Unconventional ligand activation of herpesvirus entry mediator signals cell survival

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The herpesvirus entry mediator (HVEM; TNFRSF14) activates NF-κB through the canonical TNF-related cytokine LIGHT, serving as a costimulatory pathway during activation of T cells. HVEM also functions as a ligand for the Ig superfamily members B and T lymphocyte attenuator (BTLA) and CD160, both of which limit inflammatory responses initiated by T cells. Emerging evidence indicates BTLA also promotes T cell survival, but its structural differences from LIGHT intimate BTLA is unlikely to function as an activator of HVEM. We demonstrate here that BTLA, CD160, and herpes simplex virus envelope glycoprotein D (gD) function as activating ligands for HVEM, promoting NF-κB activation and cell survival. Membrane-expressed BTLA and CD160, as well as soluble dimeric receptor surrogates BTLA-Fc and gD-Fc specifically activated HVEM-dependent NF-κB. BTLA and CD160 engagement induced recruitment of TNF receptor-associated factor 2 (TRAF2), but not TRAF3, to HVEM that specifically activated the RelA but not the RelB form of NF-κB in a mucosal epithelial tumor cell line. Moreover, *Btla*$^{-/-}$ T cells survived poorly following activation but were rescued with BTLA-Fc, indicating HVEM-BTLA bidirectional signaling may serve as a critical cell-survival system for lymphoid and epithelial cells.

Homeostasis of the immune system depends on cellular interactions mediated by cytokines and cell surface receptors that activate either stimulatory or inhibitory pathways. The TNF superfamily member herpesvirus entry mediator (HVEM; TNFRSF14) (1) activates both stimulatory and inhibitory pathways, serving as a molecular switch by engaging 2 distinct classes of molecules: the TNF-related cytokines and the members of the Ig superfamily. HVEM engages the canonical TNF-related ligand LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes, or TNFSF14) and lymphotoxin-α (LT-α) (2). LIGHT initiates a strong costimulatory signal, promoting inflammation and enhancing immune responses (3, 4). By contrast, HVEM engagement of the Ig superfamily members B and T lymphocyte attenuator (BTLA) (5, 6) and CD160 (7) activates inhibitory signaling in lymphoid cells (8).

Activation of TNF receptor family members is initiated by their cognate, trivalent, TNF-related ligand, clustering multiple receptors, which in turn recruits the cytosolic TNF receptor-associated factor (TRAF) adaptors, leading to serine kinase activation of the NF-κB system (9, 10). Clustering of receptors is the key activating event, as supported by findings that bivalent antibodies can supplant the need for the native trimeric ligands. On the other side, HVEM binding to BTLA induces phosphorylation of the immunotyrosine inhibitory motif and recruitment of the tyrosine phosphatases SHP1 and SHP2, which attenuate signaling initiated by tyrosine kinases involved in promoting cellular activation and growth (5, 11, 12). However, the existing biophysical evidence indicates the ectodomains of both BTLA and HVEM are monomeric and engage each other with a 1:1 stoichiometry (13, 14), suggesting that both of them lack an oligomeric structure required for clustering receptors. Moreover, structural and functional studies of HVEM (13, 15, 16) revealed the binding site for LIGHT is topographically distinct from that for BTLA (5, 6, 17, 18), CD160 (7), and the herpes simplex virus envelope glycoprotein D (gD) (15). Both BTLA and gD bind to a large interface in the first cysteine-rich domain (CRD1) located at the N terminus on the face opposite that on which LIGHT binds HVEM. These features call into question whether BTLA could function as an agonist for HVEM.

Several experimental models support the concept that the HVEM-BTLA inhibitory pathway functions as a communication system mediating unidirectional signaling from HVEM to BTLA when expressed in adjacent cells (5, 19, 20). For instance, signaling between distinct tissues was revealed in a model of intestinal inflammation in which the transfer of naïve CD4^{+} T cells into genetically matched recipients induced inflammatory colitis. In this model, donor CD4 T cells react to endogenous microflora, which activate innate inflammatory cells in the recipients, causing colitis. Genetic deletion of HVEM in the recipients results in a dramatically accelerated disease, and agonist BTLA antibodies provided protection from this accelerated intestinal damage (21). These results provide an example of unidirectional signaling between distinct tissues, where expression of HVEM in epithelial cells or other radioresistant cells of the host mediates inhibitory signaling through BTLA expressed in inflammatory T cells and host innate cells. The extensive damage in *Hvem*$^{-/-}$ tissues (21) suggests a potential role for HVEM as a protective factor, perhaps via a bidirectional pathway in which BTLA activates HVEM. Another unexpected observation revealed a paradoxical role for BTLA in sustaining effector T cell survival in a graft-versus-host disease model (22). *Btla*$^{-/-}$ effector T cells showed equivalent engraftment and expansion during the first week of the allogeneic response, but they failed to sustain inflammation, with a sharp decline in effector T cell numbers (22). Survival of T cells was not cell-autonomous, suggesting BTLA provided by other cells was important in signaling survival. Thus, a simple, unidirectional signaling model seems insufficient to explain at least some cellular responses mediated by the HVEM-BTLA pathway.

