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The TNF Superfamily Molecule LIGHT Promotes the Generation of Circulating and Lung-Resident Memory CD8 T Cells following an Acute Respiratory Virus Infection

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The transition of effector T cells or memory precursors into distinct long-lived memory T cell subsets is not well understood. Although many molecules made by APCs can contribute to clonal expansion and effector cell differentiation, it is not clear if clonal contraction and memory development is passive or active. Using respiratory virus infection, we found that CD8 T cells that cannot express the TNF family molecule lymphotoxin-like, exhibits inducible expression, competes with HSV glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes (LIGHT) are unimpaired in their initial response and clonally expand to form effector cell pools. Thereafter, LIGHT-deficient CD8 T cells undergo strikingly enhanced clonal contraction with resultant compromised accumulation of both circulating and tissue-resident memory cells. LIGHT expression at the peak of the effector response regulates the balance of several pro- and antiapoptotic genes, including Akt, and has a preferential impact on the development of the peripheral memory population. These results underscore the importance of LIGHT activity in programming memory CD8 T cell development, and suggest that CD8 effector T cells can dictate their own fate into becoming memory cells by expressing LIGHT. ***The Journal of Immunology*****, 2018, 200: 000–000.**

Memory CD8$^+$ T cells are critical in limiting the successful establishment of secondary infections (1, 2). It is now known that remarkable diversity can exist within the memory T cell pool, which was initially divided only into CD62L$^+$CCR7$^+$ central memory T cells (T_{CM}) and CD62L$^-$CCR7$^-$ effector memory T cells (T_{EM}) (3). This led to the hypothesis that T_{EM} cells that patrol nonlymphoid tissue and rapidly produce effector cytokines act as a first line of defense, whereas T_{CM} cells, because of their ability to proliferate extensively, act as a second line of defense by generating secondary effector cells to bolster the ongoing response (4). However, subsequent studies have found that greater heterogeneity exists even within the T_{CM} and T_{EM} populations (5–7), and the markers CXCR3, CD27, and CD43 have been proposed to refine the functional characteristics of memory subsets (8–10). Adding to this growing complexity, resident memory T cells (T_{RM}) can become committed to permanent retention within the tissue to which they localize (11), and they have been suggested to enhance the recruitment of circulating memory T cells to the site of infection (12). More recently, CX3CR1 has also been used to divide effector CD8$^+$ T cells and their memory counterparts into three distinct subsets (CX3CR1^{neg}, CX3CR1^{int}, CX3CR1^{hi}) (13, 14), with CX3CR1^{int} cells (peripheral memory cells) considered to be responsible for patrolling nonlymphoid tissue and to possess the highest potential for self-renewal and differentiation (13).

Altogether, the effectiveness of protective immunity against pathogens provided by memory T cells likely depends on the extent of their generation, localization, and survival (7, 15). Following the resolution of acute infection, only a small fraction of activated (effector and memory-precursor) CD8$^+$ T cells present at the peak of the immune response survive as long-lived memory cells. This suggests it is crucial to understand the precise molecular signals that regulate the transition of effector CD8$^+$ T cells or activated memory-precursor CD8$^+$ T cells, into the diverse memory subsets that can accumulate and persist at high frequencies.

A number of costimulatory and coinhibitory molecules in the Ig superfamily (e.g., CD28, PD-1) or the TNF receptor superfamily (e.g., OX40 and CD27) have been described to control effector T cell accumulation and differentiation, along with cytokines such as IL-12 and type 1 interferons; and thus indirectly contribute to memory development, because often the size of the memory pool is

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Abbreviations used in this article: DC, dendritic cell; HVEM, herpes virus entry mediator; LIGHT, lymphotoxin-like, exhibits inducible expression, competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes; LTβR, lymphotoxin β-receptor; NP, nucleoprotein; rVacV-WR-OVA, VacV-WR expressing full-length OVA; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_{RM}, resident memory T cell; VacV-WR, recombinant vaccinia virus Western Reserve strain; WT, wild type.

a corresponding fraction of the effector pool (16, 17). Furthermore, it is well established that the cytokines IL-15 and IL-7 provide essential survival signals to already differentiated memory cells (18). However, the factors that dictate the conversion of effector cells or memory-precursor cells into long-lived memory populations are not well appreciated. Some have hypothesized that clonal contraction and memory development is a passive and random process brought about by withdrawal of the antigenic stimulus and is preprogrammed within the first few days of infection or Ag encounter (12, 17, 19). Additionally, the contribution of pro- versus antiapoptotic molecules signals through death receptors (e.g., Fas, TNFR1 or TRAILR) and autophagy to clonal contraction, and hence the number and type of subsequent memory cells generated, has been debated with no clear consensus (18, 20). Thus, it is not clear whether there are individual molecular interactions that are major contributors to the formation, divergence, or persistence of distinct memory subpopulations, at or after the peak of the effector response.

In this study, we show that the TNF superfamily molecule lymphotoxin-like, exhibits inducible expression, competes with HSV glycoprotein D for herpes virus entry mediator (HVEM), a receptor expressed by T lymphocytes (LIGHT) is one protein that may impart essential signals for memory $CD8^+$ T cell development. LIGHT (also known as TNFSF14) is an inducible molecule that can be expressed on the surface of activated/effector $CD8^+$ and $CD4^+$ T cells, as well as other immune cells such as dendritic cells (DCs), monocytes, and neutrophils (21, 22). It has two receptors, the HVEM and lymphotoxin β-receptor (LTβR), which are found on a wide variety of hematopoietic and nonhematopoietic cells (23). LIGHT is proinflammatory as seen in studies of knockout animals that show less severe disease in models of intestinal inflammation, graft versus host disease, transplantation, and asthma (24–27). LIGHT has also been shown to be important for resistance against parasitic infection of mice such as *Leishmania* and *Chlamydia* (28–30). However, the exact source, role, and target of LIGHT activity in many of these studies has not been well defined and is likely to be diverse.

In this report, we focused on the role of LIGHT, expressed specifically by $CD8^+$ T cells, in controlling memory development in response to respiratory virus infection. We show that $CD8^+$ T cell–derived LIGHT does not control the expansion of effector cells, but rather acts at and after the peak of the primary response to profoundly influence the development of resident and circulating memory $CD8^+$ T cells of all subtypes, and in particular $CD27^{hi}$ $CD43^{hi}CX3CR1^{int}$ cells. These studies suggest that by expressing LIGHT, $CD8^+$ T cells can dictate their own fate into becoming memory cells, and imply that LIGHT might be a valuable target for promoting long-lived $CD8^+$ T cell immunity.

Materials and Methods

Mice

Female 8–12 wk old C57BL/6 (CD45.2) and B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1/Ly5.1) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). $HVEM^{-/-}$ mice, wild type (WT), and $LIGHT^{-/-}$ OT-I transgenic mice were bred and maintained at the University of Florida animal facility. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Florida (Assurance # A3377-01; OLAW).

Viruses and infections

Recombinant vaccinia virus Western Reserve strain (VacV-WR) and virus expressing full-length OVA (rVacV-WR-OVA) were grown in HeLa cells and subsequently titered on VeroE6 cells as described previously (31). Recombinant influenza virus expressing SIINFEKL peptide (PR8-OTI) was generated as previously described (32). Mice were infected with 2×10^4 PFU of VacV-WR-OVA via the intranasal route in a volume of 10 μl. For challenge experiments, PR8-SIINFEKL was administered at a normally lethal dose of 2×10^7 PFU in 40 μl volume via the intranasal route.

$CD8^+$ T cell adoptive transfer

For adoptive transfer experiments, 5×10^4 naive WT or $HVEM^{-/-}$ OT-I CD8 T cells were purified from spleens with MACS technology (Miltenyi Biotec) and transferred into WT C57BL/6 mice via the i.v. route as described previously (31). Enriched cells were 99% specific $CD8^+$ TCR-transgenic T cells. Then, 1 d later mice were infected with rVacV-WR-OVA as above. OT-I expansion and effector formation were detected by flow staining of the transgenic TCR-α– and TCR-β–chains after gating on $CD8^+$ T cells. In some cases, CD8 T cells were labeled with CFSE (Molecular Probes) by incubating 10^7 purified cells per ml with 5 μM CFSE for 10 min at 37°C. Cells were then washed three times in HBSS containing 2.5% FCS.

Flow cytometry

Preparation of cells, extracellular/intracellular staining, data acquisition, and data analysis were performed as described previously (10, 33, 34). To label all cells in compartments contiguous with the vasculature, animals were injected (i.v) with anti-mouse CD45.2-APC eFluor (clone104; eBioscience), which was allowed to circulate for 3 min prior to sacrifice. Following sacrifice, single-cell preparations of lung tissue were stained with Abs against CD8, CD44, Vα2, Vβ5, CD69, and CD103α Abs. Lung DC and macrophage stains included Abs for CD11c, CD11b, MHC class II, CD103, and Siglec-F. Other innate cells such as NK cells, inflammatory monocytes, and neutrophils present in the lungs and mediastinal lymph nodes were identified by staining with CD3, CD11b, NKp46, NK1.1, Ly6C, and Ly6G. The mediastinal lymph node DC staining panel included B220, CD3, CD11b, CD11c, MHC class II, CD8, and CD4 (35).

Immunofluorescence studies

At various time points postinfection with VacV, spleens or lungs were harvested and immediately snap frozen in OCT on dry ice, and the procedure was performed as previously described (10, 31). The OCT-embedded 5 μm thick cryosections of spleen were cut using a Microm HM 505E cryostat and prepared on super frost glass slides for immunofluorescence microscopy. Each slide of cryosections was washed with 1 ml PBS and nonspecific binding was blocked by incubating sections with 2% horse serum for 30 min at 4°C. After blocking, the sections were washed with cold PBS and incubated overnight in the dark at 4°C with fluorophore-conjugated Abs as indicated in the figure legends. Sections were washed three times with PBS, mounted with Cytoseal, and covered with a glass coverslip. The stained sections were observed and analyzed at wavelength 488 nm for FITC (green), 543 nm for PE (red), and 647 nm for APC (purple) labeling, using an EVOS *fl* (Advanced Microscopy Group inverted immunofluorescence microscope), and images were captured by 4×, 10×, 20×, and 40× objectives, keeping all the conditions of the microscope and settings of the software identical for all treatments and controls.

