCAAGGGT; For ELC, forward: ATGCGGAAGACTGCTGCCT, reverse: GGCTTTCACGATGTTCCCAG; For VCAM-1, forward: AATCTCTTGTTTGCCTCGCT, reverse: GGGCAACGTTGACATAAAGA; For LIGHT, forward: CCTGAGACTGCATCAACGTC, reverse: TTGGCTCCTGTAAGATGTGC; For beta actin, forward: ACACCCGCCACCAGTTCGC, reverse: ATGGGGTACTTCAGGGTCAGGATA.

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. The statistical significance threshold was set at *P* value less than .05.

Results

LIGHT is required for LN hypertrophy upon CFA immunization

LIGHT$^{-/-}$ mice develop a complete set of primary and secondary lymphoid organs including peripheral and mesenteric LN (MLNs) and Peyer's patches (PP) with unaltered microarchitecture (11). To determine whether LIGHT plays a role in immune responses, LIGHT$^{-/-}$ or WT mice were intradermally (i.d.) immunized with CFA. LIGHT KO mice demonstrated smaller draining LNs (DLN) within days of immunization. To more carefully determine the role of LIGHT, the total cellularity of draining inguinal LN, which reflects the extent of LN hypertrophy, was compared between WT and LIGHT$^{-/-}$ mice on day 0, 3 and 7 post-immunization. A significantly lower total DLN cellularity in LIGHT$^{-/-}$mice was found at day 3 and day 7 compared with DLN of WT mice (Figure 1A). Further analysis revealed a general reduction of several major subsets of cells including CD4+ T cells, CD8+ T cells, B, NK cells, and DC (Figure 1B and data not shown). Thus, LIGHT$^{-/-}$ mice have a defect which leads to significantly impaired LN hypertrophy, indicating that LIGHT is required for normal LN hypertrophy upon 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 utilized 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 (Figure 2A), indicating that LIGHT signaling at the time of immunization regulates LN hypertrophy. To further examine the kinetics of LIGHT signaling required for LN hypertrophy, HVEM-Ig was administered at different time points after CFA immunization. It was found that only simultaneous HVEM-Ig treatment with CFA inhibits LN hypertrophy, while HVEM-Ig treatment 1 day after CFA has no impact on LN hypertrophy (Figure 2B). 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 utilized HVEM$^{-/-}$mice to study LN hypertrophy since LTβR$^{-/-}$ mice lack lymph nodes. 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 post-immunization (Figure 2C). 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 intradermally via adenovirus vector (Ad-LIGHT). Although adenovirus infection itself (Ad-null) was able to induce LN hypertrophy, Ad-LIGHT delivery induced significantly more LN hypertrophy than Ad-null (Figure 2D), 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, LIGHT$^{-/-}$ mice were immunized with CFA followed by local treatment of either Ad-null or Ad-LIGHT. 3 days later, Ad-LIGHT was found to induce more LN hypertrophy than Ad-null in LIGHT$^{-/-}$ mice (Figure 2E), 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 lymphocytes and DC migration into LN

Reduced number of cells in the LIGHT$^{-/-}$ draining LN could be attributed to reduced proliferation, increased apoptosis, or balance of migration in and out of LN. Since approximately 90% of the lymphocytes in DLN are naïve nonproliferating cells at such an early stage (day3) after CFA immunization (Supplementary Figure 1 and (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, WT and LIGHT$^{-/-}$ mice were immunized with CFA as described before. All mice were immediately adoptively transferred (i.v.) with Ly5.1 splenocytes. 24 hours later, accumulation of Ly5.1^{+} lymphocytes in iLNs was counted and analyzed. A significantly higher number of Ly5.1^{+} B

and T cells were found accumulated in WT LN compared to LIGHT$^{-/-}$ LN (Figure 3A). 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, Ly5.1^{+} splenocytes were treated with FTY720 as described (16) before adoptive transfer and CFA immunization in WT versus LIGHT$^{-/-}$ mice. FTY720 treatment downregulates sphingosine 1-phosphate (S1P) 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 hours. We found dramatically more accumulation of FTY720 treated Ly5.1 B and T cells in DLN of WT mice than that in LIGHT$^{-/-}$ mice (Figure 3B). This strongly suggests that LIGHT regulates the influx of lymphocytes in LNs after CFA immunization.

