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CD160 Activation by Herpesvirus Entry Mediator Augments Inflammatory Cytokine Production and Cytolytic Function by NK cellsi

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Abstract

Lymphocyte activation is regulated by costimulatory and inhibitory receptors of which both B and T lymphocyte attenuator (BTLA) and CD160 engage Herpesvirus entry mediator (HVEM). Notably, it remains unclear how HVEM functions with each of its ligands during immune responses. Here, we show that HVEM specifically activates CD160 on effector NK cells challenged with virus-infected cells. Human CD56^{dim} NK cells were costimulated specifically by HVEM, but not by other receptors that share the HVEM ligands LIGHT, Lymphotoxin-α, or BTLA. HVEM enhanced human NK cell activation by type I IFN and IL-2, resulting in increased IFN-γ and TNF-α secretion, and tumor cell-expressed HVEM activated CD160 in a human NK cell line causing rapid hyper-phosphorylation of serine kinases ERK1/2 and AKT, and enhanced cytolysis of target cells. In contrast, HVEM activation of BTLA reduced cytolysis of target cells. Together, our results demonstrate that HVEM functions as a regulator of immune function that activates NK cells via CD160, and limits lymphocyte-induced inflammation via association with BTLA.

Introduction

Natural killer (NK) cells are an essential component of the innate immune system that protect against a wide range of pathogens, particularly against herpesviruses. During the early stages of immune responses to viruses, NK cells are primed by cytokines expressed by pathogen sensing cells such as macrophages and dendritic cells (1, 2). Upon maturation, NK cells express a diverse array of receptors that activate cytolysis and cytokine release (3–5). NK cell activation is restrained by a variety of inhibitory receptors that prevent uncontrolled cytolysis and inflammation through the recognition of self MHC molecules expressed in

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healthy, uninfected cells (6). While many herpesviruses have manipulated the balance between inhibitory and activating signaling in order to prevent clearance of infected cells allowing for viral evasion and replication (7, 8), many of the host and pathogen factors that regulate NK cell activation remain unidentified.

The β-herpesvirus, CMV, expresses numerous genes that modulate host immune responses and, specifically, NK cell activation (9). In human CMV many of these genes are encoded within the unique long genomic subregion (U_L)/b' that is not essential for *in vitro* replication (10). The UL144 open reading frame contained within the (U_L)/b' locus was first identified as an expressed transcript encoding a type 1 transmembrane protein and as an ortholog to cellular herpesvirus entry mediator (HVEM; TNFRSF14), a member of the TNF receptor superfamily (11). HVEM binds the TNF-related ligands LIGHT (TNFSF14) and LT-α (12), and the immunoglobulin domain-containing receptors, B and T lymphocyte attenuator (BTLA) (13, 14) and CD160 (15, 16). While UL144 does not bind LIGHT or LT-α presumably because it lacks the third and fourth cysteine-rich domains (CRD) contained in HVEM, it does bind and activate BTLA via CRD1 to attenuate T cell proliferation (17). BTLA activation results in phosphorylation of its cytoplasmic tyrosines and recruitment of the tyrosine phosphatases Src homology domain 2 containing phosphatase-1 (SHP1) and 2, resulting in diminished antigen receptor signaling in T cells and B cells (13, 14, 18). BTLA-expressing T cells are inhibited by HVEM expressed by antigen presenting cells, regulatory T cells, or by mucosal epithelium (16, 19, 20).

The role of CD160 in lymphocyte activation remains unclear. CD160 functions as an inhibitory receptor in a subset of $CD4^+$ T cells (15), while increased CD160 expression with reduced BTLA expression in $CD8^+$ T cells is associated with T cell exhaustion in hosts with chronic viral infections (21–23). In contrast, CD160 cross-linking by MHC ligands (HLA-C) costimulates $CD8^+$ T cells and activates NK cell cytotoxicity and cytokine production (24–27). Activation of HVEM signaling by LIGHT, BTLA, or CD160 enhances antigen-induced T cell proliferation and cytokine production (28–31), and epithelial cell expression of host defense genes in response to bacterial infection (32). Thus, the HVEM-LIGHT-BTLA-CD160 signaling axis may result in productive or aborted lymphocyte signaling depending upon which receptor is activated, and upon the cellular context of activation. Furthermore, the nature of the selective pressures mitigated by UL144 as CMV coevolved with primate hosts remains elusive.

Here, we employ HVEM and UL144 as molecular probes to elucidate differences in human NK cell signaling pathways triggered by viral infection. We observed greater activation of NK cells by HVEM as compared with viral UL144, which reflects the inability UL144 to bind CD160. The uniquely high expression of CD160 by primary $CD56^{dim}$ NK cells in the absence of other HVEM ligands efficiently costimulates NK cell effector functions in response to HVEM binding. In contrast, HVEM binding to NK cells coexpressing BTLA and CD160 inhibits effector functions such as cytolysis. Thus, CD160 and BTLA regulate NK cell activation through costimulatory and inhibitory pathways activated by HVEM expressing-cells in the microenvironment. These findings reconcile the contextual activity of HVEM through BTLA and CD160 and provide a framework by which this network can be manipulated to control inflammatory responses in human infection and chronic disease, such as cancer.

Materials and Methods

Human PBMC isolation and activation with Fc proteins

Fresh human blood was collected from healthy donors giving written informed consent at The Scripps Research Institute Normal Blood Donor Service, and all handling was approved

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by the Sanford|Burnham Medical Research Institute Internal Review Board. Samples were mixed 1:1 with PBS and overlaid onto Ficoll (GE Healthcare, Uppsala, SE) for density gradient centrifugation. PBMC were isolated from buffy coats and washed twice with PBS. NK cells were further purified using EasySep Human NK Cell Enrichment Kit (Stemcell Technologies, Vancouver, CA) and confirmed to be > 95% pure by CD56 staining. Cells resuspended to 1–2 × 10^6 cells/ml in RPMI supplemented with 10% heat-inactivated FBS, antibiotics, L-glutamine and 50 μM 2-ME were first incubated on ice 15–30 minutes with Fc fusion proteins or $hIgG_1$ control. For infectious co-culture experiments normal human dermal fibroblasts (NHDF) cells were infected with CMV (laboratory strain AD169) at an MOI=1 for 6 hours, washed with PBS, and mixed with pretreated PBMC at a ratio of 100:1 (PBMC:NHDF). Productive infection was validated by RT-PCR (Supplemental Fig. 1B). Alternatively, pretreated cells were then activated at 37 °C in flat-bottomed plates for the indicated times and concentrations of recombinant human IFN-β (R & D systems, Minneapolis, MN), IL-2 (rhIL-2, Biogen, Cambridge, MA), or anti-NKG2D (eBioscience, San Diego, CA).

