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LIGHT Is Critical for IL-12 Production by Dendritic Cells, Optimal CD4$^+$ Th1 Cell Response, and Resistance to *Leishmania major*[1]

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Although studies indicate LIGHT (lymphotoxin (LT)-like, exhibits inducible expression and competes with HSV glycoprotein D for herpes virus entry mediator (HVEM), a receptor expressed by T lymphocytes) enhances inflammation and T cell-mediated immunity, the mechanisms involved in this process remain obscure. In this study, we assessed the role of LIGHT in IL-12 production and development of CD4$^+$ Th cells type one (Th1) in vivo. Bone marrow-derived dendritic cells from LIGHT$^{-/-}$ mice were severely impaired in IL-12p40 production following IFN-γ and LPS stimulation in vitro. Furthermore, blockade of LIGHT in vitro and in vivo with HVEM-Ig and LT β receptor (LTβR)-Ig leads to impaired IL-12 production and defective polyclonal and Ag-specific IFN-γ production in vivo. In an infection model, injection of HVEM-Ig or LTβR-Ig into the usually resistant C57BL/6 mice results in defective IL-12 and IFN-γ production and severe susceptibility to *Leishmania major* that was reversed by rIL-12 treatment. This striking susceptibility to *L. major* in mice injected with HVEM-Ig or LTβR-Ig was also reproduced in LIGHT$^{-/-}$ $\rightarrow$ RAG1$^{-/-}$ chimeric mice. In contrast, *L. major*-infected LT$\beta^{-/-}$ mice do not develop acute disease, suggesting that the effect of LTβR-Ig is not due to blockade of membrane LT (LTα1β2) signaling. Collectively, our data show that LIGHT plays a critical role for optimal IL-12 production by DC and the development of IFN-γ-producing CD4$^+$ Th1 cells and its blockade results in severe susceptibility to *Leishmania major*. *The Journal of Immunology,* 2007, 179: 6901–6909.

Members of the TNF superfamily of cytokines and their receptors play important roles in the development of the immune system and in immune regulation, inflammation, and autoimmunity (1, 2). Acting through specific cellular receptors, these cytokines initiate signaling cascades that regulate cell death, survival, and differentiation. There are four closely related members of this family, which include lymphotoxin (LT)[4] α (LTα, LTβ, TNF-α, and LIGHT (LT-like, exhibits inducible expression and competes with HSV glycoprotein D for herpes virus entry mediator (HVEM), a receptor expressed by T lymphocytes)). Their cognate receptors include TNFR1, TNFR2, LTβR, and HVEM, respectively.

LIGHT signaling is important for cell survival, inflammation, and tumor eradication (3). In addition, signals transmitted via LIGHT-HVEM interaction may have costimulatory effects on T cells thereby enhancing T cell proliferation and cytokine production (4–6). Consistent with this, overexpression of LIGHT on T cells results in extensive T cell proliferative disorders characterized by massive polyclonal expansion of CD4$^+$ and CD8$^+$ T cells (7, 8). In contrast, blockade or disruption of LIGHT interaction with its receptors prevents or ameliorates graft-vs-host disease, allograft rejection, and autoimmune disorders including colitis and arthritis (9–12). Together, these studies indicate that LIGHT plays a crucial role in T cell-mediated immunity. However, the precise mechanism(s) through which LIGHT enhances T cell immunity is still poorly understood. Furthermore, whether LIGHT plays a functional role in resistance to infectious diseases remains unclear.

Resistance or susceptibility to *Leishmania major* infection in mice is dependent on the type of CD4$^+$ Th cell subset that is induced (13–15). Healing in resistant mice is associated with the development of IFN-γ-producing Th1 cells which activate macrophages to produce NO, an effector molecule for killing intracellular parasites. In contrast, susceptible mice produce early IL-4, which promotes the development and expansion of Th2 cells that produce IL-4 and IL-10, cytokines that deactivate macrophages and inhibit intracellular parasite killing. A key factor in determining resistance and susceptibility to *L. major* in mice is the production of, and responsiveness to, IL-12 (16, 17). The highly susceptible BALB/c mice produce less and respond poorly to IL-12 due to down-regulation of IL-12Rβ chain (18). In contrast, resistant mice produce more and maintain their IL-12 responsiveness (19). The pathways that lead to early IL-12 production during *L. major* infection (and hence Th1 response and resistance) are still poorly

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[4] Abbreviations used in this paper: LT, lymphotoxin; HVEM, herpes virus entry mediator; LIGHT, LT-like, exhibits inducible expression and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes; LN, lymph node; dLN, draining LN; DC, dendritic cell; SLA, soluble *Leishmania* Ag; WT, wild type; BMDC, bone marrow-derived DC; ODN, oligodeoxynucleotide; BTLA, B and T lymphocyte attenuator.

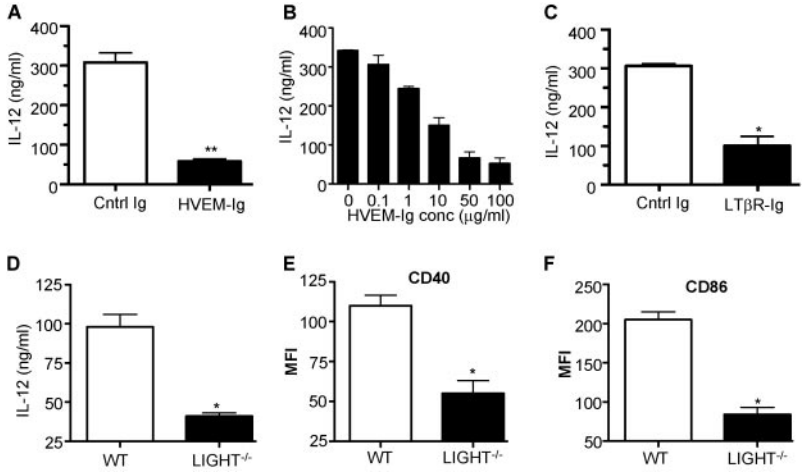

FIGURE 1. LIGHT is critical for DC maturation and IL-12 production. BMDC were differentiated from bone marrow cells of C57BL/6 (WT) mice (see *Materials and Methods*), primed with IFN-γ (100 U/ml) for 6 h, and stimulated in vitro with LPS (1 μg/ml) in the presence or absence of HVEM-Ig (50 μg/ml). After 48 h, the supernatant fluids were collected and assayed for IL-12p40 by ELISA (*A*). *B*, Dose-dependent inhibitory effect of HVEM-Ig on LPS-induced IL-12 production by WT BMDC. *C*, LTβR-Ig blocks IL-12 production by WT BMDC. BMDC from WT mice generated as in *A* above were stimulated for 48 h with LPS in the presence or absence of LTβR-Ig (50 μg/ml) and the supernatant fluids were assayed for IL-12p40 by ELISA. *D*, Impaired IL-12p40 production by BMDC from LIGHT$^{-/-}$ mice. BMDC from WT and LIGHT$^{-/-}$ mice were stimulated with LPS and assessed for IL-12 production as in *A* above. *E* and *F*, Deficiency of LIGHT impairs expression of costimulatory molecules by DCs. BMDC from WT and LIGHT$^{-/-}$ mice were stimulated in vitro with LPS (1 μg/ml) for 24 h and the expression of CD40 (*E*) and CD86 (*F*) was assessed by flow cytometry. The results presented are representatives of two to four different experiments with similar results. *, $p < 0.05$; **, $p < 0.01$.

understood although CD40-CD40L interaction may be important (20, 21).

