Evolutionarily divergent herpesviruses modulate T cell activation by targeting the herpesvirus entry mediator cosignaling pathway


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The herpesvirus entry mediator (HVEM), a member of the TNF receptor (TNFR) superfamily, can act as a molecular switch that modulates T cell activation by propagating positive signals from the TNF-related ligand LIGHT (TNFR superfamily 14), or inhibitory signals through the Ig superfamily member B and T lymphocyte attenuator (BTLA). Competitive binding analysis and mutagenesis reveals a unique BTLA binding site centered on a critical lysine residue in cysteine-rich domain 1 of HVEM. The BTLA binding site on HVEM overlaps with the binding site for the herpes simplex virus 1 envelope glycoprotein D, but is distinct from where LIGHT binds, yet glycoprotein D inhibits the binding of both ligands, potentially nullifying the pathway. The binding site on HVEM for BTLA is conserved in the orphan TNFR, UL144, present in human CMV. UL144 binds BTLA, but not LIGHT, and inhibits T cell proliferation, selectively mimicking the inhibitory cosignaling function of HVEM. The demonstration that distinct herpesviruses target the HVEM–BTLA cosignaling pathway suggests the importance of this pathway in regulating T cell activation during host defenses.

cytokines | immune evasion | T lymphocytes | costimulation

Efficient activation and differentiation of T cells depends on recognition of antigen and cooperating signals (cosignaling) that provoke either positive or inhibitory effects. Inhibitory pathways help maintain immune tolerance to self tissues. In the absence of inhibitory signals or with sustained positive cosignaling, tolerance can be overridden, leading to autoimmune responses. Two major groups of cosignaling receptors are recognized, those with an Ig-like fold, such as CTLA-4 (1), CD28 (2), PD1 (3), and B and T lymphocyte attenuator (BTLA) (4, 5), and those belonging to the TNF receptor (TNFR) superfamily, including OX40, 41BB, CD27, CD30, and herpesvirus entry mediator (HVEM; TNFR superfamily 14) among others (6–9).

Generally, positive cosignaling receptors in the Ig family act by sustaining antigen receptor-associated kinase activity, whereas their inhibitory counterparts contain an immunoreceptor tyrosine-based inhibitory motif in BTLA, but not LIGHT, and inhibits T cell proliferation, selectively mimicking the inhibitory cosignaling function of HVEM.

Abbreviations: BTLA, B and T lymphocyte attenuator; hBTLA, human BTLA; mBTLA, mouse BTLA; BTLA-T, BTLA tetramer; CRD, cysteine-rich domain; dG, glycoprotein D; HSV, herpes simplex virus; HVEM, herpesvirus entry mediator; hHVEM, human HVEM; mHVEM, mouse HVEM; NLIGHT, human LIGHT; LIGHT66, LIGHT truncated at G66; LTα, lymphotoxin α; TNF, TNF receptor.

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Materials and Methods

Fc Fusion Proteins, HVEM Mutants, and UL144 Variants. Fc fusion proteins were constructed between the ectodomain of the individual TNFR and the Fc region of human IgG1 as described in detail (22, 23). The extracellular domain of human BTLA (hBTLA) was synthesized by PCR using phi2 DNA polymerase (Stratagene) and hBTLA cDNA as a template. A HindIII restriction site was introduced into the forward primer (5'-CGCCGCGCACTCTACGGCTTCGACCTAGTGAACAGCACATGCTGCAT-3'), and a SalI site was introduced into the reverse primer (5'-GGCTGGTACTCCGACGTGGCCACTTGTTCCTGGG-3') to facilitate vector-insert ligation. The pCR3 vector (Invitrogen) containing the Fc region of human TNFR and the Fc region of human IgG1 as described in detail (22, 23) was ligated with the BTLA insert. Human HVEM (hHVEM) and mouse HVEM (mHVEM)-Fc and LTβ-Fc were expressed in insect cells by using a baculovirus system; hBTLA-Fc and UL144-Fc were expressed in 293T cells. These Fc proteins were purified by protein G affinity chromatography. hHVEM-Fc was biotinylated by using the NHS-PEO2-Biotin reagent according to the manufacturer's protocol (Pierce). The biotinylation reaction yielded a product of two biotin molecules per HVEM-Fc as determined by MS (SELDI, Ciphergen Biosystems, Fremont, CA). HSV-1 gD-Fc (rabbit IgG1) was produced in Chinese hamster ovary cells (24), and clarified supernatants were used in binding assays. Purified recombinant soluble gD (gD1-Δ 290-299) (25) was provided by G. Cohen (University of Pennsylvania, Philadelphia). Mouse BTLA tetramer (mBTLA-T) was made as described (11). Recombinant soluble human LIGHT (hLIGHT) truncated at G66 (LIGHTt66) was produced in 293T cells and purified as described (18). Purified human IgG (Gamma-gard, clinical grade, Baxter Health Care, Mundelein, IL) was used as a control for Fc fusion proteins.