Here, we demonstrate that BTLA, CD160, and gD function as unconventional ligands for HVEM that specifically activate NF-κB RelA, providing a mechanism independent of LIGHT that pro-

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motes stimulatory cosignaling. Mutations in HVEM that block BTLA and gD binding and NF-κB activation also have an impact on the function of CD160 as an activating ligand, but they do not alter LIGHT signaling. BTLA forms homooligomers in native membranes, thus providing a mechanism initiating HVEM clustering and signal propagation. The importance of HVEM regulating cell survival is demonstrated with *Btla*-deficient T cells, which fail to accumulate following activation but can be rescued by ligation of HVEM with BTLA. These findings indicate HVEM and BTLA form a bidirectional signaling pathway regulating cell survival and inhibitory responses between interacting cells.

Results

BTLA Activates HVEM Signaling. To determine whether Ig superfamily members serve as activating ligands, we stably expressed HVEM in 293T cells (293T-HVEM) and transfected these cells with an NF-κB-dependent luciferase reporter to monitor receptor activation. BTLA stably expressed in 293T cells (293T-BTLA) or LIGHT in EL4 cells (EL4-LIGHT), and purified BTLA-Fc fusion or soluble LIGHT (LIGHTt66) proteins were used to ligate HVEM. As expected, EL4-LIGHT cells specifically induced activation of the NF-κB reporter in 293T-HVEM cells more robustly than soluble LIGHT (Fig. 1*A*). In comparison, membrane BTLA expressed in 293T cells when mixed with 293T-HVEM cells also specifically activated the NF-κB reporter (Fig. 1*B*), but BTLA was unable to activate 293T cells expressing HVEM mutated in the first cysteine-rich domain (CRD1) at position tyrosine-61 to alanine (Fig. 1*C Left*). The Y61A substitution specifically alters the binding of BTLA but not LIGHT (16, 17) and, as expected from their topographical distinct binding sites, HVEM-Y61A was competent for NF-κB activation in response to ligation by membrane LIGHT (Fig. 1*C Right*). The resulting activation of NF-κB by BTLA was specifically inhibited by the addition of an anti-human BTLA antibody (J168) that blocks HVEM binding (Fig. 1*D*). In direct comparison, membrane-bound LIGHT provided a substantially stronger activating signal than cell-expressed BTLA (Fig. S1).

In previous results, soluble LIGHT dose-dependently enhanced binding of BTLA-Fc to membrane HVEM (17). Soluble LIGHT and BTLA can simultaneously occupy their respective binding sites on membrane HVEM. The enhanced binding of BTLA-Fc may reflect oligomerization of HVEM by soluble LIGHT, thus increasing the avidity of BTLA-Fc. To examine the ability of soluble LIGHT to enhance BTLA-mediated HVEM signaling, 293T cells transfected with HVEM and NF-κB reporter plasmids were cocultured with 293T-BTLA cells in the presence of graded concentrations of LIGHTt66. Proportional to enhanced binding, soluble LIGHT increased membrane BTLA activation of HVEM-dependent NF-κB activation, indicating that membrane BTLA and soluble LIGHT can cooperatively enhance HVEM signaling (Fig. 1*E*). Furthermore, the soluble, surrogate, dimeric version, BTLA-Fc, specifically induced NF-κB-dependent luciferase activity in 293T-HVEM cells, mimicking the effect of membrane BTLA (Fig. 1*F Left*). The activity of BTLA-Fc was dramatically enhanced when an anti-Fc antibody was added to further oligomerize BTLA-Fc (Fig. 1*F Right*).

These results indicate that the BTLA-Fc fusion protein represents a functional surrogate of membrane BTLA and suggest that the membrane form of BTLA may exist in an oligomeric state capable of clustering HVEM. To directly measure whether BTLA forms oligomers, we used fluorescence resonance energy transfer (FRET) to monitor protein interactions in native membranes. This assay used 2 constructs of BTLA: cyan fluorescent protein (CFP) fused to the cytosolic tail of BTLA (BTLA-CFP) as a donor fluorophore, and DsRed (BTLA-DsRed) as an acceptor fluorophore. The excitation and emission spectra between CFP and DsRed minimize coexcitation and emission spectral overlap, making CFP and DsRed an ideal pair of fluorophores for FRET analysis (Fig. S2). To enhance quantitative aspects of FRET, a flow cytometric detection system was used in which CFP was excited at 405 nm, and the FRET was detected at 564–606 nm. Flow cytometric analyses of BTLA-CFP and BTLA-DsRed coexpressing cells revealed strong fluorescence in the FRET channel (Fig. 1*G*), with minimal CFP spectral overlap and DsRed coexcitation. We found no evidence for nonspecific interactions between CFP or DsRed with BTLA in extensive control experiments (Fig. S3). The observed FRET (Fig. 1*G*) resulted from specific interaction between BTLA molecules. These results demonstrate that BTLA forms oligomers in the membranes of viable cells, consistent with a mechanism that clusters and activates HVEM.