Lymphocyte accumulation in lymph nodes is a balance between influx and egress. Under inflammation conditions, newly immigrated lymphocytes emigrate as early as 5 hours after entering LN and emigration peaks at 12 hours (21). We found no significant change of the lymphocyte retention rate between WT and LIGHT$^{-/-}$ mice (Figure 3C), suggesting that LIGHT does not control lymphocyte egress in the LN after immunization. Thus, reduced lymphocytes 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 CFA immunization site. 6 and 24 hours later, the numbers of FITC+ DC from collagenage-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 (Figure 3D). The reduced number of migratory DC 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 hours after CFA immunization. Therefore, the data suggests 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, i.e. upregulation of chemokines and adhesion molecules, which has been well documented downstream of LTβR signaling and to play important roles in leukocyte trafficking. CCL21 and CCL19 expression in the skin are critical for DC migration to DLN (5, 22). We tested whether production of these chemokines is impaired compared to WT in the skin of LIGHT$^{-/-}$ mice upon CFA immunization. Skin tissue at CFA immunization sites was collected 22–24 hours 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 CCL21 and CCL19 are dramatically lower in the skin of LIGHT$^{-/-}$ as compared to WT (Figure 4A). This suggests that LIGHT regulates the production of CCL21 and CCL19 from cells resident in skin tissues.

Lymphocyte migration to LN is regulated by lymph node 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 hours 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 (Figure 4B). This difference is only seen under immunization conditions, as VCAM-1 expression levels are comparable between WT and LIGHT$^{-/-}$ in un-immunized mice (Figure 4B). In accordance with this, previous studies have demonstrated an important role of VCAM-1 in lymphocyte transmigration and entry to mesenteric LN and Peyer's patches (24, 25).

Other proinflammatory cytokines have also been reported to regulate vascular endothelial cell activation. To further determine the relationship between LIGHT and other proinflammatory cytokines, we examined some typical proinflammatory cytokines expression in skin by CBA 22–24 hours after CFA immunization. We found dramatic impairment of TNF production from skin of LIGHT$^{-/-}$ mice compared with WT mice (Figure 4C), while 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 (Figure 4D).

Radioresistant cell derived LIGHT is unexpectedly required for LN hypertrophy

LIGHT is expressed on immature DC and activated T cells, both of which are bone marrow derived radiosensitive cells. To explore what cell delivers LIGHT for LN hypertrophy, bone marrow chimeric mice were generated as noted in Figure 5A. 6–8 wks later, when the hematopoietic compartment had completely reconstituted, mice were immunized with CFA as before 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 (Figure 5A). In contrast, we found that radioresistant cells are the essential LIGHT-expressing cells contributing to LN hypertrophy after CFA immunization, as WT bone marrow into LIGHT$^{-/-}$ recipient mice phenocopied the decrease in LN hypertrophy observed in LIGHT$^{-/-}$ mice (Figure 5A). This unexpected result led us to hypothesize that some non-conventional DC or T cell might deliver LIGHT signaling for LN hypertrophy.

We therefore asked whether skin Langerhans cells (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 LC by the pharmacological drug clobetasol propionate (CP) (26). Given the slow homeostatic rate of skin LC, they do not repopulate in epidermis within 2 wks of CP treatment and only partially repopulate in dermis, while conventional dermal DC is largely recovered (27–29). Therefore, mice were rested for 2 wks before CFA immunization. We found that CP treated mice showed significantly lower LN hypertrophy than vehicle treated mice (Figure 5B). Furthermore, supporting the role of LC-derived LIGHT on LN hypertrophy, LIGHT expression was significantly upregulated on skin LC after CFA immunization (Figure 5C).

LIGHT promotes adaptive antigen-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, CFA/MOG immunization was performed to examine MOG specific T cell response. 7 days after CFA/MOG immunization, DLN cells were collected and restimulated with MOG peptide for 5 days and cytokine production was measured. Dramatically reduced IL-17 and IFN-γ production was found from cells from LIGHT$^{-/-}$ mice (Figure 6A and 6B).