Gene array

Lungs from WT OT-I and $LIGHT^{-/-}$ OT-I recipient mice were harvested and $CD27^{hi}CD43^{hi}$ effector CD8 T cell subset was sort purified by FACS. Total lung RNA from this subset was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions (10, 34, 36). Total RNA was subsequently treated with DNase I (Qiagen) and further purified using an RNeasy Mini Kit (Qiagen). A total of 1 mg high-quality total RNA (RNA integrity number = 0.7) was then reverse transcribed using the First Strand Synthesis Kit (Qiagen) and subsequently loaded onto a mouse apoptotic RT2 profiler array according to the manufacturer's instructions (Qiagen). Qiagen's online Web analysis tool was used to produce comparative heat maps, and fold-change was calculated by determining the ratio of mRNA levels to control values using the D threshold cycle method (22DD threshold cycle). All data were normalized to an average of five housekeeping genes, Gusb, Hprt, Hsp90ab1, Gapdh, and Actb. The PCR conditions used were hold for 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 60 s at 60°C.

Statistical analysis

Tests were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA). Statistics were carried out using two-tailed, unpaired Student *t* test with 95% confidence intervals unless otherwise indicated. Unless otherwise indicated, data represent the mean ± SEM, with $*p < 0.05$ and $**p < 0.01$ considered statistically significant.

Results

LIGHT expressed by CD8 T cells regulates clonal contraction and memory generation

We investigated the role of LIGHT expressed by CD8 T cells during an antiviral response using T cells lacking this molecule. A small number of WT and LIGHT-deficient naive OT-I $CD8^+CD44^{lo}$ T cells expressing Vα2Vβ5 and specific for $H\text{-}2K^b/OVA_{257–264}$ were adoptively transferred into naive WT C57BL/6 mice. Recipient mice were infected intranasally the following day with rVacV-WR-OVA. $CD8^+$ T cells were then analyzed in the lungs and spleen on days 6 and 8, day 15, and day 40 postinfection, representing the acute expansion phase, the contraction phase, and the maintenance and memory phases of the primary response, respectively. Based on accumulation of the transferred cells and production of IFN-γ, the generation of the primary effector pool was found to be normal on day 6 even when the transferred CD8 T cells did not express LIGHT. A moderate decrease in numbers was seen by day 8 (Fig. 1A–D). However, this defect became more pronounced during the contraction phase of the response with ∼60% of $LIGHT^{-/-}$ $CD8^+$ T cells by day 15, followed by a greater loss of $LIGHT^{-/-}$ CD8 T cells (∼90%) by day 40, the time when memory generation to this virus is complete in the lungs (Fig. 1A–D). Similar results were also observed in the spleen (Supplemental Fig. 1). To control for the potential that adoptive transfer of OT-I $CD8^+$ T cells could alter the endogenous response to viral infection, we monitored the immunodominant $CD8^+$ T cell population specific for VacV (B8R) and observed the development of normal primary and memory responses in the lungs and spleen (Supplemental Fig. 2). These data suggested that LIGHT expression by Ag-specific $CD8^+$ T cells is critical for generating both effector memory (lung) and central memory (spleen) cells.

To determine whether LIGHT affects the T_{RM} $CD8^+$ T cell subset, we intravenously injected WT and $LIGHT^{-/-}$ $CD8^+$ T cell recipients with fluorophore-conjugated anti-CD45.2 Ab prior to sacrifice to distinguish between circulating memory cells ($CD45.2^+$; vascular) from lung-resident memory cells ($CD45.2^-$; parenchyma, airway). We observed reduced frequencies of both subsets including lower numbers of $CD103^+CD69^+$ T_{RM} cells in recipients that received $LIGHT^{-/-}$ $CD8^+$ T cells (Fig. 1E–G). However, the proportion of T_{RM} subsets was normal in these fractions (Fig. 1F), implying that LIGHT does not control T_{RM} differentiation but regulates the generation or persistence of T_{RM} cells. Overall, our data suggest that $CD8^+$ T cell–derived LIGHT plays a minor role in effector $CD8^+$ T cell expansion but is critical for the generation of large numbers of circulating and tissue-resident memory $CD8^+$ T cells and its primary activity is displayed from the peak of the effector response throughout the phase when $CD8^+$ T cells contract in numbers.

Immunohistological analyses showed that $LIGHT^{-/-}$ $CD8^+$ T cells localized normally within the lung tissue, similar to WT $CD8^+$ T cells at the peak of the response (day 8). Both WT and $LIGHT^{-/-}$ CD8 T cells were found near the parenchyma as well as in contact with the airway epithelium in the lung tissue (Fig. 1H). Also, no significant change was observed in the localization of WT or $LIGHT^{-/-}$ $CD8^+$ T cells in the spleen. $LIGHT^{-/-}$ $CD8^+$ T cells were observed both in the red pulp as well as the white pulp and bridging channel, similar to WT $CD8^+$ T cells (Supplemental Fig. 3A). At day 40, WT $CD8^+$ T cells were found at multiple sites within the lung tissue, such as close to the airway epithelium, in the airways, in the parenchyma away from the epithelium, as well as near the blood vessels (Fig. 1I). In striking contrast, very few $LIGHT^{-/-}$ $CD8^+$ T cells ($CD45.2^+$) were detected at day 40, whereas endogenous LIGHT-sufficient CD8 T cells (purple only) were detected in these lung samples (Fig. 1J). Similar results were also observed in the spleen where the transferred WT $CD8^+$ T cells were detected in the marginal zone, the red pulp, and the periarteriolar lymphoid sheath area, but $LIGHT^{-/-}$ $CD8^+$ T cells were very few in number (Supplemental Fig. 3B). These data validated the flow cytometry results. Thus, LIGHT deficiency does not impact the tissue-specific localization of $CD8^+$ T cells but seems critical for their long-term tissue persistence.

Intact early activation, proliferation, differentiation, and Ag-independent homeostatic turnover in the absence of LIGHT

As there was a small defect observed in the accumulation of LIGHT-deficient $CD8^+$ T cells in the lung tissue at day 8 postinfection, we asked if this was due to a defect in early activation, early proliferation, or differentiation. We observed no change in the expression of either CD25 or CD69 when LIGHT was not expressed by $CD8^+$ T cells, both in the lungs and the draining lymph nodes at day 6 postinfection (Fig. 2A). There were also no differences in the early (day 6) and later (day 8) proliferation, with all T cells being Ki67 positive at these time points (Fig. 2B). We then examined the expression of IFN-γ, TNF, and CD107α in response to SIINFEKL peptide stimulation to determine whether LIGHT expression imparts changes in T cell polyfunctionality (Fig. 2C). Again, no major differences in effector function were observed in the absence of LIGHT except for a small defect in IFN-γ production by $LIGHT^{-/-}$ $CD8^+$ T cells. Similarly, at day 8, the LIGHT deficiency did not significantly alter the expression of the transcription factors T-bet, Eomes, Blimp-1 or pSTAT5, which are known to play critical roles in $CD8^+$ T cell differentiation (Fig. 2D). Moreover, WT and $LIGHT^{-/-}$ $CD8^+$ T cells upregulated their expression of the lung trafficking molecule CXCR3 and downregulated their expression of CD62L and CD127 to the same extent (Fig. 2E). Overall, this shows that LIGHT does not play a major role in regulating early activation, proliferation or differentiation of effector $CD8^+$ T cells responding to virus-expressed Ag.

Impact of LIGHT deficiency on effector and memory CD8 T cell subsets

We next asked whether deficiency of LIGHT expression might affect the types of $CD8^+$ T cell subsets generated in response to infection (37). Using CD27 and CD43 expression, memory CD8 T cells can be segregated into four subsets (7). The $CD27^{hi}$ subset of memory cells is known to proliferate to a greater extent compared with $CD27^{lo}$ cells when reactivated (8–10). In contrast, $CD27^{lo}$ cells were shown to localize at the pathogen entry site within the secondary lymphoid organs and, hence, were suggested to confer better protection against blood-borne pathogens (9). In contrast, we have recently shown that $CD27^{hi}$ cells provide optimal protection against lethal respiratory VacV infection due to their localization near airway epithelium as well as their flexibility in differentiation (10). During the peak of the primary $CD8^+$ T cell response, we found that both WT OT-I $CD8^+$ T cells and $LIGHT^{-/-}$ OT-I $CD8^+$ T cells were equivalently able to differentiate into $CD27^{hi}$ and $CD27^{lo}$ effector cells in the lungs (Fig. 3A). However, by day 40 postinfection, the percentages of the $CD43^{hi}$ subsets (particularly $CD27^{hi}CD43^{hi}$ cells) were reduced in the absence of LIGHT (Fig. 3A). Although all $LIGHT^{-/-}$ $CD8^+$ memory T cell subsets were reduced by ∼10–15 fold at this time point compared with their WT counterparts, there was greater than a 30-fold reduction in the $CD27^{hi}CD43^{hi}$ subset (Fig. 3B). Similar results were found when analyzing the spleen (Supplemental Fig. 4). Thus, although LIGHT expression by $CD8^+$ T cells is required for