In this study, we report that blockade of LIGHT interaction with its receptor by HVEM-Ig or LTβR-Ig fusion proteins results in severe impairment in LPS- and CpG-induced IL-12 production by dendritic cells (DCs) in vitro and in vivo. In addition, in vivo blockade of LIGHT also leads to impaired Th1 (IFN-γ) response to a model Ag OVA and to the intracellular pathogen *L. major*, resulting in severe susceptibility in the usually resistant C57BL/6 mice. This susceptibility was reversed by intralesional treatment of infected mice with rIL-12, critically confirming that defective IL-12 production following LIGHT blockade is the cause of the enhanced susceptibility to *L. major*. Collectively, our results unravel a mechanism through which LIGHT enhances CD4$^+$ Th cell response and suggest that this pathway could provide a new target for therapeutic regulation of IL-12-dependent CD4$^+$ Th1 cell-mediated inflammatory conditions.

Materials and Methods

Mice

Six- to 8-wk-old female C57BL/6 (B6) and RAG1$^{-/-}$ B6 mice (B6.129S7-Rag1tm1Mom/J) were purchased from Charles River Laboratories and The Jackson Laboratory, respectively. The origin of LIGHT$^{-/-}$ and HVEM$^{-/-}$ mice has been previously described (11, 22). The generation of LTβ$^{-/-}$ mice used in this study has been previously described (23); and

Table I. *HVEM-Ig does not affect LPS-induced IL-6, TNF-α, and NO production by BMDC*[a]

	IL-6 (ng/ml)	IL-12 (ng/ml)	TNF-α (ng/ml)	Nitrite (μM)
Control Ig	4.3 ± 1.8	286.0 ± 13.0*	3.0 ± 0.2	22.7 ± 0.7
HVEM-Ig	4.1 ± 2.2	106.6 ± 14.5	2.9 ± 0.1	23.8 ± 0.5

[a] BMDC were stimulated with LPS (10 μg/ml) for 48 h in the presence of HVEM-Ig or control Ig (50 μg/ml) and the production of IL-6, IL-12, TNF-α, and NO was determined as described in *Materials and Methods*. Data are presented as mean ± SEM of quadruplicate wells and is a representative of three different experiments with similar results. *, $p < 0.05$.

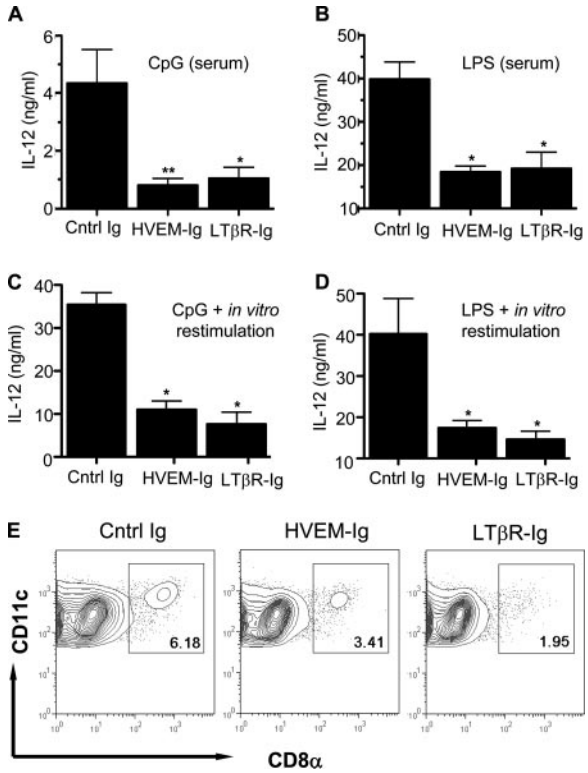

FIGURE 2. HVEM-Ig and LTβR-Ig block DC influx into the dLN and IL-12 production by dLN cells in vivo. WT mice were injected with control Ig, HVEM-Ig, or LTβR-Ig (100 μg) i.p. and challenged in the footpad with CpG ODN (100 μg/mouse) or LPS (10 mg/kg) the next day. After 3 days, mice were sacrificed and serum IL-12p40 level was measured by ELISA (*A* and *B*). At sacrifice, single-cell suspensions of the dLNs (popliteal LNs) of mice above were stimulated with anti-CD40 mAb for 48 h and the production of IL-12p40 was determined by ELISA (*C* and *D*). Some dLNs were digested with collagenase/dispase solution, and CD11c$^+$ cells were purified with magnetic beads, stained for CD8α expression, and analyzed by FACS (*E*). The results presented are representatives of two to three different experiments (n = 4–6 mice/group) with similar results. *, $p < 0.05$; **, $p < 0.01$.