Purified fusion proteins of the extracellular domains of human BTLA, HVEM, human CMV UL144 and variants and rhesus CMV UL144 with human IgG_1 Fc were produced as previously described (17).

Antibodies and Flow Cytometry

Antibodies used to identify human PBMC populations include CD3 eFluor450, CD4 PE-Cy7, CD8a APC-eFluor780, CD14 FITC, CD19 FITC, BTLA PE, HVEM PE, LIGHT APC (eBioscience, San Diego, CA), CD69 PerCP-Cy5.5, CD107a Alexa647, CD160 Alexa647, IFN-γ PE-Cy7 (Biolegend, San Diego, CA), CD25 PE, CD56 Alexa700 (BD Biosciences, San Diego, CA), and NKG2C PE (R & D systems, Minneapolis, MN).

PBMC within the live gate of flow cytometric analysis were defined as $CD19^+$ B cells ($CD14^+/CD19^+/SSC^{low}$), $CD14^+$ Monocytes ($CD14^+/CD19^+/SSC^{high}$), $CD4^+$ T cells ($CD14^-/CD19^-/CD56^-/CD3^+/CD4^+/CD8^-$), $CD8^+$ T cells ($CD14^-/CD19^-/CD56^-/CD3^+/CD8^+/CD4^-$), $CD56^{dim}$ NK cells ($CD14^-/CD19^-/CD3^-/CD56^{dim}$), and $CD56^{bright}$ NK cells ($CD14^-/CD19^-/CD3^-/CD56^{bright}$).

CD107a expression was tested by first incubating anti-CD107a and GolgiStop (BD Biosciences, San Diego, CA) during the final four hours of PBMC or NK cell activation cultures at a final dilution of 1:1000. Cells were washed and resuspended in buffer for extracellular staining, then washed, fixed in 2% paraformaldehyde, and analyzed.

Phosphatidylinositol specific phospholipase C (PI-PLC) (Invitrogen, Carlsbad, CA) was used to distinguish between the glycophosphoinositide (GPI)-linked and transmembrane forms of CD160.

Cells and surface protein expression

EL4 and 293T cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, antibiotics, L-glutamine and 50 μM 2-ME. NK92 cells were maintained in RPMI supplemented with 8% heat-inactivatated FBS, 8% equine serum, antibiotics, L-glutamine, 50 μM 2-ME and 100 U/ml IL-2.

EL4 cells were transduced with human BTLA-ires-GFP or human CD160 (Open Biosystems, Huntsville, AL) cloned into ires-GFP retroviral plasmid by PCR amplification (hCD1605BglII: AGTCAGATCTGCGTGCAGGATGCTGTTG; hCD1603XhoI: AGTCCTCGAGGGCTTACAAAGCTTGAAGGG) (33). Pseudotyped single infection retrovirus was produced by cotransfection of retroviral plasmid, pCG-VSVg envelope

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protein, and Hit60 gag-pol as previously described, or by transfection of retroviral plasmid into Phoenix-A cells. EL4 cells were sorted for GFP expression (13). 293T cells were transduced with UL144 derived from human CMV strains cloned in pND vector by calcium phosphate transfection (17). The UL144 G46K mutant was produced by site-directed mutagenesis (FUL144-G46K-5': AAACAAGGATATCGTGTTACAAAACAATGTACGCAATATACGAGT; FUL144-G46K-3': ACTCGTATATTGCGTACATTGTTTTGTAACACGATATCCTTGTTT). 293T cells were transduced with human BTLA or CD160 as described above, or with *de novo* synthesized rhesus BTLA or CD160 (Mr. Gene, Regensburg, DE) cloned into ires-GFP retroviral plasmid by PCR amplification (RhBTLABglII: AGTCAGATCTGTGCAGGAAATGAAGACATTG; RhBTLAXhoI: AGTCCTCGAGTCAGAAACAGACTTAACTCCTCACAC; RhCD160BglII: AGCTAGATCTGCGTGCAGGATGCTGATG; RhCD160XhoI: AGTCCTCGAGAAGGCTTACAAAGCTTGAAGGACC).

K562 cells were transduced with human HVEM-ires-GFP or control ires-GFP retroviral vector, and sorted for GFP expression (17).

NK92 cells were transduced with human BTLA-ires-GFP, human BTLA containing a cytoplasmic domain truncation (BTLAΔCyt)-ires-GFP, or control ires-GFP retroviral vector, and sorted for GFP expression. BTLAΔCyt mutant was produced by round-the-world PCR (hBTLAR179stop-Forward: TGCTGCCTGTGAAGGCACCAAGGAAAGC; hBTLAR179stop-Reverse: GCCTTCACAGGCAGCAGAACAGGC).

Binding Assays

Flow cytometric binding assays were performed as previously described (17). Cells were incubated with Fc ligands for 30 minutes at 4 °C in buffer (PBS with 2% FBS), washed twice and incubated with donkey anti-human Fc APC (Jackson Immunoresearch, West Grove, PA) for 15 minutes at 4 °C in buffer, washed twice and analyzed. Specific mean fluorescence intensity (MFI) was calculated by subtracting experimental cellular MFI from control cellular MFI.

Cytokine expression analysis

Supernatants from PBMC and NK activation cultures were analyzed by FlowCytomix and Procarta multiplexing kits (eBioscience, San Diego, CA) according to manufacturer instructions.

Quantitative RT-PCR Analysis

RNA was harvested from PBMC:NHDF mixtures using RNeasy® Mini kit (Qiagen). cDNA was transcribed using the iScript™ cDNA Synthesis kit (Bio-Rad), and transcripts for *HCMV IE1* (HCMV-IE1(+): CATCCACACTAGGAGAGCAGACTC; HCMV-IE1(−): GCATGAAGGTCTTTGCCCAG), *HCMV gB* (HCMV-gB(+): AACACCCACAGTACCCGTTACG; HCMV-gB(−): ATAGAGCCAGGTGCTGCCG), and *L32* (L32F: GGATCTGGCCCTTGAACCTT; L32R: GAAACTGGCGGAAACCCA) were amplified using Power SYBR® Green PCR Master Mix (Life Technologies).