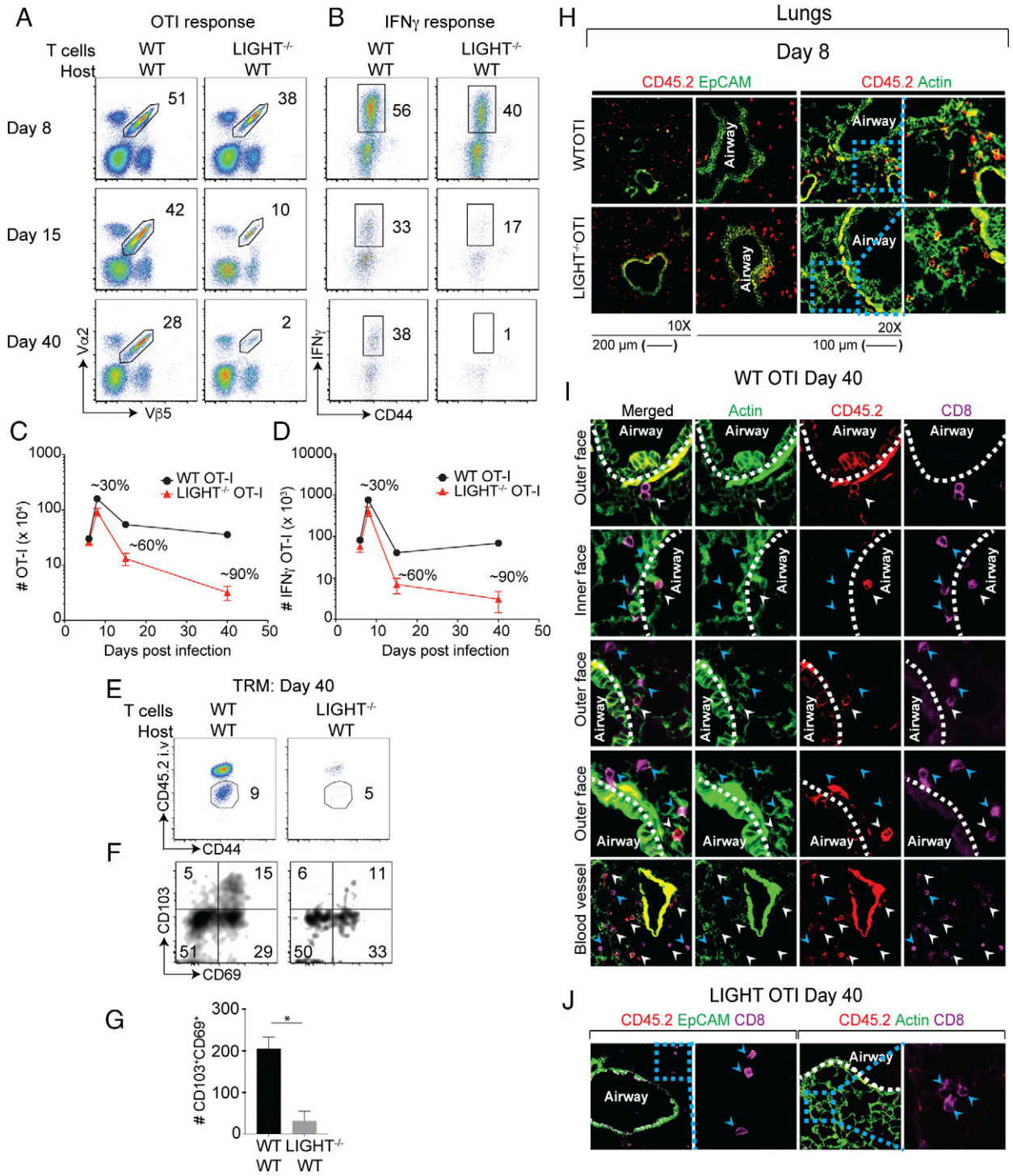

FIGURE 1. LIGHT expressed by CD8 T cells regulates formation of memory cells. Equal numbers (5×10^4) of WT and LIGHT$^{-/-}$ naive (CD44^{lo}) OT-I (Vα2$^+$Vβ5$^+$) transgenic CD8 T cells were adoptively transferred into BL/6 mice and infected with rVacV-WR-OVA [2×10^4 PFU intranasally (i.n.)] the following day. (**A**–**D**) Lungs were harvested at days 6, 8, 15, and 40 postinfection and stained for (A) CD8, CD44, Vα2, and Vβ5, and frequencies of OT-I CD8 T cells determined. (C) OT-I cells from lungs of recipients were restimulated in vitro with OVA peptide and stained intracellularly with IFN-γ. Absolute numbers of (C) OT-I cells and (D) IFN-γ+ OT-I cells from lungs were quantified postinfection. (**E**–**G**) At day 40 postinfection, recipient mice were injected with anti-CD45.2 Ab intravenously, 3 min before euthanizing. (**H**–**J**) Equal numbers (5×10^5) of WT and LIGHT$^{-/-}$ naive (CD44^{lo}) OT-I (Vα2$^+$ Vβ5$^+$) transgenic CD45.2$^+$CD8 T cells were adoptively transferred into CD45.1$^+$SJL mice and infected with rVacV-WR-OVA (2×10^4 PFU i.n.) the following day. Immunofluorescence analysis of frozen (H) lung sections from recipient mice stained for CD45.2 (red) and CD8 (magenta) at day 8 postinfection. Similar analysis for was done at day 40 postinfection in (I) and (J) the lungs. ActinGreen or EpCAM (green) were used to visualize lung morphology. Scale bars, 200 and 100 μm respectively. (H) Zoomed images in column four, ×20 of images in the other columns. (I) Original magnification ×100. (J) Columns one and three, original magnification ×20; zoomed images in columns two and four, ×20 of images in columns one and three. Similar results were obtained in two independent experiments. Representative plots of CD45.2 and CD44 were pregated on CD8$^+$OT-I (Vα2$^+$Vβ5$^+$) cells. The gated CD45.2 negative cells were analyzed for the expression of CD103 and CD69 using flow cytometry and total numbers of cells calculated in lungs. Similar results were obtained in four independent experiments. Results are mean ± SEM (n = 3 mice per group). *$p < 0.05$.

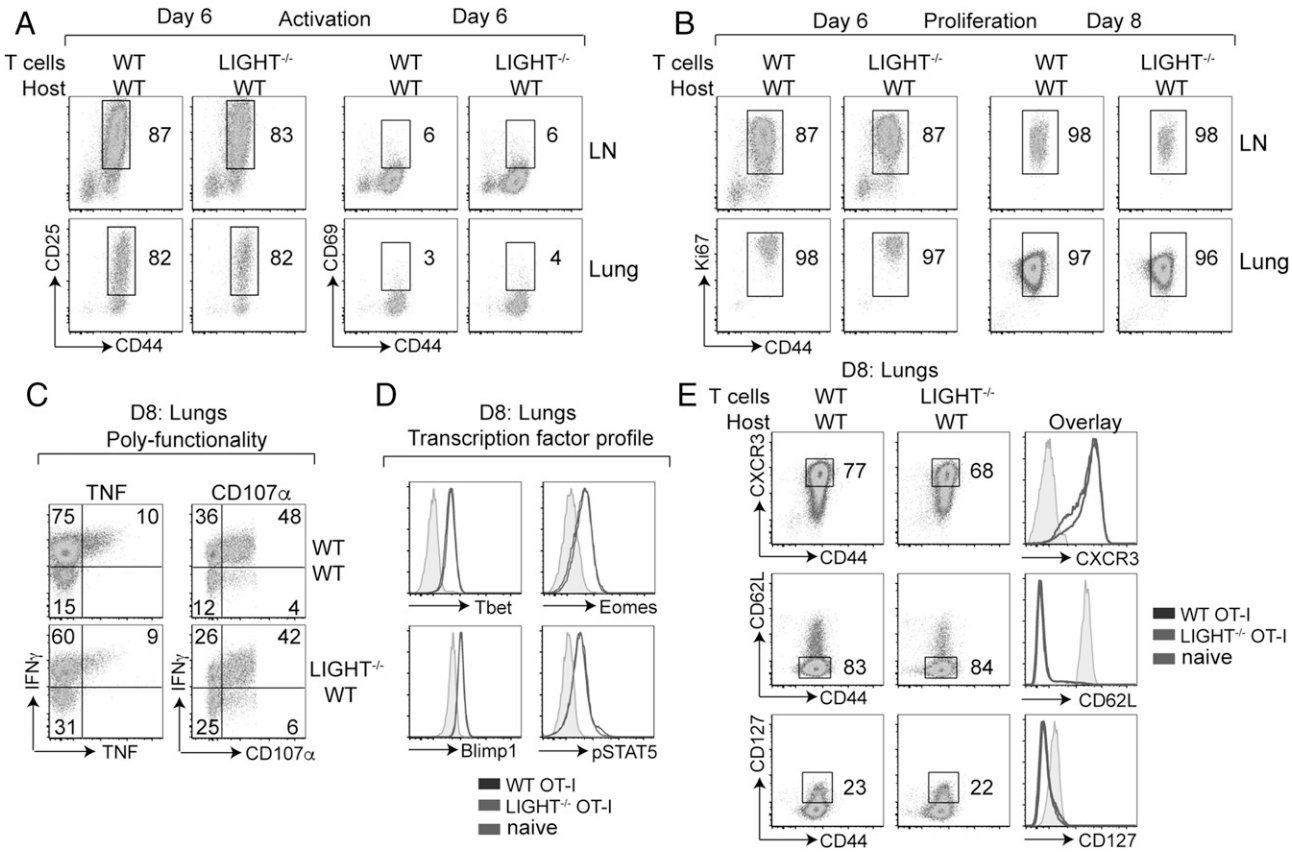

FIGURE 2. Intact early activation, proliferation, polyfunctionality, and differentiation of CD8 T cells in the absence of LIGHT. Mice that received WT or LIGHT$^{-/-}$ CD8 T cells in Fig. 1 were analyzed on day 6 and 8 postinfection. (**A**–**E**) Lung cells were stained extracellularly with Abs to CD8, Vα2, Vβ5, CD44, CD25, CD69, CXCR3, CD62L, IL7Rα (CD127); (B) intranuclearly with Ki67; (C) intracellularly with Abs to TNF, IFN-γ following restimulation with SIINFEKL peptide during which CD107α was added; and (D) intranuclearly with Abs to *t*-bet, eomes, blimp1, pStat5, without peptide restimulation.

the generation of all memory subsets, it has a greater impact on the development of the CD27^{hi}CD43^{hi} memory subset, which has been previously been implicated in providing optimal protection against lethal respiratory virus infection (10).

Effector CD8$^+$ T cells have been characterized based on differential expression of the fractalkine receptor CX3CR1 (13, 14). CX3CR1^{hi} cells are the most terminally differentiated whereas CX3CR1^{neg} cells are the least differentiated. Effector cells with intermediate expression of CX3CR1 have distinct surveillance properties in nonlymphoid tissue exhibiting the highest potential for self-renewal and differentiation (13). At the peak of the effector response, we found that CD8$^+$ T cells regardless of being WT or LIGHT-deficient, formed CXCR3$^+$CX3CR1^{neg}, CXCR3$^+$CX3CR1^{int}, and CXCR3$^-$CX3CR1^{hi} populations, but there was a significant reduction in the CXCR3$^+$CX3CR1^{neg} population in the absence of LIGHT (Fig. 3C). Similar results were also observed when CXCR3 was replaced by CD27 (Supplemental Fig. 4C). These data show that the small defect in accumulation of CD8$^+$ T cells in the lungs at the peak of the effector response (Fig. 1A–D) was mainly confined to reduced numbers of the less-differentiated CX3CR1^{neg} cells. The CD27^{hi}CD43^{hi} cells, which were most depleted at late time points, both in the lungs and the spleen of LIGHT$^{-/-}$ CD8$^+$ T cells, consisted of both CX3CR1^{int} and CX3CR1^{neg} cells, but not CX3CR1^{hi} cells (Fig. 3A, 3B, Supplemental Fig. 4). Interestingly, by day 30 postinfection, there was a much greater reduction (10-fold) in the CX3CR1^{int} cell population compared with the CX3CR^{neg} and CX3CR1^{hi} subsets (5–6-fold) in LIGHT$^{-/-}$ CD8$^+$ T cells compared with their WT counterparts (Fig. 3D). Overall, these data show that, initially (at the peak of the CD8 T cell response), LIGHT plays an important role in the accumulation of CXCR3$^+$CX3CR1^{neg} effector CD8$^+$ T cells in the lungs, and later (following contraction), LIGHT is important for all CD8$^+$ memory T cell subsets.