CD160 and gD Are Activating Ligands for HVEM. CD160 is an Ig superfamily member that binds HVEM (7). CD160 Ig domain, unlike BTLA, assembles into a homotrimer that is anchored to the cell surface through a GPI linkage (23). Similar to BTLA-expressing cells, CD160-expressing EL4 cells (EL4-CD160) specially activated NF-κB reporter when cocultured with 293T-HVEM cells (Fig. 2*A*). CD160 failed to bind HVEM-Y61A-Fc when EL4-CD160 cells were incubated with the fusion protein (Fig. 2*B*) and, consequently, EL4-CD160 cells failed to activate NF-κB luciferase reporter in mutant 293T-HVEM-Y61A cells (Fig. 2*C*). Moreover, the Fc fusion protein of envelope gD (gD-Fc) of herpes simplex virus also activated NF-κB luciferase reporter (Fig. 2*D*). Together, these data indicate that the cellular and viral Ig superfamilies of HVEM-binding proteins serve as functional ligands for HVEM but act through a binding site distinct from that for LIGHT.

The Ig Ligands Specifically Activate NF-κB RelA via TRAF2 in Human HT29 Intestinal Epithelial Tumor Cells. LIGHT-mediated HVEM signal transduction recruits TRAF2 to the cytoplasmic tail of HVEM and initiates a TRAF-dependent serine kinase cascade that activates NF-κB (10). To investigate whether ligation of HVEM by BTLA or CD160 induced recruitment of TRAF2 and RelA activation, we used the human colon adenocarcinoma cell line HT29, which naturally expresses HVEM and lymphotoxin-β receptor (LTβR) (10) but not BTLA, CD160, or LIGHT. As expected, LTβR engagement with an agonistic antibody induced activation of both the RelA and RelB forms of NF-κB, as well as processing of p100 to p52; however, anti-HVEM agonistic antibody only activated the RelA form of NF-κB in HT29 cells (Fig. 3*A*). BTLA-Fc and cell-associated CD160 specifically induced the recruitment of TRAF2 to HVEM (Fig. 3*B*, lanes 2 and 4). As visualized by immunohistochemistry, RelA rapidly translocated to the nucleus of HT29 cells following incubation with anti-HVEM, BTLA-Fc, or cell-associated CD160, but not by their specific controls (Fig. 3*C*).

BTLA Mediates Survival of Activated T Cells. To evaluate whether BTLA activation of HVEM has an impact on primary T lymphocytes, we assessed T cell proliferation in culture by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution, and survival by incorporation of 7-aminoactinomycin D (7-AAD). CD4 and CD8 T cells isolated from WT or *Btla*-deficient mice were cultured in the presence of anti-CD3/anti-CD28 antibodies and mouse BTLA-Fc or IgG1 as a negative control. Three days after culture, WT and $Btla^{-/-}$ CD4 and CD8 T cells treated with the IgG control had divided at least 4 times. $Btla^{-/-}$ T cells proliferated substantially more than WT T cells (Fig. 4*A*). These results are in agreement with the inhibitory role that BTLA plays in T lymphocytes, which has been described extensively in the literature (24, 25). The addition of BTLA-Fc to the cultures of CD4 and CD8 T cells substantially enhanced the percentage of $Btla^{-/-}$ cells undergoing proliferation (from 63% to 74% and from 45% to 72%, respectively). BTLA-Fc treatment also affected WT T cell proliferation, but at a more modest effect than that observed in $Btla^{-/-}$ T cells (Fig. 4*A Right*). Note that the number of cell divisions did not change with BTLA-Fc treatment, suggesting that the fusion protein did not affect the rate of T cell proliferation. In contrast, BTLA-Fc