HVEM point mutants were made with a QuikChange site-directed mutagenesis kit (Stratagene). Incorporation of the correct amino acid substitution was confirmed by DNA sequencing of the entire coding region.

CMV genomic DNA was extracted from cells infected with CMV clinical strains representing each of the UL144 sequence groups, 1A, 1B, 1C, 2, and 3. The UL144 ORF was amplified by PCR from genomic templates representing each group by using the same set of primers. The forward primer contained a BamHI restriction site: 5'-AGGTGATCTTGGGGATATAAACCCACCGGAGATT-3', and the reverse primer contained an XhoI restriction site: 5'-AGGCTCTTGAGACTCAGACACGGTCTGGTAA-3'. The amplified UL144 products were cloned into the pND expression vector (gift of P. Barry, University of California, Davis) (26), and each cloned UL144 product was sequenced to verify the previously determined UL144 group sequence.

Flow Cytometry-Based Binding Assays. Flow cytometry-based binding assays were carried out as described (23, 27) and yield values for those ligands that match with other immobilized ligand binding assays (ELISA and plasmon resonance). Expression plasmids for BTLA, HVEM, HVEM mutants, and UL144 variants were transfected into 293T cells, and full-length hLIGHT was expressed in EL4 cells by retroviral vector transduction (pMIG, gift of D. Baltimore, California Institute of Technology, Pasadena, CA). BTLA-expressing human dermal fibroblasts (Clonetics, San Diego) were generated by transduction with hBTLA- or mBTLA-expressing retroviral vectors (11) that were generated by transient transfection of 293T cells (28, 29). For saturation binding and competition inhibition assays, graded concentrations of recombinant proteins [hHVEM-Fc, mHVEM-Fc, hBTLA-Fc, hLIGHTt66, gD-Fc, soluble gD, and mouse anti-hLIGHT recombinant “Omniclone” antibody (30)] were diluted in binding buffer (2% FBS in PBS, pH 7.4 with 0.02% NaN3) and incubated for 60 min at 4°C. Goat anti-human Fc fragment (IgG)-specific antibody conjugated with R-phcoeythrin or goat anti-rabbit Ig antibody was used for detecting the Fc fusion proteins; anti-FLAG M2 mAb (Sigma) was used to detect hLIGHTt66, and phycoerythrin-conjugated streptavidin was used to detect biotinylated hHVEM-Fc. Specific mean fluorescence intensity was obtained by subtracting the background fluorescence staining of the nontransfected cells or isotype-matched control antibody (negative control) from the experimental group. The K0 values were calculated by nonlinear regression analysis with PRISM (version 4, GraphPad, San Diego), and the molecular mass of the purified protein was determined by MS.

T Cell Proliferation Assays. Human blood was obtained from healthy donors with ethical approval, and mononuclear cells were isolated by density gradient centrifugation. Flat-bottomed plates were incubated with varying concentrations of anti-CD3 (clone UCHT1, BD Pharmingen) and 5 μg/ml anti-human IgG1 Fc antibody (Caltag, Burlingame, CA) overnight at 4°C. Human IgG or various TNFR-Fc proteins were preincubated at 37°C for 2 h with different concentrations. Purified CD4+ T cells obtained by negative immunomagnetic selection (Miltenyi Biotec, Auburn, CA) were added at a concentration of 2 × 10^6 cells per ml in DMEM with 5% heat-inactivated human AB serum, antibiotics, and 1 μg/ml soluble anti-CD28 (R & D Systems) and cultured for 72 h with 1 μCi of [3H]thymidine added during the last 12 h.