Intact homeostatic proliferation in absence of LIGHT

Although we found no difference in proliferation of LIGHT-deficient effector CD8$^+$ T cells at day 8, we observed a strong reduction in the proportion of highly proliferating LIGHT$^{-/-}$ CD8$^+$ T cells at day 15, as assessed by the intensity of Ki67 staining (Fig. 4A, top). There was also a significant reduction in the total numbers of both Ki67^{hi} and Ki67^{lo} LIGHT$^{-/-}$ CD8$^+$ T cells at this time point (Fig. 4A, bottom). To test whether the LIGHT deficiency led to impaired homeostatic turnover in an Ag-independent manner, we adoptively transferred T cells into RAG-deficient mice, which have surplus endogenous IL-7 and IL-15, enabling cells to undergo proliferation without the need for an antigenic stimulus. Based on CFSE dilution, WT and LIGHT-deficient OT-I CD8$^+$ T cells underwent similar homeostatic proliferation in the absence of Ag over the initial 4 d (Fig. 4B). Moreover, the accumulation of the transferred CD8$^+$ T cells and the percentages of proliferating cells (based on a high intensity of Ki67 staining) were similar in the RAG$^{-/-}$ hosts at all time points tested over 40 d (Fig. 4C, 4D). Therefore, enhanced contraction of LIGHT$^{-/-}$ CD8$^+$ T cells (Fig. 1A–D) was not due to their impaired ability to undergo Ag-independent homeostatic proliferation. Rather, it was likely due to a greater propensity to undergo

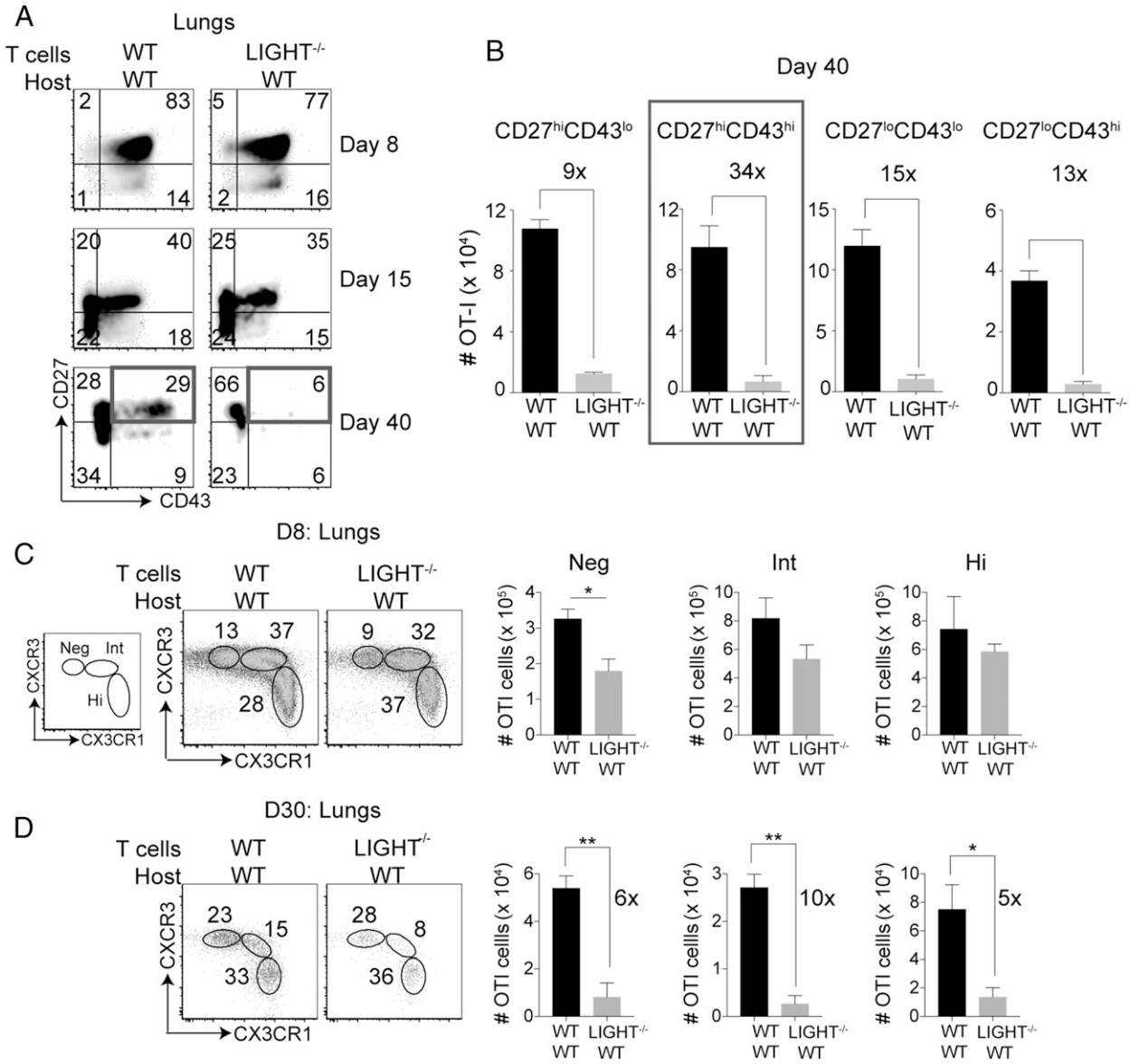

FIGURE 3. Differential impact of a LIGHT deficiency on memory CD8 T cell subsets. (**A**) Mice that received WT or LIGHT$^{-/-}$ CD8 T cells as in Fig. 1 were analyzed on day 8, 15, and 40 postinfection. Lung cells were stained with extracellular Abs for CD8, Vα2, Vβ5, CD44, CD27, CD43. (**B**) Absolute cell numbers of CD27 versus CD43 subsets at day 40 postinfection were quantified. Similar results were obtained in three independent experiments and results are the mean ± SEM ($n = 3$ mice per group). (**C** and **D**) Lungs of WT or LIGHT$^{-/-}$ CD8 T cell recipient mice were harvested at (C) day 8 and (D) day 30 postinfection, and stained for CD8, Vα2, Vβ5, CD44, CXCR3, and CX3CR1 and corresponding cell numbers of subsets were quantified. Single experiment with four mice in a group and results are mean ± SEM. Statistical significance was performed using Student *t* test. $*p > 0.05$, $**p > 0.005$.

apoptosis during the contraction phase of the response and, hence, related to a defect in their capacity to survive, during and following the peak of the primary effector response to viral infection.

LIGHT regulates expression of pro- and antiapoptotic factors

We then asked whether LIGHT deficiency affects the balance of pro- versus antiapoptotic molecules in the virus-responding CD8$^+$ T cells. Because the most severe defect was in the CD27^{hi}CD43^{hi} memory subset, we isolated cells based on these markers at day 8 (the peak of the effector response), a time point when the initial loss was observed in LIGHT$^{-/-}$ CD8$^+$ T cells (Fig. 5A). Comparing fold-change between LIGHT$^{-/-}$ CD8$^+$ T subset and WT CD8$^+$ T cell subset, we found that the expression of the majority of genes associated with apoptosis were not changed more than 2-fold (Fig. 5B). However, three genes that promote apoptosis were highly upregulated in LIGHT-deficient subset, namely Caspase-2, Bid, and Bad. Correspondingly, four genes that enhance survival were downregulated: Akt1, Atf5, Igfr1, and Nol3 (Fig. 5B). In particular, Caspase-2 was upregulated ~60-fold whereas Akt1 gene expression was reduced ~30-fold (Fig. 5C). Thus, LIGHT expression regulates the balance of pro- and antiapoptotic genes in CD8$^+$ T cells and, thereby, provides an essential survival signal allowing a proportion of effector CD8$^+$ T cells to transition into long-lived memory cells.

HVEM as a ligand for CD8 T cell–expressed LIGHT

We next asked which LIGHT receptor (HVEM or LTβR) was required to generate memory cells. Mice that lack LTβR are devoid of lymph nodes and have other abnormalities associated with LTβR expression on stromal/tissue structural cells (38), hence we focused on HVEM. WT OT-I CD8$^+$ T cells were adoptively transferred into either naive WT or HVEM$^{-/-}$ recipient mice and their response compared with LIGHT$^{-/-}$ OT-I CD8$^+$ T cells