LIGHT expression on T cells has been well accepted as a T cell costimulatory molecule and the impaired T cell immune response found above could be due to 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 while the host 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 10μg of OVA/CFA at the tail base. At day 3 after immunization, iLN cells were harvested for CFSE dilution and IFN-γ production analysis. For this assay, iLN cells were restimulated with OT-II peptide for 14 hours and IFN-γ production was measured using CBA. We found that LIGHT$^{-/-}$ iLNs have dramatically reduced OT-II cell proliferation and reduced production of IFN-γ compared with that in WT mice (Figure 6C and 6D). 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 TNFSF members, membrane LT and LIGHT. LT but not LIGHT is essential for normal lymphoid tissue organogenesis (17, 18, 30, 31). While 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, since both LT KO and LTβR KO mice lack peripheral LN due to the developmental defect. However, rescued LN from LT KO mice treated at embryonic stage with LTβR agonistic antibody 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 while 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 BMDC have been reported, LIGHT expressing T cells or BMDC are not essential. Unexpectedly, a radioresistant cell (likely Langerhans cell) expressing LIGHT controls LN hypertrophy upon CFA immunization. Importantly, LIGHT$^{-/-}$ mice have also exhibit a defect in T cell response after immunization.

Peripheral DC has been demonstrated to play a critical role for LN hypertrophy upon CFA immunization using the CD11c-DTR DC depletion system (3). Several DC subsets exist in the skin, Langerhans cells in the epidermis, Langerin+ DC and Langerin- DC in the dermis. While Langerhans cells are radioresistant, both Langerin+ dermal DC and Langerin- dermal DC are radiosensitive (27, 32). In the CD11c-DTR system, the major DC depleted by DT treatment is dermal DC while Langerhans cells are preserved due to low CD11c expression (33). These pieces of information together led us to suspect that radioresistant Langerhans cells 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 Langerhans cells in the skin are required, we used a topical treatment of clobetasol propionate to deplete Langerhans cells (26), and found that LN hypertrophy was dramatically reduced. Furthermore, LC actually increased LIGHT expression after CFA immunization in stark contrast to conventional DC that reduce LIGHT expression after activation (34), further implicating LIGHT as a signal delivered by Langerhans cells 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 knockout 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 draining LN and is essential for LN hypertrophy (40, 41). Thus, LIGHT could have both direct role and 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 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). Since 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 antibody response and CTL response was found in LIGHT$^{-/-}$ and WT mice after VSV infection (11). In another study, only a minor role for LIGHT was found in CD8, but not CD4, T cell response generation after SEB immunization (12). Antibody response in LIGHT$^{-/-}$ mice after TNP-KLH 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 antigen was used for immunization subcutaneously in the skin, while mice were vaccinated/infected with high dose antigen/virus intravenously in previous studies. The significance of LIGHT could therefore be more critical under sub-optimalconditions. In fact, when a high dose of antigen (100μg/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 antigen, 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 antigen 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 antigen-specific lymphocytes and antigen presenting cells. Since pathogens can replicate exponentially early after infection, 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 DC, B cells and mast cells have also been found to play important roles in LN hypertrophy induced by CFA or *E. 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 only essential for the first 24 hours 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. Since LT controls LN hypertrophy and significant B cell migration at 2–3 days 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 above, it will be interesting to investigate whether LIGHT regulates hypertrophy of LNs under infection conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Mendy Miller for editing of this paper.

This work was supported by grants from the National Institutes of Health (R01 CA141975 to Y.X.F.), National Natural Science Foundation of China (General Program 81072414 to M.Z.) and Chinese Academy of Sciences (Innovative Program 2010- Biols-CAS-02 and the Hundred Talents Program to M.Z.)

Abbreviations

DC	dendritic cell
EpCAM	Epithelial cell adhesion molecule
HEV	high endothelial venue
HVEM	herpes virus entry mediator
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 beta receptor
WT	wild type

References

1. von Andrian UH, Mempel TR. Homing and cellular traffic in lymph nodes. Nat Rev Immunol. 2003; 3:867. [PubMed: 14668803]
2. McConnell I, Hopkins J. Lymphocyte traffic through antigen-stimulated lymph nodes. I. Complement activation within lymph nodes initiates cell shutdown. Immunology. 1981; 42:217–223. [PubMed: 6906330]