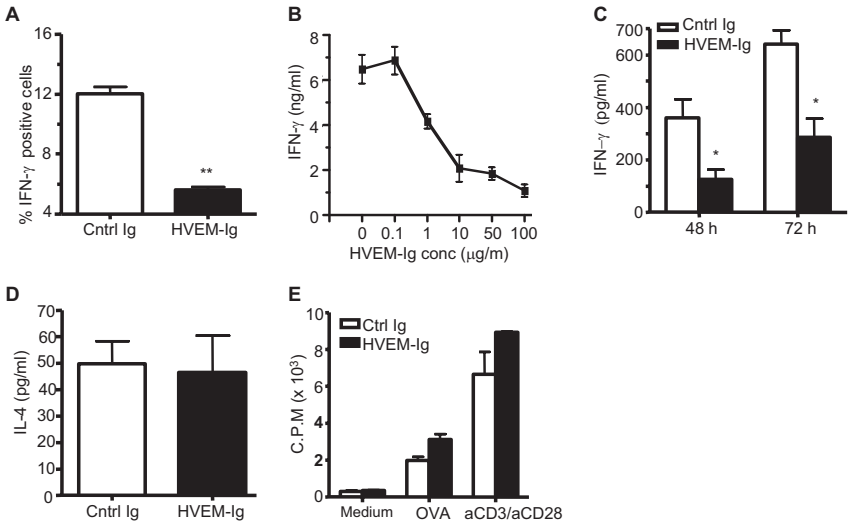

FIGURE 3. HVEM-Ig causes impairment in Th1 response in vitro and in vivo. *A* and *B*, Impaired IFN-γ (Th1 cytokine) production after polyclonal T cell activation. Splenocytes from C57BL/6 mice were stimulated in vitro with ant-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) in the presence or absence of varying concentrations of HVEM-Ig (0–100 μg/ml). After 48 h, the frequency of IFN-γ-producing $CD4^+$ T cells was determined by flow cytometry (*A*, 50 μg/ml HVEM) and the supernatant fluids were collected and assay for IFN-γ by ELISA (*B*). *C–E*, LIGHT blockade impairs Th1 response to a model Ag, OVA. C57BL/6 mice were injected with HVEM-Ig (100 μg/mouse) and immunized the next day i.p. with OVA (2 μg/mouse) adsorbed unto alum. Five days after immunization, mice were sacrificed and their splenocytes were restimulated in vitro with OVA (300 μg/ml) and the production of IFN-γ (*C*) and IL-4 (*D*) was determined at the indicated times by ELISA. Some cells were also stimulated with anti-CD3/anti-CD28 (1 μg/ml) or OVA for 4 days, pulsed overnight with [^{3}H]thymidine (0.5 μCi/well), and used to measure proliferation (*E*). The results presented are representatives of three (*A* and *B*) and two (*C–E*, $n = 6$ mice/group) different experiments with similar results. *, $p < 0.05$; **, $p < 0.01$.

Dr. J. Gommerman (University of Toronto, Toronto, Canada) provided the breeding pairs. All mouse experiments were approved by the University of Manitoba Animal Care Committee in accordance with the regulation of the Canadian Council on Animal Care.

Creation of LIGHT$^{-/-}$ → RAG1$^{-/-}$ and HVEM$^{-/-}$ → RAG1$^{-/-}$ B6 chimeras

Bone marrow cells from B6 (wild-type (WT)), LIGHT$^{-/-}$, and HVEM$^{-/-}$ mice (also on B6 background) were adoptively transferred by i.v. (10^7 cells/mouse) into lethally irradiated (9.5 Gy) RAG1$^{-/-}$ mice. Chimeric mice were infected with *L. major* 5 wk after reconstitution when T cell engraftment was ≥75% of the unmanipulated (control) B6 mice.

Parasites, infection, HVEM-Ig, LTβR-Ig, and rIL-12 treatment and estimation of parasite burden L. major

Leishmania major (MHOM/IL/80/Friedlin) were grown in Grace's insect medium (Invitrogen Life Technologies) supplemented with 20% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Mice were infected by injecting 5×10^6 (high dose) or 10^5 (low dose) stationary phase promastigotes (day 7) suspended in 50 μl of PBS into the right-hind footpad. One day before infection, some mice were injected with HVEM-Ig (11) or LTβR-Ig (24) fusion proteins (100 μg/mouse) or control Ig (human IgG1 Fc fragment, 100 μg; Sigma-Aldrich) i.p. and once weekly for 5 wk. Some mice also received intralesional injection of rIL-12 (0.3 μg/mouse; PeproTech) three times a week for 2 wk (25). After infection, the development and progression of footpad lesion was monitored weekly by measuring the diameter of the infected footpad with calipers (Fisher Scientific). Lesion sizes are presented as the difference in measurement between infected and uninfected contralateral feet. In accordance with the institutional animal care policy, mice were sacrificed when the lesion reaches 3.5 mm in diameter or begins to ulcerate. Parasite burden in the footpad, spleen, and lymph node (LN) was quantified by limiting dilution analysis (25).

In vitro recall response, proliferation assay, and intracellular cytokine staining

At various times after infection, single-cell suspensions of spleens and draining LNs (dLNs) from infected mice were cultured in complete medium (DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin), in 24-well tissue-culture plates (Falcon; VWR). Cells were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) or soluble leishmanial Ag (SLA; 50 μg/ml) for 72 h and the supernatant fluids were assayed for cytokines by ELISA. Some cells were stimulated with PMA (50 ng/ml), ionomycin (500 ng/ml), and BFA (10 μg/ml) for 3–5 h and used for intracellular cytokine staining as previously described (25). For proliferation, some cells were labeled with CFSE (Molecular Probes) as previously described (25), stimulated with SLA, or anti-CD3, and anti-CD28 (1 μg/ml) for 5 days and analyzed by flow cytometry.

Cytokine ELISAs and measurement of NO

The levels of IL-2, IL-6, IL-10, IL-12, TNF-α, and IFN-γ in culture supernatant fluids were determined by sandwich ELISA using Ab pairs from BD Pharmingen according to manufacturer's suggested protocols. NO levels in culture supernatant fluids were measured by the Griess assay as previously described (26).

Bone marrow-derived DCs (BMDC)

BMDC were derived from B6, LTβ$^{-/-}$, and LIGHT$^{-/-}$ mice as previously described (27). Briefly, bone marrow cells were differentiated in petri dishes in the presence of rGM-CSF (20 ng/ml; PeproTech). Immature DCs were harvested on day 7 and assessed for expression of CD11c, CD40, CD80, CD86, and MHC class II by flow cytometry (LIGHT$^{-/-}$). The BMDC were primed with IFN-γ (100 U/ml; PeproTech) for 6 h, stimulated for 48 h in vitro with LPS (10 μg/ml; Sigma-Aldrich), CpG oligodeoxynucleotide (ODN; 100 μg/ml; Coley Pharmaceuticals), or anti-CD40 mAb (5 μg/ml; BD Pharmingen) in the presence or absence of varying concentrations of HVEM-Ig, LTβR-Ig, or control IgG and the production of cytokines (IL-6, IL-12p40, and TNF-α) was determined by ELISA.

CpG- and LPS-induced IL-12 production in vivo

C57BL/6 mice were injected in the footpad with class A CpG ODN 1826 (100 μg/mouse; Coley Pharmaceuticals) or LPS (10 mg/kg; Sigma-Aldrich) with or without HVEM-Ig or LTβR-Ig (100 μg/mouse). Three days later, mice were sacrificed by euthanasia and serum was collected and assayed for IL-12p40 and IL-12p70 by ELISA. The dLNs were pooled, made into single-cell suspensions, stimulated in vitro with 5 μg/ml anti-CD40 mAb for 48 h and the supernatant fluids were assayed for IL-12p40 by ELISA. To isolate $CD11c^+$ cells (DC), the dLNs were cut into small pieces and digested at 37°C with 1 mg/ml collagenase D/Dispase (Roche Scientific) and 50 μg/ml DNase I (Sigma-Aldrich) in 1 ml of RPMI 1640 medium (Invitrogen Life Technologies) containing 2% FBS. Frequent mixing at room temperature for 20 min was sufficient to disperse the cells, after

which 600 μl of 0.1 M EDTA (pH 7.2), was added and stirred continuously to break up DC-T cell conjugates. The digested LN cell suspensions were pooled, labeled with anti-CD11c Ab-coated microbeads, and passed through an autoMACS separator (Miltenyi Biotec) according to the manufacturer's suggested protocols. Purified DCs were counted, stained for CD8α expression, and analyzed by flow cytometry. The purity of the isolated DC was between 90 and 95% ($CD11c^+$ cells).

