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_J Immunol_ 2009; 183:7286-7296; Prepublished online 13 November 2009; doi: 10.4049/jimmunol.0902490
http://www.jimmunol.org/content/183/11/7286

Supplementary Material
http://www.jimmunol.org/content/suppl/2009/11/13/jimmunol.0902490.DC1

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
T Cell Intrinsic Heterodimeric Complexes between HVEM and BTLA Determine Receptivity to the Surrounding Microenvironment

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The inhibitory cosignaling pathway formed through the TNF receptor herpesvirus entry mediator (HVEM, TNFRSF14) and the Ig superfamily members, B and T lymphocyte attenuator (BTLA) and CD160, limits the activation of T cells. However, BTLA and CD160 can also serve as activating ligands for HVEM when presented in cis by adjacent cells, thus forming a bidirectional signaling pathway. BTLA and CD160 can directly activate the HVEM-dependent NF-κB RelA transcriptional complex raising the question of how NF-κB activation is repressed in naïve T cells. In this study, we show BTLA interacts with HVEM in cis, forming a heterodimeric complex in naïve T cells that inhibits HVEM-dependent NF-κB activation. The cis-interaction between HVEM and BTLA is the predominant form expressed on the surface of naïve human and mouse T cells. The BTLA ectodomain acts as a competitive inhibitor blocking BTLA and CD160 from binding in trans to HVEM and initiating NF-κB activation. The TNF-related ligand, LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes, or TNFSF14) binds HVEM in the cis-complex, but NF-κB activation was attenuated, suggesting BTLA prevents oligomerization of HVEM in the cis-complex. Genetic deletion of BTLA or pharmacologic disruption of the HVEM-BTLA cis-complex in T cells promoted HVEM activation in trans. Interestingly, herpes simplex virus envelope glycoprotein D formed a cis-complex with HVEM, yet surprisingly, promoted the activation NF-κB RelA. We suggest that the HVEM-BTLA cis-complex competitively inhibits HVEM activation by ligands expressed in the surrounding microenvironment, thus helping maintain T cells in the naïve state. The Journal of Immunology, 2009, 183: 7286–7296.

Both Ag recognition and cooperating signaling pathways that activate either stimulatory or inhibitory responses contribute to T cell homeostasis. Cosignaling pathways initiated by members in the Ig superfamily or the TNF receptor (TNFR) superfamily can independently initiate either positive or inhibitory signaling pathways. An important intersection between these cosignaling families occurs in the engagement of the TNFR,

4 Abbreviations used in this paper: TNFR, TNF receptor; HVEM, herpesvirus entry mediator; BTLA, B and T lymphocyte attenuator; LIGHT, related inducible ligand that competes for glycoprotein D binding to HVEM on T cells; LT, lymphotoxin; gD, glycoprotein D; DC, dendritic cell; LIGHT+, LIGHT truncated at G66; herpesvirus entry mediator (HVEM, TNFRSF14) (1) with the Ig superfamily member, B and T lymphocyte attenuator (BTLA) (2, 3). HVEM binds the conventional TNF-related ligands, LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes, or TNFSF14) and lymphotoxin (LT)-α (4), and an additional Ig superfamily members, CD160 (5) and the herpes simplex virus envelope glycoprotein D (gD) (1).

HVEM serves as a molecular switch activating both stimulatory and inhibitory pathways crucial for immune homeostasis. The LIGHT-HVEM system initiates a strong costimulatory signal promoting inflammation and enhancing immune responses (6, 7), by initiating activation of the prosurvival transcription factor NF-κB through a TRAF-dependent serine kinase cascade (8, 9). By contrast, HVEM engagement of BTLA and CD160 activates inhibitory signaling in lymphoid cells (2, 5, 10) through recruitment of SHP-1 and SHP-2 phosphatases, which attenuate tyrosine kinases activated by TCR Ag recognition (2, 11, 12). LIGHT engages HVEM at a topographically distinct site from the common site bound by BTLA (2, 3, 13–17), CD160 (5), and gD (13). However, membrane LIGHT noncompetitively inhibits the HVEM-BTLA interaction, suggesting a role for LIGHT as a regulator of HVEM-BTLA inhibitory signaling.

Previous results indicated the physiological context of inhibitory signaling initiated through the HVEM-BTLA pathway proceeds in a unidirectional fashion, with HVEM activating inhibitory

CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; CRD1, cysteine-rich domain; TRAF2, TNF receptor-associated factor 2.

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trans-signaling in adjacent cells expressing BTLA. In a model of colitis, trans-signaling between HVEM in host intestinal cells and BTLA in T cells prevented accelerated colitis (18). However, emerging evidence indicated BTLA can also initiate survival signals for effector T cells (19). Indeed, Bila−/− T cells reactive to alloantigens in a graft vs host disease setting failed to survive, although the initial response was normal. The colitis model also revealed BTLA as a survival factor based on the findings that Bila−/− T cells transferred into Rag−/− hosts failed to accumulate, with the reduced number of effector T cells in the recipients, ultimately affecting the onset of colitis (18). Although the structural features of BTLA raised doubts of whether it could activate HVEM, recent evidence demonstrated that BTLA, CD160, and gD function as activating ligands for HVEM promoting NF-κB activation and cell survival (20). Moreover, Bila−/− T cells survived poorly following activation, however, substitution with a soluble surrogate of BTLA, BTLA-Fc, activated NF-κB and rescued Bila−/− T cells from activation-induced apoptosis. These results indicate the HVEM-BTLA trans-complex forms a bidirectional signaling system that may serve as both an inhibitory and cell survival system for lymphoid and epithelial cells. However, the direct activation of HVEM-dependent NF-κB RelA in naive Bila−/− T cells, but not wild-type T cells raised the question of how NF-κB activation is controlled in naive T cells.

The specific context of ligand receptor engagement dramatically alters signaling by HVEM. Previously, we showed that HVEM-BTLA limits the proliferation of conventional dendritic cell (DC) subsets in the spleen, countering the proliferation signals mediated through LTβR (21, 22). HVEM or BTLA-deficient CD4+ DC showed a dramatic increase in repopulation compared with wild-type DC (21). Interestingly, the control of DC proliferation required intrinsic expression of HVEM and/or BTLA, however, HVEM and BTLA in the supporting stroma also affected DC, suggesting both cis- and trans-interactions between HVEM and BTLA limit DC proliferation.