3. Webster B, Ekland EH, Agle LM, Chyou S, Ruggieri R, Lu TT. Regulation of lymph node vascular growth by dendritic cells. J Exp Med. 2006; 203:1903–1913. [PubMed: 16831898]
4. Angeli V, Ginhoux F, Llodra J, Quemeneur L, Frenette PS, Skobe M, Jessberger R, Merad M, Randolph GJ. B cell-driven lymphangiogenesis in inflamed lymph nodes enhances dendritic cell mobilization. Immunity. 2006; 24:203–215. [PubMed: 16473832]
5. Martin-Fontecha A, Sebastiani S, Hopken UE, Uguccioni M, Lipp M, Lanzavecchia A, Sallusto F. Regulation of Dendritic Cell Migration to the Draining Lymph Node: Impact on T Lymphocyte Traffic and Priming. J Exp Med. 2003; 198:615–621. [PubMed: 12925677]
6. Browning JL, Allaire N, Ngam-Ek A, Notidis E, Hunt J, Perrin S, Fava RA. Lymphotoxin-beta receptor signaling is required for the homeostatic control of HEV differentiation and function. Immunity. 2005; 23:539–550. [PubMed: 16286021]
7. Chyou S, Ekland EH, Carpenter AC, Tzeng TC, Tian S, Michaud M, Madri JA, Lu TT. Fibroblast-type reticular stromal cells regulate the lymph node vasculature. J Immunol. 2008; 181:3887–3896. [PubMed: 18768843]
8. Liao S, Ruddle NH. Synchrony of high endothelial venules and lymphatic vessels revealed by immunization. J Immunol. 2006; 177:3369–3379. [PubMed: 16920978]
9. Mauri DN, Ebner R, Montgomery RI, Kochel KD, Cheung TC, Yu GL, Ruben S, Murphy M, Eisenberg RJ, Cohen GH, Spear PG, Ware CF. LIGHT, a New Member of the TNF Superfamily, and Lymphotoxin [alpha] Are Ligands for Herpesvirus Entry Mediator. Immunity. 1998; 8:21. [PubMed: 9462508]
10. Carl FW. Targeting lymphocyte activation through the lymphotoxin and LIGHT pathways. Immunological Reviews. 2008; 223:186–201. [PubMed: 18613837]
11. Scheu S, Alferink J, Potzel T, Barchet W, Kalinke U, Pfeffer K. Targeted Disruption of LIGHT Causes Defects in Costimulatory T Cell Activation and Reveals Cooperation with Lymphotoxin {beta} in Mesenteric Lymph Node Genesis. J Exp Med. 2002; 195:1613–1624. [PubMed: 12070288]
12. Tamada K, Ni J, Zhu G, Fiscella M, Teng B, van Deursen JMA, Chen L. Cutting Edge: Selective Impairment of CD8+ T Cell Function in Mice Lacking the TNF Superfamily Member LIGHT. J Immunol. 2002; 168:4832–4835. [PubMed: 11994431]
13. Wang Y, Subudhi SK, Anders RA, Lo J, Sun Y, Blink S, Wang Y, Wang J, Liu X, Mink K, Degrandi D, Pfeffer K, Fu YX. The role of herpesvirus entry mediator as a negative regulator of T cell mediated responses. The Journal of Clinical Investigation. 2005; 115:711–717. [PubMed: 15696194]
14. Wang J, Lo JC, Foster A, Yu P, Chen HM, Wang Y, Tamada K, Chen L, Fu YX. The regulation of T cell homeostasis and autoimmunity by T cell-derived LIGHT. J Clin Invest. 2001; 108:1771–1780. [PubMed: 11748260]
15. Wang YG, Kim KD, Wang J, Yu P, Fu YX. Stimulating Lymphotoxin {beta} Receptor on the Dendritic Cells Is Critical for Their Homeostasis and Expansion. J Immunol. 2005; 175:6997–7002. [PubMed: 16272360]
16. Ledgerwood LG, Lal G, Zhang N, Garin A, Esses SJ, Ginhoux F, Merad M, Peche H, Lira SA, Ding Y, Yang Y, He X, Schuchman EH, Allende ML, Ochando JC, Bromberg JS. The sphingosine 1-phosphate receptor 1 causes tissue retention by inhibiting the entry of peripheral tissue T lymphocytes into afferent lymphatics. Nat Immunol. 2008; 9:42–53. [PubMed: 18037890]
17. Randall TD, Carragher DM, Rangel-Moreno J. Development of Secondary Lymphoid Organs. Annual Review of Immunology. 2008; 26:627.
18. Fu YX, Chaplin DD. Development and maturation of secondary lymphoid tissues. Annu Rev Immunol. 1999; 17:399–433. [PubMed: 10358764]
19. Soderberg KA, Payne GW, Sato A, Medzhitov R, Segal SS, Iwasaki A. Innate control of adaptive immunity via remodeling of lymph node feed arteriole. Proceedings of the National Academy of Sciences. 2005; 102:16315–16320.
20. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu Rev Immunol. 2005; 23:127–159. [PubMed: 15771568]
21. Srikusalanukul W, De Bruyne F, McCullagh P. Modelling of peripheral lymphocyte migration: System identification approach. Immunol Cell Biol. 2000; 78:288. [PubMed: 10849118]