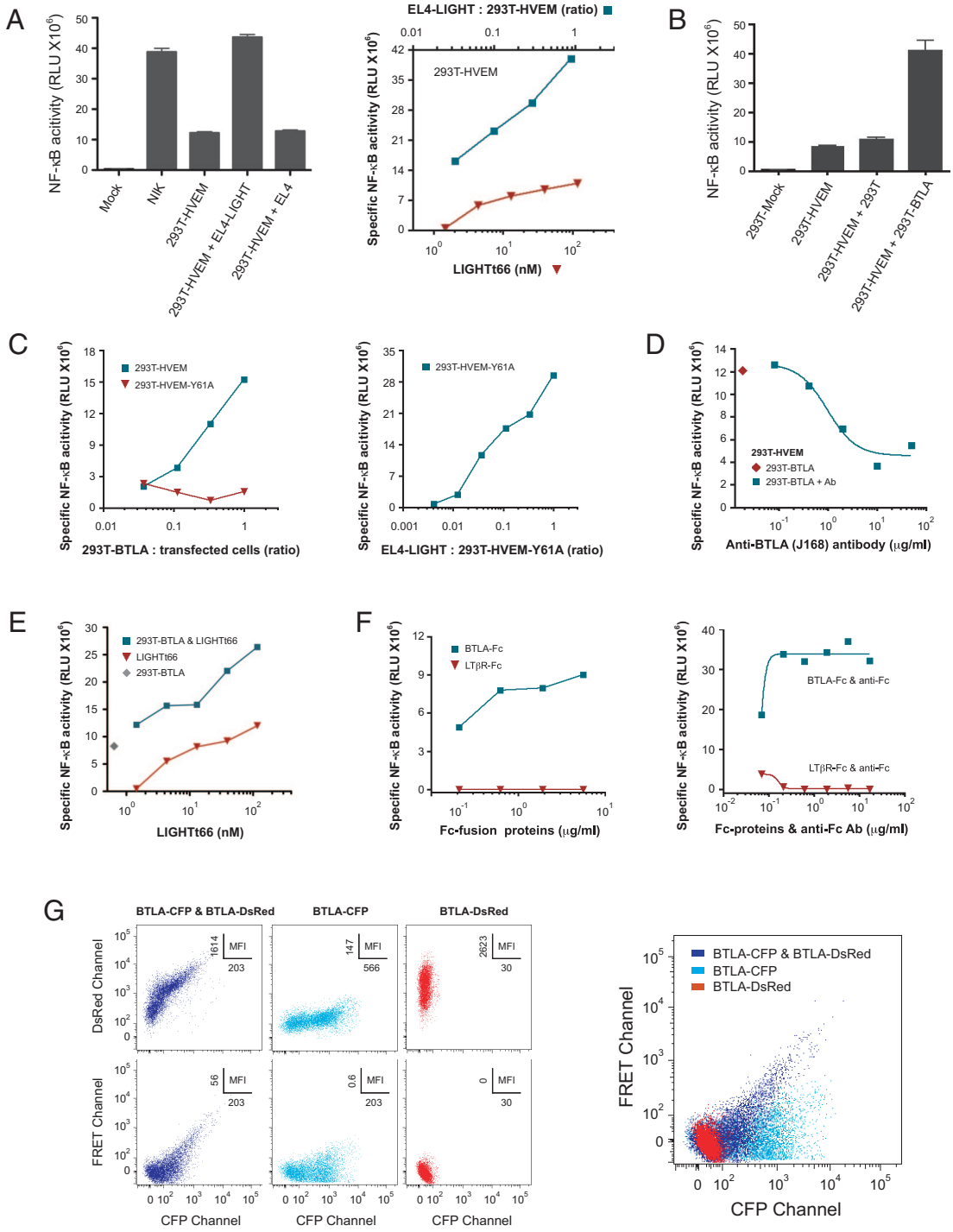

Fig. 1. BTLA activates HVEM-dependent NF-κB. (*A*) LIGHT activates HVEM signaling. NF-κB-dependent luciferase reporter vector was transfected into 293T-HVEM and 293T-HVEM-BTLA coexpressing cells. LIGHT-expressing EL4 cells (EL4-LIGHT) were cocultured with 239T-HVEM cells for 24 h and then assessed for luciferase activity in cell lysates (*Left*). Dose–response: EL4-LIGHT cells or soluble LIGHTt66 were incubated at the indicated ratio or concentration with 293T-HVEM, and HVEM signaling was assessed by using a luciferase reporter assay (*Right*). (*B*) 293T-BTLA or 293T cells were cocultured with either 293T-HVEM cells (1:1 ratio) transfected with luciferase NF-κB reporter or mock-transfected cells. NF-κB activation was measured as in *A*. (*C*) 293T-BTLA cells were cocultured at the indicated ratios with transfected 293T cells expressing HVEM-Y61A mutant or WT HVEM, along with luciferase reporter (*Left*). EL4-LIGHT cells cocultured with 293T-HVEM-Y61A cells expressing luciferase reporter (*Right*). (*D*) Mouse anti-human BTLA mAb (J168 clone) was added to 293T-BTLA cells cocultured with 293T-HVEM cells transfected with NF-κB reporter plasmids, and luciferase activity was measured 24 h later. (*E*) 293T cells transfected with HVEM and NF-κB reporter plasmids were cocultured with 293T-BTLA cells with LIGHTt66 as indicated. (*F*) Graded concentrations of BTLA-Fc or LTβR-Fc were incubated with 293T-HVEM cells transfected with NF-κB reporter plasmids (*Left*). Anti-Fc IgG was added to BTLA-Fc or LTβR-Fc and incubated with 293T-HVEM cells (*Right*). (*G*) Assessment of BTLA oligomerization by FRET. 293T cells transfected individually or cotransfected with BTLA-CFP and BTLA-DsRed 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). Note that at high levels of CFP and DsRed expression, with reference to the HVEM-CFP and BTLA-DsRed coexpressing cells (*Upper Left*), there was minimal CFP spectral overlap and DsRed coexcitation detected in the FRET channel (*Lower Left*). (*Right*) An overlay of the FRET channel.