Results

Spatially Distinct BTLA and LIGHT Binding Sites on HVEM. To determine the specificity and molecular topography of the HVEM–BTLA interaction we constructed Fc fusion proteins with the ectodomain of HVEM or BTLA as surrogates of their cell-bound receptors (23). hHVEM-Fc bound with a saturable profile (K0 = 112 nM) to hBTLA expressed in 293T cells as detected by flow cytometry (Fig. L4), but failed to bind mBTLA over this concentration range. By contrast, mHVEM-Fc bound both hBTLA (K0 = 27 nM) and mBTLA (K0 = 24 nM) with similar affinities (Fig. 1B) in agreement with species restriction previously observed (11). Reciprocally, hBTLA-Fc-bound HVEM expressed in 293T cells (K0 = 636 nM), but less efficiently than when BTLA was positioned in the membrane (Fig. 1C). In a similar FACS assay, hHVEM-Fc bound hLIGHT expressed in EL4 thymoma cells with a K0 of 11 nM (data not shown). A soluble form of recombinant hLIGHT (LIGHTt66) also bound with high affinity to cell-expressed hHVEM (K0 = 13 nM) (Fig. 1D) yet failed to inhibit binding of BTLA-Fc to HVEM, and as the concentration approached saturation (>60 nM) LIGHT enhanced BTLA-Fc binding to HVEM (Fig. 1E), suggesting the formation of a ternary complex, similar to results recently reported by Gonzalez et al. (12). In the mouse system, LIGHTt66 similarly did not block the binding of mHVEM to mBTLA-T (Fig. 1F), although mHVEM-Fc binding to membrane-expressed LIGHT was effectively competed. These results indicate that LIGHT and BTLA have substantially different binding affinities and occupy spatially distinct sites on HVEM.

A fourth reactant with HVEM, envelope gD from HSV-1, can bind both hHVEM and mHVEM (15, 24). With the BTLA site also located in the first CRD, we hypothesized that gD might serve as a useful tool to further probe the specific structural requirements for HVEM–BTLA interaction. A soluble deletion mutant of HSV-1 gD inhibited the binding of BTLA-T to cell-expressed mHVEM, yet also blocked binding of hLIGHTt66 to membrane LIGHT with similar dose–response (K0 = 250 nM) (Fig. 1G) (see also ref. 14). However, previous experiments have shown that gD did not block the binding of soluble LIGHT or LTα to HVEM-Fc in a plate binding format (31). This difference in competitive action of gD with soluble vs. transmembrane-anchored LIGHT indicates that the membrane position sterically restricts HVEM binding to LIGHT when gD is present. Similarly, BTLA-Fc inhibited the binding of HVEM-Fc to membrane LIGHT in a dose-dependent manner (Fig. 1H), suggesting that gD is a viral mimic of BTLA.
Together, these results indicate that LIGHT and BTLA occupy distinct sites on HVEM and identify the BTLA binding site as topographically close to the site occupied by gD in CRD1.

**BTLA and gD Bind a Distinct, but Overlapping, Site on HVEM.** To address whether BTLA occupies the gD binding site on HVEM, alanine substitutions were introduced into hHVEM in residues within CRD1 and CRD2 (Fig. 2C). None of the mutants affected expression of HVEM on the cell surface (Fig. 2D) or total protein as detected with a polyclonal anti-HVEM in Western blots (data not shown). Mutations Y61F and K64A in CRD1 were particularly informative. The K64A, but not Y61F, mutation abolished binding to BTLA, yet both resulted in a complete loss of gD-Fc binding and virus infectivity as measured by gD expression and viral protein expression [Fig. 2D and data not shown; in concurrence with Connolly et al. (32)]. These mutants indicate that the BTLA binding site on HVEM is distinct from, but overlaps, that of gD. Saturation binding analysis of the HVEM mutants revealed decreased binding affinity of BTLA-Fc to HVEM mutants R62A and E65A (2- to 3-fold increase in KD) and K64A, but not to several other mutants in CRD1 or CRD2 (Table 1). As expected, none of the HVEM mutants affected the affinity of LIGHT66 binding, further indicating that the mutations were unlikely to have altered the global conformation of HVEM. These results lead to a model in which the gD and BTLA binding sites are located primarily within CDR1, yet are topographically close, but distinct.