22. Randolph GJ, Angeli V, Swartz MA. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. Nat Rev Immunol. 2005; 5:617. [PubMed: 16056255]
23. Bajenoff M, Egen JG, Qi H, Huang AYC, Castellino F, Germain RN. Highways, byways and breadcrumbs: directing lymphocyte traffic in the lymph node. Trends in Immunology. 2007; 28:346. [PubMed: 17625969]
24. Faveeuw C, Di Mauro ME, Price AA, Ager A. Roles of {alpha}4 integrins/VCAM-1 and LFA-1/ICAM-1 in the binding and transendothelial migration of T lymphocytes and T lymphoblasts across high endothelial venules. Int Immunol. 2000; 12:241–251. [PubMed: 10700459]
25. Berlin-Rufenach C, Otto F, Mathies M, Westermann J, Owen MJ, Hamann A, Hogg N. Lymphocyte Migration in Lymphocyte Function-associated Antigen (LFA)-1 eficient Mice. The Journal of Experimental Medicine. 1999; 189:1467–1478. [PubMed: 10224287]
26. Grabbe S, Steinbrink K, Steinert M, Luger TA, Schwarz T. Removal of the majority of epidermal Langerhans cells by topical or systemic steroid application enhances the effector phase of murine contact hypersensitivity. J Immunol. 1995; 155:4207–4217. [PubMed: 7594576]
27. Merad M, Ginhoux F, Collin M. Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. Nat Rev Immunol. 2008; 8:935–947. [PubMed: 19029989]
28. Bursch LS, Wang L, Igyarto B, Kissenpfennig A, Malissen B, Kaplan DH, Hogquist KA. Identification of a novel population of Langerin+ dendritic cells. J Exp Med. 2007; 204:3147–3156. [PubMed: 18086865]
29. Kissenpfennig A, Henri S, Dubois B, Laplace-Builhe C, Perrin P, Romani N, Tripp CH, Douillard P, Leserman L, Kaiserlian D, Saeland S, Davoust J, Malissen B. Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. Immunity. 2005; 22:643–654. [PubMed: 15894281]
30. Drayton DL, Liao S, Mounzer RH, Ruddle NH. Lymphoid organ development: from ontogeny to neogenesis. Nat Immunol. 2006; 7:344–353. [PubMed: 16550197]
31. Mebius RE. Organogenesis of lymphoid tissues. Nat Rev Immunol. 2003; 3:292. [PubMed: 12669020]
32. Nagao K, Ginhoux F, Leitner WW, Motegi SI, Bennett CL, Clausen BE, Merad M, Udey MC. Murine epidermal Langerhans cells and langerin-expressing dermal dendritic cells are unrelated and exhibit distinct functions. Proceedings of the National Academy of Sciences. 2009; 106:3312–3317.
33. Jung S, Unutmaz D, Wong P, Sano G-I, De los Santos K, Sparwasser T, Wu S, Vuthoori S, Ko K, Zavala F, Pamer EG, Littman DR, Lang RA. In Vivo Depletion of CD11c+ Dendritic Cells Abrogates Priming of CD8+ T Cells by Exogenous Cell-Associated Antigens. Immunity. 2002; 17:211. [PubMed: 12196292]
34. Tamada K, Shimozaki K, Chapoval AI, Zhai Y, Su J, Chen SF, Hsieh SL, Nagata S, Ni J, Chen L. LIGHT, a TNF-Like Molecule, Costimulates T Cell Proliferation and Is Required for Dendritic Cell-Mediated Allogeneic T Cell Response. J Immunol. 2000; 164:4105–4110. [PubMed: 10754304]
35. Lo JC, Basak S, James ES, Quiambo RS, Kinsella MC, Alegre ML, Weih F, Franzoso G, Hoffmann A, Fu YX. Coordination between NF-kappaB family members p50 and p52 is essential for mediating LTbetaR signals in the development and organization of secondary lymphoid tissues. Blood. 2006; 107:1048–1055. [PubMed: 16195333]
36. Stopfer P, Mannel DN, Hehlgans T. Lymphotoxin-{beta} Receptor Activation by Activated T Cells Induces Cytokine Release from Mouse Bone Marrow-Derived Mast Cells. J Immunol. 2004; 172:7459–7465. [PubMed: 15187124]
37. Ji RC. Lymphatic endothelial cells, inflammatory lymphangiogenesis, and prospective players. Curr Med Chem. 2007; 14:2359–2368. [PubMed: 17896984]
38. Naldini A, Carraro F. Role of inflammatory mediators in angiogenesis. Curr Drug Targets Inflamm Allergy. 2005; 4:3–8. [PubMed: 15720228]
39. Galli SJ, Grimbaldeston M, Tsai M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. Nat Rev Immunol. 2008; 8:478–486. [PubMed: 18483499]

