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LIGHT, a TNF-Like Molecule, Costimulates T Cell Proliferation and Is Required for Dendritic Cell-Mediated Allogeneic T Cell Response

Koji Tamada,‡ Koji Shimozaki,‡ Andrei I. Chapoval,* Yifan Zhai,‡ Jeffery Su,‡ Su-Fang Chen,§ Shie-Liang Hsieh,§ Shigekazu Nagata,§ Jian Ni,§ and Lieping Chen§*

LIGHT is a recently identified member of the TNF superfamily and its receptors, herpesvirus entry mediator and lymphotoxin β receptor, are found in T cells and stromal cells. In this study, we demonstrate that LIGHT is selectively expressed on immature dendritic cells (DCs) generated from human PBMCs. In contrast, LIGHT is not detectable in DCs either freshly isolated from PBMCs or rendered mature in vitro by LPS treatment. Blockade of LIGHT by its soluble receptors, lymphotoxin β receptor-Ig or HVEM-Ig, inhibits the induction of DC-mediated primary allogeneic T cell response. Furthermore, engagement of LIGHT costimulates human T cell proliferation, amplifies the NF-κB signaling pathway, and preferentially induces the production of IFN-γ, but not IL-4, in the presence of an antigenic signal. Our results suggest that LIGHT is a costimulatory molecule involved in DC-mediated cellular immune responses. The Journal of Immunology, 2000, 164: 4105–4110.

Accumulating evidence demonstrates that two different signals are necessary for the optimal activation of T cell response, a first signal delivered from TCR and a second signal from costimulatory molecules such as CD80 and CD86 (1). Costimulation is known to play important roles in numerous immune responses both in vitro and in vivo. For example, antigenic stimulation of T cells in the absence of costimulation induces an unresponsive state known as anergy (2). In vivo activation of costimulatory pathway by gene transfer (3) or an agonistic Ab (4) enhances antigen immune response leading to regression of tumor. On the other hand, blockade of costimulatory pathways can ameliorate undesirable immune responses such as autoimmunity or graft-versus-host response (5, 6). Costimulatory molecules B7-1 and B7-2 are expressed on professional APCs, particularly on dendritic cells (DCs), and play a critical role in the initiation and regulation of primary T cell response (7).

Recent studies show that several molecules belonging to the TNF superfamily function as costimulatory molecules for the induction, differentiation, and survival of the immune cell (8). For example, 4-1BB and OX-40 can costimulate T cell growth and affect the differentiation of subsets of Th cells (9, 10). Administration of anti-4-1BB mAbs elicits curative antitumor immune responses against established tumors in several mouse models (4), indicating that costimulatory functions of the TNF superfamily can be manipulated toward therapeutic benefit. LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for herpesvirus entry mediator (HVEM), a receptor expressed by T lymphocytes) is a recently identified member of TNF superfamily (11). Several studies showed that MLR can be enhanced by inclusion of soluble LIGHT (12) and can be inhibited by neutralization of LIGHT (13, 14). These results suggest that LIGHT is involved in T cell activation. However, the effector cells and precise mechanisms of LIGHT in T cell activation remain unknown. Therefore, we study the expression of LIGHT on human DC and its role in costimulation of T cell responses.

Materials and Methods

Fusion proteins and Abs

To prepare human (h)LIGHT as a soluble protein, a 542-bp fragment of extracellular domain of hLIGHT (Q60–V240) was amplified from the full-length LIGHT cDNA clone (11) by PCR (sense primer, 5′-GCGGGATCCACGCTGCACTGGCGTCTAG-3′; antisense primer, 5′-GCGGGATTCACCATGAAAGCCCGGA-3′). After digestion with BamHI, the PCR product was cloned into pc4CK8-IgFc vector, which contains CK8 signal peptide (15), to yield a construct encoding amino acid residues Q60–V240 of the LIGHT sequence fused at the C terminus with the 234-residue hinge of the CH2 and CH3 regions of hIgG1 (16). The construct was then transfected into Chinese hamster ovary cells, and the hLIGHT-Ig fusion protein was purified from the conditioned media by a Sepharose-Protein A affinity column. Isolated hLIGHT-Ig was greater than 95% purity, and LPS content was less than 0.1 ng/mg of purified protein according to Limulus amebocyte lysate assays (Sigma, St. Louis, MO).