**The BTLA Binding Site Is Conserved in CMV UL144.** Mutational analysis indicated K64 is a major determinant in the ability of HVEM to engage BTLA with additional contributions from R62 and E65. These three residues form a charged ridge on the solvent-exposed surface of HVEM that is part of the loop formed by disulfide bonds C57–C75 and C67–C54 in CRD1 (Fig. 2C). The sequence of CRD1, including the positioning of the cysteines and the equivalent K64 residue, is highly conserved between hHVEM and mHVEM (62% overall identity in CRD1) (Fig. 3). Interestingly, a viral protein encoded by the UL144 ORF in human CMV showed significant homology to HVEM in CRD1 (Fig. 3). We previously described UL144 as a member of the TNFR family that contained only two CRDs, exhibiting the closest sequence homology to HVEM and TRAILR2; however, UL144 failed to bind any of the known members of the TNF ligand family, including LIGHT (22), thus its function remained elusive. The conservation of UL144 with HVEM in this region suggested the possibility that UL144 may function as a BTLA binding protein.

Sequence hypervariation exists in the ecto domain of UL144 from human CMV isolated from different clinical sources that can
be categorized into five major groups: 1A, 1B, 1C, 2, and 3 (33) (Fig. 3). Expression plasmids encoding representatives of each UL144 group were transfected into 293T cells, and the binding of hBTLA-Fc was examined by flow cytometry. Binding profiles revealed specific interactions between hBTLA-Fc with cells transfected with each of the UL144 variants from human CMV (Fig. 4A). Reciprocally, UL144-Fc generated from the Fiala(F) strain of human CMV (a group 3 sequence) (22) specifically bound hBTLA, but not mBTLA (data not shown). Somewhat surprisingly, hBTLA-Fc bound to cell-expressed UL144 from each group with similar affinity ($K_D/H_11005/H_9262/H_20862/H_20862$) despite the sequence variation in CRD1, although binding was weaker than that seen for HVEM ($K_D/H_11015/H_325/H_20862$-5-fold). hHVEM-Fc effectively competed with cell-expressed UL144(1C) for binding BTLA-Fc (Fig. 4B), indicating they engage a spatially related interaction site on BTLA.

The functional similarity of UL144 and HVEM was observed in the ability of UL144-Fc to inhibit the proliferation of human CD4$^+$ T cells when activated with limiting amounts of anti-CD3 and anti-CD28 in the presence of immobilized fusion proteins. HVEM-Fc and UL144-Fc, but not LTBR-Fc, were effective at inhibiting proliferation (Fig. 5A); however, UL144-Fc was significantly more potent than HVEM-Fc in this assay (Fig. 5B). Both HVEM-Fc and UL144-Fc were most potent in blocking T cell proliferation when immobilized, indicating that crosslinking is probably needed for those proteins to be effective. In contrast to hHVEM and mHVEM, UL144(F) did not function as an entry factor for HSV-1 and did not bind LIGHT (22).

**Discussion**

The potential of HVEM to serve as a molecular switch for positive or inhibitory signaling during T cell activation will depend on which of its four ligands is engaged. We defined the hierarchy of occupancy of HVEM by BTLA and LIGHT, which

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**Table 1. Binding analysis of BTLA, LIGHT, and gD to HVEM**

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<tr>
<th>Binding partners</th>
<th>HVEM mutants</th>
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<tr>
<td></td>
<td>Y47F</td>
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<tr>
<td>BTLA-Fc ($K_D/nM$)*</td>
<td>636</td>
</tr>
<tr>
<td>LIGHTt66 ($K_D/nM$)*</td>
<td>13</td>
</tr>
<tr>
<td>gD-Fc†</td>
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293T cells were transfected with WT or mutant HVEM expression plasmids. Binding analyses were performed on day 3 after transfection. BTLA-Fc, the extracellular domain of hBTLA was fused to Fc of human IgG; LIGHT-t66, FLAG epitope-tagged soluble LIGHT. The amino acid residues in HVEM or individual substitution mutants were stained with anti-HVEM antibody, hBTLA-Fc (100 μg/ml), soluble hLIGHT (400 nM), and gD-Fc (0.4 μg/ml). Binding analyses were performed by flow cytometry. Binding profiles of HVEM ligands to HVEM-293T cells (dark lines) and mock-transfected 293T parental cells (light lines) are shown.