Immunization of mice with OVA and recall response

C57BL/6 mice were injected i.p. with HVEM-Ig or control Ig (100 μg/mouse) and 1 day later immunized with OVA (2 μg; ICN Biochemicals) adsorbed unto 2 mg $Al(OH)_3$ (Alum, Imject; Pierce Biochemicals). Five days after immunization, mice were sacrificed and their spleen cells were stimulated with OVA (300 μg/ml) or anti-CD3 and anti-CD28 (1 μg/ml) for 48 h (IL-4) and 72 h (IFN-γ) and the supernatant fluids were assayed for cytokines by ELISA. For proliferation, the cultures were incubated for 4 days, pulsed with 0.5 μCi [^{3}H]thymidine/well for the last 16 h, and the radioactivity was determined by scintillation counter.

Statistical analysis

A two-tailed Student *t* test was used to compare means of lesion sizes, parasite burden, and cytokine production from different groups of mice. Significance was considered if $p \leq 0.05$.

Results

LIGHT is critical for optimal IL-12 production by DCs

Several studies show that LIGHT enhances T cell (Th1)-mediated immunity (6, 7, 28) but the precise mechanism(s) remain unclear. Because IL-12 production by DC is critically important for the development and maintenance of IFN-γ-producing $CD4^+$ Th1 cells (29–31), we hypothesized that the Th1-promoting effect of LIGHT is mediated by enhancing IL-12 production by DC. Therefore, we determined IL-12 production by IFN-γ-primed LPS-stimulated BMDC in the presence or absence of HVEM-Ig, a fusion protein that blocks the interaction of LIGHT with its receptors (32). As shown in Fig. 1*A*, HVEM-Ig significantly blocked LPS-induced IL-12 production by BMDC in vitro. The inhibitory effect of HVEM-Ig on IL-12 production by BMDC was dose dependent (Fig. 1*B*).

HVEM-Ig can potentially block LIGHT, LTα3, and B and T cell lymphocyte attenuator molecule (BTLA) signaling (all bind to HVEM) (33). Therefore, we investigated whether LTβR-Ig (which blocks LIGHT signaling without affecting LTα3 and BTLA) has similar inhibitory effect on LPS-induced IL-12 production by BMDCs. Similar to the results obtained with HVEM-Ig, LTβR-Ig also severely impaired LPS-induced IL-12 production by BMDC in vitro (Fig. 1*C*). Furthermore, BMDC from $LIGHT^{-/-}$ mice were impaired in their ability to produce IL-12 after LPS stimulation (Fig. 1*D*). To test whether LIGHT blockade globally affected DC maturation and cytokine production, we assessed the expression of costimulatory molecules and production of NO, IL-6, and TNF-α by BMDC from $LIGHT^{-/-}$ after LPS stimulation. Deficiency of LIGHT did not affect the differentiation of bone marrow cells into DCs ($CD11c^+$ cells) or the expression of MHC class II, CD40, CD80, and CD86 on immature DC in vitro (data not shown). In contrast, the expression of CD40 and CD86 by mature (LPS-stimulated) $LIGHT^{-/-}$ BMDCs was severely impaired (Fig. 1, *E* and *F*). However, HVEM-Ig or LIGHT deficiency has no effect on the production of NO, IL-6, and TNF-α by DCs (Table I and data not shown). Taken together, these results show that the absence or blockade of LIGHT interaction with its receptors in vitro leads to specific impairment in DC maturation and IL-12 production.

HVEM-Ig and LTβR-Ig block CpG- and LPS-induced IL-12 production in vivo

Previous studies show that unmethylated CpG motifs (34–36) and LPS (37–39) induce IL-12 production by DC in vivo. Injection of HVEM-Ig or LTβR-Ig before challenge with LPS and CpG caused a significant decrease in serum levels of IL-12p40 (Fig. 2, *A* and *B*). Injection of HVEM-Ig or LTβR-Ig alone into mice (without LPS or CpG challenge) has no significant effect on the serum levels of IL-12p40 as these mice had background serum levels of IL-12p40 (similar to those treated with control Ig or PBS; data not shown). In addition, HVEM-Ig or LTβR-Ig significantly decreased cellular influx into the LN draining the challenge site (dLN; data not shown) and these cells produced significantly less IL-12p40 following stimulation with anti-CD40 (Fig. 2, *C* and *D*). Interestingly, HVEM-Ig and LTβR-Ig treatment also significantly reduced the total number of DCs (particularly $CD11c^+CD8\alpha^+$ DC) in the dLN following CpG or LPS challenge (Fig. 2*E*). Together, these results suggest that interaction of LIGHT with its receptor is important for influx of cells and $CD11c^+CD8\alpha^+$ DC into the dLN and optimal IL-12 production in vivo.

