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Reciprocal Expression of the TNF Family Receptor Herpes Virus Entry Mediator and Its Ligand LIGHT on Activated T Cells: LIGHT Down-Regulates Its Own Receptor


The TNF receptor (TNFR) superfamily plays a central role in the development of the immune response. Here we describe the reciprocal regulation of the recently identified TNFR superfamily member herpes virus entry mediator (HVEM) (TR2) and its ligand LIGHT (TL4) on T cells following activation and the mechanism of this process. T cell activation resulted in down-regulation of HVEM and up-regulation of LIGHT, which were both more pronounced in CD8+ than CD4+ T lymphocytes. The analysis of HVEM and LIGHT mRNA showed an increase in the steady state level of both mRNAs following stimulation. LIGHT, which was present in cytoplasm of resting T cells, was induced both in cytoplasm and at the cell surface. For HVEM, activation resulted in cellular redistribution, with its disappearance from cell surface. HVEM down-regulation did not rely on de novo protein synthesis, in contrast to the partial dependence of LIGHT induction. Matrix metalloproteinase inhibitors did not modify HVEM expression, but did enhance LIGHT accumulation at the cell surface. However, HVEM down-regulation was partially blocked by a neutralizing mAb to LIGHT or an HVEM-Fc fusion protein during activation. As a model, we propose that following stimulation, membrane or secreted LIGHT binds to HVEM and induces receptor down-regulation. Degradation or release of LIGHT by matrix metalloproteinasises then contributes to the return to baseline levels for both LIGHT and HVEM. These results reveal a self-regulating ligand/receptor system that contributes to T cell activation through the interaction of T cells with each other and probably with other cells of the immune system. The Journal of Immunology, 2000, 165: 4397–4404.
Confocal immunofluorescence analysis

Cells were deposed on coverslips at a concentration of 1 \times 10^6/ml and fixed in 3% paraformaldehyde (Fluka, St. Quentin Fallavier, France). Then cells were indirectly stained with biotinylated anti-HVEM 12C5 (Smith-Kline Beecham) or anti-LIGHT (SmithKline Beecham) mAbs, followed by streptavidin-Ala 488 (Molecular Probes, Eugene, OR). For membrane staining, we preincubated cells for 2 min with the red fluorescence Cell-Tracker CM-Dil (Molecular Probes). Serial optical sections were obtained using the TCS 4D laser scanning confocal microscope (Leica, Heidelberg, Germany). Microscope settings were adjusted in to black level values when cells were stained with the mouse isotypic Ig control.

Protein synthesis inhibitor and matrix metalloprotease (MMP) inhibitors

Inhibitors were added at the beginning of the culture. The protein synthesis inhibitor cycloheximide (CHX; Sigma) was used at 10 \mu g/ml. The MMP inhibitor KB8301 (PharMingen), which was shown to block FasL cleavage (26, 27) was used at 1 \mu M according to the manufacturer’s instructions. The BB94 broad spectrum MMP inhibitor (27) (a gift from B. Mang, Institut National de la Sante et de la Recherche Medicale, Nice, France) was used at 1 \mu M. Cell viability was assessed by trypan blue exclusion and by propidium iodide staining for flow cytometry analysis.

Nucleic acid preparation, RT-PCR amplification, and semiquantitative analysis

Total RNA was isolated from 1.5 to 5 \times 10^6 cells for each sample by suspension in Trizol (Life Technologies, Grand Island, NY) and extraction by phenol-chloroform, as recommended by the manufacturer. Total RNA (2.5 \mu g) was reverse transcribed using Moloney murine leukemia virus Superscript reverse transcriptase and random hexamers according to the manufacturer’s instructions (Life Technologies). For PCR, 2.5 \mu l of this cDNA was used as the target in a total volume of 25 \mu l containing 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.5), 200 \mu M each of dNTP, 1 pmol/\mu l of primers, and 1.25 U of Taq polymerase (Perkin-Elmer, Norwalk, CT). The amplification was performed in a Touchdown Temperature Cycling System (Hybaid, Teddington, U.K.); the first cycle was at 94°C for 3 min, then annealing at 65°C for 30 s, extension at 72°C for 30 s, and denaturation at 94°C for 30 s (25–35 cycles for HVEM and LIGHT), terminating with 10 min at 72°C. Evaluation of the transcripts was performed by gel analysis using the Bio-Imaging Analyzer MacBAS V2.5 (Fuji Photo Film, Kodak Systems, Tokyo, Japan). Local background is subtracted for each signal. Results are expressed in arbitrary units (A.U.) as the ratio of signal intensity by \beta-actin signal intensity.