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engaged distinct sites on HVEM. The viral ligand for HVEM, HSV gD, acted as a dual antagonist by competitive displacement of BTLA and noncompetitive blockade of LIGHT. Moreover, the molecular definition of the BTLA binding site on HVEM provided the key clue, revealing a function for the orphaned TNFR encoded by the UL144 ORF in human CMV. These two viral proteins provided insight into the mechanisms regulating the HVEM molecular switch.

The BTLA binding site on HVEM is energetically centered on K64 and adjacent residues R62 and E65 embedded within the loop formed by disulfide bonds at C57–C75 and C67–C54 in CRD1. This result positions the BTLA binding site on the face opposite the LIGHT binding site on HVEM, similar to HSV-1 gD. We refer to this region as DARC (gD and BTLA binding site on the TNFR HVEM in CRD1). Based on structural models of several TNF–TNFR complexes (19), LIGHT and HVEM must be on juxtaposed membranes for binding to occur, with the N terminus of HVEM proximal to the membrane in which LIGHT resides. The ability of HVEM to activate BTLA signaling when presented in trans from another cell suggests the juxtaposition of HVEM and BTLA in distinct membranes is sufficient for proper orientation (11), but does not exclude the possibility of an interaction in cis. The noncompetitive interactions of BTLA-Fc and LIGHTt66 suggest both molecules can simultaneously occupy HVEM, consistent with recent findings in another study (12). Moreover, the binding of soluble LIGHTt66 to HVEM at levels approaching saturation enhanced binding of BTLA-Fc (Fig. 1E), and, as shown elsewhere (12), the binding of HVEM-Fc was also enhanced when cells coexpressed LIGHT and BTLA. These results are consistent with the ability of the soluble reactants to form a trimolecular complex.

However, all of the experimental evidence for a trimolecular LIGHT–HVEM–BTLA complex has been generated with one or more reactants in soluble form. Whether this trimolecular complex forms in their normal membrane-anchored positions remains untested. Three findings suggest LIGHT will displace the BTLA–HVEM interaction, indicating a trimolecular complex is unlikely to form. First, the affinity of the LIGHT–HVEM interaction is an order of magnitude greater than for the observed HVEM–BTLA complex (K_D = 11 nM, HVEM–Fc binding membrane LIGHT; K_D = 112 nM, HVEM–Fc binding membrane BTLA). This difference in affinity indicates that the LIGHT–HVEM interaction will predominate when HVEM is the limiting reactant, which may occur when HVEM is down-modulated after T cell activation, concurrent with the induction of LIGHT (11, 14, 34). Second, the viral inhibitor gD may set a precedent in this system for influencing ligand binding without directly occupying the binding site (noncompetitive inhibition). gD inhibited the interactions of HVEM with BTLA in a competitive fashion, supported by the fact their binding sites overlap. However, gD inhibited HVEM binding to LIGHT only when LIGHT was in its membrane-anchored position (Fig. 1G); soluble LIGHT was not blocked by gD (31, 35). This noncompetitive blockade of HVEM–LIGHT by gD parallels the behavior of...
BTLA in blocking HVEM-Fc binding to membrane LIGHT. These results suggest the possibility that the proximity of the membrane sterically excludes HVEM from binding LIGHT when gD or BTLA occupies its binding site in the DARC (gD and BTLA binding site on the TNFR HVEM in CRD1) region (noncompetitive behavior). Promoted by high-affinity binding, the LIGHT–HVEM complex, may in turn, sterically exclude membrane BTLA from binding HVEM, thus acting in a noncompetitive fashion to disrupt inhibitory signaling by BTLA.

A third line of evidence supporting the ability of LIGHT to act as a noncompetitive inhibitor of the HVEM–BTLA complex is provided by UL144. Surprisingly, UL144-Fc was far more efficient than HVEM-Fc in blocking T cell proliferation, even though its binding affinity for BTLA was measurably less (5-fold). One possibility to account for the enhanced antiproliferative activity of UL144 relative to HVEM could be its inability to bind LIGHT, possibly to account for the enhanced antiproliferative activity of LIGHT during T cell activation (14) and occupancy of HVEM, thus acting in a noncompetitive fashion to disrupt inhibitory signaling by BTLA.

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