40. McLachlan JB, Hart JP, Pizzo SV, Shelburne CP, Staats HF, Gunn MD, Abraham SN. Mast cell-derived tumor necrosis factor induces hypertrophy of draining lymph nodes during infection. Nat Immunol. 2003; 4:1199–1205. [PubMed: 14595438]

41. Kunder CA, St John AL, Li G, Leong KW, Berwin B, Staats HF, Abraham SN. Mast cell-derived particles deliver peripheral signals to remote lymph nodes. J Exp Med. 2009; 206:2455–2467. [PubMed: 19808250]

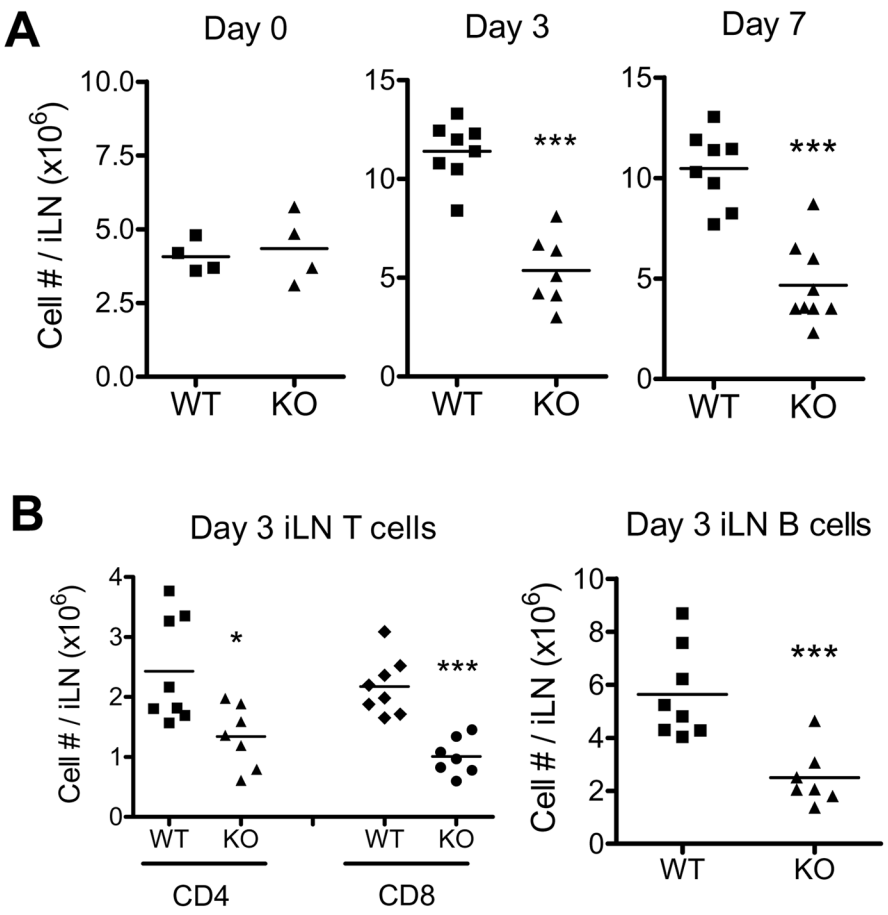

Figure 1. Impaired hypertrophy of DLN in LIGHT$^{-/-}$ mice

A. WT and LIGHT$^{-/-}$ mice were immunized with CFA/PBS (v:v=1:1) i.d. at tail base. At day0 (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. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. Representative of at least three experiments.