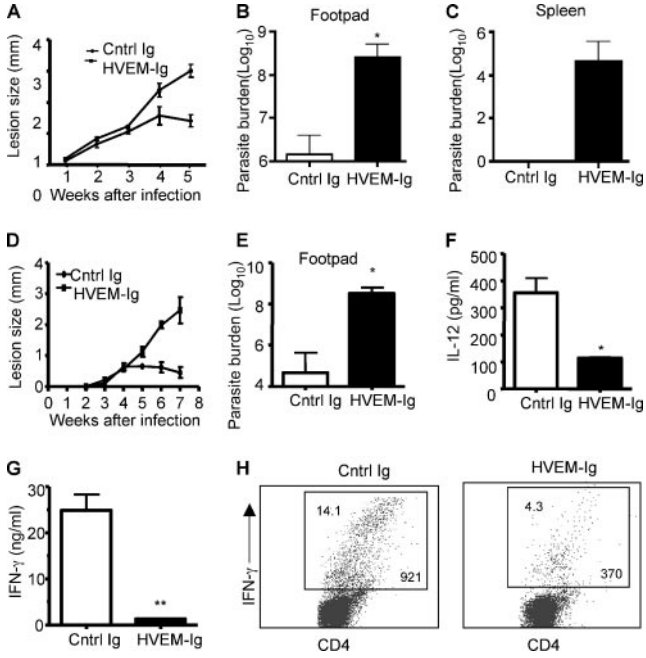

FIGURE 4. Injection of HVEM-Ig into the resistant C57BL/6 mice results in severe susceptibility to *Leishmania major* and impairment in IFN-γ response. C57BL/6 mice were injected with HVEM-Ig or control Ig (100 μg/ml) and infected with high (5×10^6, *A–C*) or low (10^5, *D* and *E*) dose *L. major* the next day followed by weekly injection of the fusion protein for 5 (high dose) or 7 (low dose) additional weeks. The progression of cutaneous lesion in the infected feet was monitored weekly with calipers (*A* and *D*). At 5 (high dose) or 7 (low dose) wk after infection, mice were sacrificed to estimate parasite burden in the infected footpad (*B* and *E*) and spleen (*C*). At the time of sacrifice, cells from the dLNs of infected mice (high dose) were stimulated with SLA (50 μg/ml) for 72 h and the production of IL-12 (*F*) and IFN-γ (*G*) was determined by ELISA. Some of the cells were stained for intracellular expression of IFN-γ and analyzed by flow cytometry (*H*). Numbers in the box represent the percentage of $CD4^+$ IFN-γ-producing cells (*upper*) and mean florescence intensity (MFI, *bottom*) of IFN-γ-positive cells. The results presented are representatives of four (*A–C* and *F–H*) and two (*D* and *E*) independent experiments ($n = 4–6$ mice/group) with similar results. *, $p < 0.05$; ND, Not detectable.

Blockade of LIGHT leads to impaired Th1 response in vitro and in vivo

Given that deficiency of LIGHT or treatment with HVEM-Ig and LTβR-Ig leads to impairment in DC maturation and IL-12 production in vitro and in vivo, we hypothesized this will critically

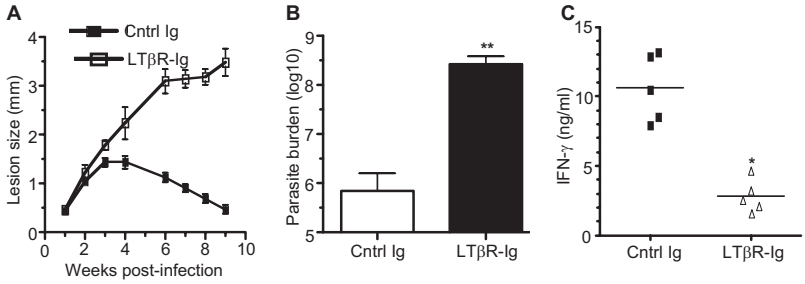

FIGURE 5. Injection of LTβR-Ig into the resistant C57BL/6 mice results in severe susceptibility to *L. major*. C57BL/6 mice were injected with LTβR-Ig or control Ig (100 μg/mouse) and infected with *L. major* the next day followed by weekly injection of the fusion protein for 5 wk. The progression of cutaneous lesion in the infected feet was monitored weekly with calipers (*A*). At 9 wk after infection, mice were sacrificed to estimate parasite burden in the footpad (*B*). At the time of sacrifice, cells from the dLNs of infected mice were stimulated with SLA for 72 h and the production of IFN-γ (*C*) was determined by ELISA. The results presented are representatives of two independent experiments ($n = 5$ mice/group) with similar results. *, $p < 0.05$; **, $p < 0.01$.

impair Th1 cell development in vitro and in vivo. In agreement with this hypothesis, HVEM-Ig inhibited polyclonal differentiation of splenocytes into Th1 cells in a dose-dependent manner (Fig. 3, *A* and *B*). Furthermore, injection of HVEM-Ig before immunization with OVA drastically impaired IFN-γ recall response without affecting IL-4 production and polyclonal or OVA-specific T cell proliferation (Fig. 3, *C–E*). Similar results were obtained in LTβR-Ig-treated mice that were subsequently immunized with OVA (data not shown). Taken together, these results suggest that LIGHT is important for optimal Th1 response in vitro and in vivo.

Injection of HVEM-Ig into the resistant C57BL/6 mice results in severe susceptibility to L. major *infection*

Resistance to *L. major* is mediated by IFN-γ-producing $CD4^+$ Th1 cells whose induction is dependent on IL-12 production by DC (40, 41). Given that HVEM-Ig and LTβR-Ig impaired IL-12 and polyclonal and Ag-specific IFN-γ production in vivo (Figs. 2 and 3), we hypothesized this will result in susceptibility to *L. major* in the normally resistant mice. C57BL/6 mice injected with HVEM-Ig fusion protein became highly susceptible to *L. major* infection as manifested by the development of uncontrolled progressive lesion, which became ulcerative after 5–6 wk necessitating sacrifice and termination of the experiment (Fig. 4*A*). Analysis of lesion parasite burden revealed HVEM-Ig-treated mice contained significantly high parasite load in their infected footpad (Fig. 4*B*), spleens (Fig. 4*C*), and livers (data not shown). Because parasite dose influences the outcome of *L. major* infection (25, 42), we investigated the effect of HVEM-Ig during a relatively low-dose infection. As with high-dose (5×10^6) infection, treatment with HVEM-Ig abolished healing in the usually resistant B6 mice following low-dose (10^5) infection (Fig. 4, *D* and *E*).

Analysis of cytokine response at the time of sacrifice showed HVEM-Ig treatment significantly inhibited IL-12 and IFN-γ production in infected mice (Fig. 4, *F–H*). As with OVA immunization, both *Leishmania*-specific and polyclonal (anti-CD3) T cell proliferation and IL-2 production were unaffected by HVEM-Ig treatment (data not shown). Taken together, these results show that LIGHT is important for optimal Th1 response and resistance to *L. major* infection in mice. They suggest that defective $CD4^+$ Th1 response possibly resulting from impaired IL-12 production may be responsible for the susceptibility of HVEM-Ig-injected C57BL/6 mice to *L. major*.

Injection of LTβR-Ig also results in severe susceptibility to L. major *in C57BL/6 mice*

To further determine whether the in vivo effects of HVEM-Ig were mediated primarily via blockade of LIGHT (and indirectly exclude the role of other HVEM ligands such as BTLA and LTα3), we treated *L. major*-infected C57BL/6 mice with LTβR-Ig (which targets only LIGHT and LTα1β2) and monitored the outcome of *L. major* infection over several weeks. As with HVEM-Ig, *L. major*-infected mice treated with LTβR-Ig developed severe and acute progressive disease associated with extensive and uncontrolled parasite proliferation and dissemination to the spleen and liver (Fig. 5, *A* and *B*, and data not shown). Furthermore, the production of IL-12 (data not shown) and IFN-γ (Fig. 5*C*) by cells from LTβR-Ig-treated mice was significantly low compared with the control groups. Taken together, these results strongly suggest