Western Blotting

Biochemical activation of the NK92 model cell line was triggered by K562 target cells. To block Bcr-Abl induced signaling K562 cells were treated with 10 μM Imatinib for 60–90 minutes, then washed prior to incubation with NK92 cells. In some experiments to block PI3K induced signaling NK92 cells were treated with 1 μM Wortmannin for 60 minutes, then washed prior to incubation with K562 cells. In experiments using fusion proteins NK92

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cells were coated with control human IgG1, LTβR-Fc, HVEM-Fc, UL144-Fc, or HVEM Y61A-Fc for at least 15 minutes on ice prior to activation. NK92 cells were aliquoted to 2×10^6 cells per condition in 100 μl and mixed with an equal volume of K562 target cells aliquoted to 2×10^5 cells per condition. Cell mixtures were activated at 37 °C for the indicated times, quenched with ice cold PBS and lysed in RIPA buffer at 4 °C for 20 minutes and centrifuged at 14,000 rpm, 4 °C. Extracts were boiled in SDS loading buffer containing 1% β-mercaptoethanol for 5 minutes and resolved by SDS-PAGE on 10% Bis-Tris gels (Bio-Rad, Hercules, CA). Proteins were transferred using tank method to PVDF membrane and blocked with 1% ovalbumin in TBS-T buffer, and blotted with phospho-AKT (S473), phospho-extracellular-signal regulated kinase (ERK) 1/2, total AKT (Cell Signaling, Danvers, MA), and total ERK2 (Santa Cruz, Santa Cruz, CA), followed by anti-rabbit HRP (GE Healthcare), and visualized by enhanced chemiluminescence (Thermo Scientific, Rockford, IL).

Cytotoxicity assays

NK92 cytotoxicity was assayed using a flow cytometry based killing assay (34). K562 target cells transduced with HVEM or GFP vector control were labeled with Cell Proliferation Dye e450 (eBioscience, San Diego, CA) according to the manufacturer's instructions. NK92 cells transduced with BTLA, BTLAΔCyt, or GFP vector control were co-cultured with labeled K562 cells at various effector:target ratios for 3 hours prior to staining with 7-AAD. Specific lysis was calculated as described. Primary NK cell cytotoxicity was assayed using JAM protocol (35). K562 target cells were radiolabeled with ^{3}H-thymidine, washed, and incubated with IL-2 activated purified human NK cells treated with fusion proteins or control Ig at various effector:target ratios for 4 hours. Cultures were harvested onto filtermats and ^{3}H-thymidine incorporation was counted. Specific lysis was calculated as described.

Results

HVEM co-stimulates NK responses to human CMV

To test how HVEM and its viral ortholog, UL144, function to regulate immune responses during a viral infection, we monitored expression of activation markers in cells from human PBMC mixed with CMV-infected fibroblasts (Fig. 1). As expected, these co-cultures were marked by early expression of inflammatory cytokines including IFN-γ, LT-α, TNF-α, IL-6, IL-8, and IL-17A that drive innate cellular activation (Supplemental Fig. 1A). Expression of the S1P1 dependent regulator of lymphocyte egress, CD69, was induced on all PBMC subsets and steadily increased throughout the duration of the co-cultures. Notably, CD69 expression was uniquely upregulated on CD56^{dim} NK cells through days one and three in cells treated with HVEM-Fc, a bivalent soluble fusion protein of the HVEM ectodomain and Fc region of IgG1 (Fig. 1A). We observed a similar upregulation of CD107a expression on CD56^{dim} NK cells by HVEM-Fc after one day of culture (Fig. 1B). Importantly, HVEM induction of CD69 expression was not associated with demographic factors such as age, sex, or CMV seropositive status of donors (Supplemental Fig. 1B–D). We do note, however, that within CMV seropositive donors, the ability of HVEM-Fc to costimulate responses to CMV-infected cells was inversely correlated with anti-CMV titers and NKG2C expression in CD56^{dim} NK cells (Fig. 1C–E), both hallmarks of adaptive and innate memory responses to CMV, respectively (36–38). Nevertheless, CD56^{dim} NK cells from all but one donor were responsive to HVEM-Fc costimulation. Thus, HVEM-Fc specifically enhances early activation of CD56^{dim} NK cells during responses to CMV.

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Human CD56^{dim} NK cells uniquely express CD160 among HVEM ligands

To identify which HVEM ligands were expressed on lymphocytes, we examined expression of BTLA, CD160, LIGHT, and HVEM in human peripheral blood (Fig. 2A–D). In this regard, B cells showed high BTLA expression and low CD160 expression while T cells and monocytes expressed intermediate levels of both BTLA and CD160. Importantly, CD56^{dim} NK cells showed the highest surface expression of CD160, confirming previously reported data (39), and the lowest BTLA expression among all PBMC, while CD56^{bright} NK cells expressed low levels of both BTLA and CD160. LIGHT was specifically expressed by monocytes, CD8^{+} T cells, and weakly by NK cells. In contrast, HVEM was broadly expressed by all PBMC.

We next sought to distinguish the expression of CD160 splice variants encoding glycophosphoinositide (GPI) or transmembrane cellular linkages using phosphoinositol-specific phospholipase C (PI-PLC) treatment which removes GPI-linked proteins (40). We found that while most primary NK cells retained some uncleavable fraction of CD160 similar to levels observed on the NK92 cell line, nearly all surface-expressed CD160 was cleaved from primary NKG2C^{+} NK cells (Fig. 2E, F). Finally, human NK cells upregulated expression of BTLA in response to stimulation through the activating receptor NKG2D, similar to BTLA expression on T cells stimulated through the T cell receptor (Fig. 2G) (41). Thus, in resting human NK cells, CD160 is the predominant HVEM receptor, a portion of which is not GPI-linked, while in activated NK cells BTLA and CD160 are coexpressed and potentially compete for HVEM binding.