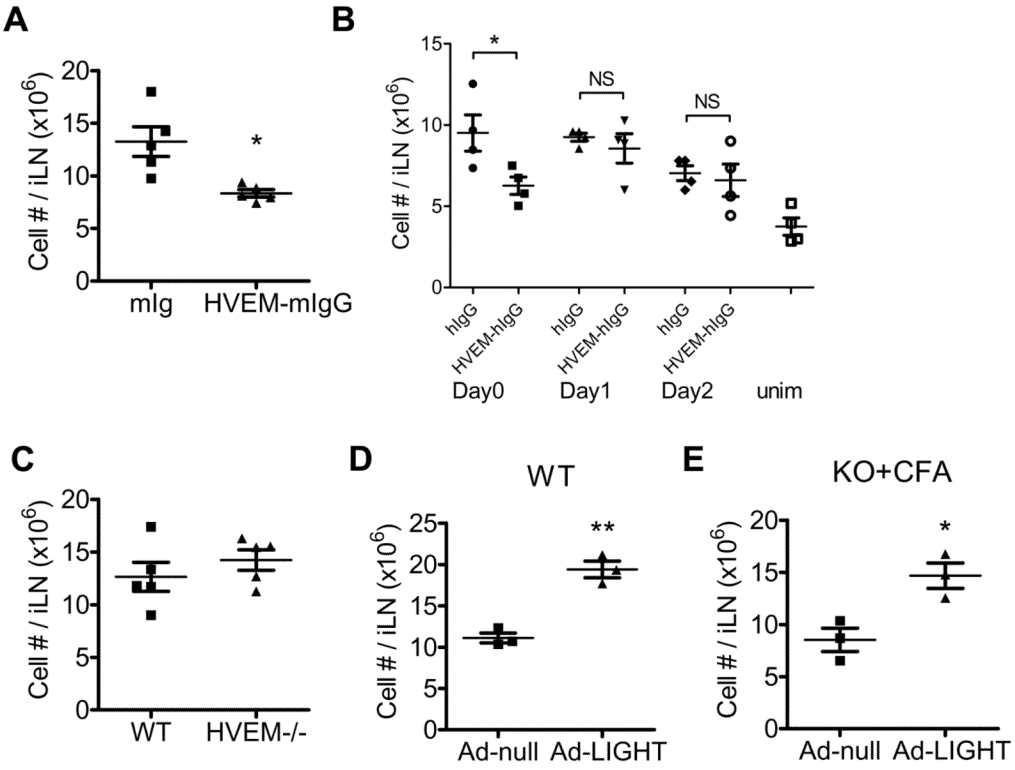

Figure 2. LIGHT signaling is not only essential but can also enhance LN hypertrophy

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. $P < 0.05$. Representative of two experiments. **B.** WT mice were treated i.d. with HVEM-IgG or control IgG, at day 0, 1, 2 of CFA immunization. iLN cellularity was determined at day 3. *, $P < 0.05$, NS, not significant. 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. $P < 0.05$. 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. *, $P < 0.05$, **, $P < 0.01$. Representative of two experiments.

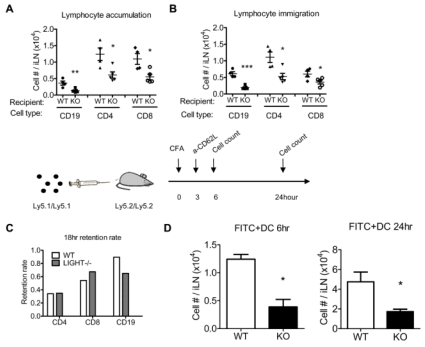

Figure 3. LIGHT is required for lymphocytes and DC migration to inflamed LN

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

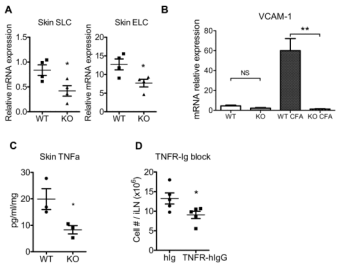

Figure 4. Impaired vascular activation and local TNF production in LIGHT$^{-/-}$ mice after CFA immunization