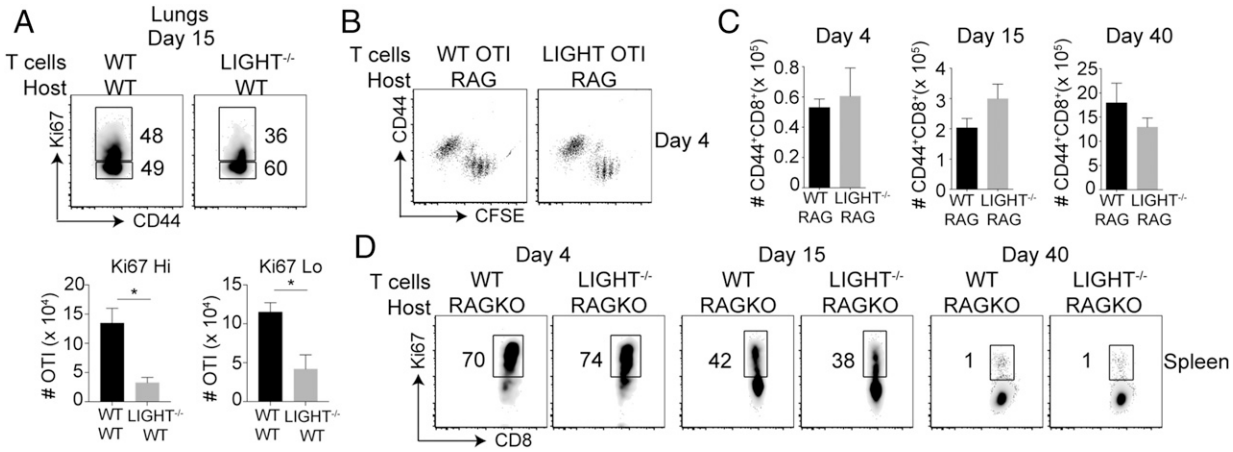

FIGURE 4. Intact homeostatic proliferation in the absence of LIGHT. (**A**) Lung cells from mice that received WT or LIGHT$^{-/-}$ CD8 T cells were analyzed on day 15 postinfection with VacV as in Fig. 1. After staining cells for CD8, Vα2, Vβ5, and CD44, cells were stained intranuclearly with Ki67. (**B**–**D**) Equal numbers (5×10^5) of WT and LIGHT$^{-/-}$ naive (CD44^{lo}) OT-I (Vα2$^+$Vβ5$^+$) transgenic CD8 T cells were CFSE labeled and adoptively transferred into RAG-deficient mice without infection. Spleens were harvested from recipient mice at day 4, 15, and 40 postinfection, and cells were stained extracellularly for CD8, Vα2, Vβ5, CD44, and intranuclearly with Ki67. Similar results were obtained in two independent experiments and results are mean ± SEM (n = 3 mice per group). *$p < 0.05$.

transferred into WT recipients. Strikingly, HVEM$^{-/-}$ hosts receiving WT CD8$^+$ T cells exhibited a severe defect in the percentages of memory CD8$^+$ T cells, identical to the defect observed in WT recipients that received LIGHT$^{-/-}$ CD8$^+$ T cells (Fig. 6A). This defect was also reflected in the total memory CD8$^+$ T cell numbers, both in the lungs and the spleen (Fig. 6B). This suggests that HVEM on endogenous cells plays an important role as a ligand for LIGHT expressed by CD8$^+$ T cells. However, whether along with HVEM, LTβR is also involved in imparting signals to LIGHT on CD8$^+$ T cells cannot be completely excluded. Nevertheless, HVEM seems to play a nonredundant role as a ligand because even though LIGHT-LTβR interactions are presumably intact in HVEM$^{-/-}$ mice, memory CD8 T cell development in the lungs is severely compromised.

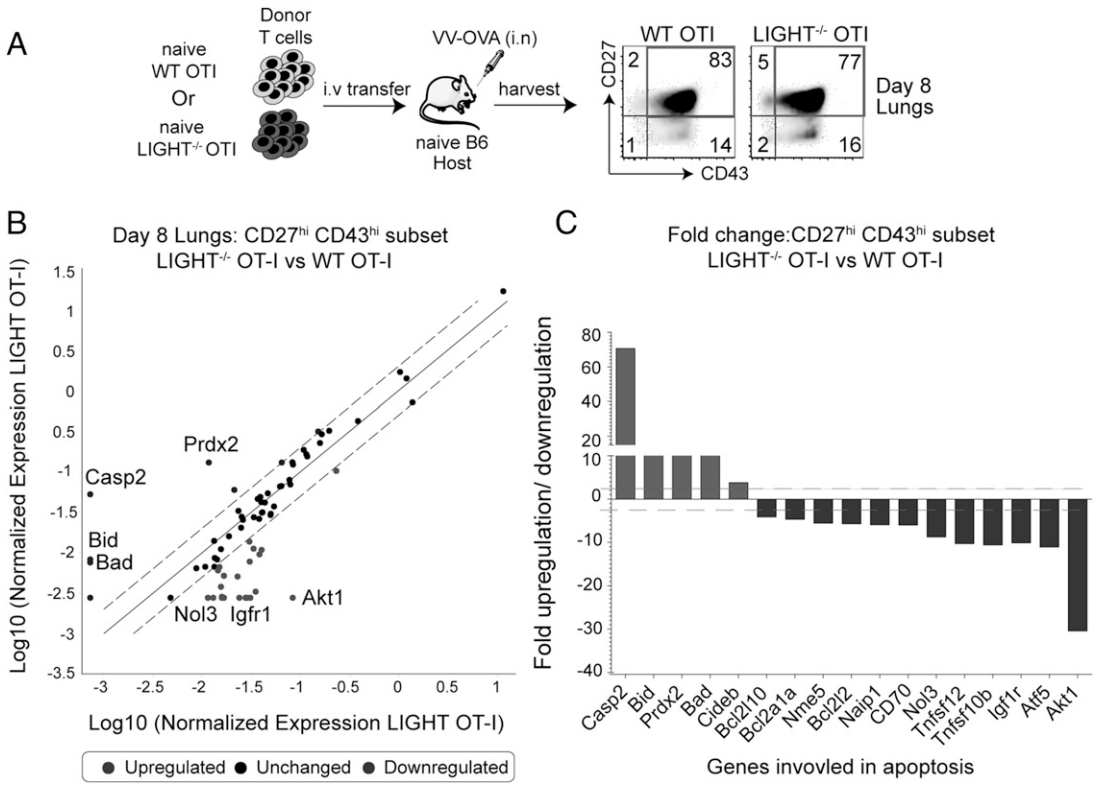

FIGURE 5. LIGHT regulates the survival of effector CD8 T cells. (**A**) Lungs from mice that received WT or LIGHT$^{-/-}$ CD8 T cells were harvested at day 8 postinfection with VacV and stained for CD8, Vα2, Vβ5, CD44, CD27, and CD43. The CD27^{hi}CD43^{hi} subset was sort purified and total mRNA was isolated. (**B**) Transcript levels of apoptotic genes were measured using Affymetrix mouse apoptotic gene arrays and presented as a scatter plot with dotted lines representing 2-fold differences between the two groups. (**C**) Bars represent fold change in transcript levels of the CD27^{hi}CD43^{hi} subset between LIGHT-deficient CD8 T cells and WT CD8 T cells. Inset, Lung cells from WT and LIGHT$^{-/-}$ OT-I recipient mice were stained intranuclearly with pAKT.

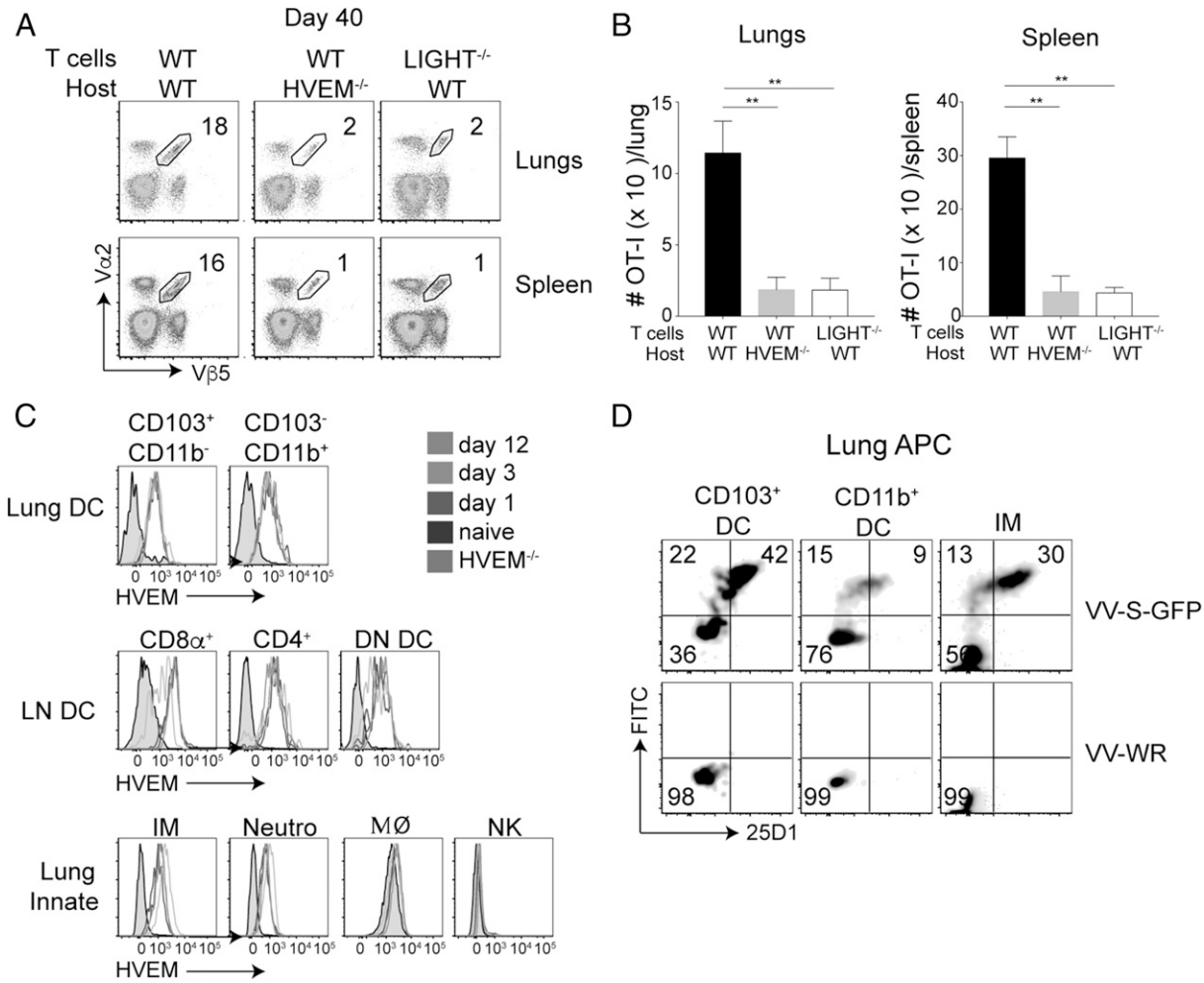

FIGURE 6. Endogenous HVEM acts as a ligand for LIGHT made by CD8 T cells. (**A** and **B**) Equal numbers (5×10^4) of WT CD8 T cells were adoptively transferred into naive WT and HVEM$^{-/-}$ mice, and compared with LIGHT$^{-/-}$ CD8 T cells transferred into WT mice. All recipient mice were infected with rVacV-WR-OVA (2×10^4 PFU intranasally) the following day. At day 40 postinfection, lung and spleen cells were stained for CD8, Vα2, Vβ5, and CD44 and analyzed by flow cytometry. Similar results were obtained in two independent experiments and results are the mean ± SEM ($n = 3$ mice per group). $^{**}p < 0.01$. (**C**) Lungs and mediastinal lymph nodes from WT CD8 T cell recipient mice were harvested on day 0 (naive), 1, 3, and 12 postinfection. DCs and various innate cells were stained for HVEM. Gray histogram represents cells from HVEM$^{-/-}$ mice. (**D**) Lung cells were incubated in vitro with either recombinant vaccinia virus–SIINFEKL–OVA–GFP or rVacV-WR at a multiplicity of infection of 1. Then 6 h postincubation, cells were stained with Abs specific for lung DC subsets and inflammatory monocytes and 25D1 that recognizes SIINFEKL–MHC class I complexes.