Production and preparation of hHVEM-Ig (17) and human lymphotoxin β receptor (hLTβR)-Ig (18) were previously described. hCTLA4Ig was constructed following the protocol described previously (19) and was purified from the culture supernatant of Chinese hamster ovary cells by a Sepharose-Protein A affinity column. A similar method was used for preparation of murine (m)4-1BB-Ig.

Rabbit anti-LIGHT Ab (ML69) was prepared at Cocalico Biologicals (Reamstown, PA) by immunizing rabbits with a keyhole limpet hemocyanin-conjugated synthetic peptide encoding mLIGHT 59–69 (HLPDGGKQG...).
PBMCs were separated from healthy donors by Ficoll-Hypaque (Phar-macia Biotech, Uppsala, Sweden) density gradient centrifugation and cultured in the complete medium consisting of RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% FBS (HyClone, Logan, UT), 25 mM HEPES, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate. To purify T cells, adherent cells were first eliminated by incubation on plastic tissue culture plates for 2 h at 37°C, and nonadherent cells were collected and passed through nylon wool columns (Robbins Scientific, Sunnyvale, CA). Purity of isolated human T cells was >85%.

CD3+ T cells were generated from adherent PBMCs by culture in the complete medium in the presence of 800 U/ml GM-CSF (Immunex, Seattle, WA) and 500 U/ml IL-4 (R&D Systems, Minneapolis, MN) for 7 days, as previously reported (21). For induction of allogeneic MLR, purified T cells (2 x 10^6 cells/ml) were cocultured with 40 Gy-irradiated allogeneic DCs (2 x 10^5 cells/ml) in flat-bottom 96-well microplates in the presence of indicated fusion proteins or control IgGl (hlgGl; Sigma). For anti-CD3 mAb-induced T cell proliferation assay, flat-bottom 96-well microplates were first coated with the indicated doses of an anti-CD3 mAb diluted in 50 µl PBS at 4°C for 18 h. After washing, the plates were further coated with hLIGHT-Ig or hlgGl at 37°C for 4 h, and then purified T cells (1 x 10^5 cells/ml) were added to the wells in the presence or absence of the fusion proteins.

In all proliferation assays, the cells were cultured for 72 h, and [3H]TdR at 1 µCi/well was added during the last 15 h. Incorporation of 1[3H]TdR was counted by a MicroBeta TriLux liquid scintillation counter (Wallac, Turku, Finland).

Flow cytometric analysis of DCs

Human immature DCs were generated from adherent PBMCs as described above. To prepare mature activated DCs, immature DCs were further incubated in the presence of LPS (1 µg/ml; Sigma) or anti-human CD40 mAb (clone G28.5 at 5 µg/ml) for 24 h. Immature or mature DCs were stained at 4°C for 30 min with either 2 µg of HVEM-Ig or hLTβR-Ig or 1 µg of anti-LIGHT Ab in 100 µl of PBS supplemented with 3% FBS and 0.02% azide. Cells were washed and further incubated at 4°C for 30 min with either FITC-conjugated goat anti-human IgG (BioSource, Camarillo, CA) or FITC-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL). DCs were also stained with FITC-conjugated anti-CD86 mAb as the positive control. Human peripheral blood fresh DCs were enriched and identified as previously reported (22) with some modifications. Briefly, T, B, and NK cells and macrophages were depleted from PBMCs by passing through a magnetic field twice after staining with a cocktail of FITC-conjugated anti-CD3, -CD19, -CD16, and -CD14 mAbs and then with anti-FITC magnetic beads (Miltenyi Biotec, Auburn, CA). After depletion, negatively enriched cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-HLA-DR mAbs. A subpopulation, which represents large and bright cells in forward and side scatter and is equivalent to 25% of the enriched cells (see Fig. 1C), was identified to express CD4 and HLA-DR. The subpopulation was gated and analyzed for the expression of LIGHT by ML69.

