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

John R. Sedý, Ryan L. Bjordahl, Vasileios Bekiaris, Matthew G. Macauley, Brian C. Ware, Paula S. Norris, Nell S. Lurain, Chris A. Benedict and Carl F. Ware

*J Immunol* 2013; 191:828-836; Prepublished online 12 June 2013; doi: 10.4049/jimmunol.1300894
http://www.jimmunol.org/content/191/2/828

Supplementary Material http://www.jimmunol.org/content/suppl/2013/06/12/jimmunol.1300894.DC1

References This article cites 57 articles, 31 of which you can access for free at: http://www.jimmunol.org/content/191/2/828.full#ref-list-1

Subscription Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
CD160 Activation by Herpesvirus Entry Mediator Augments Inflammatory Cytokine Production and Cytolytic Function by NK Cells

John R. Šedý,* Ryan L. Bjordahl,* Vasileios Bekiaris,* Matthew G. Macauley,* Brian C. Ware,* Paula S. Norris,* Nell S. Lurain,† Chris A. Benedict,‡ and Carl F. Ware*

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. In this study, we show that HVEM specifically activates CD160 on effector NK cells challenged with virus-infected cells. Human CD56dim 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 hyperphosphorylation of serine kinases ERK1/2 and AKT and enhanced cytolyis of target cells. In contrast, HVEM activation of BTLA reduced cytolyis 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. The Journal of Immunology, 2013, 191: 828–836.

Natural killer 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 cytolyis and cytokine release (3–5). NK cell activation is restrained by a variety of inhibitory receptors that prevent uncontrolled cytolyis and inflammation through the recognition of self-MHC molecules expressed in healthy, uninfected cells (6). Although many herpesviruses have manipulated the balance between inhibitory and activating signaling 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.

*Infectious and Inflammatory Disease Center, Sanford|Burnham Medical Research Institute, La Jolla, CA 92037; †Department of Immunology and Microbiology, Rush University Medical Center, Chicago, IL 60612; ‡Division of Immune Regulation, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037

Received for publication April 3, 2013. Accepted for publication May 13, 2013.

This work was supported by grants from the National Institutes of Health (AI-033068, AI48073, and CA164679 to C.F.W.) and AI069298 to C.A.B.), postdoctoral training fellowships from the National Institutes of Health (T32AI06536 to J.R.S. and T32CA121949 to R.L.B.), a Diabetes and Immune Disease National Research Institute Fellowship (DIDNRI/10 to J.R.S.), a Type 1 Diabetes Clinical Research Fellowship (T1D-CRF to J.R.S.), and a gift from the Perkins Family Foundation (to C.F.W.).

Address correspondence and reprint requests to Dr. Carl F. Ware, Infectious and Inflammatory Disease Center, Sanford|Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037. E-mail address: cware@sanfordburnham.org

The online version of this article contains supplemental material.

Abbreviations used in this article: BTLA, B and T lymphocyte attenuator; GPI, glycosylphosphatidylinositol; HVEM, herpesvirus entry mediator; MFI, mean fluorescence intensity; NHDF, normal human dermal fibroblast; PI-PLC, phosphatidylinositol-specific phospholipase C.
Furthermore, the nature of the selective pressures mitigated by UL144 as CMV coevolved with primate hosts remains elusive. In this study, we used 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 compared with viral UL144, which reflects the inability of 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 cytolyis. 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 by the SanfordBurnham 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. PBMCs were isolated from buffy coats and washed twice with PBS. NK cells were further purified using an 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 1640 supplemented with 10% heat-inactivated FBS, antibiotics, l-glutamine, and 50 μM 2-ME were first incubated on ice for 15–30 min with Fe fusion proteins or human IgG control. For infectious coculture experiments, normal human dermal fibroblast (NHDF) cells were infected with CMV (laboratory strain AD169) at a multiplicity of infection of 1 for 6 h, washed with PBS, and mixed with pretreated PBMCs at a ratio of 100:1 (PBMCs/NHDFs). Productive infection was validated by RT-PCR (Supplemental Fig. 1B). Alternatively, pretreated cells were activated at 37˚C in flat-bottom plates with recombinant human IFN-γ (R&D Systems, Minneapolis, MN), recombinant human IL-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 IgG1 Fc were produced as previously described (17).

Abs and flow cytometry

Abs used to identify human PBMC populations include CD3 eFluor 450, CD4 PE-Cy7, CD8a allophycocyanin eFluor 780, CD14 FITC, CD19 FITC, BTLA PE, HVEM PE, LIGHT allophycocyanin (eBioscience), CD69 PerCP-Cy5.5, CD107a Alexa Fluor 647, CD160 Alexa Fluor 647, IFN-γ PE-Cy7 (BioLegend, San Diego, CA), CD25 PE, CD56 Alexa Fluor 700 (BD Biosciences, San Diego, CA), and NKG2C PE (R&D Systems).