In this study, we present evidence indicating BTLA and HVEM form a cis-complex in T cells, with BTLA directly limiting the activation HVEM by its cellular ligands. We demonstrate that native human and mouse T cells express a stable heterodimeric cis-complex of HVEM and BTLA that uses the same binding interaction as the trans-complex. The HVEM-BTLA cis-complex competitively inhibits trans-signaling by all its cellular ligands, providing a mechanism maintaining T cells in a resting state. In opposite fashion, the viral ligand for HVEM, HSV envelope gD activates HVEM in cis. These results demonstrate the physiologic context of HVEM expression with its various ligands determines signaling outcome: bidirectional signaling in trans can provide survival signaling for activated T cells, and cis-interactions in naive T cells limits receptivity to signals from cells in the surrounding microenvironment.

Materials and Methods

Reagents and cell lines

Abs used included: mouse anti-BTLA mAb (J168, IgG1; BD Bioscience), mouse anti-human HVEM mAb (clone 94801, R&D Systems), mouse anti-mouse BTLA (6F7), Armenian Hamster anti-HVEM mAb (LH1), mouse anti-human HVEM (eBioHVEM-122), and mouse anti-human BTLA (MH26) (eBioscience); mouse anti-FLAG mAb (M2 clone, Sigma-Aldrich), rabbit-anti-RelA/p65 Ab (C-20), anti-RelB (C-19) and anti-TRAF3 Ab (H-122) (Santa Cruz Biotechnology) and rat anti-TRAF2 mAb (6F8 clone, MBL). Rat anti-BTLA mAb (6F4, IgG1e), goat anti-HVEM and anti-LTβR IgG2 were made in-house against purified receptor Fc proteins as described (23). Purified Fc fusion proteins: HVEM-Fc, BTLA-Fc and LTβR-Fc, of mouse or human origin, were produced and purified as described (16). Recombinant soluble human LIGHT truncated at G66 (LIGHT66), eliminating the cytosolic and transmembrane regions, was purified and characterized as described (9).

Recombinant cyan fluorescent protein tagged HVEM (HVEM-CFP) plasmid was generated by inserting the full-length HVEM sequence upstream of the ECFP gene of the pECP-N1 expression vector (Clontech Laboratories). Recombinant red fluorescent protein tagged BTLA plasmid (BTLA-DsRed) was constructed by inserting the full-length BTLA sequence into the pDsRed vector (Clontech). The BTLA-A117-pcDNA3 was used in all experiments except in Fig. 3 where the BTLA-Y117 variant was used. HVEM-Y61A and HVEM-Y61F mutants were made with a QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing of the entire coding region.

The retroviral vector, pMIG-GFP, was used to introduce LIGHT or CD160 into EL4 cells, and BTLA into human dermal fibroblasts (16). The AD5/Babe retroviral vector was used for stable expression of HVEM in 293T cells, and BTLA-pMIG was used to create stable 293T-HVEM-BTLA coexpressing cells. Calcium phosphate precipitation was used to transiently transfect 293T cells with BTLA-pcDNA3 or NF-κB luciferase reporter. The mouse PE16 T cell hybridoma was derived from fusion of BW50 T thymoma cells with T cells from AN0 transgenic mice crossed with OX40−/− mice (a gift from T. So and M. Croft, La Jolla Institute for Allergy and Immunology, La Jolla, CA).

T cell cotransfers into Hvem- and Btla-deficient Rag−/− recipients

C57BL6/Rag−/− (C57BL6 background), and C57BL6/SJL CD45.1 congenic mice were purchased from The Jackson Laboratory. Bila−/−, Hvem−/−, and Rag−/− Hvem- or Btla-deficient mice have been described previously (18). Mice were maintained under specific pathogen-free conditions and used at 7–12 wk of age under protocols approved by the Institutional Animal Care and Use Committee of the La Jolla Institute for Allergy and Immunology. CD4+ CD45RBlow T cells were enriched from splenocyte suspensions by positive selection using anti-CD4 (L3T4) microbeads and further purified by cell sorting. For cotransfer experiments, 5 × 10^4 CD4+ CD45RB+ T cells isolated from congenic CD45.1+ mice were mixed with 5 × 10^4 CD4+ CD45RB+ T cells (CD45.2+) obtained from Bila−/−, Hvem−/−, or double deficient mice, and injected i.v. into Hvem−/− or Btla−/− Rag−/− recipients as described (18). Transferred mice were monitored regularly for signs of disease, including weight loss, hunched appearance, piloerection of the coat, and diarrhea.

HVEM-BTLA cis-complex assay

T cells were enriched from mouse spleens by negative selection using biotinylated anti-mouse B220-, DX5-, CD11b-, CD11c- and Ter119 mAbs (R&D Systems; Bioxcell). Recombinant red fluorescent protein tagged BTLA plasmid was generated by inserting the full-length HVEM sequence upstream of the ECFP gene of the pECFP-N1 expression vector (Clontech) and purified and characterized as described (9).

Cellular assays

293T cells were cotransfected with the dual-luciferase reporter plasmids (pSVβ, Stratagene, pRL-TK, Promega) and various combinations of ligands added to cells cultures overnight. The surface expression levels of HVEM, BTLA, CD160, gD, and their mutants in transfected 293T cells were monitored by flow cytometry. Cell lysates were prepared and the luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega). The data are presented as the mean of duplicate samples with a SE of <1.7% variation.