Primers

The housekeeping gene \beta-actin was used as a control to assess RT and PCR efficiency using the following primers: sense, 5'-ggc atc gtt atg gac tcc gtc a-3' and antisense, 5'-gtc gga agg tgg aca gca g-3'. The primer set for detection of HVEM was: sense, 5'-gct cat cet gtc gea tgg gtt cc-3'; and antisense, 5'-act tgg tct ggt gtt gac att cct-3'. The primer set for LIGHT was: sense, 5'-gag cga agg tgt cag gtc a-3' and antisense, 5'-cca ggc gtt cat cca gca ca-3'.

Results

Regulation of HVEM and LIGHT expression in purified T lymphocytes

We first tested the regulation of HVEM and LIGHT surface expression during T cell stimulation in vitro using the superantigens SEA plus SEB, which have specificity for V\beta8 and V\beta3, respectively. As shown in Fig. 1, we observed a down-regulation of HVEM in the activated (CD25+) T lymphocyte subpopulation (Fig. 1C) compared with unstimulated (CD25+) T lymphocytes (Fig. 1A). At the same time, we observed an up-regulation of LIGHT in the stimulated T cells (Fig. 1D) compared with unstimulated T lymphocytes (Fig. 1B).

To gain insight into this apparent reciprocal regulation of HVEM and LIGHT, we evaluated their expression in several in vitro activation systems. CD28 mAbs, CD2 mAbs, PHA, IL-2, ionomycin, or PMA were tested alone or in combination. The most

Materials and Methods

Blood samples and cell separation

PBMCs from healthy donors were isolated on Ficoll-Hypaque gradients (24). T lymphocytes were isolated as the CD2-positive PBMC population, corresponding to cells that adhere to sheep erythrocytes (25) in the E-rosetting technique, but fail to adhere to plastic dishes after overnight incubation in medium and 30% FCS.

For RT-PCR analysis, positive CD4+ and CD8+ T cell isolation was performed by flow cytometry using fluorescent anti-CD4 or anti-CD8 mAbs on FACSvantgage cell sorter (Becton Dickinson, Mountain View, CA). The purity of the sorted CD8+ and CD4+ cells, evaluated by reanalysis, was \geq 99%.

For flow cytometric experiments, we isolated CD4+ and CD8+ T cell by two rounds of negative selection using magnetic beads (Beckman Coulter, Mingen, San Diego, CA). The purity of the isolated CD8+ and CD4+ cells by flow cytometry analysis was \geq 95%.

Culture conditions and dendritic cell (DC) generation

Culture experiments were performed in RPMI 1640 (Bioproducts, Walkersville, MD) with 10% FBS (Bioproducts). T lymphocytes were cultured at 10^6/ml. For DC generation, PBMCs were depleted of nonadherent cells by 4-h adhesion on plastic dishes. Adherent cells were then cultured in 10% RPMI 1640 (Bioproducts) with GM-CSF (Sandoz, Copenhagen, Denmark) at 100 ng/ml and IL-4 (Genzyme, Cambridge, MA) at 10 ng/ml for 6 days. The medium was replenished with cytokines every 3 days. On day 6 final maturation was induced by the addition of 50 ng/ml TNF-\alpha (PromoCell, Heidelberg, Germany) for an additional 72 h.

T cell stimulation

For superantigen stimulation, T lymphocytes were incubated with mature autologous DCs at a 10:1 ratio, in the presence of staphylococcal enterotoxins A (SEA; Toxin Technology, Sarasota, FL) and E (SEE; Toxin Technology) at 10 ng/ml. The other stimuli used were 1 ng/ml PMA (Sigma, St. Louis, MO) and 1 \mu M ionomycin (Sigma). In some experiments, the neutralizing LIGHT mAb 2C8 or recombinant HVEM-Fc was added at 5 \mu g/ml at the start of the culture.

Flow cytometric studies

For surface staining, cells were processed following standard procedures, and analysis was performed on a FACSscan flow cytometer (Becton Dickinson). The mAbs directed against HVEM (12C5 and 2D4, both murine IgG1) and LIGHT (2C8, murine IgG2b) were generated at Smith-Kline Beecham by conventional hybridoma methodology from mice immunized with the respective recombinant proteins and screening the hybridomas by ELISAs. The mAbs for CD1a, CD3, CD4, CD8, CD14, CD19, CD25, CD56, CD69, and CD83 were obtained from Beckman Coulter (Brea, FL). The mAb for CD80 was purchased from Becton Dickinson, and the mAbs for CD86, CD40L, and FasL were obtained from PharMingen (San Diego, CA).