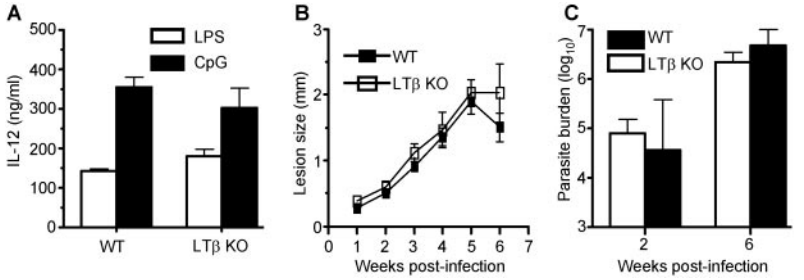

FIGURE 6. LT$\beta^{-/-}$ mice do not develop acute fatal leishmaniasis. *A*, Unimpaired IL-12 production by BMDC from LT$\beta^{-/-}$ mice. BMDC from WT and LT$\beta^{-/-}$ mice were stimulated with LPS (1 μg/ml) or CpG (10 μg/ml) for 48 h and the production of IL-12 was determined by ELISA. WT and LT$\beta^{-/-}$ mice were infected in the footpad with *L. major* (5×10^6) and the progression of cutaneous lesion in the infected feet was monitored weekly with calipers (*B*). At 6 wk after infection, mice were sacrificed to estimate parasite burden in the footpad (*C*). The results presented are representatives of two independent experiments ($n = 4–6$ mice/group) with similar results.

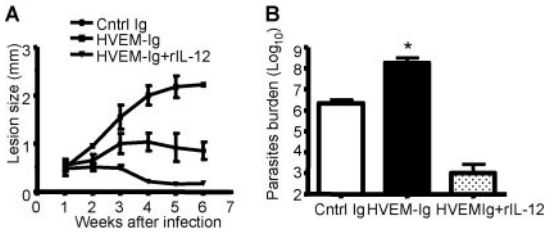

FIGURE 7. Administration of rIL-12 to *L. major*-infected mice reverses susceptibility caused by HVEM-Ig fusion protein. C57BL/6 mice were injected with HVEM-Ig or control Ig (100 μg/mouse) and infected with 5×10^6 *L. major* the next day. Infected mice were further treated once weekly with fusion protein for additional 6 wk. Some HVEM-Ig-treated mice were given rIL-12 intralesionally (0.3 μg/mouse, three times a week) for 2 wk and lesion size was monitored weekly (*A*). Six weeks after infection, mice were sacrificed and parasite burden in the infected footpad was determined (*B*). The results presented are representatives of two different experiments ($n = 4$–5 mice/group) with similar results. *, $p < 0.05$.

that the effects of HVEM-Ig treatment were most likely mediated via blockade of LIGHT.

The susceptibility of LTβR-Ig-treated mice to L. major *is not due to blockade of membrane LT (LTα1β2) signaling*

A recent report shows that the interaction of T cell expressed LTα1β2 with DC-expressed LTβR is critical for DC activation and expression of costimulatory molecules (43). Therefore, we investigated the role of LTα1β2 (membrane LT) in our in vivo system of resistance. As shown in Fig. 6*A* (and in contrast to LIGHT$^{-/-}$ DC, see Fig. 1*C*), LPS- and CpG-stimulated BMDC from LT$\beta^{-/-}$ mice (which has intact LIGHT but lacks membrane LT), produced similar levels of IL-12 as their WT controls. Upon infection with *L. major*, LT$\beta^{-/-}$ mice controlled early lesion developed and parasite proliferation (Fig. 6, *B* and *C*), and their early immune response was normal and comparable to WT controls (data not shown). Taken together, these results exclude the involvement of LTα1β2 for optimal IL-12 production by DC and resistance to *L. major* in our system.

Administration of rIL-12 to L. major*-infected mice reverses susceptibility caused by HVEM-Ig fusion protein*

Blockade of LIGHT with HVEM-Ig or LTβR-Ig in vitro and in vivo impairs IL-12 production by DC, Th1 response and leads to susceptibility to *L. major* infection in the normally resistant C57BL/6 mice. To determine whether this impaired IL-12 production was responsible for the defective Th1 response and susceptibility to *L. major*, we treated mice given HVEM-Ig with recombinant murine IL-12 (rIL-12) and assessed the outcome of *L. major* infection. As shown in Fig. 7, HVEM-Ig treated mice given rIL-12 exhibited enhanced resistance to *L. major* as evidenced by minimal lesion development and effective parasite control. In fact, the rIL-12-treated groups were more resistant than the control Ig-treated group possibly because the dose of IL-12 we used may be several folds higher than the amount produced in control mice following *L. major* infection. Indeed, mice given control Ig and treated with rIL-12 did not develop any lesion and parasite burden was undetectable, suggesting that despite rIL-12 treatment, HVEM-Ig treated mice were still relatively more susceptible than control-Ig groups (data not shown). Taken together, these results strongly indicate that the susceptibility of HVEM-Ig-treated C57BL/6 mice to *L. major* is due to impaired IL-12 production by DC, which results in defective Th1 response.

Direct evidence for involvement of LIGHT in resistance to L. major

To directly investigate the contribution of LIGHT in resistance to *L. major*, we reconstituted lethally irradiated RAG1$^{-/-}$ mice with bone marrow cells from WT, LIGHT$^{-/-}$, and HVEM$^{-/-}$ mice and infected them with *L. major* 5 wk later. Whereas WT chimeric mice developed lesions that began to heal by week 4, LIGHT$^{-/-}$ chimeric mice developed progressive nonhealing lesion that began to ulcerate after 5 wk necessitating sacrifice of infected mice (Fig. 8*A*). Compared with WT and LIGHT$^{-/-}$ chimeras, the lesions in HVEM$^{-/-}$ chimeric mice were slow to develop, but grew rapidly once started and began to ulcerate as in LIGHT$^{-/-}$ reconstituted mice. Analysis of parasite burden shows massive parasite proliferation in the LN and spleen of LIGHT$^{-/-}$ and HVEM$^{-/-}$ reconstituted mice, which was significantly higher than control WT or WT→WT chimeric mice (Fig. 8, *B* and *C*). This inability to control lesion growth and parasite replication was associated with impaired IL-12 and IFN-γ production by spleen cells from LIGHT$^{-/-}$ and HVEM$^{-/-}$ reconstituted mice (Fig. 8, *D* and *E*). Furthermore, although IL-4 was undetectable, spleen cells from LIGHT$^{-/-}$ and HVEM$^{-/-}$ reconstituted RAG1$^{-/-}$ mice produced more IL-10 than those from WT or WT→WT chimeric