Mouse bone marrow cells were harvested from Rag2−/− (H2O2) or mouse T cell hybridoma (PE16) cells were cultured on chambered coverglass (Nalge Nunc International). Cells were treated with ligands for the indicated time, then fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton

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X-100/PBS for 15 min. After blocking with 1% BSA/0.1% Triton X-100/PBS for 1 h, slides were incubated with anti-RelA/p65 and detected with Cy5 or Cy3 conjugated anti-rabbit Ab (Jackson ImmunoResearch Laboratories). Cells were counter stained with DAPI solution (1 μg/ml) and visualized with a Marcanius fluorescence microscope using 4×04 or ×63 1.3 numerical aperture oil immersion objective (Carl Zeiss) and images were analyzed with SlideBook software (version 4.2,09). Cultures of T cells (1×10^7/ml) negatively selected WT or Btla^−/−CD4^+ or CD8^+CD25^− T cells were labeled with CFSE and cultured in 96-well plates coated with 1 μg/ml anti-CD3e mAb and in medium 0.5 μg/ml anti-CD28 mAb and 10 μg/ml mouse BTLA-Fc or IgG1 isotype control. Cell proliferation and apoptosis was determined by flow cytometric monitoring CFSE dilution at day 3, and 7-aminoactinomycin D cell viability dye exclusion at day 5 of culture, respectively.

Fluorescence resonance energy transfer (FRET)

HVEM-CFP and BTLA-DsRed were expressed in 293T cells by transient transfection. Detection of fluorescence was performed using an LASR II flow cytometry system (BD Biosciences) fitted with solid state diode lasers (Coherent). HVEM-CFP and BTLA-DsRed coexpressing cells, HVEM-CFP cells, and BTLA-DsRed cells were detected at the CFP channel (excitation at 405 nm, emission at 425–475 nm), DsRed channel (excitation at 488 nm, emission at 562–588 nm with a 535 nm long pass filter), and FRET channel (excitation at 405 nm, emission at 564–606 nm with a 550 nm long pass filter). The data were analyzed with FlowJo software (version 8.5.3; Tree Star). Specific FRET fluorescence was calculated as: FRET = Total emission collection at the FRET channel – CFP spectral overlap – DsRed emission at 405 nm laser excitation. The amount of CFP fluorescence spectral overlap subtracted was derived from the mean fluorescence observed in the FRET channel from 293T cells expressing HVEM-CFP (293T-HVEM-CFP). To measure the CFP fluorescence of the spectral overlap was subtracted from the HVEM-CFP and BTLA-DsRed coexpressing cells, 293T-HVEM-CFP should have equal or slightly higher levels of CFP expression compared with coexpressing cells. Similar accounting was considered for DsRed background fluorescence. Control experiments showed no nonspecific interactions between CFP and DsRed, CFP and BTLA or HVEM, or DsRed and BTLA or HVEM (Ref. 20 and data not shown).

HVEM-CFP and BTLA-DsRed subcellular localization in transfected 293T cells were observed by confocal microscopy using a Bio-Rad confocal system (Bio-Rad) fitted to a Nikon microscope with a 40×1.3 numerical aperture oil immersion objective (Nikon Instruments). The pinhole was set at 1 Airy disc unit. HVEM-CFP and BTLA-DsRed expressing cells were visualized by illumination using a Coherent Enterprise Kr-Ar-visible laser (Coherent) with the laser line set at 488 nm for CFP; 568 nm for DsRed. The fluorescence was detected at an emission window of 504–540 nm for CFP; 589–621 nm for DsRed. The image was recorded at a frame-average of five.

Results

Intrinsic BTLA and HVEM complex in T cells

We examined the expression patterns of HVEM and BTLA in mouse and human naive T cells isolated from spleen or peripheral blood respectively (Fig. 1A). All CD3^+ T cells obtained from human and mouse coexpressed HVEM and BTLA, indicating a conserved pattern of expression. Both major subsets of mouse T lymphocytes coexpressed HVEM and BTLA with CD4^+ T cells expressing relatively more BTLA than CD8^+ T cells (Fig. 1B).

Coexpression of HVEM and BTLA in T cells suggested this ligand and receptor pair may interact in cis. To determine whether HVEM and BTLA form a complex when coexpressed in the same cell, we modeled interactions in 293T cells by transfecting cDNA encoding BTLA, HVEM, or both. HVEM was immunoprecipitated from detergent lysates with the aid of a Flag-epitope tag (HVEM-Flag) and Western blotted for BTLA. BTLA (47 kDa) specifically coimmunoprecipitated with HVEM-Flag (50 kDa) in lysates obtained from cells that coexpressed both HVEM and BTLA (Fig. 2A, lane 4), but not from a mixture of cells that expressed either HVEM (293T-HVEM) or BTLA (293T-BTLA) (Fig. 2A, lane 3). Thus, HVEM and BTLA can form a stable heterodimeric complex in cis, but not in trans. Importantly, HVEM also coimmunoprecipitated with BTLA in lysates obtained from the mouse CD4^+ T cell hybridoma PE16, which coexpressed HVEM and BTLA at levels comparable to those observed in primary T cells (Fig. 2B and supplemental Fig. 1). This result indicates formation of the HVEM-BTLA complex in cis occurs at cellular physiological levels.

To measure HVEM and BTLA interactions in membranes of viable cells, we used FRET to monitor protein interactions (20). This assay used CFP fused to the cytosolic tail of HVEM (HVEM-CFP) as a donor fluorophore, and DsRed fused at the cytosolic tail of BTLA (BTLA-DsRed) as an acceptor fluorophore (Fig. 2C). As expected, both HVEM-CFP and BTLA-DsRed localized to the plasma membrane, while the nonconjugated CFP and DsRed proteins showed a uniform cytoplasmic pattern when visualized by confocal microscopy (Fig. 2D). To enhance quantitative aspects of FRET, a flow cytometric detection system excited CFP at 405 nm, and detection of FRET at 564–606 nm. Flow cytometric analyses of HVEM-CFP and BTLA-DsRed coexpressing cells revealed strong fluorescence in the FRET channel (Fig. 2E, left) with minimal CFP spectral overlap and DsRed coexcitation (Fig. 2E, right) and ~80% of the specific fluorescence in the FRET channel (see supplemental Fig. 2 for experimental details). The specific FRET

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FIGURE 1. Coexpression of HVEM and BTLA in T cells. A. Coexpression analysis of HVEM and BTLA in naive T cells. T cells enriched from naive C57Bl/6 spleen (left) and human blood (right) were identified with anti-CD3 and analyzed by flow cytometry. T cells isolated from splenocytes of HVem^−/−; Btla^−/−; and HVem^−/−; Btla^−/− mice were used as background staining (left) and Ig isotype proteins were used as negative control for the staining of human T cells (right). This profile for human T cells was observed in multiple donors; the staining profile for mouse T cells was conducted four or more times. B, HVEM and BTLA expression by naive CD4^+ and CD8^+ mouse T cells. T cells enriched from C57Bl/6 spleen (open histogram) and Hvem^−/−; Btla^−/− spleen (closed histogram) were stained for CD3, CD4, CD8, and HVEM (left) or BTLA (right) and analyzed by flow cytometry.