We then assessed the expression of HVEM in the lung and draining lymph nodes during the response to intranasal VacV infection. We found that HVEM was highly expressed on both CD103$^+$ and CD11b$^+$ DCs (CD11c$^+$ MHC class II$^+$) within the lungs and all three DC subsets (CD8α$^+$DCs, CD4$^+$DCs, and CD8α$^-$CD4$^-$ double-negative DCs) within the mediastinal lymph nodes (Fig. 6C). HVEM was also highly expressed on inflammatory monocytes (Ly6C^{hi}CD11b$^+$), and neutrophils (Ly6G^{hi}CD11b$^+$), but not on macrophages (SiglecF^{hi}CD11b$^-$) or NK cells (NKp46$^+$CD11b^{int}NK1.1$^+$) (Fig. 6C). This suggests that multiple cell types in the lung or lymph nodes have the potential to engage CD8$^+$ T cell–expressing LIGHT via HVEM. However, as we did not observe a role for LIGHT in homeostatic Ag-independent responses (Fig. 4), we hypothesized that HVEM expression would be most relevant on APCs.

To understand the potential for lung APCs to present virus-derived antigenic peptides, mouse lung APCs were infected in vitro with recombinant VacV expressing GFP and the SIINFEKL peptide of OVA. GFP fluorescence and staining with the 25D1 Ab to detect SIINFEKL peptide-MHC complexes showed that CD103$^+$ DCs and inflammatory monocytes were most susceptible to infection and capable of presenting the antigenic peptide (Fig. 6D). Overall, these results suggest that HVEM expressed on endogenous innate immune cells, most likely CD103$^+$ DCs or inflammatory monocytes, provides a *trans*-activating signal when it engages LIGHT on CD8$^+$ T cells to directly or indirectly alters the balance of pro- versus antiapoptotic factors and to allow for the survival and development of long-lived memory cells.

LIGHT is important for the generation of memory CD8 T cells in response to respiratory influenza virus infection

Finally, we asked if the requirement for LIGHT expressed by CD8$^+$ T cells was only restricted to VacV infection or if LIGHT was essential for memory to other respiratory viruses. Experiments were carried out as described above with one exception: recipient mice were infected with a recombinant influenza virus PR8 expressing the OVA peptide SIINFEKL, genetically unrelated positive-sense RNA virus. Transfer of LIGHT$^{-/-}$ OT-I CD8$^+$ T cells into WT recipients again showed a severe defect in memory cell development at day 40 in the lungs, compared with

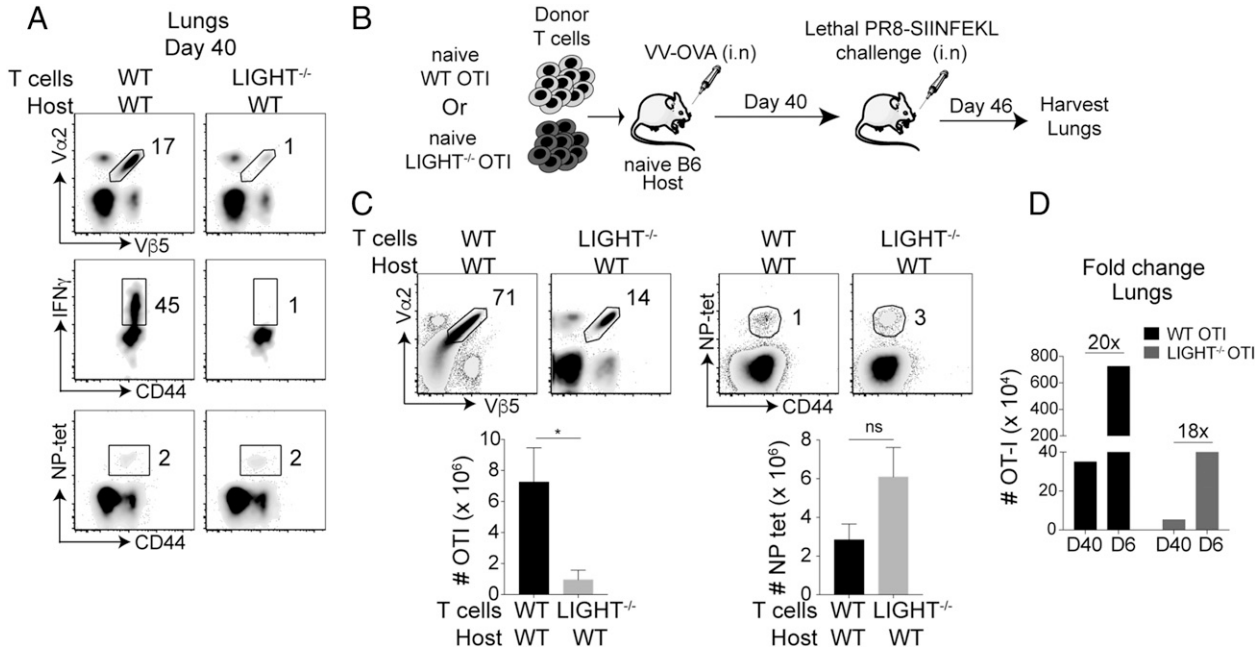

FIGURE 7. LIGHT controls memory and recall responses to influenza virus infection. (**A**) Equal numbers (5×10^4) of WT and LIGHT$^{-/-}$ naive (CD44^{lo}) OT-I (Vα2$^+$Vβ5$^+$) transgenic CD8 T cells were adoptively transferred into WT mice and infected with PR8-SIINFEKL (intranasally, i.n.) the following day. Lungs were harvested at day 40 postinfection and stained for CD8, CD44, Vα2, Vβ5, and NP-tetramer (top and bottom). OT-I cells from lungs of recipients were restimulated in vitro with OVA peptide and stained intracellularly with IFN-γ (middle). (**B** and **C**) WT and LIGHT$^{-/-}$ CD8 T cell recipient–mice that were previously infected with rVacV-WR-OVA (i.n.) were reinfected with PR8-SIINFEKL (i.n.) after 40 d from primary infection. Lungs were harvested at day 6 and stained for CD8, CD44, Vα2, Vβ5, and NP-tetramer. (**D**) Numbers and fold change in the cell numbers between WT CD8 T cells and LIGHT$^{-/-}$ CD8 T cells from day 40 postprimary infection to day 6 postreinfection was quantified. Similar results were obtained in two independent experiments and results are mean ± SEM (n = 3 mice per group). *$p < 0.05$.

their WT counterparts (Fig. 7A). A control for viral reactivity also showed that endogenous CD8$^+$ T cell memory to viral nucleoprotein (NP) was unaffected in the recipients of LIGHT$^{-/-}$ CD8$^+$ T cells (Fig. 7A). Thus, LIGHT expressed by CD8$^+$ T cells plays a crucial role in generating CD8$^+$ T cell memory to two genetically distinct respiratory viruses.

To further expand on this, we assessed the response to secondary influenza virus challenge. Recipients of WT or LIGHT$^{-/-}$ OT-I CD8$^+$ T cells previously infected with rVacV-OVA were reinfected with PR8-SIINFEKL after 40 d (Fig. 7B). At day 6 post-PR8 infection, there was a dramatic defect in the accumulation of LIGHT$^{-/-}$ CD8 T cells in the lungs compared with WT T cells (Fig. 7C). As a control for virus reactivity no change was observed in the response of endogenous LIGHT-sufficient NP-reactive cells (Fig. 7C). Also, during this time period, no gross difference was observed in the fold expansion of LIGHT$^{-/-}$ memory CD8$^+$ T cells compared with WT memory CD8$^+$ T cells (Fig. 7D), even though the total numbers of transferred CD8 T cells that accumulated in both recipients at day 46 were different. This shows the defect in the secondary response was due to the low memory CD8$^+$ T cell precursor frequency at the time of virus rechallenge. Collectively, our data indicate that LIGHT does not participate in the expansion phase of the primary or secondary responses to respiratory viruses, but plays a critical role in determining the frequency and type of memory CD8$^+$ T cells that are generated and survive the phase of clonal contraction.

Discussion

The development of pulmonary memory CD8$^+$ T cells is a vital component of immunity to respiratory viruses (8, 10, 33, 39–41). Therefore, understanding the molecular signals necessary for the generation of long-lived memory CD8$^+$ T cells in the lungs is of potential importance to the design of effective immunization strategies against respiratory viruses. In this study, our work identified a crucial role for LIGHT, a TNF family molecule expressed by activated CD8$^+$ T cells, in regulating the development of memory CD8$^+$ T cell subsets in the lungs. LIGHT$^{-/-}$ CD8$^+$ T cells exhibited no gross defects in their activation, proliferation, functionality or trafficking to the lungs in response to respiratory virus infection, however, a very low number of LIGHT$^{-/-}$ CD8$^+$ T cells survived after the peak of the effector response resulting in a severely compromised memory compartment. Although LIGHT regulated all memory subsets, it had a particularly strong impact on the accumulation of cells with the phenotype CD27^{hi}CD43^{hi}CX3CR1^{int}, which comprise the recently designated peripheral memory pool (13). Importantly, this phenomenon was not restricted to a single virus infection and we showed the crucial role of LIGHT with two genetically distinct viruses, suggesting that LIGHT activity might be a common feature for development of strong cellular immunity to many respiratory viral infections.