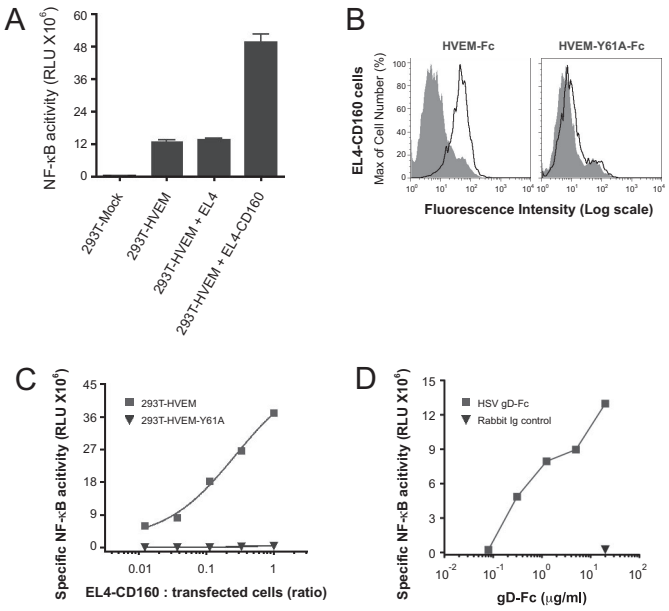

Fig. 2. CD160 activates HVEM-dependent NF-κB. (*A*) CD160-mediated HVEM signaling. CD160-expressing EL4 cells (EL4-CD160) were cocultured with 293T-HVEM cells, and NF-κB reporter luciferase assay was performed after 24 h of incubation. (*B*) CD160-binding site on HVEM. HVEM-Y61A-Fc fusion protein was incubated with EL4-CD160 cells, and binding was assessed by flow cytometry. (*C*) Specificity of BTLA-mediated HVEM signaling. EL4-CD160 cells were cocultured with 293T-HVEM or HVEM-Y61A cells transfected with NF-κB reporter plasmids at the indicated ratios. (*D*) Herpes simplex virus gD-mediated HVEM signaling. Graded concentrations (0.3–20 μg/mL) of gD-Fc were incubated with 293T-HVEM cells transfected with NF-κB reporter plasmids. Rabbit IgG (20 μg/mL) was used as a negative control.

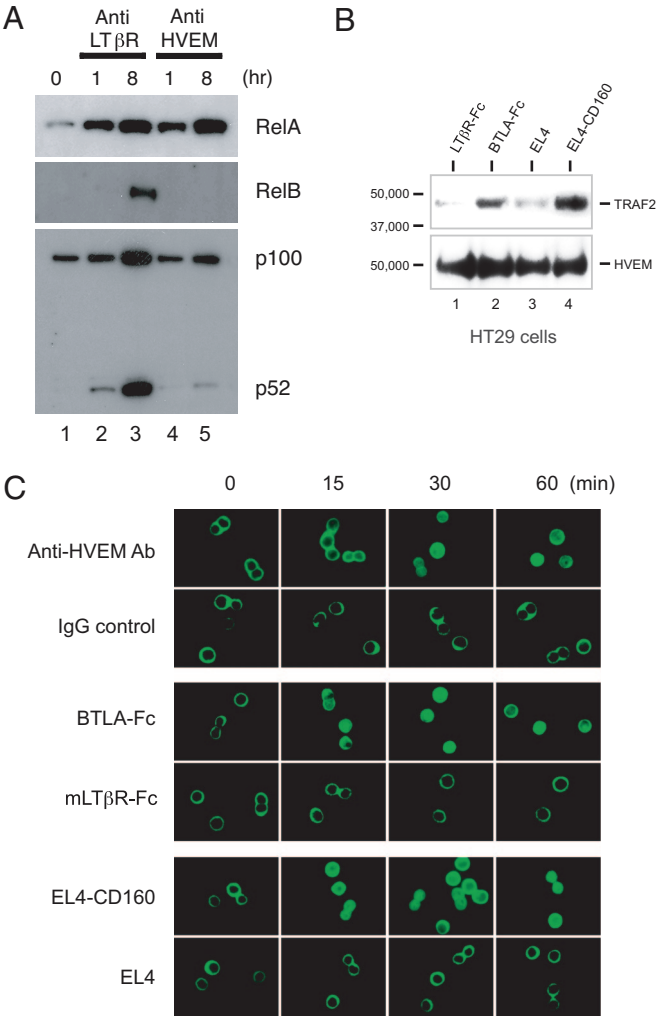

Fig. 3 Activation of HVEM signaling by BTLA and CD160. (*A*) HVEM-dependent RelA nuclear translocation in human HT29 cells. HT29 cells were incubated with agonistic antibodies to HVEM or LTβR, lysed, nuclear fractions isolated, and Western blotted for NF-κB RelA (*Top*) or RelB (*Middle*), or the cytosolic fraction for the processing of p100 to p52 (*Bottom*). (*B*) HVEM-dependent TRAF2 recruitment in HT29 cells. HT29 cells were stimulated with BTLA-Fc (BTLA-Fc and anti-Fc antibody, each at 5 μg/mL, lanes 1 and 2) or cocultured with EL4-CD160 cells (1:1 ratio) for 60 min (lane 4), lysed and immunoprecipitated with anti-HVEM, and Western blotted for TRAF2 or HVEM. LTβR-Fc and EL4 cells (lanes 1 and 3) were used as negative controls. (*C*) BTLA- and CD160-mediated, HVEM-dependent RelA nuclear translocation in HT29 cells. Immunocytochemical staining for RelA translocation was performed on HT29 cells following incubation with anti-HVEM antibody (5 μg/mL), goat IgG (5 μg/mL), BTLA-Fc (15 μg/mL), mouse LTβR-Fc (15 μg/mL), EL4-CD160 (1:5 ratio), or EL4 cells (1:5 ratio). (Magnification: 400×.)