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The online version of this article contains supplemental material.
signal was detected over a two-orders of magnitude range of HVEM-CFP. The FRET signal inherently requires that both fluorophores are in a functional conformation and located within 100 Å. At this distance, fluorescence emission will be possible only when the two molecules specifically interact. These results demonstrate that HVEM and BTLA proteins form a molecular complex in \textit{cis} in the membranes of viable cells.

\textit{cis}-association restricts \textit{trans}-interactions between HVEM and BTLA

To investigate whether \textit{cis}- and \textit{trans}-interactions between HVEM and BTLA can occur simultaneously, we assessed the binding of HVEM-Fc, a soluble chimera of ectodomain of HVEM and human Fc region of IgG, to cells expressing either BTLA or both HVEM and BTLA. HVEM-Fc bound specifically and with a saturable dose response to BTLA (Fig. 3A, left and right). However, coexpression of both HVEM and BTLA in 293T cells (with HVEM in slight excess) blocked HVEM-Fc binding to cell surface BTLA (Fig. 3A, right), suggesting a high level of stability of the \textit{cis}-complex. These results imply the HVEM-BTLA \textit{cis}-complex may serve as an inhibitor of \textit{trans}-interactions, thus blocking signaling cues from neighboring cells.

We used the BTLA-Fc blocking effect to quantify the level of \textit{cis}-complex on the surface of T cells deficient in BTLA, HVEM, or both. Mouse BTLA-Fc bound to BTLA-deficient T cells with a 4–5-fold increase above normal T cells (MFI ratio of Btla$^{-/-}$ T cells to WT T cells = 4.4) (Fig. 3B, left histogram). At saturating conditions, WT T cells specifically bound a low level of BTLA-Fc,

![Figure 2](https://via.placeholder.com/150)

**FIGURE 2.** \textit{cis}-interaction between HVEM and BTLA. A, Commmunoprecipitation of HVEM and BTLA. 293T cells were transfected with HVEM (Flag tagged) and BTLA, or individually with expression plasmids. HVEM was isolated by immunoprecipitation using anti-Flag mAb (M2) and Western blotted to visualize BTLA with anti-human BTLA mAb (J168) (lane 4, \textit{top}) and HVEM with biotinylated mouse anti-human HVEM (lane 4, \textit{bottom}). 293T cells individually transfected with HVEM or BTLA were mixed at 1:1 ratio, centrifuged to establish contact, incubated for 30 min and subjected to immunoprecipitation as above (lane 3). Recombinant HVEM-Fc and BTLA-Fc were used as positive controls (lanes 1 and 2). Mouse IgG was used as isotype control for immunoprecipitation (lane 5). B, Whole cell extract was prepared from the mouse T cell hybridoma and the extract precleared (PC) with protein G Sepharose (lane 2). Endogenous BTLA-HVEM complex was isolated by immunoprecipitation using mouse anti-mBTLA mAb (6F7 clone, lane 3) and Western blotted to visualize HVEM with goat anti-mHVEM Ab. HVEM-Fc (50 ng) was used as positive control (lane 1). This experiment was conducted twice. C, Schema of the FRET system using HVEM-CFP (donor fluorophore) and BTLA-DsRed (acceptor fluorophore) to detect HVEM-BTLA \textit{cis}-interaction. 293T cells were transfected individually or cotransfected with these fluorophores. Fluorescence was detected at the FRET channel with the detection window at 564–606 nm using a BD LSRII flow cytometry system. D, Subcellular localization of HVEM-CFP and BTLA-DsRed detected by confocal microscopy. HVEM-CFP and BTLA-DsRed expressing 293T cells were visualized using an Ar-Kr laser with the laser line set at 488 nm, and the fluorescence is colored in cyan for CFP and in red for DsRed. Cells expressing CFP or DsRed 293T cells were used as controls (bottom). E, Assessment of the \textit{cis}-association of HVEM and BTLA by FRET assay. HVEM-CFP and BTLA-DsRed coexpressing cells, HVEM-CFP cells, and BTLA-DsRed cells were detected at the CFP channel (excitation at 405 nm, emission at 425–475 nm), DsRed channel (excitation at 488 nm, emission at 562–588 nm), and FRET channel (excitation at 405 nm, emission at 564–606 nm). HVEM-CFP and BTLA-DsRed coexpressing cells (blue), HVEM-CFP cells (cyan), and BTLA-DsRed cells (red). Note that at similar levels of CFP and DsRed expression with reference to the HVEM-CFP and BTLA-DsRed coexpressing cells (top left), there was minimal CFP spectral overlap and DsRed coexcitation detected in the FRET channel (bottom left). E, right panel, overlay of the FRET channel. This result is representative of more than four independent experiments.
suggesting naive T lymphocytes expressed a limited amount of free HVEM on the surface that was not engaged in a cis-interaction with BTLA (Fig. 3B, left histogram). However, when measured with specific Abs (which do not block binding), T cells from gene-deficient mice displayed the same level of surface protein as T cells from WT mice (Fig. 3B, middle and right histograms). These results indicate that HVEM-BTLA cis-complex is the predominant form of HVEM and BTLA expressed in primary naive T cells. Furthermore, the results indicate that the cis-complex limited trans-interactions between HVEM and BTLA.