Human CMV UL144 binds BTLA but not CD160

UL144 is a structural ortholog of cellular HVEM, however, the engagement of CD160 by UL144 has not been determined in NK cells. We measured UL144 binding to cells expressing human BTLA or CD160 using purified HVEM-Fc or UL144-Fc proteins (Fig. 3A, B). We found that UL144-Fc only bound cells expressing BTLA but not CD160, while HVEM-Fc bound both BTLA- and CD160-expressing cells with similar disassociation constants and required overlapping surfaces of HVEM to bind these receptors as binding to both receptors was abrogated by the Y61A HVEM mutant (Fig. 3C) (42). We sought to determine whether the failure to detect UL144-CD160 binding was due to a low affinity of UL144 for CD160 using a UL144 mutant (G46K) identified while mapping the binding surface of UL144 that bound BTLA with high affinity (manuscript in preparation). In this regard, CD160 failed to show any binding to UL144 (Fig. 3D), although BTLA showed robust binding to the UL144 G46K mutant. The ectodomain of UL144 is highly polymorphic across primate CMV with five distinct human CMV isoforms diverging up to 36% in their amino acid sequences (43). We examined UL144 selectivity for BTLA throughout these diverse sequences using representative UL144 variants derived from clinical human CMV strains (Fig. 3E). Despite the extensive sequence divergence, BTLA-Fc bound all UL144 variants (17), whereas CD160-Fc failed to bind any of the UL144 variants. We note one exception in which UL144 from rhesus CMV bound human and rhesus CD160 with low affinity (Fig. 3D, F). This interaction likely represents a divergence between viral species and not host species as primate BTLA and CD160 are highly homologous (unpublished observations). Together, these data show that UL144 is a highly selective molecule that mimics HVEM binding, yet discriminates between BTLA and CD160, and selectively utilizes the inhibitory BTLA pathway. These results suggest that HVEM engagement and activation of CD160 may serve as a critical regulatory pathway for human NK cells.

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HVEM-Fc co-stimulates cytokine activation of NK cells

The production of cytokines, particularly type I IFN, during early virus infection promotes NK cell differentiation into active effector cells to help limit infection (2, 44, 45). To test how HVEM and its CMV viral ortholog UL144 regulate cytokine-mediated activation of NK cells, we monitored expression of activation markers in lymphoid cells from human peripheral blood stimulated with IFN-β or IL-2 (46). Notably, HVEM-Fc consistently enhanced the number of $CD56^{dim}$ NK cells expressing CD69, with or without IFN-β stimulation in 18 h cultures (Fig. 4A–B), and in 8 h cultures stimulated with IL-2 (Fig. 4C–D). Additionally, in purified NK cells stimulated with IFN-β or IL-2, HVEM-Fc co-stimulated expression of the high affinity IL-2 receptor α subunit, CD25, and the cytolytic granule marker, CD107a, in the $CD56^{dim}$ subset (Fig. 5A–D). Furthermore, HVEM-Fc co-stimulation specifically increased the levels of secreted IFN-γ and TNF-α in IFN-β treated NK cells (Fig. 5E). Together, these data indicated that accessory cells were not required for the activity of HVEM-Fc in NK cells. In contrast, UL144-Fc did not promote NK cell activation. Thus, HVEM-Fc directly co-stimulates cytokine-induced activation and expression of inflammatory cytokines by $CD56^{dim}$ NK cells.

HVEM-CD160 activates AKT and ERK1/2 phosphorylation in response to target cells

We next tested whether activation of NK cells by target cells was affected by HVEM ligation by CD160 using the NK92 cell line as a model of activated NK cells. Similar to results obtained using peripheral blood $CD56^{dim}$ NK cells, NK92 cells expressed abundant CD160 and low BTLA levels (Fig. 2E, Supplemental Fig. 2A). We observed rapid ERK1/2 phosphorylation followed by AKT phosphorylation in NK92 cells mixed with K562 target cells (Fig. 6A). Notably, ERK1/2 and AKT were hyperphosphorylated in NK92 cells coated with HVEM-Fc but not with control Ig, LTβR-Fc, or the Y61A mutant HVEM-Fc. In contrast, ERK1/2 and AKT phosphorylation in NK92 cells was reduced upon UL144 treatment as compared with control Ig (Fig. 6A, B). Since LIGHT ligation does not co-activate ERK1/2 and AKT signaling, and because BTLA ligation inhibits ERK1/2 and AKT signaling, we reasoned that HVEM-induced ERK1/2 and AKT activation occurs via CD160, consistent with previous reports of CD160 signaling (40, 47). To rule out a role for Fc receptor binding in HVEM-CD160 costimulation, we used target cells expressing high levels of HVEM (Supplemental Fig. 2B). Similar to NK92 cells coated with HVEM-Fc, K562 cells expressing high levels of HVEM stimulated robust ERK1/2 and AKT phosphorylation in NK92 cells (Fig. 6C). Importantly, we confirmed that AKT phosphorylation was occurring in the NK92 cells and not in the target cells, as pretreatment of NK92 cells with the phosphoinositide-3 kinase (PI3K) inhibitor Wortmannin blocked target cell-induced AKT activation (Fig. 6D). Together, these results demonstrate that HVEM engagement of CD160, and not other receptors, promotes signaling downstream of target cell recognition in NK cells.

HVEM-CD160 enhances NK lysis of target cells

We next tested whether enhanced biochemical activation of NK cells by HVEM-CD160 engagement was correlated with enhanced NK cell lytic function. Additionally, we determined whether the presence of BTLA alters lytic activity using NK92 cells expressing high levels of BTLA or a BTLA mutant lacking the BTLA signaling domain (BTLAΔCyt) (Supplemental Fig. 2C). In control NK92 cells, we observed increased lysis of target cells expressing HVEM as compared to control K562 cells, consistent with HVEM costimulation through CD160 (Fig. 7A). Interestingly, NK92 cells expressing high levels of BTLA showed reduced lysis of target cells expressing HVEM as compared to control K562 cells, consistent with HVEM inhibition through BTLA. Importantly, in NK92 cells expressing high levels of BTLAΔCyt, we again observed increased lysis of target cells expressing HVEM as compared to control K562 cells, demonstrating that BTLA signaling could inhibit lytic

activity of NK cells. We also measured lytic function of IL-2 activated primary NK cells treated with fusion proteins targeting HVEM ligands (Fig. 7B). In this regard, NK cells treated with HVEM-Fc showed greater lytic activity as compared to NK cells treated with LTβR-Fc, UL144-Fc, or the Y61A HVEM-Fc mutant, consistent with HVEM costimulation of lytic activity through CD160 (26). Thus, HVEM costimulates or inhibits NK cytolysis depending on whether CD160 or BTLA is activated.