Over the years, a number of TNF family molecules have been shown to participate in responses to viruses, and the principal model emerging from these studies proposes that the molecules act together (concurrent use) and in a temporal manner (kinetic use) to drive and sustain clonal expansion in both primary and secondary T cell responses allowing for the development of large populations of effector CD8$^+$ and CD4$^+$ T cells (42). In this regard, previous work has highlighted roles of several TNF family interactions such as CD27/CD70, OX40/OX40L, 4-1BB/4-1BBL, and GITR/GITRL during systemic infection with lymphocytic choriomeningitis virus, murine CMV, or VacV, and intranasal infection with influenza virus (15, 16, 43). The general consensus is that these molecules act predominantly during the early phase of the antiviral T cell response, as effector cells begin to accumulate in the draining lymph nodes and infected tissue, because the lack of

these molecules often causes a significant defect in accumulation of high numbers of T cells at the peak of the primary or secondary response. In many infection models, this early defect has also been accompanied by a parallel effect on the size of the subsequent memory cell pool. However, none of the molecules studied to date had an effect strictly during the later phase of the response (i.e., clonal contraction and memory development). In that sense, our data highlight that LIGHT clearly has a distinct and nonredundant function compared with other TNF family proteins because LIGHT deficiency had a negligible effect on early effector cell differentiation and accumulation over the first 6 d of either a primary or secondary response. These findings further substantiate the use of kinetic model of temporal activity for TNF family molecules. Importantly, they demonstrate the essential prosurvival or antiapoptotic effect of the LIGHT-HVEM interaction on differentiated effector CD8 T cells as they mature and transition into long-lived memory cells.

Previously, no gross changes in either T cell or B cell responses were reported when LIGHT-deficient hosts were intravenously infected with vesicular stomatitis virus or i.p. infected with influenza virus (38, 44), in contrast to the notable phenotype observed in our study. This discrepancy could be explained by the use of $CD8^+$ T cell–specific LIGHT-deficiency in our system rather than the use of hosts completely deficient in LIGHT. LIGHT is expressed by several immune cells including T cells, DCs, monocytes, and neutrophils, and though the mechanism is not well understood, increasing evidence suggests that LIGHT also has anti-inflammatory properties in addition to its proinflammatory activities (45, 46). Moreover, because LIGHT can interact with two receptors, multiple cell-cell interactions might be abrogated in whole LIGHT-deficient mice complicating the interpretation of the prior virus studies or otherwise obscuring a phenotype.

Another important observation was with respect to the impact of LIGHT on the balance of alternate memory subsets. The role of TNFR molecules in the development of memory T cell subsets has not been studied in great detail. Increasing evidence suggests that specific subsets within the memory population might have superior protective characteristic against certain pathogens emphasizing the need to understand the molecular signals that regulate the development of these subsets (9, 10). $CD27^{hi}CD43^{hi}$ T cells are known to localize close to pathogen entry sites in the lungs and have better proliferative capacity than $CD27^{lo}$ T cells; hence, loss of these cells is expected to strongly compromise protective immunity against respiratory virus infection (10). More recently, Gerlach et al. (13) described a memory subset expressing intermediate levels of CX3CR1 called peripheral memory cells that predominantly surveyed the peripheral tissue at steady state. These cells are also largely $CD27^{hi}CD43^{hi}$ and therefore may be critical in orchestrating recall responses against pathogens that infect mucosal sites such as the lung. Intriguingly, LIGHT deficiency caused a significant reduction in the $CD27^{hi}CD43^{hi}CX3CR1^{int}$ peripheral memory cell compartment, further supporting the importance of LIGHT in controlling the development of memory subsets crucial for fighting pulmonary infections. In recent years, another subset of noncirculating memory cells called T_{RM} have been demonstrated to permanently reside within tissues including the lung, and provide immunity against respiratory virus challenge such as influenza (40, 41). Rather than being cytolytic, this subset seems to mediate its effect by enhancing local innate immunity as well as rapidly recruiting memory cells from circulation. LIGHT expression on $CD8^+$ T cells also greatly contributed to the accumulation of optimal numbers of lung T_{RM}, further substantiating its overall importance. Recently it was suggested that T cells generated around the peak of the effector response seed lung T_{RM} cells (47). Hence, it is likely that the defect in CD8 T cell numbers in the lungs at day 8 postinfection contributes to the T_{RM} defect. In line with this, we observed that at day 8 postinfection the defects in lung $CD8^+$ T cell numbers were mainly due to the specific reduction in the $CXCR3^+CX3CR1^{neg}$ population, which is known to give rise to T_{RM} cells (13). These findings raise the question of whether other TNF family interactions also play similar or distinct roles in memory subset development. A recent report demonstrated a role for 4-1BBL in the generation of T_{RM} cells in the context of intranasal influenza virus infection, suggesting other molecules, in addition to LIGHT, can also be relevant (48).

A focus for future studies is how the LIGHT-HVEM interaction controls memory development. $CD8^+$ T cells transferred into HVEM-deficient hosts recapitulated the defect observed when LIGHT-deficient T cells were transferred into intact hosts, suggesting that LIGHT expressed on the surface of the effector CD8 T cell interacts with HVEM expressed on endogenous cells. The most logical cells would be APCs, which are infected with the virus following respiratory infection, such as $CD103^+$ DCs or inflammatory monocytes, as we implied. However, because HVEM is widely expressed on many different innate immune cells, future studies with conditional deletion of HVEM in specific cell types will be required to understand the essential cell type that provides HVEM signal. Furthermore, at present, it is not clear whether HVEM is functional and transmits a signal when it engages LIGHT. This again is the most logical hypothesis as HVEM can signal through several proinflammatory pathways, including NF-κB, and has the potential to induce maturation, differentiation, survival, and inflammatory activity in the cell that expresses it, including DCs or monocytes. An alternative hypothesis is that LIGHT itself is functional on $CD8^+$ T cells. Similar to other TNF family proteins, LIGHT has only a short cytoplasmic domain; nevertheless, it has the potential to transmit intracellular signals (21, 23). Little is known about whether signaling through LIGHT can affect T cell responses in vivo, but in vitro studies have shown that engagement of LIGHT expressed on the surface of T cells can induce proliferation (49, 50). Experiments with macrophages have substantiated the idea that LIGHT can signal because its engagement on these cells was found to induce some inflammatory molecules and enhance activation of several pathways including those involving ERK, PI3-kinase, Akt, and NF-κB (51). In our studies, when transferring $LIGHT^{-/-}$ $CD8^+$ T cells into WT hosts, the endogenous VacV and influenza-specific $CD8^+$ T cell responses were normal despite the profound defect in transferred $LIGHT^{-/-}$ $CD8^+$ T cell responses. Although difficult to interpret, because endogenous LIGHT-sufficient T cells were responding in these hosts, the results might imply that HVEM signaling in APCs or other cells was intact, leading to the conclusion that the defect in T cell memory was specifically due to the lack of signals through LIGHT into the responding T cells.

In summary, our findings provide new insights into the role of LIGHT expressed specifically by $CD8^+$ T cells in the generation of memory CD8 T cell subsets. Because the factors controlling the development of the mucosal memory $CD8^+$ T cell compartment are not fully understood, our novel results suggest that targeting LIGHT and its binding partners is worth considering when formulating the design of vaccines to generate strong immune memory.

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Disclosures

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Supplemental 1

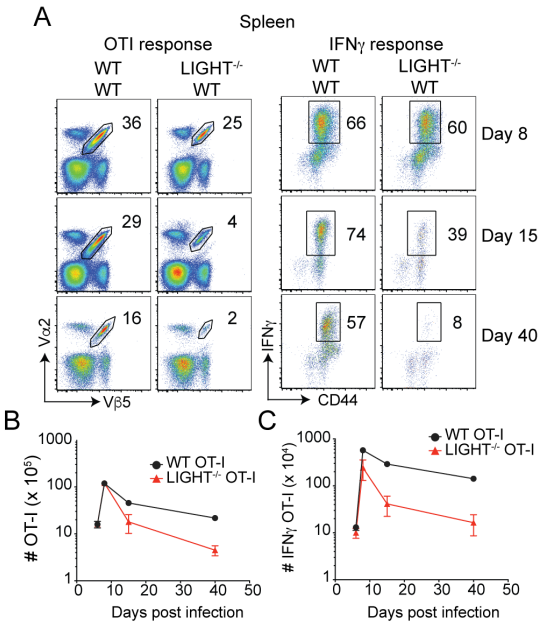

Figure S1. LIGHT expressed by CD8 T cells is important for formation of splenic memory cells after respiratory vaccinia virus infection. (A-D) Equal numbers (5 x 10^4) of WT and LIGHT$^{-/-}$ naïve (CD44^{lo}) OT-I (Vα2$^+$Vβ5$^+$) transgenic CD8 T cells were adoptively transferred into C57BL/6 mice and infected with rVacV-WR-OVA (2 x 10^4 PFU i.n) the following day. (A) Spleens were harvested at days 8, 15 and 40 post-infection and stained for CD8, CD44, Vα2, and Vβ5 and frequencies of OT-I CD8 T cells and IFN-γ$^+$ OT-I CD8 T cells determined. Absolute numbers of (C) OT-I CD8 T cells and (D) IFN-γ$^+$ OT-I CD8 T cells from spleens of recipients quantified. Results are the mean +/- SEM (n = 3 mice/group). Similar results were obtained in four independent experiments.