BTLA engages HVEM in its first cysteine-rich domain (CRD1) located at the N-terminal region (15, 16). To determine whether the formation of the cis-complex occurs through the same binding region, we introduced mutations into CRD1 of HVEM substituting tyrosine-61 to alanine (Y61A) or phenylalanine (Y61F). The Y61A substitution specifically inhibits the interaction between wild type HVEM, HVEM-Y61A and BTLA. BTLA and HVEM-Y61F mutant were cotransfected into 293T cells (293T-BTLA-HVEM-Y61F). Graded concentrations HVEM-Fc were added to the cells in binding buffer (PBS with 2% FBS) for 45 min, washed, and stained with RPE conjugated goat anti-human IgG Fcγ, 293T-BTLA and 293T-BTLA-HVEM that have a similar level of BTLA expression (based on the GFP expression) were used for the assessment HVEM-Fc binding (Fig. 3A, right). B. Staining of purified mouse T cells with mBTLA-Fc or anti-BTLA (6F7) and anti-HVEM (LH1) Abs. Splenic T cells isolated from Hvem−/−Btla−/− (filled histogram), Btla−/−Hvem−/− (unfilled histogram) or mock transfected 293T cells (filled histogram). C. Left, Flow cytometric staining of 293T-BTLA-HVEM cells with rat anti-BTLA mAb (6F4 clone, 5 μg/ml; unfilled histogram) or mock transfected 293T cells (filled) or BTLA-Fc (25 μg/ml) (bottom) to the HVEM mutants. D. Competition of cis- and trans-interaction between wild type HVEM, HVEM-Y61A, and BTLA. BTLA and HVEM-Y61A mutant were cotransfected into 293T cells (293T-BTLA-HVEM-Y61A). Left, Flow cytometric staining of 293T-BTLA-HVEM cells with rat anti-BTLA mAb (6F4 clone, 5 μg/ml, unfilled histogram) or goat anti-HVEM Ab (10 μg/ml, unfilled histogram) or mock transfected 293T cells (filled histogram). Right, Saturation binding analysis for HVEM-Fc binding to 293T-BTLA-HVEM-Y61A. Graded concentrations HVEM-Fc were added to the cells in binding buffer, and the saturation binding assay was carried as in A. E. Competition of cis- and trans-interaction between wild-type HVEM, HVEM-Y61F and BTLA. BTLA and HVEM-Y61F mutant were cotransfected into 293T cells (293T-BTLA-HVEM-Y61F). Cell surface staining for BTLA and HVEM was performed as in D, left. Saturation binding assay for HVEM-Fc binding to 293T-BTLA-HVEM-Y61F was conducted as in D, right. These results are the representative of a minimum of two independent experiments.

coexpression of the HVEM-Y61A mutant with BTLA allowed HVEM-Fc binding to membrane BTLA (Fig. 3D). This result indicated that HVEM-Y61A mutant did not associate with BTLA in cis (or trans), leaving BTLA available for HVEM-Fc binding. In contrast, coexpression of the phenylalanine mutation, HVEM-Y61F, which retains BTLA binding in cis, inhibited trans-binding between HVEM-Fc and BTLA, indicating the occupation of membrane BTLA molecules by HVEM-Y61F (Fig. 3E). These experiments demonstrate that cis-and trans-interactions between HVEM and BTLA occur in the same region of the N-terminal CRD1 of HVEM, and implicate the cis-complex competitively inhibits trans-interactions.

HVEM-BTLA cis-complex modulates LIGHT-mediated HVEM signaling

LIGHT engages HVEM in a topographically distinct site from the BTLA site, yet membrane LIGHT can disrupt HVEM-BTLA interactions in trans (16). To determine whether the HVEM-BTLA cis-complex altered LIGHT binding to HVEM, we measured the...
binding of soluble LIGHT (LIGHT66) at saturating concentrations to 293T cells coexpressing both HVEM and BTLA. The results show that cis-association between HVEM and BTLA did not inhibit binding of soluble LIGHT, which bound HVEM-BTLA cis-complexes as efficiently as HVEM expressed independently of BTLA (Fig. 4A).

Although the HVEM-BTLA cis-complex did not block the binding of soluble LIGHT, it remained unclear whether the HVEM-BTLA cis-complex modulated LIGHT-dependent HVEM activation. To address this question, we used an NF-κB-dependent luciferase reporter assay to monitor HVEM activation. To this end, we used an NFκB-dependent luciferase reporter vector that expresses LIGHT (LIGHT66) to assess whether LIGHT bound to HVEM-BTLA complexes.

We reasoned that the cis-configuration attenuated the magnitude of NF-κB activation by membrane LIGHT (Fig. 4C, right).

We reasoned that the cis-configuration favored the equilibrium toward the HVEM-BTLA complex, which was then able to activate HVEM and NFκB. We previously demonstrated that an Ab to BTLA inhibited LIGHT-mediated HVEM signaling (Fig. 4B). We also showed that the anti-BTLA Ab efficiently blocked LIGHT-mediated NFκB activation by LIGHT66 in direct proportion to the concentration of LIGHT (Fig. 4D). In contrast, this Ab partially diminished the FRET signal of the cis-HVEM-BTLA complex (Fig. 4E), suggesting the relative inaccessibility of the BTLA epitope in the cis-configuration (Fig. 3A). However, the anti-BTLA Ab dramatically enhanced the induction of NFκB by EL4-LIGHT cells in direct proportion to the concentration of LIGHT (Fig. 4F). Minimal induction of NFκB occurred in 293T cells expressing membrane LIGHT or in EL4-LIGHT cells expressing membrane LIGHT-BTLA. This result indicated that the anti-BTLA Ab acted in concert with membrane LIGHT, shifting the equilibrium toward free HVEM molecules on the cell surface, allowing LIGHT to fully activate HVEM.

HVEM-BTLA cis-complex inhibits TRAF2-dependent HVEM signaling

LIGHT-mediated HVEM signal transduction recruits the TNF receptor-associated factor 2 (TRAF2) to the cytoplasmic tail of HVEM and initiates a TRAF-dependent serine kinase cascade that activates the RelA form of NFκB (9). We previously
showed that ligation of HVEM by BTLA in trans-reruited TRAF2, and induced NF-κB RelA activation (20). To investigate whether ligation of HVEM by BTLA in cis also induced recruitment of TRAF2, we transfected 293T cells with HVEM and BTLA expression plasmids. As expected, trans-activation of HVEM by BTLA led to TRAF2, but not TRAF3 recruitment, to HVEM (Fig. 5A, lane 6). Interestingly, coexpression of HVEM and BTLA did not lead to TRAF2 recruitment (Fig. 5A, lane 3) consistent with activation of both RelA and RelB NF-κB complexes. These results demonstrated that BTLA can activate HVEM in trans, but not in cis. Furthermore, the results indicate that HVEM-BTLA cis-complex limited NF-κB activation at the initial activation step in HVEM signaling, principally preventing TRAF2 recruitment to HVEM. In addition, a BTLA mutant lacking the cytoplasmic domain (BTLAΔcyto) inhibited HVEM activation, revealing cell surface interactions were sufficient to inhibit HVEM signaling in trans (Fig. 5B).