Discussion

Here, we report that HVEM interaction with CD160 on NK cells results in costimulation of NK cell effector function (Fig. 8). Specifically, HVEM binding to CD160 enhances CD69 expression, inflammatory cytokine expression, degranulation as measured by CD107a expression, and cytolysis by NK cells. Importantly, we show that HVEM activity is not the result of HVEM association with other ligands such as LIGHT or BTLA, as these proteins are poorly expressed on NK cells, and because LTβR, the Y61A mutant HVEM, and the viral protein UL144 all fail to induce costimulatory activity on NK cells. NK cells express abundant levels of the HVEM ligand CD160, which has been shown to activate NK cells when engaged with MHC related ligands (39). Interestingly, we find that the viral protein UL144 selectively binds BTLA and not CD160, thus exclusively activating inhibitory signaling through BTLA (Fig. 8).

Our results are consistent with the idea that CD160 is an activating receptor. Recent work has demonstrated the presence of an alternative splice variant of CD160 coding for a tyrosine-containing cytoplasmic tail which may recruit Fyn, SHC1, or the p85 subunit of PI3K (48), and is required for induction of ERK1/2 phsophorylation and proliferation in Jurkat cells (40, 47). We identified PI-PLC-resistant CD160 in NKG2C^{-} NK cells, while the majority of CD160 was cleaved in NKG2C^{+} NK cells. Notably, it is difficult to estimate the proportion of GPI- or transmembrane- linked CD160 as antibodies do not recognize these forms equivalently (unpublished observations) (40). Nevertheless, the increased proportion of transmembrane-linked CD160 suggests that NKG2C^{-} cells are poised to respond to HVEM coupled with inflammatory stimuli. Together with the extensive reports that in HCMV infected individuals there is an expansion of the NKG2C^{+} NK cell subset (36), we propose that as the NK compartment adapts to HCMV these cells become less reliant on non-specific cues from the environment such as HVEM. Thus, HVEM-CD160 may be a pathway to activate antigen inexperienced NK cells.

The ubiquitous expression of the CD160 ligand, HVEM (15), and MHC proteins (24, 26) in lymphoid and non-lymphoid cells including mucosal epithelia cells, raises the possibility of constitutive NK cell activation. It is important to note that HVEM induces robust NK cell effector activity only in conjunction with cytokines (IFNβ or IL2) or direct target cell contact. Thus, the HVEM-CD160 interaction functions to costimulate NK cell activity in the context of inflammation. Additionally, BTLA upregulation competes with CD160 to provide negative feedback for NK cell activation, although it is unclear whether BTLA antagonizes CD160 signaling directly. In our model, UL144 selectivity for BTLA prevents costimulation of NK cells and thus may attenuate anti-viral functions to promote viral replication and spread.

CD160 was shown to act as an inhibitory receptor in a fraction of CD4^{+} T cells notably lacking a transmembrane variant of CD160, although it remains unclear how GPI-linked proteins initiate inhibitory signaling (15). BTLA activation reduces CD3ζ phosphorylation in T cells and Syk, BLNK, and PLCγ2 phosphorylation in B cells (18, 49). Thus, human CMV has evolved UL144 as a BTLA-specific ligand to inhibit lymphocyte activation, and to prevent NK cell activation of effector functions associated with HVEM and CD160. Of

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note, activation of CD160 by HVEM may be primate-specific as CD160 alternative transcripts have not been identified and CD160 loci do not encode tyrosine-containing cytoplasmic domains in non-primates (unpublished observations). Additionally, mouse NK cells may not be activated through CD160 (50). Thus, we suggest that primate CMV may have coevolved the expression of UL144 in response to the evolution of CD160 as an activating receptor in primates.

The association between different strains of CMV and disease outcome in congenital or postnatal infection is controversial (51, 52). Nevertheless, there continue to be reports that specific CMV variants encoding unique UL144 sequences may be associated with termination of pregnancy, or newborn viremia, symptomatic infection, and developmental sequelae (53, 54). The uniform BTLA selectivity among all UL144 variants implies that UL144 has a particularly forgiving structure, however, the factors that drive hypervariability of the UL144 ectodomain remain elusive (43). In addition, UL144 can regulate NF-κB-dependent signaling pathways (55), and has been recently revealed to be expressed in human myeloid cells latently infected with specific isolates of CMV (56), strongly suggesting multiple functions for this viral HVEM ortholog. HVEM may also be a proinflammatory factor in tumors. In this regard, in follicular lymphoma, the most common secondary karyotypic alteration at 1p36 is due to deletions or mutations in *TNFRSF14*, which are associated with poor prognosis (57). In accordance with the cancer immunoediting model (58), HVEM-deficient tumors may escape immunosurveillance by CD160-expressing NK cells in order to acquire additional mutations. Thus, regulation of HVEM-BTLA-CD160 may represent a common immune evasion mechanism utilized both by viruses and by tumors, and suggests that manipulation of this regulatory network may serve as a potential therapeutic target to control inflammatory responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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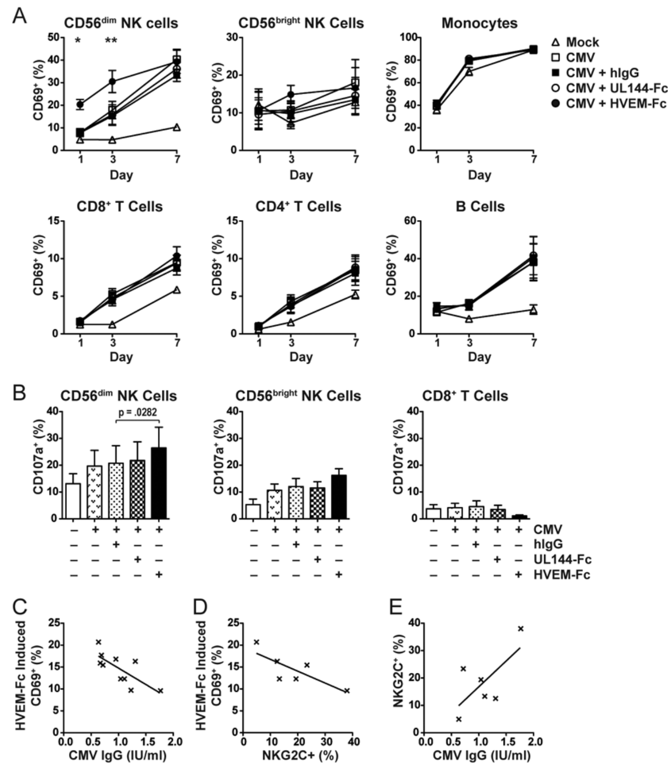

Figure 1. HVEM-Fc enhances activation of human CD56^{dim} NK cells in response to virus infected cells