For intracellular detection, cells were washed twice in 1× PBS, 0.5% (w/v) BSA (Sigma), and 0.1% (w/v) saponin (Sigma). Then, 5 \times 10^4 cells in 100 \mu l were stained in the same medium with the relevant mAbs. Cells were washed once in the same medium, once in PBS-BSA and then fixed for 10 min at room temperature in 1× PBS and 0.5% formaldehyde (Sigma). All data are presented after subtraction of the background represented by corresponding isotypic control mAbs.
potent regulation of HVEM and LIGHT was observed using ionomycin along with PMA, shown in Fig. 2. At the beginning of culture, HVEM was expressed on all T cells, and in medium alone it remained at a constant level from time 0 through day 8 (Fig. 2A). In contrast, LIGHT was not detected in freshly isolated cells, and incubation with culture medium alone did not induce its expression (Fig. 2C). Costimulation with PMA and ionomycin induced the loss of HVEM from days 2–5, followed by a return to its original level (Fig. 2B). In contrast, PMA and ionomycin induced a strong expression of LIGHT that peaked on day 2 and then returned to an undetectable level on day 5 (Fig. 2D). A similar effect was observed with the PHA and IL-2 stimulation, although the extent of change in HVEM and LIGHT expression was less (data not shown).

Several studies were performed to rule out that the down-regulation of HVEM was not an artifact resulting from blockade of the receptor Ab by binding of the ligands LIGHT or LTα3. Addition of LTα3, soluble LIGHT, or PMA/ionomycin-activated T lymphocyte supernatants did not mask HVEM. Similar results were obtained with both HVEM mAbs, which have different epitope specificities. The 12C5 mAb inhibits the binding of LIGHT to HVEM, whereas the 20D4 mAb does not inhibit this interaction (R. Costello, Y. Morel, and D. Olive, unpublished observations). Finally, flow cytometric experiments using acid washing to eliminate noncovalent interactions failed to restore the detection of HVEM (data not shown). We thus conclude that the observed decrease in HVEM is the result of receptor down-modulation.

HVEM- and LIGHT-specific RNA regulation and cellular localization

To examine the mechanism by which HVEM and LIGHT protein expression is regulated, we first measured the levels of specific transcripts using semiquantitative RT-PCR. The mitogenic stimulus PMA/ionomycin induced a delayed increase in the expression of HVEM mRNA beginning around day 3 compared with the level at time zero or for incubation in medium alone (Fig. 3, top row). This timing precedes the reappearance of HVEM protein on the
cell surface, as described above. In contrast, a rapid increase in LIGHT mRNA was observed following PMA/ionomycin stimulation (Fig. 3, second row), coincident with the increased cell surface expression of the LIGHT protein.

We then determined the cellular content and localization of HVEM and LIGHT during T lymphocyte activation using confocal microscopy. As shown in Fig. 4A, HVEM was initially distributed around the whole cell, in accordance with flow cytometric data. After a 48-h activation with PMA/ionomycin, HVEM expression was decreased but was still detected, with a “capping” phenomenon (Fig. 4B). The baseline expression of LIGHT protein was low, and it was localized in the cytoplasm rather than at the cell membrane (Fig. 4C), in agreement with the flow cytometry data. After PMA/ionomycin activation, a marked increase in LIGHT expression was observed both at the membrane and in the cytoplasm (Fig. 4D).

**Regulation of HVEM and LIGHT in the CD4^+ and CD8^+ subpopulations**

We then examined the differential regulation of HVEM and LIGHT in CD4^+ and CD8^+ T lymphocyte subsets. As shown in Fig. 5, PMA/ionomycin induced a more potent decrease in HVEM expression in the CD8^+ subpopulation compared with that in CD4^+ cells, in terms of both the fraction of expressing cells (data not shown).

**FIGURE 4.** HVEM and LIGHT cellular localization in T lymphocytes by confocal microscopy. Resting (A) or PMA/ionomycin-activated (B) T lymphocytes were plated on coverslips and stained with the membrane marker CellTracker CM-Dil (red fluorescence) and anti-HVEM 12C5 (green fluorescence). Resting (C) or PMA/ionomycin-activated (D) T lymphocytes were plated on coverslips and stained with the membrane marker CellTracker CM-Dil (red fluorescence) and anti-LIGHT 2C8 (green fluorescence). Serial optical sections were obtained using the TCS 4D laser scanning confocal microscope (Leica). Microscope settings were adjusted to black level values when cells were stained with the mouse isotypic Ig control.