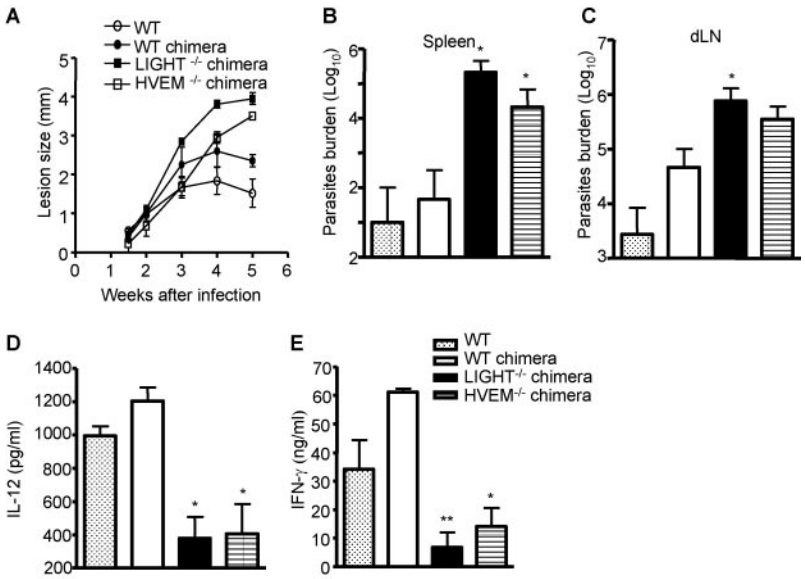

FIGURE 8. The protective anti-*Leishmania* effect of LIGHT is possibly mediated via interaction with HVEM. Bone marrow cells from WT, LIGHT$^{-/-}$, and HVEM$^{-/-}$ mice were transferred by i.v. into lethally irradiated RAG1$^{-/-}$ mice. Five weeks posttransfer, the resulting chimeras were infected with *L. major* and footpad lesion was measured weekly (*A*). Five weeks after infection, the chimeras were sacrificed to estimate parasite burden in the spleens (*B*) and dLN (*C*). Single-cell suspensions of the spleens were stimulated with SLA for 72 h and the levels of IL-12 (*D*) and IFN-γ (*E*) in the culture supernatant fluids were determined by ELISA. The results presented are representatives of two different experiments ($n = 3$–4 mice/group) with similar results. *, $p < 0.05$; **, $p < 0.01$.

mice (WT, 103 ± 26 pg/ml; WT→WT, 156 ± 44 pg/ml; LIGHT$^{-/-}$ → RAG1$^{-/-}$, 1346 ± 109 pg/ml; HVEM$^{-/-}$ → RAG1$^{-/-}$, 1625 ± 239 pg/ml). Treatment of LIGHT$^{-/-}$ → RAG1$^{-/-}$ chimeras with rIL-12 results in cure of cutaneous lesions and parasite control (data not shown). Taken together, these results indicate that the effects of HVEM-Ig and LTβR-Ig were most likely mediated via disruption of LIGHT-HVEM interaction/signaling.

Discussion

These studies report a critical role for LIGHT in IL-12 production by DCs, the development of Th1 cells and resistance to *L. major* infection in mice. This conclusion is based on results from studies using soluble HVEM-Ig and LTβR-Ig fusion proteins, which inhibit the interaction of LIGHT with its receptors, and were further confirmed using LTβ$^{-/-}$ (which excludes a role for membrane LT) and RAG1$^{-/-}$ mice reconstituted with LIGHT$^{-/-}$ and HVEM$^{-/-}$ bone marrow cells. First, LPS-induced production of IL-12 by DCs from LIGHT$^{-/-}$ mice was severely impaired. This impairment was reproduced in DC from WT mice in the presence of HVEM-Ig or LTβR-Ig fusion proteins. Second, in vivo CpG-, LPS-, and *L. major* infection-induced DC recruitment in the dLN and IL-12 production were dramatically blocked by a single injection of HVEM-Ig or LTβR-Ig. Third, following immunization with OVA, injection of HVEM-Ig significantly impaired OVA-specific CD4$^+$ T cell response and IFN-γ production. Fourth, the usually resistant C57BL/6 mice treated with HVEM-Ig or LTβR-Ig became highly susceptible to *L. major* infection. This susceptibility was reproduced in LIGHT$^{-/-}$ → RAG1$^{-/-}$ and HVEM$^{-/-}$ → RAG1$^{-/-}$ chimeras (but not in LTβ$^{-/-}$ mice) and could be reversed by administration of rIL-12, indicating that this defective CD4$^+$ Th1 cell development and susceptibility to *L. major* was due to the impaired IL-12 production.

LIGHT is primarily expressed by activated T cells and immature DCs (4, 5, 44) and binds to three receptors: LTβR, HVEM and in humans, the decoy receptor, DcR3/TR6 (4, 45). HVEM is expressed on resting T cells, NK cells, monocytes, and immature DC (46–48), and has three known ligands: LIGHT, BTLA, and LTα (reviewed in Ref. 33). In contrast, LTβR is expressed mostly on stromal cells, DC, and endothelial cells (49–51) and interacts with LIGHT and LTα1β2 (membrane LT) (1, 52). This shared use of ligands and receptors by members of this family may suggest functional redundancy and presents a challenging problem in dissecting the specific role(s) of these molecules and their ligands in immune response. For instance, HVEM-Ig could, in addition to LIGHT, potentially block the interaction of BTLA and LTα with HVEM. However, the fact that we obtained similar results with LTβR-Ig (which does not affect the interaction of BTLA and LTα with HVEM) suggests that BTLA and LTα may not be playing significant roles in this model. Similarly, LTβR-Ig could potentially bind to LIGHT and LTα1β2 and block their interaction with LTβR. We found that BMDC from LTβ$^{-/-}$ mice (which lack LTα1β2), unlike those from LIGHT$^{-/-}$ mice, were not impaired in IL-12 production in vitro. Furthermore, unlike HVEM-Ig treated WT or LIGHT$^{-/-}$ → RAG1$^{-/-}$ chimeras, LTβ$^{-/-}$ mice do not develop acute leishmaniasis and their early IL-12 and IFN-γ responses following *L. major* infection were unimpaired (Fig. 6). Importantly, our bone marrow chimera experiments support our conclusion that LIGHT is the primary pathway affected in our system. For example, in LIGHT$^{-/-}$ chimeras, the expression of LIGHT is absent whereas HVEM, LTα, and LTα1β2 are intact. Hence, BTLA-HVEM, LTα-HVEM, and LTα1β2-LTβR signaling should be unaffected. In contrast, in HVEM$^{-/-}$ chimeras, LIGHT, BTLA, and LTα signaling via HVEM are disrupted whereas LIGHT-LTβR and LTα1β2-LTβR signaling are intact. Because the outcome of infection in LIGHT$^{-/-}$ and HVEM$^{-/-}$ chimeras is similar, and LTβ$^{-/-}$ mice show no defect in early resistance to *L. major*, the results collectively implicate LIGHT as playing a major role and suggest that BTLA, LTα, and LTα1β2 may only play minor (if any) roles in our system.