Supplemental 2

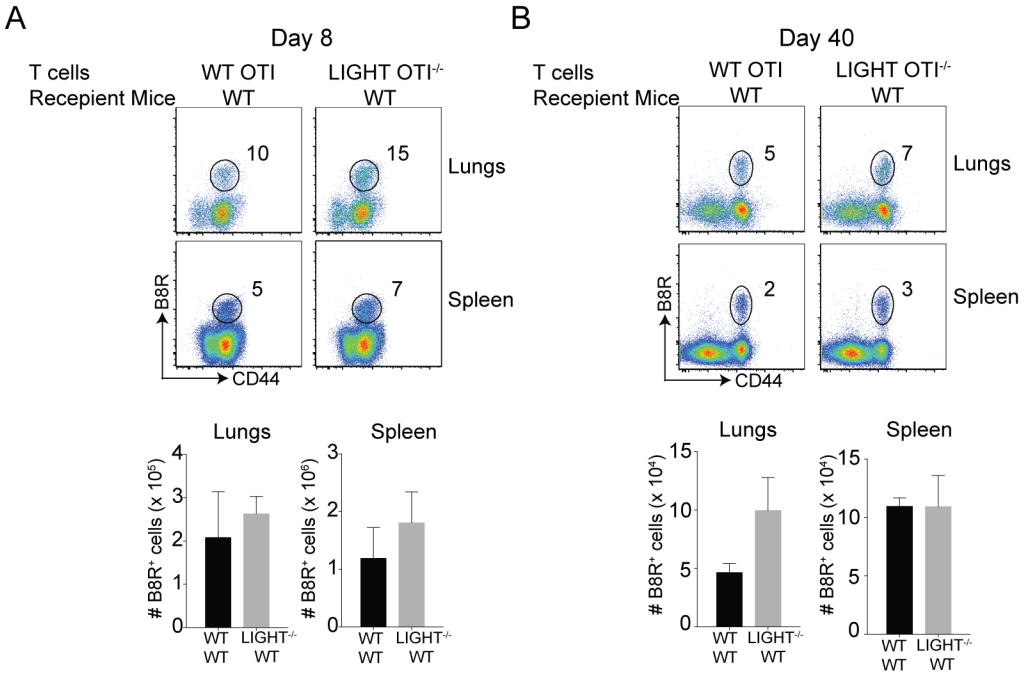

Figure S2. Normal endogenous B8R response in WT OTI and LIGHT$^{-/-}$ OTI recipient mice. As in figure 1, lung and spleen cells at day 8 and day 40 post-infection were stained with vaccinia specific immunodominant epitope B8R tetramer and their frequencies and absolute cell numbers were quantified.

Supplemental 3

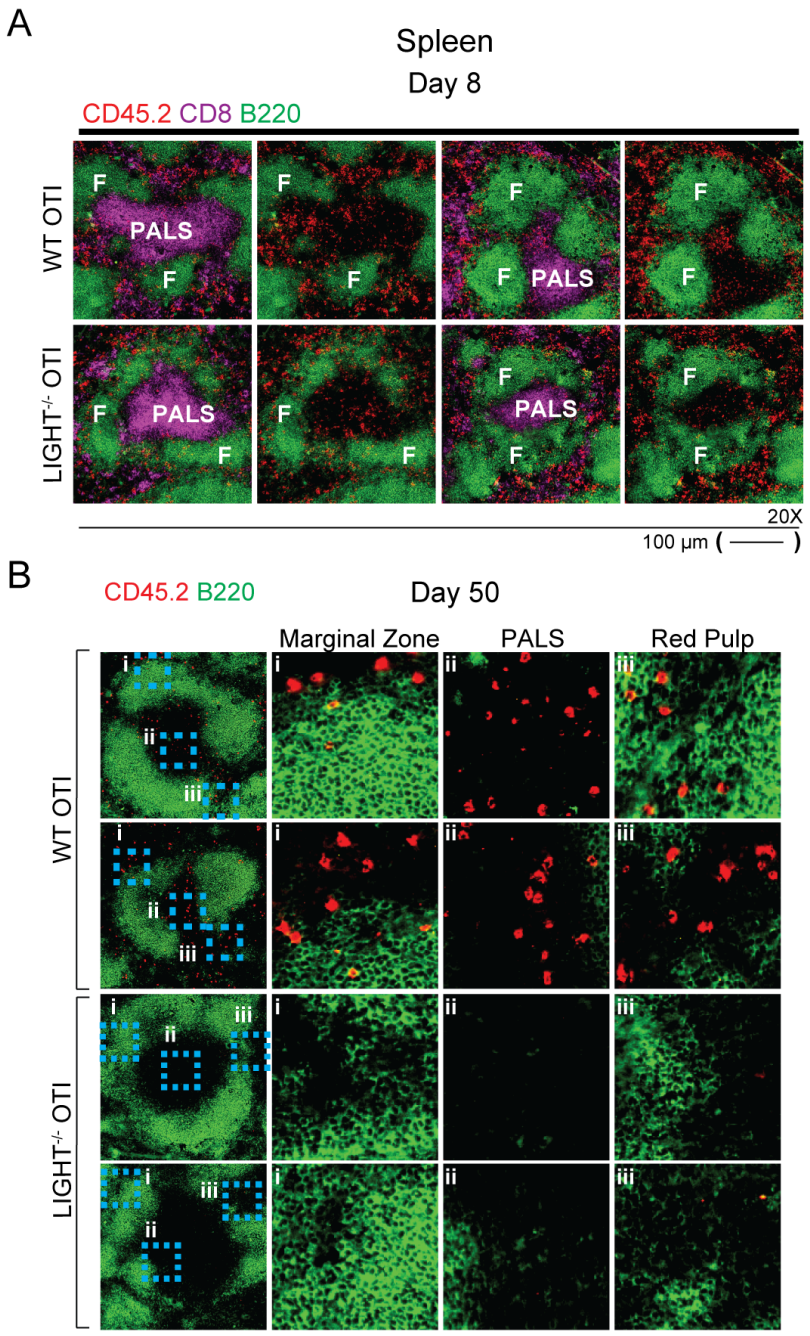

Figure S3. LIGHT deficient CD8 T cells have similar homing preference as WT CD8 T cells at the peak of the effector response but are completely absent at memory phase. Equal numbers (5 x 10^5) of WT and LIGHT$^{-/-}$ naive (CD44^{lo}) OT-I (Vα2$^+$Vβ**5$^+$**) transgenic CD45.2$^+$CD8 T cells were adoptively transferred into CD45.1$^+$SJL mice and infected with rVacV-WR-OVA (2 x 10^4 PFU i.n) the following day. (A) IF analysis of frozen spleen sections from recipient mice stained for CD45.2 (red) and CD8 (magenta) at day 8 post-infection. (B) Similar analysis for was done at day 40 post-infection in the spleen. ActinGreen or EpCAM (green) were used to visualize lung morphology. Bars, 200 µm & 100 µm respectively. Similar results were obtained in two independent experiments.

Supplemental Figure 4

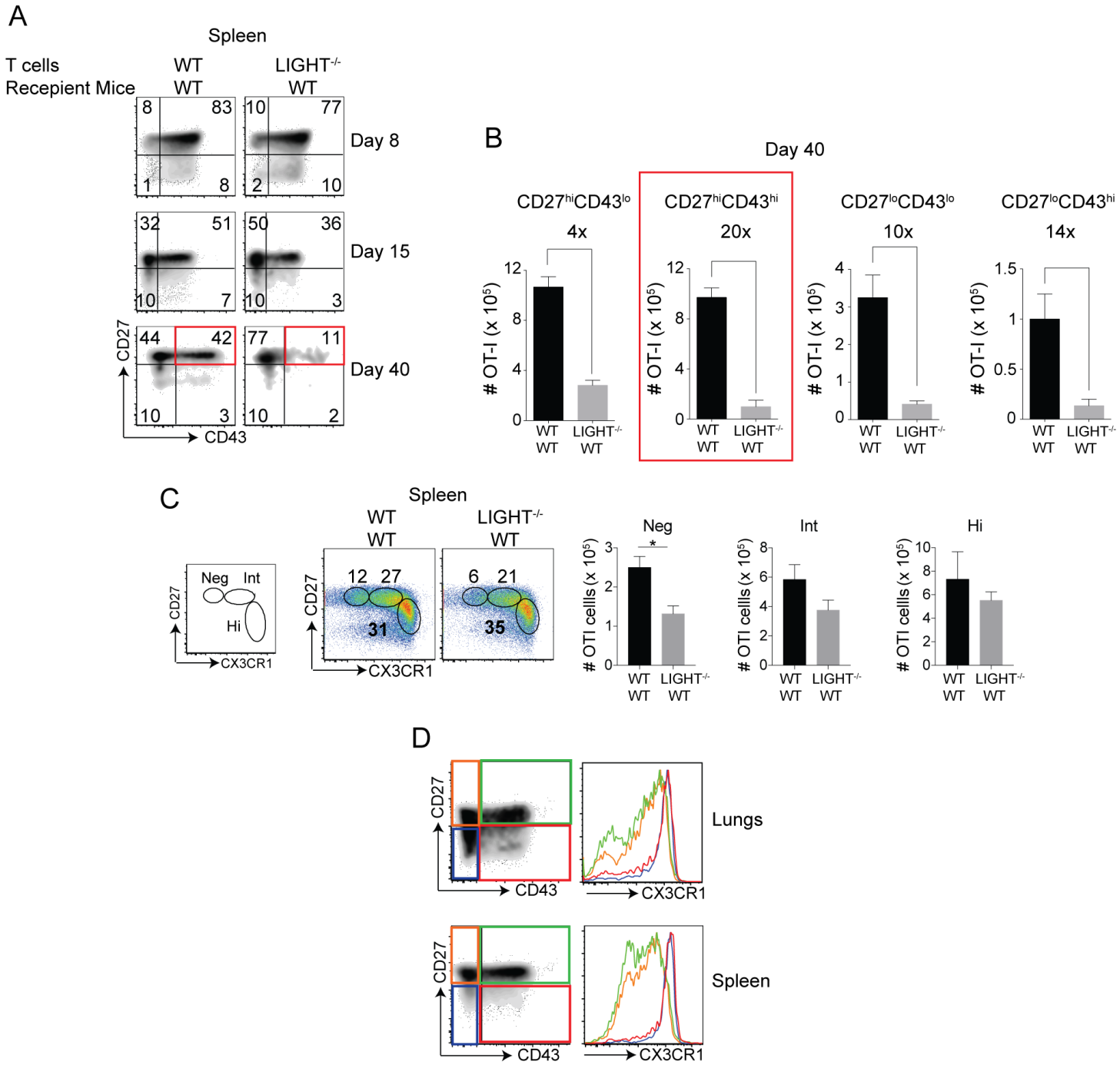

Figure S4. Differential impact of LIGHT deficiency on splenic memory CD8 T cell subsets. (A) Similar to figure 3A, spleen cells were also stained with CD27, CD43, CD8, Vα2, Vβ5 and CD44 at days 8, 15 and 40 post-infection and (B) absolute numbers of CD27 vs. CD43 subset was quantified. Similar results were obtained in two independent experiments and results are the mean SEM (n = 3 mice/group). (C) Similar to figure 3C lung cells from WT or LIGHT$^{-/-}$ CD8 T cell recipient mice were stained with CD27 instead of CXCR3. (D) At day 8 post-infection, lung cells were stained with CD8, Vα2, Vβ5, CD44, CD27, CD43 and CX3CR1. CX3CR1 expression was determined on each of the CD27 vs. CD43 subsets.