**FIGURE 5.** Flow cytometric and RT-PCR analysis of HVEM and LIGHT expression by purified CD4^+ and CD8^+ T lymphocyte subsets. Purified T lymphocytes were further separated in CD4^+ (X) and CD8^+ (■) subsets by two rounds of negative selection with magnetic beads as described in Materials and Methods. The purity of the preparation, assessed by flow cytometric analysis of separated cells, was >95% in all experiments. Purified CD4^+ and CD8^+ T lymphocytes were separately incubated with PMA/ionomycin, and the expression of HVEM and LIGHT was assessed by flow cytometry from the beginning of the culture to day 6. Data correspond to the MFI of HVEM expression (A) and LIGHT (B) after subtraction of the background, corresponding to the isotypic control. These data correspond to one representative experiment from three performed with different healthy blood donors. We performed semiquantitative RT-PCR in the highly purified CD4^+ and CD8^+ subpopulations using variable numbers of cycles to compare specific signals to control β-actin in nonsaturating conditions (C). The results shown correspond to 30 cycles, while parallel experiments using 33 and 36 cycles were also performed (data not shown). The choice of the number of PCR cycles was made to show high visibility, but nonsaturating, signals. The results presented here correspond to one representative experiment from three performed, using different healthy blood donor samples.
LIGHT, but not HVEM, modulation is partially mediated by metalloprotease

MMPs have been implicated in the cleavage, release, and function of several members of the TNF/TNF family, including FasL (27), CD40L and TNF-α (28), RANKL (29), and TNFRII (30). We thus tested the effects of two MMP inhibitors on the modulation of HVEM and LIGHT at the surface of activated T cells, using FasL and CD40L as positive controls. BB94 is a broad spectrum protease inhibitor, whereas MMP inhibitor (MMPI) is more selective for mitogen-activated protein kinases. The expression of both FasL and CD40L was increased by both protease inhibitors; BB94 had a greater effect (Fig. 6B) than MMPI (Fig. 6A). As a negative control, the surface expression of CD25/IL-2Ra was not affected by MMPI (Fig. 6A) or BB94 (Fig. 6B). Both inhibitors increased the expression of LIGHT (Fig. 6), and, as observed for FasL and CD40L, BB94 had the greatest effect. This protease effect on LIGHT surface expression is consistent with the detection of soluble LIGHT protein by immunoprecipitation from the supernatant of radiolabeled CD4+ T lymphocytes activated with PHA/PHA, but not from resting cells (not shown). Neither inhibitor affected the expression of HVEM on either unstimulated or PMA/ionomycin-stimulated T lymphocytes.

Up-regulation of cell surface LIGHT is partially independent of protein synthesis

The dependence of the cell surface regulation of HVEM and LIGHT on protein synthesis was tested with the inhibitor CHX. Incubation of T lymphocytes with CHX did not modify either baseline or PMA/ionomycin HVEM expression (data not shown). In contrast, the up-regulation of LIGHT expression following PMA/ionomycin stimulation was reduced to a great extent, but not completely, by CHX preincubation (Fig. 7B). As controls, the expression of CD25/IL-2Ra, which is induced upon T cell stimulation, was completely inhibited by CHX (Fig. 7C), while the expression of the early activation marker CD69 was less affected (Fig. 7D).

The incomplete blockade of LIGHT up-regulation by CHX suggests that the increase in surface expression results in part from the recolocalization of preformed molecules in the cytoplasm in addition to de novo protein synthesis. In agreement with this hypothesis and in line with the confocal microscopy results (Fig. 4), we observed expression of intracellular LIGHT by flow cytometry in resting T lymphocytes (Fig. 8). Moreover, the levels of intracellular LIGHT were greater in the CD8+ subset (Fig. 8, lower panel) than in the CD4+ subset (Fig. 8, upper panel), suggesting that this differential baseline content contributes to the differences in membrane expression observed between these subsets.

LIGHT ligation causes HVEM down-regulation

Having observed a reciprocal regulation of HVEM and one of its ligands, we tested whether engagement of HVEM by either of its TNF family ligands was responsible for its down-regulation on activated T cells. Down-modulation of HVEM was not induced by the addition of recombinant LTα, and it was not reduced by the addition of an inhibitory LTα mAb (data not shown). In contrast, incubation with the neutralizing LIGHT mAb 2C8 or with recombinant HVEM-Fc inhibited to a great extent the down-regulation of HVEM (Fig. 9). Thus, the induced expression of LIGHT on the cell surface or in soluble form contributes by its interaction to the down-modulation of its receptor HVEM.
Discussion