(A–B) Freshly isolated PBMC cultured with mock- or CMV-infected NHDF cells were left untreated or were treated with HVEM-Fc, UL144-Fc, or human Ig control. Graphs indicate the percent of cells expressing CD69 within CD3$^+$CD8$^+$, CD3$^+$CD4$^+$, CD19$^+$, CD56^{dim}, CD56^{bright}, or CD14$^+$ gates over one week of culture in panel A, or the percent of cells expressing surface CD107a within CD56^{dim}, CD56^{bright}, and CD3$^+$CD8$^+$ cells following overnight culture in panel B. Results are representative of at least two separate experiments with at least 4 donors each. * $p<0.05$, ** $p<0.01$ between Ig and HVEM-Fc in panel A. Graphs show mean +/− SEM, significant p values are shown in panel B. (C–D) Costimulation of CD56^{dim} NK cells with HVEM-Fc was plotted against CMV IgG titers in panel C and the frequency of NKG2C expression within CD56^{dim} cells in CMV seropositive donors (CMV IgG > 0.5 IU/ml) in panel D. Costimulation of CD56^{dim} NK cells is calculated as the percent expression of CD69 with HVEM-Fc treatment minus the percent expression of CD69 with control Ig treatment (HVEM-Fc Induced CD69 (%)). (E) CMV IgG titers in CMV seropositive donors are plotted against frequency of NKG2C expression within CD56^{dim} cells.

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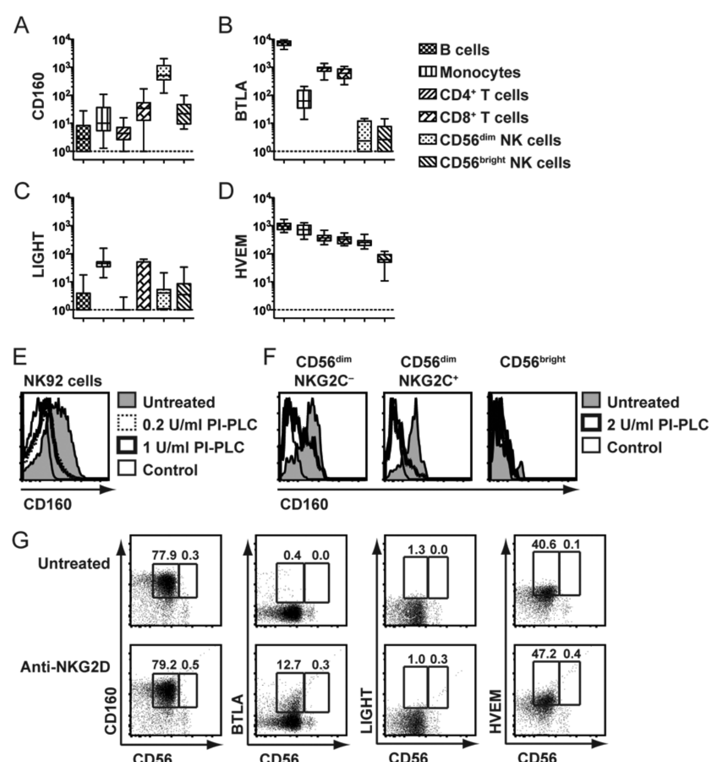

Figure 2. Expression of HVEM and its ligands in human CD56^{dim} NK cells

(A–D) Box and whisker plots show MFI of CD160 in panel A, BTLA in panel B, LIGHT in panel C, or HVEM in panel D in PBMC gated on CD19^{+} B cells, CD14^{+} Monocytes, CD3^{+}CD4^{+} T cells, CD3^{+}CD8^{+} T cells, CD56^{dim}, and CD56^{bright} NK cells. (E–F) NK92 cells or PBMC were left untreated or treated with the indicated doses of PI-PLC. Overlaid histograms show CD160 expression in NK92 cells in panel E or in NKG2C$^{+/-}$ CD56^{dim} and CD56^{bright} NK cells in panel F with and without PI-PLC treatment. (G) CD56^{+} cells purified from whole blood were left untreated or activated with anti-NKG2D for two days and then analyzed for surface marker expression. Two color histograms show CD56 plotted against BTLA, CD160, HVEM and LIGHT with receptor positive CD56^{dim} and CD56^{bright} fractions indicated. Results are representative of three independent donors.

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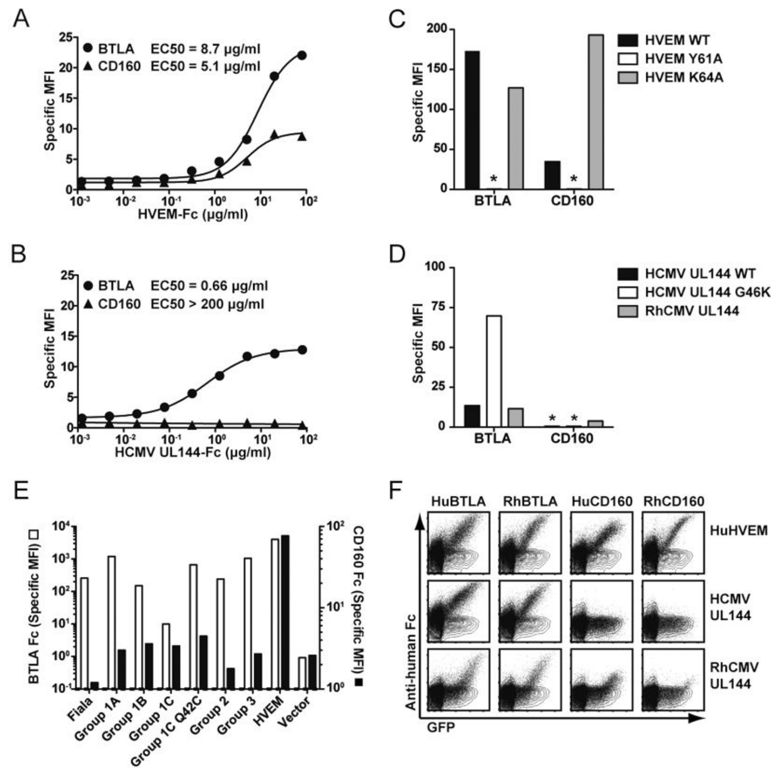

Figure 3. HVEM but not its ortholog UL144 binds CD160

(A–B) Human BTLA- or CD160-expressing EL4 cells were stained with the indicated concentrations of HVEM-Fc or human CMV UL144-Fc. EC50 values calculated using four parameter (variable slope) analysis. (C) Cells used above were stained with 20 µg/ml of wild-type, Y61A, or K64A HVEM-Fc. (D) Cells used above were stained with 20 µg/ml of Fiala strain human CMV, G46K, or rhesus CMV UL144-Fc. (E) Representative human CMV group UL144- or HVEM-expressing 293T cells were stained with 50 µg/ml of BTLA-Fc (white) or CD160-Fc (black). (F) Human or rhesus BTLA- or CD160-expressing 293T cells were stained with 20 µg/ml of HVEM-Fc, human CMV UL144-Fc, or rhesus CMV UL144-Fc. Dot plots of GFP plotted against anti-human Fc show selective loss of interaction between CD160 and human CMV UL144. * No staining.