We found that blockade of LIGHT severely impaired IFN-γ response but had no effect on T cell proliferation. In contrast, previous reports using allogeneic MLR system show that LIGHT may be important for T cell costimulation and optimal T (CD8$^+$) cell-mediated immune responses (5, 9, 53, 54). However, some reports also show that Ag-specific T cell proliferation is normal in LIGHT-deficient mice or following LIGHT blockade by fusion proteins (10, 55). It is possible that these differences may be related to the frequency of Ag-specific T cells in these systems. The frequency of allospecific T cells, unlike those for model Ags and peptides derived from infectious agents, is unusually very high. The high numbers of allospecific T cells in MLR culture systems may allow HVEM-LIGHT interaction among T cells (T-T interactions) leading to effective T-T cell cooperation and help (and hence minimal DC help). Such interactions may be limited in vivo, especially during model Ag- or infection-driven responses (as in our system), with T-DC interaction providing the major source of costimulation and help to T cells.

Several studies show that LIGHT enhances IFN-γ production and T cell-mediated immunity (5, 7, 9, 53) although the precise mechanisms remain largely unexplored. Recently, it was shown in a mixed alloproliferation system that LIGHT enhancement of allogeneic T cell proliferation and IFN-γ production involves direct activation of T cells and is independent of IL-12 (53). Similar to these studies, we found that deficiency of LIGHT (as in LIGHT$^{-/-}$ → RAG$^{-/-}$ chimeric mice) or its blockade with HVEM-Ig or LTβR-Ig dramatically affected IFN-γ production in vitro (anti-CD3 stimulation) and in vivo (OVA immunization and *L. major* infection). However, unlike Brown et al. (53), we found that the impaired IFN-γ response following LIGHT blockade was due to defects in IL-12 production. Treatment of HVEM-Ig-treated WT mice or LIGHT$^{-/-}$ → RAG$^{-/-}$ chimeras with rIL-12 abolished the impaired IFN-γ response and reversed their susceptibility to *L. major*, strongly suggesting that LIGHT enhancement of T cell immunity and IFN-γ response in mice infected with *L. major* is mediated via IL-12. This is consistent with previous report showing that LIGHT enhancement of allogeneic T cell proliferation, cytokine secretion, and CTL responses is mediated via its effect on DC maturation including expression of costimulatory molecules and cytokine (including IL-12) release (56).

Of crucial importance is the mechanism by which LIGHT enhances IL-12 production by DCs following stimulation with various ligands in vitro and in vivo. It has been shown that the interaction of LIGHT expressed on T cells and HVEM on DC or vice versa is important for T cell-mediated maturation of DC and IL-12 production (56, 57). In contrast, a recent report indicates that the interaction of LTα1β2 expressed on T cells and LTβR on DC is critical for DC activation, expression of costimulatory molecules, and enhancement of T cell response (43). Recently, Smith et al. (58) showed that human HSV6 blocks LPS-induced IL-12p40 production by DCs, an effect that most likely was mediated via binding of HSV glycoprotein D to HVEM (4). Our data are consistent with the reports showing that LIGHT enhances optimal IL-12 production by DC. Furthermore, our studies suggest that the effect of LIGHT may be to enhance agonistic signals including TLR (e.g., LPS and CpG) and non-TLR (e.g., anti-CD40 mAb) on DC leading to enhanced IL-12 production. In line with this, we found that HVEM-Ig or LTβR-Ig did not completely block IL-12 production

by DC following stimulation with LPS, CpG, or anti-CD40 mAb and the expression of costimulatory molecules and IL-12 production by LIGHT$^{-/-}$ LPS-stimulated BMDC was not completely abolished. Thus, as in previous reports, our results indicate that LIGHT cooperates with DC stimulatory/agonistic signals leading to enhanced DC maturation and IL-12 production (56, 57). Furthermore, because these effects were seen in in vitro cultures of pure DC, our results suggest a possible role for direct LIGHT receptor interaction on APCs (i.e., DC-DC interaction) for LIGHT-mediated enhancement of DC maturation and IL-12 production. However, it is conceivable that in the microenvironment of the peripheral lymphoid organs in vivo, both T cell-DC and DC-DC LIGHT-LIGHT receptor interactions may be operational for optimal IL-12 production by DC following stimulation with various agonists including LPS, CpG, and CD40L.

Our finding that LIGHT enhances resistance to *L. major* and this effect is mediated via enhancement of IL-12 production leading to optimal CD4$^+$ Th1 cell response is a critically important and novel. Injection of HVEM-Ig or LTβR-Ig into the usually resistant B6 mice results in impaired IL-12 production and susceptibility to both high- and relatively low-dose *L. major* infection, which was reversed by rIL-12 administration (Figs. 4 and 7). Furthermore, *L. major*-infected LIGHT$^{-/-}$ $\rightarrow$ RAG$^{-/-}$ chimeras develop severe ulcerative disease associated with defective IL-12- and Ag-specific IFN-γ responses (Fig. 8). Previous studies suggest that CD40-CD40L interaction is the major pathway involved in IL-12 production by DC during *L. major* infection and disruption of this pathway in mice leads to susceptibility to cutaneous leishmaniasis (20, 21, 59). However, recent reports suggest that this pathway may be dispensable particularly during low-dose *L. major* infection (60, 61), strongly suggesting that other pathways may contribute to IL-12 production in leishmaniasis. The fact that CD40-CD40L pathway is intact in the LIGHT$^{-/-}$ $\rightarrow$ RAG$^{-/-}$ chimeras or WT mice treated with HVEM-Ig fusion proteins suggest that LIGHT plays a hitherto unknown major role in resistance to *L. major* infection in mice. However, it is possible that signals generated by LIGHT interaction with its receptor(s) may serve to augment CD40-CD40L signal leading to optimal IL-12 production in APC.

In summary, we have demonstrated that signals mediated via LIGHT interaction with its receptor are critically important for DC maturation (costimulatory molecule expression) and optimal IL-12 production following stimulation with anti-CD40 (T cell-mediated signal), LPS (TLR-4), CpG ODN (TLR-9), and *L. major*. Interestingly, blockade of this pathway does not affect other DC effector functions (e.g., IL-6, TNF-α, and NO production). Most importantly, disruption of this interaction leads to a significant impairment in polyclonal and Ag-specific CD4$^+$ Th1 cell-mediated (IFN-γ) responses without affecting Th2 cell responses. In this regard, LIGHT could provide a new therapeutic target for regulation of IL-12 production in vivo and for controlling various CD4$^+$ Th1 cell-mediated autoimmune and inflammatory diseases such as arthritis, diabetes, colitis, etc.

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Disclosures

The authors have no financial conflict of interest.

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