CD160 also engages HVEM in the same region as BTLA (5, 20), suggesting formation of a cis-complex that may be inhibitory to HVEM trans signaling. Supporting this contention, we found that CD160 and HVEM coexpressed in 293T cells attenuated NF-κB activation mediated by membrane-bound CD160 expressed in EL4 cells, in contrast to HVEM alone, which showed a strong NF-κB luciferase signal (Fig. 5C). However, HVEM-BTLA coexpression completely suppressed CD160 activation of HVEM. This result indicates that the HVEM-BTLA cis-complex inhibits trans-signaling by all of the cellular ligands for HVEM.

**Herpes simplex virus-1 gD manipulates the HVEM-BTLA cis-complex**

Herpes simplex virus-1 gD, the namesake ligand, engages HVEM in the same site as the cellular ligands BTLA and CD160, and thus gD provided another probe for evaluating the function of the HVEM-BTLA cis-complex. To examine the effect of HVEM-BTLA cis-complex on gD-HVEM interaction, we assessed the binding of gD-Fc to 293T cells expressing HVEM, or HVEM and BTLA. As expected, gD-Fc bound specifically and with a saturable dose response to HVEM (Fig. 5D, left). However, in cells coexpressing both HVEM and BTLA (with BTLA in slight excess), gD-Fc was unable to bind HVEM (Fig. 5D, left). Coexpression of gD with HVEM in 293T cells also blocked BTLA-Fc binding, indicating that HVEM and gD form stable cis-complexes when coexpressed in the same cells (Fig. 5D, right). These results suggest that the HVEM-BTLA or HVEM-gD cis-complexes reciprocally serve as inhibitors of trans-signaling.

To determine whether the HVEM-gD cis-complex modified HVEM signaling, EL4-LIGHT cells or gD-Fc were used to activate 293T cells expressing HVEM, gD or both. Cotransfection of gD with HVEM strongly activated NF-κB dependent signaling (Fig. 5E, left and Fig. 5F, left). In contrast to HVEM-BTLA cis-complex, the HVEM-gD cis-complex inhibited membrane LIGHT from activating HVEM (Fig. 5E, right). Similarly, coexpression of gD and HVEM made 293T cells unresponsive to further stimulation with gD-Fc (Fig. 5F, right). These results indicate that gD in cis-association with HVEM inhibits trans-activation of HVEM.

**The impact of the HVEM-BTLA cis-complex on T cell activation**

To determine whether the cis-complex limits HVEM-dependent NF-κB activation in T cells, we used the mouse PE16 T cell hybridoma, which expresses the cis-HVEM-BTLA complex. Disruption of the HVEM-BTLA cis-complex with a direct blocking Ab to mouse BTLA (as in Fig. 4, D–F) should predictably shift the equilibrium toward free HVEM. Concurrently, we added human BTLA-Fc (lacking cross reactivity with anti-mouse BTLA 6A6 or 6F7 mAb) to initiate trans-engagement of HVEM, and assessed the level of RelA nuclear translocation in PE16 cells (Fig. 6A). In a time-dependent fashion, the combination of the blocking anti-mouse BTLA mAb (6A6) and human BTLA-Fc specifically induced RelA nuclear localization in PE16 T cells. In contrast, the nonblocking anti-BTLA mAb (6F7) combined with human BTLA-Fc failed to induce nuclear accumulation of RelA, as did any of the reagents alone. This result indicates that the HVEM-BTLA cis-complex is an inhibitor of trans-activation of HVEM in T cells.

In the colitis model, Btla−/− T cells transferred into Rag−/− hosts failed to accumulate, with the reduced number of effector T cells in the recipients ultimately affecting the onset of colitis (18). We performed T cell cotransfer experiments to determine whether T cell accumulation in this model also depended on HVEM. Equal numbers of wild type naive CD4+CD45RBhigh T cells (CD45.2+) and allelic-marked CD45.1+CD45.2+ CD4+CD45RBhigh T cells isolated from Btla−/− or Hvem−/− mice were transferred into Rag−/− mice or Rag−/− mice deficient for either Hvem or Btla. The use of Hvem−/−Btla−/− and Btla−/−Rag−/− recipients restricted the expression of HVEM and HVEM specifically to the donor T cells, which allowed direct evaluation in T cells. The results demonstrated that T cells from mice deficient in Btla or Hvem failed to accumulate in the different recipients. Indeed, following naive T cell cotransfer into the different Rag−/− hosts, higher percentages of wild-type T cells accumulated in spleens, MLN, lamina propria, and intestinal intraepithelium of the transferred animals than allelic-marked deficient T cells (Fig. 6B).

The cotransfer of wild-type T cells with Btla−/−Hvem−/− double deficient T cells yielded similar results (supplemental Fig. 3A). Importantly, Rag−/− recipients cotransferred with Btla−/− (CD45.2+) or Hvem−/− (CD45.2+) T cells and congenic marked (CD45.1+) wild-type T cells also accumulated lower percentages of the knockout T cells (supplemental Fig. 3B), demonstrating that reduced T cell accumulation in Rag−/− mice was unrelated to the presence of the congenic gene in the T cells. These results indicated that the accumulation of pathogenic effector T cells in Rag−/− recipients required both BTLA and HVEM. Given that neither the host environment, nor wild-type T cells influenced the accumulation of the cotransferred HVEM- and/or BTLA-deficient T cells suggested a requirement for the expression of both molecules in the same cell.