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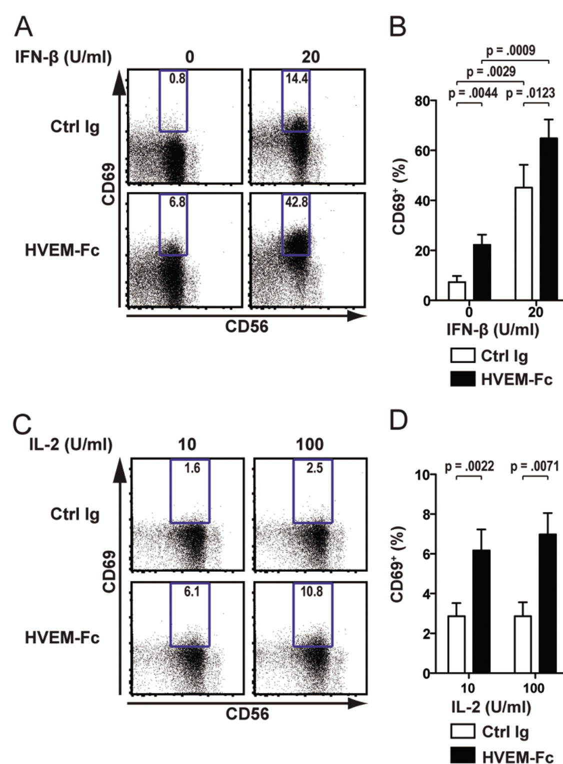

Figure 4. HVEM-Fc costimulates type I IFN and IL-2 activation of CD56^{dim} NK cells within PBMC

Freshly isolated PBMC were treated with HVEM-Fc or human Ig control and stimulated with 20 U/ml of IFN-β 18 hours or 10 and 100 U/ml of IL-2 for 8 hours. (A–D) The percentage of CD56^{dim} cells that are CD69^{+} within gated CD14^{-}/CD19^{-}/CD3^{-} cells are shown in dot plots of CD56 versus CD69 with the percentage of CD56^{dim}/CD69^{+} cells indicated for representative donors in panels A,C, and graphed in panels B,D. Results are representative of at least three separate experiments with at least 4 donors each. Graphs show mean + SEM, specific p values are shown.

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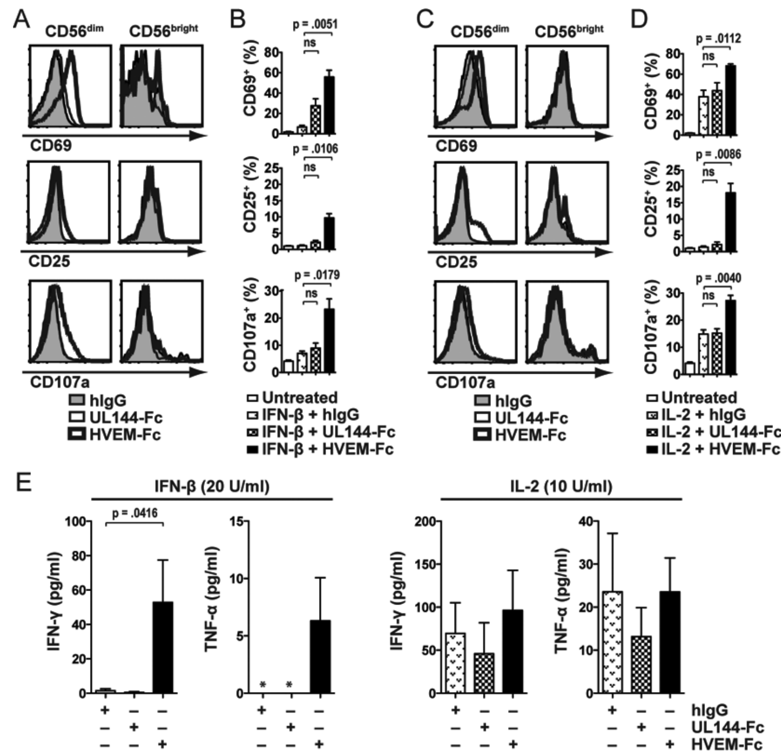

Figure 5. HVEM-Fc co-activates type I IFN and IL-2 induction of inflammatory effectors by CD56^{dim} NK cells

Purified CD56^{+} cells from whole blood were untreated or treated with HVEM-Fc, UL144-Fc or human Ig control and stimulated overnight with 20 U/ml of IFN-β or 10 U/ml IL-2. (A–D) Overlaid histograms of cells from representative donors show expression of CD69 (top row), CD25 (middle row), or CD107a (bottom row) in CD56^{dim} and CD56^{bright} NK cells in panels A,C. Graphs show the percent of CD56^{dim} cells expressing CD69 (top), CD25 (middle), and CD107a (bottom) in panels B,D. Results are representative of two separate experiments with at least 4 donors each, mean + SEM, specific p values are shown. (E) Culture supernatants were collected and assayed for the presence of IFN-γ and TNF-α. Graphs show mean + SEM from two experiments, significant p values are shown. * none detected.