PBMCs within the live gate of flow cytometric analysis were defined as CD19 + B cells (CD14 +/CD19+/SSClow), CD14 + monocytes (CD14+/CD19+/SSChigh), CD4 + T cells (CD14−/CD19−/CD56−/CD3+/CD4+/CD8−), CD8 + T cells (CD14−/CD19−/CD56−/CD3+/CD4−/CD8+), and CD8 − T cells (CD14−/CD19−/CD56−/CD3−/CD4−/CD8−).

FIGURE 1. HVEM-Fc enhances activation of human CD56 dim NK cells in response to virus-infected cells. (A and B) Freshly isolated PBMCs cultured with mock- or CMV-infected NHDF cells were left untreated or were treated with HVEM-Fc, UL144-Fc, or human Ig control. Graphs show the percentage of cells expressing CD69 within CD3−CD8−, CD3−CD4−, CD19−, CD56 dim, CD56 bright, or CD14+ gates over 1 wk of culture (A) or the percentage of cells expressing surface CD107a within CD56 dim, CD56 bright, and CD3−CD8− cells following overnight culture (B). Results are representative of at least two separate experiments with at least four donors each. Graphs show mean ± SEM. Significant p values are shown in (B). Costimulation of CD56 dim NK cells with HVEM-Fc was plotted against CMV IgG titers (C) and the frequency of NKG2C expression within CD56 dim cells in CMV-seropositive donors (CMV IgG > 0.5 IU/ml) (D). Costimulation of CD56 dim NK cells is calculated as the percentage expression of CD69 with HVEM-Fc treatment minus the percentage expression of CD69 with control Ig treatment (HVEM-Fc–induced CD69 [%]). (E) CMV IgG titers in CMV-seropositive donors are plotted against the frequency of NKG2C expression within CD56 dim cells. *p < 0.05, **p < 0.01, between Ig and HVEM-Fc.
CD107a expression was tested by first incubating anti-CD107a and GolgiStop (BD Biosciences) during the final 4 h of PBMC or NK cell activation cultures at a final dilution of 1:1000. Cells were washed and resuspended in buffer for extracellular staining and then washed, fixed in 2% paraformaldehyde, and analyzed.

Phosphatidylinositol-specific phospholipase C (PI-PLC; Invitrogen, Carlsbad, CA) was used to distinguish between the glycoprophoinositide (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, t-glutamine, and 50 μM 2-ME. NK92 cells were maintained in RPMI 1640 supplemented with 8% heat-inactivated FBS, antibiotics, t-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 (RhBTLABglII: 5'-GTCAGAAGGACC-3'; RhCD160BglII: 5'-GTCAGATCTGCAGGAAATGAAGACATTG-3'; RhCD160XhoI: 5'-AGTCCTCGAGGGCTT-9-3'; FUL144-G46K: 5'-AAACAAGGATATCGTGTTACAAAACAATGTACGCAATATACAGGAG-3').

Pseudotyped single-infection retroviral plasmid by PCR amplification (bCD160BglII: 5'-AGTCAGATCTGCAGGAAATGAAGACATTG-3'; bCD160XhoI: 5'-AGTCCTCGAGGGCTT-9-3'; hBTLAR179stop-forward: 5'-GTCAGAAACAGACTTAACTCCTCACAC-3'; hBTLAR179stop-reverse: 5'-GCCTTCACAGGCCAGCAGGACC-3').

Cyt mutant was produced by round-the-world PCR

Flow cytometric binding assays were performed as previously described (17). Cells were incubated with Fc ligands for 30 min at 4˚C in buffer (PBS with 2% FBS), washed twice, incubated with donkey anti-human Fc allophycocyanin (Jackson ImmunoResearch, West Grove, PA) for 15 min 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), according to the manufacturer's instructions.
Quantitative RT-PCR analysis