Flow cytometry was analyzed by FACS Calibur flow cytometry (Becton Dickinson, Mountain View, CA) with CellQuest software (Becton Dickinson). All mAbs used for FACS analysis were purchased from PharMingen.

NF-kB assay

The activation of NF-kB was determined by gel shift assay as described previously (23) but with modifications. Human T cells (1 x 10^6 cells/ml) purified from PBMCs were incubated in the plate, which was precoated with 2 µg/ml of anti-CD3, in the presence or absence of soluble hLIGHT for 20 min. Cells were pelleted, suspended in 150 µl HEPES-KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT supplemented by protease and phosphatase inhibitors (0.1 mM Na2VO4, 10 mM NaF, 1 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml pepstatin A, and 2 µg/ml leupeptin), and lysed by adding 10 µl of 10% Nonidet P-40. Nuclei were collected by centrifugation, suspended in 25 µl buffer B (20 mM HEPES-KOH (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT), and supplemented with the phosphatase and protease inhibitors. After incubation at 4°C for 20 min, the nuclei were centrifuged for 10 min at 15,000 x g, and the supernatants were recovered as nuclear extracts. A set of complementary oligonucleotides (5'-TC GAGAGTGAAGGGGGCTTCCAGG-3') that carry the NF-kB binding site were annealed and end-labeled with [γ-32P]deoxycytidine 5'-triphosphate (3,000 Ci/mmole) and the Klenow fragment of Escherichia coli DNA polymerase. The reaction mixture for the gel shift assay contained 20 mM HEPES buffer (pH 7.8), 80 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, 2 µg poly(dI-dC), 10 µg of the nuclear extracts, and 62.5 fmol probe DNA (132,000 cpm) in a total volume of 17 µl. After incubation at room temperature for 20 min, the products were resolved by electrophoresis on a 4% polyacrylamide gel in 0.25 x TBE buffer. Soluble hLIGHT was produced in COS cells by transfecting with pEF-BOS-EX vector (24) inserted with full-length hLIGHT cDNA (11). The culture supernatant was collected at 72 h after transfection and used as the soluble hLIGHT.

Cytokine assay by ELISA

Human purified T cells (1 x 10^6 cells/ml) were stimulated with plate-coated hLIGHT-Ig (10 µg/ml) or control hlgGl (10 µg/ml) in the presence of indicated doses of plate-coated anti-CD3 mAb. After 2, 3, 4, and 5 days, the culture supernatants were harvested, and the cytokine concentration was assayed by sandwich ELISA for IFN-γ and IL-4 according to the manufacturer’s instructions (PharMingen).

Results

Selective expression of LIGHT on human immature DCs

The expression of LIGHT on human DCs during differentiation was determined by FACS analysis. Immature human DCs were generated from adherent PBMCs of healthy donors by standard method (21). After 7 days of culture in the presence of GM-CSF + IL-4, contaminated macrophages (CD14+ and B cells (CD19+) were typically less than 6 and 4%, respectively (data not shown). Immature DCs exhibited a low-level expression of CD86 (Fig. 1A, right panel) and few dendrites (data not shown). Staining of immature DCs with a soluble receptor of LIGHT, hLTβR-Ig, and hHVEM-Ig demonstrated significant binding (Fig. 1A, left panel). We also stained cells with an anti-LIGHT Ab, ML69, specific for both human and mLIGHT (data not shown). A significant staining with ML69 was demonstrated on immature DCs (Fig. 1A, center panel).

The expression of LIGHT on mature DCs was examined after further stimulation of immature DCs with LPS or anti-CD40 mAb. Consistent with previous studies (25, 26), the expression level of CD86 on activated DCs increased significantly. However, the expression of LIGHT was remarkably down-regulated on the surface of mature DCs after exposure to LPS or anti-CD40 mAb (Fig. 1B).