**Discussion**

The conformational arrangement of HVEM in cis or trans with its Ig superfamily ligands fundamentally changes the signaling activity of HVEM, and determines receptivity to cellular signals present in the surrounding microenvironment. The results presented here reveal a novel signaling paradigm, the HVEM-BTLA cis-complex—a cell autonomous acting heterodimer that inhibits signaling from adjacent cells. In the cis-conformation, BTLA competitively blocks trans-activation of HVEM by its cellular ligands. BTLA may act by preventing spontaneous multimerization of HVEM, consistent with the observation that the cis-conformation prevented recruitment of TRAF2, an early event in HVEM activation of NF-κB. Perhaps not surprisingly, the viral ligand gD when coexpressed with HVEM, constitutively activated NF-κB, yet blocked trans-signaling by the other
FIGURE 5. Inhibition of NF-κB activation by HVEM through BTLA and CD160 cis-complexes and activation by herpesvirus gD. A, Recruitment of TRAF molecules to HVEM in the context of BTLA-mediated HVEM signaling. 293T-LT/R (lane 3), 293T (lane 4), 293T-HVEM-BTLA cells (cis) (lane 5), as well as 293T-HVEM-Flag and 293T-BTLA (trans) (lane 6) were lysed and immunoprecipitated with anti-Flag (M2) or with anti-LT/R and Western blotted for TRAF2 and TRAF3 (lanes 3 and 6). 293T-HVEM and 293T-BTLA cells were cocultured at 1:1 ratio and incubated for 30 min before lysis (lane 6). HVEM-Fc was used as a positive control (lane 1) and the M2 mAb were used as a negative control (lane 2). B, Inhibition of HVEM trans-activation by HVEM-BTLAcyto cis-complex. NF-κB dependent luciferase reporter was transfected into 293T-HVEM, 293T-HVEM-BTLA, and 293T-HVEM-BTLAcyto coexpressing cells. BTLA-Fc (5 μg/ml) and anti-FcAb (1:1 ratio) were incubated with the transfected cells for 24 h and then assessed for luciferase activity in cell lysates. Error bars indicate the SE generated from the average of two data points from a representative experiment repeated at least twice. C, HVEM-BTLA cis-interaction inhibits CD160-mediated HVEM signaling. EL4-CD160 cells were cocultured with 293T-HVEM, 293T-HVEM-CD160, or 293T-HVEM-BTLA cells that were transfected with NF-κB reporter plasmid. Luciferase activities were measured after 24 h. D, Saturation binding assay for gD-Fc binding to 293T-HVEM or 293T-HVEM-BTLA. 293T cells were transfected with HVEM and/or BTLA expression plasmids. Graded concentrations of gD-Fc were added to the transfected cells in binding buffer (PBS with 2% FCS) for 45 min, washed, and stained with PE conjugated anti-rabbit IgG (left). Saturation binding assay for BTLA-Fc binding to 293T-HVEM or 293T-HVEM-gD. 293T cells were transfected with HVEM and/or gD expression plasmids. Graded concentrations of BTLA-Fc were added to the transfected cells in binding buffer. Binding analyses were conducted as in the left panel. RPE conjugated goat anti-human IgG Fcγ was used as secondary Ab (right). E, HVEM-gD cis-interaction alters LIGHT-mediated HVEM signaling. NF-κB dependent luciferase reporter vector was transfected into 293T-HVEM, 293T-gD, and 293T-HVEM-gD coexpressing cells. LIGHT expressing EL4 cells (EL4-LIGHT) were cocultured with the prepared cells for 24 h and then assessed for luciferase activity in cell lysates. EL4 cells were used as negative controls (left). Error bars indicate the SE generated from the average of two data points from a representative experiment repeated at least twice. The level of NF-κB activation mediated by EL4-LIGHT was determined by the equation as follows: (EL4-LIGHT-mediated RLU/EL4-mediated RLU) – 1. For comparison, the level of EL4-LIGHT-mediated NF-κB activation on 293T-HVEM cells was set at 100 U (right). F, HVEM-gD cis-interaction alters gD-mediated HVEM signaling. Transfected cells were prepared as in E. gD-Fc (20 μg/ml) was added to the cells for 24 h and then luciferase activity was assessed in cell lysates. Rabbit IgG (20 μg/ml) were used as negative control (left). Error bars indicate the SE generated from the average of two data points from a representative experiment repeated at least twice. The level of NF-κB activation mediated by gD was determined by the equation above (right).
ligands. Taken together, the results suggest the inhibitory function of the cis-complex may be important to limit trans-signaling by LIGHT, BTLA, and CD160 that are expressed in adjacent activated cells, perhaps reflecting a mechanism of maintaining peripheral tolerance of naive T cells.

Naïve T cells constitutively coexpress HVEM and BTLA as heterodimer that functions to limit HVEM signaling by all of its cellular ligands, including LIGHT, BTLA, and CD160. We estimated that the cis-complex represents ~80% of HVEM and BTLA expressed on the surface of naïve T cells (Fig. 3B). Commun precipitation and FRET analyses indicated the HVEM-BTLA cis-complex is relatively stable, and localized to the cell surface. The HVEM Y61A mutant demonstrated both the cis- and trans-configurations use the same binding motif, indicating a competitive basis for blocking BTLA and CD160 binding. Moreover, deletion of the cytosolic domain of BTLA did not impact the inhibitory function of the cis-complex, indicating that the interaction of the ectodomains determines the functional outcome. Accommodating the cis-complex into the structural model of HVEM suggests a distinct conformation for the interacting partners, perhaps involving conformational flexibility in HVEM. The ~100Å distance determined by FRET and the elongated structure of HVEM ectodomain compared with the compact Ig-domain of BTLA suggests HVEM may need to “bend” to accommodate BTLA in the same membrane plane. The structural flexibility of HVEM in the membrane proximal CRD3 and 4, which contain nearly 60 residues disordered in the crystal structure (13, 15), could theoretically contribute to a supine contortion of HVEM necessary to accommodate BTLA in cis.