RNA was harvested from PBMC/NHDF mixtures using an RNeasy Mini kit (QIAGEN). cDNA was transcribed using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA), and transcripts for HCMV IE1 (HCMV-IE1[+]; L32F: 5'-CATCCACTAGTAGACGACAGTCT-3', HCMV-IE1[-]; 5'-GCATGAGGTCCTGCCAG-3'), HCMV gB (HCMV-gB[+]; 5'-AATACCC-CACAGTACCCGTTACG-3', HCMV-gB[-]; 5'-ATAGACCCAGTCTGGTCGCGGAAACCCA-3') 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 min and 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 min and washed prior to incubation with K562 cells. In experiments using fusion proteins, NK92 cells were coated with control human IgG1, LTBR-Fc, HVEM-Fc, UL144-Fc, or HVEM Y61A-Fc for ≥15 min on ice prior to activation. NK92 cells were aliquoted to 2 x 10^6 cells/condition in 100 μl and mixed with an equal volume of K562 target cells aliquoted to 2 x 10^5 cells/condition in 100 μl and mixed with an equal volume of K562 target cells aliquoted to 2 x 10^5 cells/condition on ice prior to activation. K562 cells were aliquoted to 2 x 10^6 cells/condition in a 100 μl mixture with an equal volume of K562 cells. Cell mixtures were activated at 37°C for the indicated times, quenched with ice-cold PBS, lysed in RIPA buffer at 4°C for 20 min, and centrifuged at 14,000 rpm at 4°C. Extracts were boiled in SDS loading buffer containing 1% 2-ME for 5 min and resolved by SDS-PAGE on 10% Bis-Tris gels (Bio-Rad). Proteins were transferred using the tank method to polyvinylidene difluoride membrane, blocked with 1% OVA in TBST buffer, and blotted with phospho-AKT (S473), phospho-ERK1/2, total AKT (Cell Signaling, Danvers, MA), and total ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA), followed by anti-rabbit HRP (GE Healthcare), and visualized by ECL (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), according to the manufacturer’s instructions. NK92 cells transduced with BTLA, BTLAΔCyt, or GFP vector control were cocultured with labeled K562 cells at various E:T ratios for 3 h prior to staining with 7-amino-actinomycin D. Specific lysis was calculated as described. Primary NK cell cytototoxicity was assayed using the JAM protocol (35). K562 target cells were radiolabeled with [3H]thymidine, washed, and incubated with IL-2–activated purified human NK cells treated with fusion proteins or control Ig at various E:T ratios for 4 h. Cultures were harvested onto filters, and [3H]thymidine incorporation was counted. Specific lysis was calculated as described.

Results

HVEM costimulates 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 the expression of activation markers in cells from human PBMCs mixed with CMV-infected fibroblasts (Fig. 1). As expected, these cocultures were marked by early expression of inflammatory cytokines, including IFN-γ, LT-α, TNF-α, IL-6, IL-8, and IL-17A, which 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 cocultures. Notably, CD69 expression was uniquely upregulated on CD56dim NK cells through day 3 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 CD56dim NK cells by HVEM-Fc after 1 d 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). However, we do note 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 CD56dim NK cells (Fig. 1C–E), both hallmarks of adaptive and innate memory responses to CMV, respectively (36–38). Nevertheless, CD56dim NK cells from all but one donor were responsive to HVEM-Fc costimulation. Thus, HVEM-Fc specifically enhances early activation of CD56dim NK cells during responses to CMV.

Human CD56dim NK cells uniquely express CD160 among HVEM ligands

To identify which HVEM ligands were expressed on lymphocytes, we examined the expression of BTLA, CD160, LIGHT, and HVEM in human peripheral blood (Fig. 2A–D). B cells showed high BTLA expression and low CD160 expression, whereas T cells and monocytes expressed intermediate levels of both BTLA and CD160. Importantly, CD56dim NK cells showed the highest surface expression of CD160, confirming previously reported data (39), and the lowest BTLA expression among all PBMCs, whereas CD56bright NK cells expressed low levels of both BTLA and CD160. LIGHT was specifically expressed by monocytes and CD8+ T cells and weakly by NK cells. In contrast, HVEM was broadly expressed by all PBMCs.

We next sought to distinguish the expression of CD160 splice variants encoding GPI or transmembrane cellular linkages using PI-PLC treatment, which removes GPI-linked proteins (40). We found that, although 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, 2F). Finally, human NK cells upregulated the expression of BTLA in response to stimulation through the activating receptor NKG2D, similar to BTLA expression on T cells stimulated through the TCR (Fig. 2G) (41). Thus, in resting human NK cells, CD160 is the predominant HVEM receptor, a portion of which is not GPI linked, whereas 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, 3B). We found that UL144-Fc only bound cells expressing BTLA and not CD160, whereas HVEM-Fc bound both BTLA- and CD160-expressing cells with similar dissociation constants and required overlapping surfaces of HVEM to bind these receptors, because 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 (J.R. Šedý, W. Smith, I. Nemčovičová, P.S. Norris, D. Zajonc, C.F. Ware, 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. We note one exception: UL144 from rhesus CMV bound human and rhesus CD160 with low affinity (Fig. 3D, 3F). This interaction likely represents a divergence between viral species and not host species, because primate BTLA and CD160 are highly homologous (J.R. Šedý and C.F. Ware, 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 uses 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.