We further investigated the expression of LIGHT on freshly isolated DCs. CD3+, CD14+, CD16+, and CD19+ cells were depleted from PBMCs of healthy donors with magnetic beads and subsequently were stained with mAbs to HLA-DR and CD24 (22, 27, 28). An HLA-DR "CD4" subset of large and bright cells (Fig. 1C, left panel) after depletion was identified as containing peripheral blood DCs (Fig. 1C, center panel). Anti-LIGHT Ab ML69 did not stain this subset (Fig. 1C, right panel), indicating that LIGHT is not expressed on peripheral blood DCs.

Inhibition of DC-induced allogeneic T cell response by blockade of LIGHT

To determine the role of LIGHT in DC-induced primary allogeneic T cell response, purified T cells were stimulated with allogeneic DCs in the presence of hHVEM-Ig or hLTβR-Ig. As shown in Fig. 2, DCs induce a high level of proliferation of allogeneic T cells. However, inclusion of hHVEM-Ig or hLTβR-Ig significantly inhibited the allogeneic T cell response in a degree similar to that in hCTLA4-Ig, a soluble receptor for B7 molecules (19), whereas neither hlgGl nor m4-1BB-Ig control protein exhibited inhibitory effects. Also, both hHVEM-Ig and hLTβR-Ig saturate at concentrations of 0.625-1.25 µg/ml, and the inhibitory effect plateaus at
FIGURE 1. LIGHT is expressed on immature but not on mature DCs and peripheral blood DCs. A, Human immature DCs were prepared from adherent PB-MCs by culture with 800 U/ml GM-CSF and 500 U/ml IL-4 for 7 days. B, Mature DCs were generated from the immature DCs by the stimulation with 1 µg/ml LPS or 5 µg/ml anti-CD40 mAb for an additional 24 h. These DCs were incubated with either hHVEM-Ig, LTβR-Ig, or rabbit anti-LIGHT Ab (ML69) for 30 min at 4°C and subsequently were stained with FITC-conjugated anti-human IgG or FITC-conjugated anti-rabbit IgG (solid line). DCs were also stained with FITC-conjugated anti-CD66 mAb (solid line). Normal human IgG or rabbit IgG was used as control (dashed line). C, For analysis of peripheral blood DCs, PBMCs of healthy donors were depleted with CD3-, CD14-, CD16-, and CD19-expressing cells by magnetic cell separation system. Among the resulting lineage Ag-negative cells, a subpopulation representing large and bright cells in forward and side scatters was gated as R1 (left panel). The R1 population was analyzed by staining with FITC-conjugated CD4 and PE-conjugated HLA-DR mAbs (center panel). The expression of LIGHT on R1 population was also examined with ML69 (solid line) or control rabbit IgG (dashed line) and then with FITC-conjugated anti-rabbit IgG (right panel).

Costimulatory activity of LIGHT in T cell response induced by anti-CD3 mAb

B7-1 and B7-2 are believed to be the key costimulators for DC-mediated T cell response (7). To determine whether LIGHT can act as a T cell costimulator, hLIGHT-Ig was immobilized on a 96-well plate to stimulate purified naive T cells in the presence of anti-CD3 mAb as a mimicking antigenic signal. Immobilized hLIGHT-Ig significantly enhanced the proliferation of T cells in the presence, but not in the absence, of anti-CD3. Maximal effect of LIGHT on the stimulation of T cells was shown when anti-CD3 was used in suboptimal concentrations (range, 0.02–0.08 µg/ml) (Fig. 3A), demonstrating that hLIGHT-Ig costimulates T cell growth when engagement of TCR occurs. A significant costimulatory effect could be detected using as little as 2.5 µg/ml hLIGHT-Ig, and increased concentration of the fusion protein can further stimulate T cell growth in a dose-dependent manner (Fig. 3B). Costimulatory effects of cross-linked hLIGHT-Ig can be completely abrogated by soluble hHVEM-Ig or hLTβR-Ig (Fig. 3C), but not by the control hlgG. Our results thus demonstrate that LIGHT can serve as a costimulatory molecule for stimulation of T cell growth.