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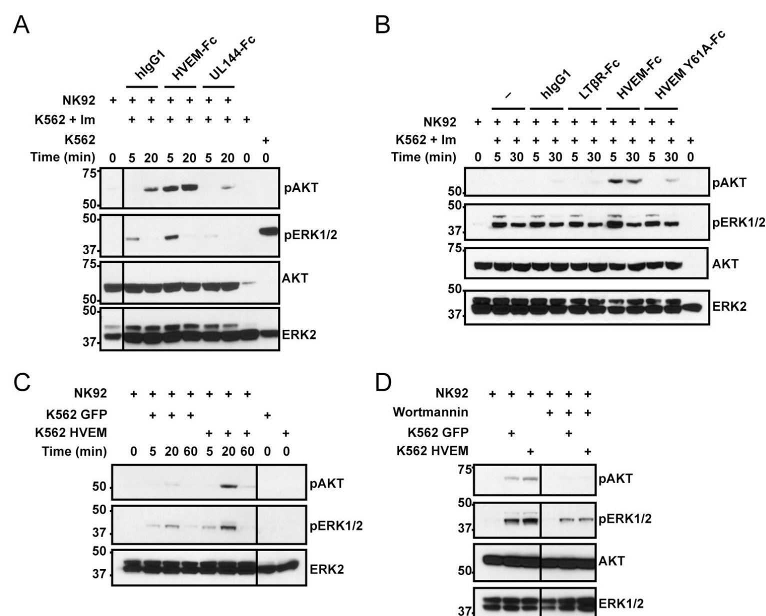

Figure 6. Target cell expressed HVEM costimulates AKT signaling in NK cells

(A–B) NK92 cells were treated with human IgG_1, HVEM-, and UL144-Fc in panel A, or human IgG_1, LTβR-Fc, HVEM-Fc, and HVEM Y61A-Fc in panel B and stimulated with Imatinib-treated K562 cells for the indicated times. (C–D) Untreated NK92 cells or Wortmannin-treated NK92 cells were stimulated with Imatinib-treated K562 cells transduced with GFP control- or HVEM-expressing vector for the indicated times in panel C or for 15 minutes in panel D. Western blots of whole cell extracts show phospho-ERK1/2 and phospho-AKT (S473) to monitor activation and total AKT and total ERK2 to control for total protein levels. K562 cells alone are shown to show target cell specific signals. Untreated or Imatinib treated K562 cells are shown where indicated to show target cell contribution to phosphorylation signals. Im, Imatinib.

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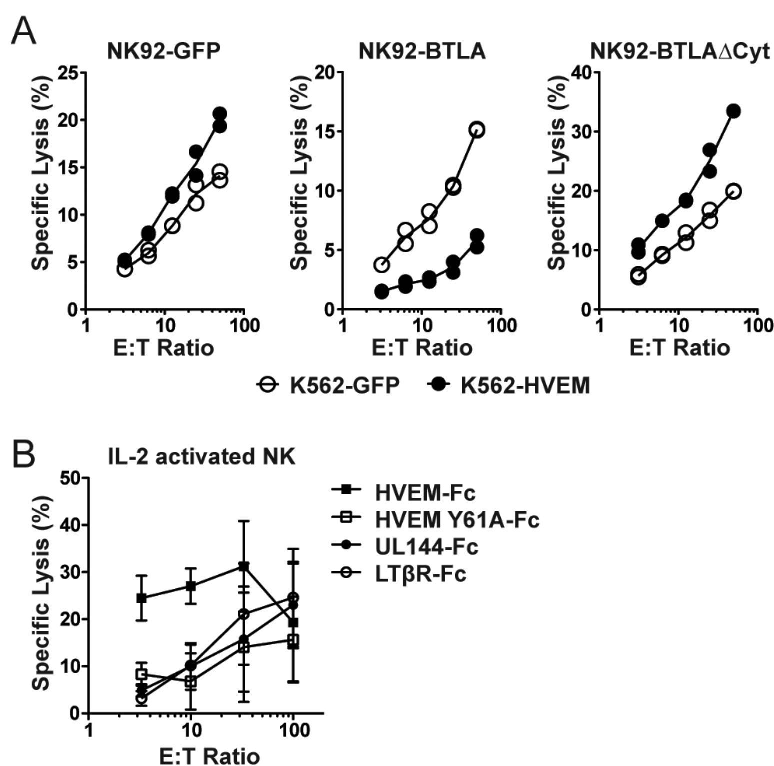

Figure 7. HVEM enhances lytic activity of NK cells

(A) Titrated GFP-, BTLA-, or BTLAΔCyt-expressing NK92 cells were incubated with labeled GFP- or HVEM-expressing K562 cells. Graphs show the specific lysis of K562 target cell lines following incubation with effector cells. Curves are mean with replicates plotted, representative of at least two experiments. (B) IL-2-activated purified human NK cells were incubated with the indicated fusion proteins and titrated onto K562 target cells. Graph shows specific lysis of target cells following incubation with effector cells. Curves are means +/– SEM, representative of at least two experiments. E:T, effector:target.

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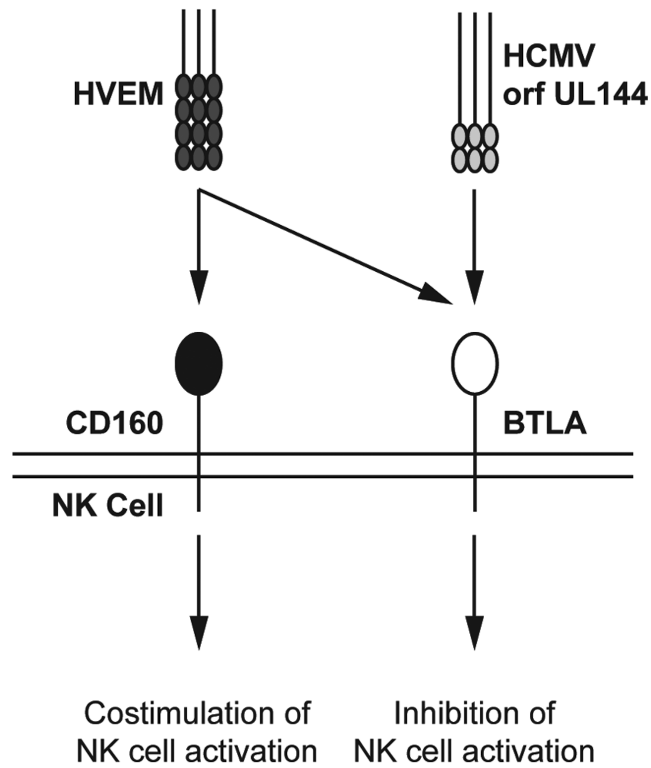

Figure 8. Model of HVEM and human CMV UL144 regulation of NK cell activation

HVEM binding to CD160 expressed by NK cells costimulates activation signals from cytokines and target cells. HVEM and human CMV UL144 binding to BTLA on NK cells inhibits NK activation resulting in attenuated effector function.