treatment led to higher numbers of cells undergoing proliferation, indicating that HVEM binding by BTLA-Fc likely affected the survival of the activated T cells. Furthermore, *Btla*$^{-/-}$ CD4 T cells treated with BTLA-Fc showed increased percentages of 7-AAD-negative, proliferating cells (38% vs. 28%; Fig. 4*B Left*) after 5 days, with >90% of CD4 T cells undergoing cell division.

BTLA-Fc induced RelA translocation into the nucleus of *Btla*$^{-/-}$ CD4 T cells, but not in T cells deficient in *Hvem* (Fig. 4*C*). Specific RelA translocation occurred within 2 h in 14% of naïve *Btla*$^{-/-}$ CD4 T cells treated with BTLA-Fc, as did T cells treated with anti-HVEM mAb. In contrast, CD4 T cells from HVEM$^{-/-}$ mice failed to respond to BTLA-Fc or anti-HVEM, indicating that RelA induction is mediated through HVEM.

These results demonstrate that the ability of BTLA to serve as a functional ligand for HVEM has a direct impact on the survival of activated T cells. This function of BTLA occurred in *Light*- and *CD160*-sufficient T cells, suggesting this HVEM-BTLA pathway is independent of these other HVEM ligands.

Discussion

The experiments presented here demonstrate that the cellular and viral Ig superfamily members BTLA, CD160, and gD serve as functional agonists for HVEM. These Ig ligands activate NF-κB RelA in epithelial and T cells similarly to the canonical TNF-related ligand LIGHT. The reciprocal ability of HVEM to activate inhibitory signaling in T cells via BTLA or CD160 establishes HVEM-BTLA as a bidirectional signaling system.

Features distinguishing these Ig molecules from LIGHT include their overall structures and a binding interface on HVEM that is distinct from LIGHT. The Y61A mutation in CRD1 of HVEM, known to have an impact on BTLA and gD binding, also inhibited CD160 binding but not LIGHT, confirming that this site in CRD1 is specific for the Ig ligands. Despite their obvious structural differences, the Ig- and TNF-related ligands share the common feature of oligomerization, which is thought to induce receptor clustering, the key initiating event in activation of TNF receptor family members (26). CD160 forms homodimers and homotrimers through intermolecular disulfide bonds, and envelope gD forms dimers in the virion membrane (23, 27). Although the existing biophysical evidence indicated that ectodomains of both BTLA and HVEM interact as monomers with a 1:1 stoichiometry (13, 14), evidence presented here shows that membrane-associated BTLA can form homooligomers. Based on FRET analysis, BTLA expression in 293T cells at modest levels revealed significant energy transfer between the distinct BTLA fluorophores that is consistent with an oligomeric structure with their cytosolic domains in close

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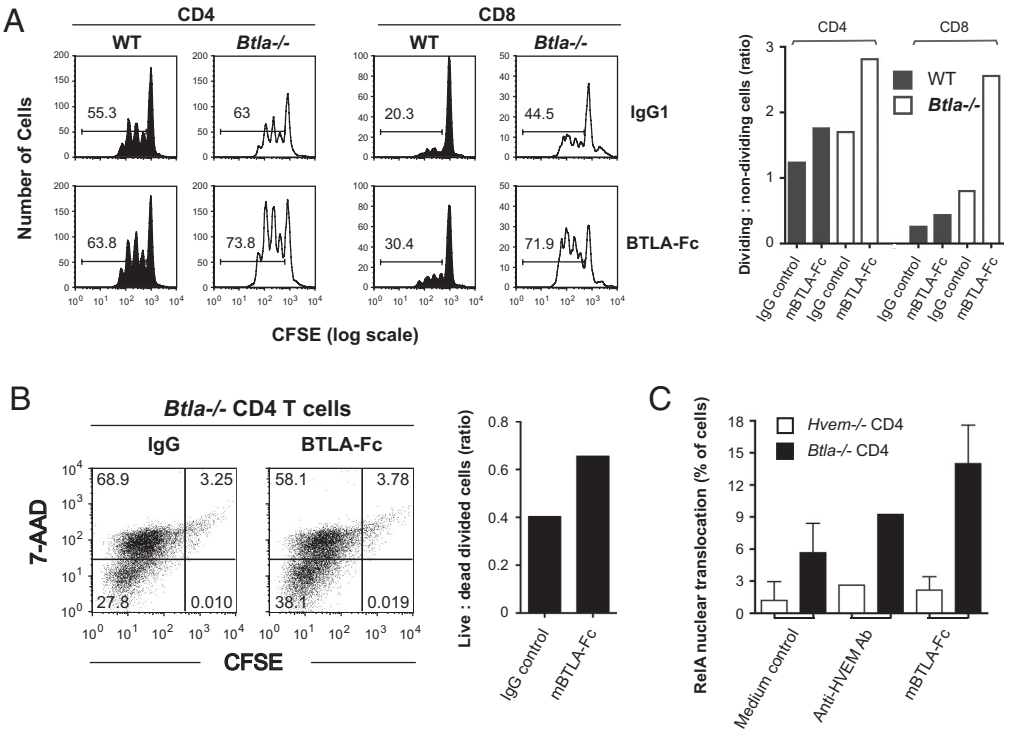