NF-κB activation on T cells by LIGHT costimulation

NF-κB activation and translocation is a crucial event in the activation of T cells (29). To determine whether LIGHT can costimulate the activation of NF-κB in T cells, we performed the gel shift assay to directly detect NF-κB translocation in nuclear extracts of activated T cells. The activated NF-κB was detectable in T cells stimulated with either anti-CD3 (Fig. 4, lane 4) or LIGHT protein (lane 3) alone. However, highest levels of NF-κB activity can be found in T cells stimulated by a combination of anti-CD3 and LIGHT protein (lane 2). The assay is NF-κB binding-specific because inclusion of an unlabeled oligonucleotide carrying the NF-κB binding site can inhibit the binding of labeled probes (lanes 5 and 6). Our result indicate that LIGHT can costimulate NF-κB activation and translocation in T cells.

Preferential induction of IFN-γ secretion by LIGHT costimulation

Secretion of IFN-γ and IL-4 from T cells after LIGHT costimulation was determined by specific sandwich ELISA. The production of IFN-γ, but not IL-4, in the culture supernatant was significantly increased in the presence of both anti-CD3 and hLIGHT-Ig in comparison to anti-CD3 alone or anti-CD3 with control hlgG (Fig. 5A). Supernatant from T cells stimulated with hLIGHT-Ig and anti-CD3 was concentrated and tested for IFN-γ and IL-4 by ELISA under the same condition (Fig. 5B, B). The results are expressed as the means ± SD of triplicate wells.
alone in the absence of anti-CD3 did not have detectable IFN-γ production (data not shown). We further examined kinetics of cytokine secretion in T cells activated by LIGHT costimulation. As shown in Fig. 5B, sustained enhancement of IFN-γ production was detected in the supernatants of T cells stimulated with anti-CD3 and hLIGHT-Ig, whereas no significant increase of IL-4 production was observed during the culture for up to 5 days. Therefore, these results suggest that LIGHT costimulation preferentially induces the production of IFN-γ.

**Discussion**

In this study, we demonstrate that LIGHT, a TNF-like molecule, is a novel costimulatory molecule for human naive T cells to proliferate and produce IFN-γ. LIGHT is expressed on human immature DC and is required to initiate primary allogeneic T cell responses induced by DC. Our studies thus suggest that LIGHT costimulation may be involved in the induction of cell-mediated immune responses.

It is generally believed that adherent PBMCs or peripheral blood monocytes give rise to immature myeloid DCs via the culture with GM-CSF and IL-4 (21). Further maturation can be driven by exposure to bacterial endotoxin or CD40 ligand (25, 26). Accompanied with their maturation, Ag uptake/processing functions of DCs reduce, whereas their capacities to stimulate T cell responses increase. This shift of the function is at least partially due to up-regulation of costimulatory molecules (7). Consistently with this observation, CD86 expression was up-regulated by LPS on mature DCs (Fig. 1, A and B). However, the expression of LIGHT decreases during the maturation of DCs in our study (Fig. 1, A and B). It is likely that down-regulation of LIGHT on mature DCs is due to a cleavage of membrane-bound form. It has been shown that the molecules of the TNF family, such as TNF-α, Fas ligand, and CD40 ligand, are known to be cleaved by matrix metalloproteinases (30, 31). In these cases, cleaved molecules remain functional.
The culture supernatants were harvested, and the concentration of IFN-γ was measured. Purified human T cells (10^6 cells/ml) were incubated in the 96-well microplates, which were first coated with indicated doses of anti-CD3 and subsequently with 10 μg/ml of either hLIGHT-Ig or control hlgG for 2 days (A). Purified human T cells (10^6 cells/ml) were similarly incubated in the 96-well microplates coated with 40 ng/ml anti-CD3 and then with 10 μg/ml of either hLIGHT-Ig or control hlgG for 3, 4, or 5 days (B). The culture supernatants were harvested, and the concentration of IFN-γ and IL-4 was assessed by sandwich ELISA. The results are expressed as the means ± SD of triplicate wells.