A. WT or LIGHT$^{-/-}$ mice were immunized with CFA, 22–24 hrs later, skin on top of the CFA site was collected and RNA was isolated. SLC and ELC expression level was determined by quantitative PCR. *, $P < 0.05$. Representative of two experiments. **B.** WT or LIGHT$^{-/-}$mice were immunized with CFA or left untreated, 22–24 hrs 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. **, $P < 0.01$. Data were representative from two experiments. **C.** Skin homogenate was made 22hrs after CFA immunization from either WT or LIGHT$^{-/-}$ mice, TNF production was measured by CBA. *, $P < 0.05$. 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. *, $P < 0.05$. Representative of two experiments.

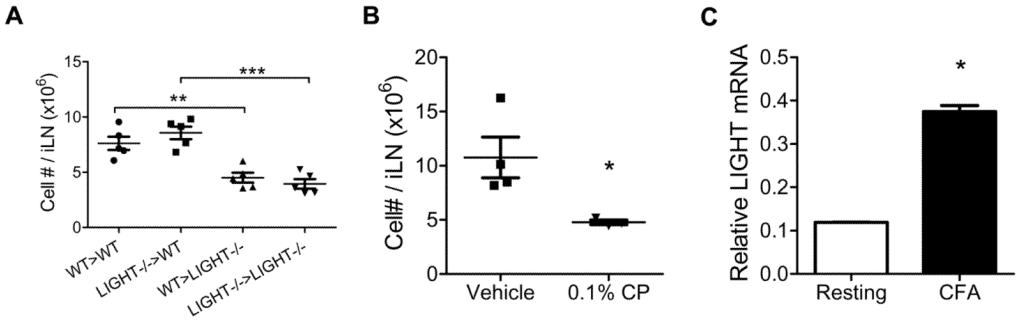

Figure 5. Radioresistant cell-derived LIGHT is required for LN hypertrophy

A, Bone marrow chimeric mice are generated with 2×10^6 bone marrow cells in 1000rad lethally irradiated recipient mice. 6–8 wks 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 2wks 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 hrs later, skin on top of the CFA site was collected and digested. Langerhans cells were isolated by FACS sorting and the expression level of LIGHT was determined by quantitative PCR. *, $P < 0.05$. Representative of two experiments.

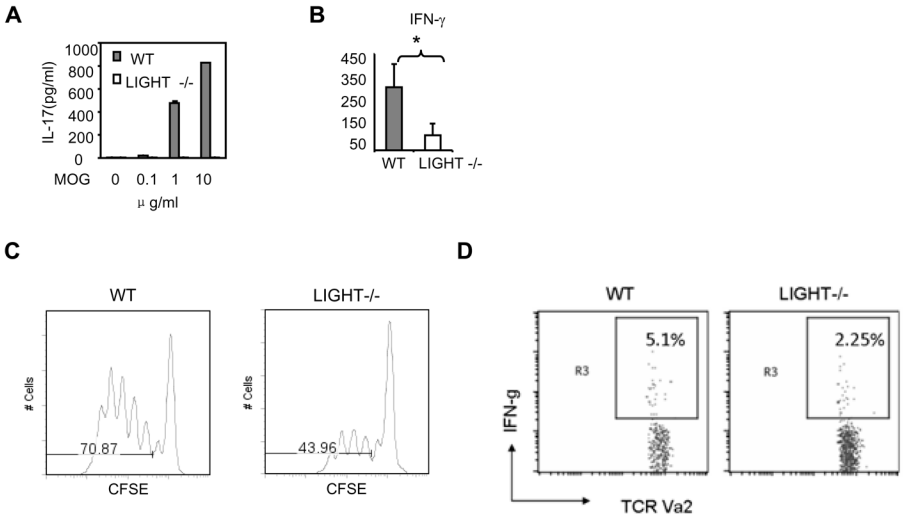

Figure 6. LIGHT-expression on non-T cells is essential for T cell response

A.B. WT and LIGHT-/- mice were immunized with 10 μg MOG/CFA i.d. in the tail base. 7 days post immunization inguinal DLN cells were re-stimulated with MOG peptide at indicated concentration for 5 days 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 10μg immunization i.d. at the tail base. 3 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 (10μg/ml) for 14 hrs. IFN-γ production was determined by intracellular staining. Representative of three experiments.