Fig. 4. BTLA-mediated HVEM signaling enhances T cell survival. (*A*) BTLA-mediated T cell proliferation. (*Left*) WT or *Btla*$^{-/-}$ naïve CD4 or CD8 T cells cultured in vitro with plate-bound anti-CD3ε (1 μg/mL) and soluble anti-CD28 mAb (0.5 μg/mL) with IgG control or BTLA-Fc (10 μg/mL). CFSE staining was performed at day 3. (*Right*) The level of T cell proliferation under each condition was calculated as the ratio of dividing cells to nondividing cells. (*B*) Effect of BTLA-HVEM transinteractions on CD4 T cell survival. (*Left*) *Btla*$^{-/-}$ naïve CD4 T cells cultured in vitro as in *A*. CFSE and 7-AAD stainings were performed at day 5. (*Right*) The level of T cell survival under each condition was calculated as the ratio of viable divided cells to dead divided cells. The experiment shown is representative of 3 similarly conducted experiments. (*C*) BTLA-mediated, HVEM-dependent NF-κB activation in CD4 T cells. CD4^{+} T cells were isolated from *Hvem*$^{-/-}$ or *Btla*$^{-/-}$ mice and treated with 20 μg/mL anti-HVEM antibody (clone 14C1.1) or 15 μg/mL mBTLA-Fc for 2 h. Immunohistochemical staining was performed as described above. The percentage of cells with specific RelA nuclear translocation was determined by subtracting nonspecific staining in WT CD4^{+} T cells.

proximity (<100 Å). The region of BTLA controlling oligomeric interactions is not known. Although a potential dimerization region was seen in the HVEM-BTLA structure, the binding interaction was too weak to observe in solution at micromolar concentrations (16). However, as a membrane resident protein, this region in BTLA might have greater potential to form weak dimers. Although dimeric BTLA-Fc was capable of directly activating NF-κB, further oligomerization via the Fc domain dramatically increased NF-κB activation. Our results also revealed the enhanced activation of HVEM by membrane LIGHT in comparison with soluble LIGHT, indicating that from their membrane position, these ligands greatly enhance HVEM activation. The finding that soluble LIGHT enhanced BTLA binding and activation of HVEM suggests the possibility that a ternary complex forms between LIGHT-HVEM-BTLA during cell-to-cell interactions. Interestingly, oligomerization of a fusion protein of CD28 ectodomain and BTLA cytosolic domain via antibodies directed to CD28 dramatically enhanced inhibitory signaling (12). This observation suggests that the reciprocal oligomerization of BTLA and HVEM will enhance bidirectional signaling in the interacting cells.

The HVEM-BTLA bidirectional signaling mechanism may operate in the intestinal compartment, accounting for the dramatically accelerated colitis observed in *Hvem*-deficient mice (21). First, absence of HVEM on host cells could reduce both the engagement of BTLA on donor T cells and the negative regulatory effects of that engagement. Second, the agonist action of BTLA could provide a mechanism for HVEM to function as a protective factor for intestinal epithelial cells through induction of NF-κB RelA, which is well known to activate an array of cell-survival genes (e.g., cIAP- and Bcl2-related proteins). Moreover, the HVEM-BTLA system provides stimulatory cooperating signals that promote survival during T cell activation in vitro, and thus it may account for the failure of effector T cell differentiation in *Btla*$^{-/-}$ mice observed during allograft rejection (22). In these models, *Light* or *CD160* sufficiency in mice did not compensate for the absence of *Btla*, indicating the HVEM-BTLA pathway can function independently of these other ligands for T cell survival. However, an alternate possibility to consider is that the interaction of BTLA-Fc with HVEM may have prevented HVEM signaling to CD160, thus conceivably blocking inhibitory signaling. Although the GPI form of CD160 lacks a clear signaling mechanism, a recent report identified an alternate splice mRNA for CD160 that encodes a transmembrane and cytosolic tail (28). The membrane form of CD160 appears capable of activating the Erk1/2 pathway through recruitment of Src-family kinase p56 (Lck) (28). These results indicate a substantial diversity in potential cellular responses activated by bidirectional signaling pathways initiated by HVEM.