HVEM-Fc costimulates 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 the 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 CD56dim NK cells expressing CD69, with or without
IFN-β stimulation, in 18-h cultures (Fig. 4A, 4B), as well as in 8-h cultures stimulated with IL-2 (Fig. 4C, 4D). Additionally, in purified NK cells stimulated with IFN-β or IL-2, HVEM-Fc costimulated expression of the high-affinity IL-2Rα subunit, CD25, and the cytolytic granule marker, CD107a, in the CD56\(^{dim}\) subset (Fig. 5A–D). Furthermore, HVEM-Fc costimulation 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 costimulates 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 the results obtained using peripheral blood CD56\(^{dim}\) NK cells, NK92 cells expressed abundant CD160 and low BTLA (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 compared with control Ig (Fig. 6A, 6B). Because LIGHT ligation does not coactivate ERK1/2 and AKT signaling, and 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 FcR 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, because pretreatment of NK92 cells with the 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 compared with 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 compared with 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 compared with 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 compared with 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 (Fig. 8).
Discussion

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, because 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 was 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 demonstrated the presence of an alternative splice variant of CD160 coding for a tyrosine-containing cytoplasmic tail that may recruit Fyn, SHC1, or the p85 subunit of PI3K (48), which is required for induction of ERK1/2 phosphorylation and proliferation in Jurkat cells (40, 47). We identified PI-PLC-resistant CD160 in NKG2C- NK cells, whereas the majority of CD160 was cleaved in NKG2C+ NK cells. Notably, it is difficult to estimate the proportion of GPI- or transmembrane-linked CD160, because Abs do not recognize these forms equivalently (J.R. Šedy and C.F. Ware, 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 there is an expansion of the NKG2C+ NK cell subset in human CMV–infected individuals (36), we propose that, as the NK compartment adapts to human CMV, these cells become less reliant on nonspecific cues from the environment, such as HVEM. Thus, HVEM–CD160 may be a pathway to activate Ag in experienced NK cells.

The ubiquitous expression of the CD160 ligand, HVEM (15), and MHC proteins (24, 26) in lymphoid and nonlymphoid 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 (IFN-β or IL-2) 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 antiviral 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 note, activation of CD160 by HVEM may be primate specific, because CD160 alternative transcripts have not been identified, and CD160 loci do not encode tyrosine-containing cytoplasmic domains in nonprimates (J.R. Šedy and C.F. Ware, 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, 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–signal- ing pathways (55), and it was 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 to acquire additional mutations. Thus, regulation of HVEM–BTLA–CD160 may represent a common immune-evasion mechanism used by both viruses and tumors and suggests that manipulation of this regulatory network may serve as a potential therapeutic target to control inflammatory responses.

Acknowledgments

We thank the personnel of the Flow Cytometry Shared Resources facility at SanfordBurnham Medical Research Institute for technical assistance and Ana V. Miletic Šedy and Daniel L. Popkin for critical reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Characterization of PBMC responses to CMV-infected fibroblasts

**A.-B.** Freshly isolated PBMC were co-cultured with mock- or CMV-infected NHDF cells. Culture supernatants were harvested after two days and analyzed for IL-1β, IL-2, IL-6, IL-8, IL-17A, IL-17F, IL-21, IL-22, IFN-β, IFN-γ, LT-α, and TNF-α. Graphs show the mean levels of cytokines present + SEM, significant p values are shown (A.). Cells were harvested after three days for RNA to synthesize cDNA for RT-PCR. Graphs show the mean expression of *HCMV IE1* and *gB* present + SEM, significant p values are shown (B.). Results are pooled from 5 donors. **C.-E.** Costimulation of CD69 expression in CD56<sup>dim</sup> NK cells with HVEM-Fc was plotted against donor age (B.), sex (C.), CMV seropositivity status (D.). Costimulation of CD56<sup>dim</sup> NK cells is calculated as in Fig.1.
Supplemental Figure 2

A. The expression of BTLA (left) and CD160 (right) is plotted in overlaid histograms with specific staining in gray. B. The expression of GFP and HVEM in K562 cells transduced with GFP- (left) or HVEM- (right) containing retroviruses is plotted in two-color overlaid histograms with specific staining in red. C. The expression of GFP and BTLA (first column), CD160 (second column), HVEM (third column), and LIGHT (fourth column) in NK92 cells transduced with GFP- (top row), BTLA- (middle row), or BTLAΔCyt-containing retroviruses is plotted in two-color overlaid histograms with specific staining in red.

Supplemental Figure 2. Expression of native and transduced BTLA, CD160, HVEM, and LIGHT by NK92 and K562 cells