Our data provide insights into the mechanisms of regulation of HVEM and one of its ligands, LIGHT, in human T lymphocytes. Upon T cell activation, HVEM and LIGHT show reciprocal modulation on the cell surface. HVEM decreases from its constitutive level on resting cells, while LIGHT increases from a baseline that is undetectable. One week after stimulation both receptors return to their original levels. The analysis of HVEM mRNA suggests that transcriptional control or mRNA stability do not play a central role in its down-regulation following T cell activation. Although, a moderate decrease in the HVEM mRNA level occurred 6 h after activation for some donors, we also cannot exclude that this phenomenon contributes to cell surface HVEM down-regulation, but it does not seem to represent the main factor. The lack of effect by inhibitors of the MMPs that are involved in the enzymatic cleavage of other TNFR members indicates that down-regulation of HVEM is not due to the cleavage of its extracellular domain. These results together with observed capping by confocal microscopy suggest that down-regulation of this receptor following T cell activation could occur by internalization. Intracellular staining at 24 h failed to detect HVEM (data not shown) upon activation, suggesting either degradation or sequestration of the protein. Moreover, the delayed reappearance of HVEM following up-regulation of its mRNA on day 3 suggests that this material is newly synthesized, rather than recycled. The fact that addition of CHX to 48-h PMA/ionomycin-activated T cells completely inhibits HVEM reappearance supports this hypothesis (data not shown). In contrast, the reciprocal regulation of LIGHT involves different mechanisms. First, RT-PCR reveals a striking up-regulation of LIGHT mRNA transcript following T cell activation, which indicates regulation at the transcriptional level or possibly mRNA stabilization, which is reported for other TNF family members (31). Confocal microscopy and intracellular flow cytometry showed the presence of intracellular LIGHT in unstimulated T cells and a marked induction both in the cytoplasm and at the cell surface following stimulation. Along with only a partial inhibition of LIGHT up-regulation by the protein synthesis inhibitor CHX, these results indicate that both de novo synthesis and intracellular redistribution contribute to LIGHT up-regulation on the cell surface following T cell stimulation. The mechanism of the redistribution of preformed LIGHT remains unknown. Therefore, LIGHT sequence does not possess a retention signal based on homology with that described for CTLA4 (32). The basis, at least in part, for the reciprocal regulation of HVEM and LIGHT was revealed by the blockade of this process by inhibitors of this receptor/ligand interaction. The greatest effect was observed with a neutralizing LIGHT mAb, with a lesser effect on the HVEM expression level by the HVEM-Fc fusion protein. The lack of effect by addition of LTα or LTα-neutralizing Ab demonstrated that this other ligand for HVEM did not contribute to

FIGURE 7. Effects of the protein synthesis inhibitor CHX on LIGHT expression and CD40L in comparison with activation markers. T lymphocyte flow cytometric analysis was performed from baseline to 96 h with different markers. Cells were incubated with medium alone (×), PMA/ionomycin (■), or PMA/ionomycin following a 1-h preincubation with CHX (○). The results are presented here with the MFI, but comparable data were obtained for the percentage of positive cells (data not shown). These graphs represent one representative experiment of three performed with samples from different healthy blood donors. Only viable cells determined by propidium iodine exclusion were analyzed.

FIGURE 8. Flow cytometric analysis of intracellular LIGHT. Purified T lymphocytes, corresponding to either the whole population (left) or purified CD4+ or CD8+ cells (right), were permeabilized and stained with anti-LIGHT mAb. White curves correspond to the negative control (isotype-matched Ig), and black curves correspond to specific staining for LIGHT. As control, we performed extracellular staining, and we failed to detect LIGHT at the cell surface of resting T lymphocytes. These data correspond to one representative experiment of three performed with different blood donors.
the receptor down-modulation. Thus, the down-regulation of HVEM in the presence of LIGHT antagonists could reflect incomplete blockade of this interaction or other mechanisms not yet apparent.

The complex regulation and interactions of HVEM and its ligands has to be compared with the other TNFR/TNF family member CD40/CD40L system, which is of crucial importance in the immune response (33), particularly in cytotoxic lymphocyte priming and anti-tumor immunity (34, 35). The CD40 molecule is not a ubiquitous receptor with either proliferative or apoptotic effects. The role of the HVEM ligands system is not completely elucidated, since we only know that this system participates in T cell activation (5) and can mediate, under particular circumstances, tumor apoptosis (17). Other functional implications will probably be rapidly discovered. Since HVEM is widely expressed in the pivotal cells of the immune system, such as B lymphocyte or dendritic cells (R. Costello, Y. Morel, and D. Olive, unpublished observations), we can hypothesize a role in T-B cell interaction or in DC physiology. This later point is of particular interest, since some recent publications have shown that the CD8+ T lymphocytes mediate a CD40-independent maturation of DCs (37).

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