The absence of BTLA compromises the survival of pathogenic T cells during inflammatory responses (21, 22). The ability of BTLA-Fc to specifically activate NF-κB RelA provides evidence for a mechanism operating via HVEM that enhances T cell survival. In this regard, HVEM behaves similarly to the other TNFR paralogs, such as OX40 and 4-1BB, which provide key cosurvival signals during T cell activation (29). These cosignaling TNFR paralogs use similar mechanisms of activating cell-survival programs via TRAF-, NF-κB-, and AKT-dependent pathways (30); however, they do not appear redundant in their individual roles in T cell differentiation, as gleaned from the distinct phenotypes in mice with specific gene deletions.

The relatively wide distribution of BTLA and HVEM throughout the hematopoietic compartment, as well as HVEM expression in epithelial cells, indicates the role of the HVEM-BTLA pathway is not limited to inhibitory signaling in T cells. For example, the growth of myeloid dendritic cells in lymphoid tissues is restricted by the HVEM-BTLA pathway, counterregulating the growth-promoting signals by LTβR (31). These findings suggest other cellular systems are regulated by the bidirectional HVEM-BTLA and related Ig ligand signaling mechanism described here. We found that gD-Fc activated HVEM signaling of NF-κB, consistent with recent observations that gD–HVEM interaction protected against death receptor-induced apoptosis and enhanced herpes simplex virus infection (32). The importance of the HVEM-BTLA

pathway in cell survival revealed here helps clarify the nature of the selective pressures guiding the evolution of herpesviruses (33), which so efficiently target this pathway.

Experimental Procedures

Reagents and Cell Lines. Antibodies used included mouse anti-human BTLA mAb (J168; IgG1κ; BD Bioscience); 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; IgG1κ), goat anti-HVEM, and anti-LTβR IgG were made in-house against purified receptor Fc proteins, as described previously (10, 34). Purified Fc fusion proteins HVEM-Fc, BTLA-Fc, and LTβR-Fc, of mouse or human origin, and HSV1 gD-Fc were produced and purified as described previously (1, 17). Recombinant soluble human LIGHT truncated at G66 (LIGHTt66), eliminating the cytosolic and transmembrane regions, was purified and characterized as described previously (10).

Recombinant CFP-tagged BTLA (BTLA-CFP) plasmid was generated by inserting the full-length HVEM sequence upstream of the ECFP gene of the pECFP-N1 expression vector (Clontech Laboratories Inc.). Recombinant red fluorescent protein-tagged BTLA plasmid (BTLA-DsRed) was constructed by inserting the full-length BTLA sequence into the pDsRed vector (Clontech Laboratories Inc.). 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 express LIGHT or CD160 in EL4 cells (17).

Binding Assays and Immunoprecipitation. Flow cytometry-based binding assays with Fc fusion proteins were carried out as described previously (17). Ligands were incubated with cells in binding buffer (PBS with 2% FBS) for 45 min, washed, and stained with Recombinant Phycoerythrin (RPE)-conjugated goat anti-human IgG Fcγ (Jackson ImmunoResearch). Immunoprecipitation was performed in nonionic detergent cell lysates, with isolation of immune complexes with immobilized Protein G-Sepharose beads (GE Healthcare) and detection of the antigen by SDS/PAGE and Western blotting as described previously (10).

Cellular Assays. The 293T cells were cotransfected with the dual-luciferase reporter plasmids (pNFκB; Stratagene; pRL-TK; Promega) and various combinations of ligands added to cell cultures overnight. Cell lysates were prepared, and the luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega).

Human colon adenocarcinoma (HT29) or mouse T cells were cultured on chambered coverglass (Nalge Nunc International). Cells were treated with ligands for the indicated times, and then fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton 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 antibody (Jackson ImmunoResearch Laboratory). Cells were counterstained with DAPI solution (1 μg/mL) and visualized with a Marianas fluorescence microscope using 40$\times$ 1.3 numerical aperture oil immersion objective (Carl Zeiss Inc.), and images were analyzed with SlideBook software (version 4.2.09; Intelligent Imaging Innovations).

T cells were purified from spleens of *Btla*-deficient (*Btla*$^{-/-}$) or *Hvem*$^{-/-}$ mice (11, 19). 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$^+$ or CD8$^+$ CD25$^-$ T cells purified by negative selection from WT or *Btla*$^{-/-}$ mice were labeled with CFSE and cultured (1×10^5 per mL) in 96-well plates coated with 1 μg/mL anti-CD3ε mAb and in medium consisting of 0.5 μg/mL anti-CD28 mAb and 10 μg/mL mouse BTLA-Fc (mBTLA-Fc) or IgG1 isotype control. Cell proliferation and apoptosis were determined by flow cytometric monitoring of CFSE dilution at day 3 and 7-AAD cell viability dye exclusion at day 5 of culture, respectively.

FRET. Detection of FRET was performed as described previously (35). Briefly, BTLA-CFP and BTLA-DsRed were expressed in 293T cells by transient transfection. Detection of fluorescence was performed by using an LSRII flow cytometry system (BD Biosciences) fitted with solid-state diode lasers (Coherent).

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