**FIGURE 5.** LIGHT costimulates T cells to produce IFN-γ but not IL-4. Purified human T cells (1 × 10^6 cells/ml) were incubated in the 96-well microplates, which were first coated with indicated doses of anti-CD3 and subsequently with 10 μg/ml of either hLIGHT-Ig or control hlgG for 2 days (A). Purified human T cells (1 × 10^6 cells/ml) were similarly incubated in the 96-well microplates coated with 40 ng/ml anti-CD3 and then with 10 μg/ml of either hLIGHT-Ig or control hlgG for 3, 4, or 5 days (B). The culture supernatants were harvested, and the concentration of IFN-γ and IL-4 was assessed by sandwich ELISA. The results are expressed as the means ± SD of triplicate wells.

It is possible that cleaved LIGHT can still costimulate T cell growth. Our preliminary experiments indicate that LIGHT is detectable as a soluble form in culture supernatants of the cells transfected with LIGHT gene and that a metalloproteinase inhibitor can decrease a release of soluble LIGHT (our unpublished data). Our results thus implicate that matured DCs can regulate T cell responses by releasing LIGHT as a cytokine.

DCs are believed to be the most potent and crucial APCs in initiating primary T cell responses. Using HVEM-Ig and LTRβR-Ig fusion proteins, we demonstrated that blockade of LIGHT can inhibit the optimal induction of primary T cell responses to allogeneic DCs (Fig. 2). Furthermore, ligation of LIGHT in the presence of TCR stimulation costimulates naïve T cells to proliferate and produce IFN-γ (Figs. 3 and 5). Our results thus extend previous findings and indicate that LIGHT is an important costimulator in DC-mediated stimulation of primary T cell responses. Costimulatory effect of LIGHT is likely mediated by its interaction with HVEM, which is expressed on the majority of PBMCs, including resting T cells (14). In addition to HVEM, two additional receptors, LTRβR and TR6, have been reported (11, 32, 33). However, LTRβR is not found on the surface of T cells (34) and TR6, a new TNFR-like molecule, is found only as a decoy receptor without membrane-anchor sequence (32, 33). Taken together with the fact that T cell activation can be inhibited by anti-HVEM blocking mAb (14), interaction between LIGHT and HVEM may be critical for the costimulatory function.

It has been shown that overexpression of HVEM in the non-lymphoid line 293 stimulates TNFR-associated factor 1, 2, 3, and 5 binding and activates NF-κB and AP-1 signaling pathways (17, 35). Furthermore, engagement of HVEM-expressing U937 cells with soluble LIGHT induced a weak increase of luciferase activity of a NF-κB-luciferase reporter gene (12). Despite these reports, it remains unknown whether LIGHT activates NF-κB in resting human T cells and whether a combined stimulation of TCR and LIGHT induces a synergistic effect on NF-κB activation. We addressed these issues and demonstrated that LIGHT costimulation induces NF-κB translocation in human resting T cells in a synergistic manner with anti-CD3 stimulation (Fig. 4).

In addition to LIGHT, several members of the TNFR superfamily, including 4-1BB and OX-40, have been shown to costimulate T cell growth and induce TNFR-associated factor binding and NF-κB activation (9, 10, 36). However, the expression and function of these molecules appear to have individual characteristics. For example, 4-1BB engagement preferentially activates CD8+ T cells compared with CD4+ T cells (10), whereas OX-40 plays a predominant role on CD4+ T cells because of its limited expression on CD4+ T cells (9). In addition, the OX-40 costimulation polarizes T cells toward Th2-type responses (37), whereas the LIGHT costimulates T cells to produce IFN-γ but not IL-4 (Fig. 5). HVEM is constitutively expressed on resting T cells and is transiently down-regulated after activation (14), which is a remarkable contrast to the inducible expression of 4-1BB and OX-40 on activated T cells (36). These results indicate the respective roles of these costimulatory molecules in the initiation and expansion phases of T cell response. Our results thus define a new costimulatory molecule of the TNF family that is potentially involved in the regulation of dendritic cell-mediated T cell activation.

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**References**