In T cells, HVEM and BTLA interact in a dynamic equilibrium between unbound HVEM and BTLA and the stable cis-heterodimer, which appears the energetically favored state. The dramatic enhancement of HVEM signaling by combining anti-BTLA, itself a weak antagonist for HVEM-BTLA cis-complex, with membrane LIGHT or BTLA-Fc (Fig. 4F and 6A) supports the concept of a dynamic equilibrium between the cis-complex and HVEM and BTLA in their unbound state. This experiment also suggests that BTLA in the cis-complex acts as a noncompetitive inhibitor of LIGHT, and reciprocally, membrane LIGHT functions as a noncompetitive antagonist of HVEM-BTLA cis-complex. The topographically distinct binding sites mapped for BTLA and LIGHT on HVEM indicate a noncompetitive mechanism controls the ability of membrane LIGHT to activate HVEM in either the trans- or cis-complex with BTLA (16). Membrane LIGHT, but not its soluble form, blocked BTLA binding without occupying the binding site, indicating LIGHT sterically displaces BTLA from HVEM, shifting the equilibrium toward free HVEM (16). The intrinsically higher avidity of LIGHT for HVEM compared with BTLA should favor the actively signaling LIGHT-HVEM complex. However, the inducible and transient expression in activated T cells suggests LIGHT is the limiting component in regulating the HVEM-BTLA cis-complex. In addition, during T cell activation, the relative expression levels of HVEM change (2), initially decreased, and then increasing after T cell activation, offering the opportunity for engagement of ligands in trans. Thus, these two distinct classes of ligands for HVEM serve as opposing forces directing the equilibrium of the HVEM-BTLA cis-complex toward inhibitory or stimulating signals. The HVEM-BTLA cis-complex may parallel regulatory receptors that recognize MHC, including the human leukocyte Ig-like receptors, which inhibit signaling via ITIM (24). The inhibitory receptor and MHC (e.g., Ly49A with H2-Dd) form both cis- and trans-complexes using the same binding site. cis-interactions may serve as a prominent feature of immunoreceptors that prevent activation of NK and T cells, thus aiding immune homeostasis.

The relatively wide distribution of BTLA and HVEM throughout the hematopoietic compartment, as well as HVEM expression in epithelial cells, indicates that the HVEM-BTLA cis-regulatory mechanism may influence other cell lineages. For example, subsets of myeloid DC differ in their relative levels of BTLA, with CD8α+ DC subset expressing ~10-fold more BTLA than detected on the CD4+ DC subset, yet both subsets express similar levels of HVEM (21). Bone marrow repopulation experiments revealed intrinsic and exogenous expression of both HVEM and BTLA influenced DC reconstitution in the spleen. These results indicate that both cis- and trans-interactions between BTLA and HVEM are physiologically important in DC and T cells.
The experiments presented here illustrate a dual role of the HVEM-BTLA system in cell-cell communication and cell autonomous regulation of T cell survival. BTLA-deficient T cells fail to survive during inflammatory responses in vivo (18, 19). Although Btlai−/− T cells responded normally in both models, effector T cells failed to accumulate resulting in loss of progressive disease. Btlai−/− and wild-type T cells showed comparable proliferation and number of divisions following stimulation in culture. BTLA-Fc specifically activated NF-κB RelA, increased the percentage of 7-AAD-negative, proliferating cells and rescued survival of Btlai−/− T cells, indicating the defect in cell survival occurs after cell division. These results provide a mechanistic explanation for the role of BTLA in T cell survival (20). In this regard, HVEM behaves akin to its TNFR paralogs, CD27, OX40, and 4-1BB, which provide key cosurvival signals during T cell activation (25). These cosignaling TNFR paralogs use similar mechanisms of activating cell survival programs via TRAF, NF-κB, and AKT dependent pathways (26), however, they are not redundant in their individual roles in T cell differentiation.

Furthermore, the results presented in this study in the mouse CD4 T cell transfer model of colitis clearly demonstrated a T cell intrinsic requirement for BTLA and HVEM in the accumulation of pathogenic T cells. The outcome of this genetic experiment (Fig. 6B) indicated that effector cell accumulation required the presence of both HVEM and BTLA, suggesting a functional role for the cis-complex in T cells. The predominant expression of the cis-complex on naive T cells implicates its role in regulating effector cell accumulation during the T cell activation program. The biochemical evidence indicates HVEM does not activate NF-κB dependent signals in the cis-complex, suggesting BTLA may provide the intrinsic survival mechanism in the cis-complex. Whether survival signaling involves the ITIM or other sites, such as the Grb2/P3K recruitment site in the cytosolic domain of BTLA (27) remains unclear at present. Studies in progress are addressing this question.

In contrast to the cellular ligands, envelope gD of HSV constitutively activated NF-κB when coexpressed with HVEM, illustrating the insidious immune evasion capacity herpesvirus. The regulation of prosurvival genes by NF-κB may provide HSV with a selective advantage early during infection. Ligand-induced clustering of HVEM serves as the activating mechanism common to all TNFR. Thus, the ability of HSV gD to activate HVEM in cis suggests gD may induce clustering of HVEM. Furthermore, this result implies that BTLA in the cis-complex may prevent the clustering of HVEM. Even though gD engages the same site on HVEM as BTLA, gD also inhibited the binding of soluble and membrane LIGHT to HVEM. The structure of gD, although substantially different from BLTA, does not reveal a clear mechanism of how it competes with LIGHT. Interestingly, gD expressed in cis with HVEM blocked trans-activation by LIGHT, BTLA, and CD160, similar to the HVEM-BTLA cis-complex, thus interfering with signals from cells in the surrounding microenvironment. Herpesvirus gD mediates viral interference in which the initial infecting pathogen blocks further infection (28, 29). Perhaps analogous to viral interference, the inhibitory function of the HVEM-BTLA cis-complex can be viewed as interference of cellular signals from the surrounding microenvironment. The remarkable diversity in viral mimicry of the HVEM-BTLA complex (30) suggests this pathway serves as a key selective pressure driving evolution of host defenses (31). Recent evidence demonstrated the infection of human T cells during herpes recrudescence (32), and thus, the NF-κB activating function of the HVEM pathway in regulating effector T cell survival may reflect this evolutionary selective pressure.

Acknowledgments
We thank Dr. John Sedy and Heather Shumway for insightful discussions and Brian Sears, Cheryl Kim, and Sacha Garcia for flow cytometry and imaging.

Disclosures
The authors have no financial conflict of interest.

References

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