Soluble Fc-Disabled Herpes Virus Entry Mediator Augments Activation and Cytotoxicity of NK Cells by Promoting Cross-Talk between NK Cells and Monocytes

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CD160 is highly expressed by NK cells and is associated with cytoytic effector activity. Herpes virus entry mediator (HVEM) activates NK cells for cytokine production and cytokytic function via CD160. Fc-fusions are a well-established class of therapeutics, where the Fc domain provides additional biological and pharmacological properties to the fusion protein including enhanced serum t1/2 and interaction with Fc receptor–expressing immune cells. We evaluated the specific function of HVEM in regulating CD160-mediated NK cell effector function by generating a fusion of the HVEM extracellular domain with human IgG1 Fc bearing CD16-binding mutations (Fc*) resulting in HVEM-(Fc*). HVEM-(Fc*) displayed reduced binding to the Fc receptor CD16 (i.e., Fc-disabled HVEM), which limited Fc receptor–induced responses. HVEM-(Fc*) functional activity was compared with HVEM-Fc containing the wild type human IgG1 Fc. HVEM-(Fc*) treatment of NK cells and PBMCs caused greater IFN-γ production, enhanced cytotoxicity, reduced NK fratricide, and no change in CD16 expression on human NK cells compared with HVEM-Fc. HVEM-(Fc*) treatment of monocytes or PBMCs enhanced the expression level of CD80, CD83, and CD40 expression on monocytes. HVEM-(Fc*)–enhanced NK cell activation and cytotoxicity were promoted via cross-talk between NK cells and monocytes that was driven by cell–cell contact. In this study, we have shown that soluble Fc-disabled HVEM-(Fc*) augments NK cell activation, IFN-γ production, and cytotoxicity of NK cells without inducing NK cell fratricide by promoting cross-talk between NK cells and monocytes without Fc receptor–induced effects. Soluble Fc-disabled HVEM-(Fc*) may be considered as a research and potentially therapeutic reagent for modulating immune responses via sole activation of HVEM receptors.

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Nat ural killer cells, a subset of lymphoid cells, are an essential component of the innate immune system that protects against viruses (e.g., human CMV, HIV, and hepatitis C virus), tumor cells, and other pathogens (1–5). NK cell innate immune responses are tightly regulated by multiple activating and inhibitory receptors. Unlike typical activating and inhibitory receptors on NK cells, CD160 is tightly regulated in two alternative splice variants: a GPI-anchored (CD160-GPI) form and a differentially spliced transmembrane form of the protein (CD160-TM) that is unique to NK cells. CD160 is part of the Ig superfamily of receptors and is predominantly expressed in peripheral blood NK cells, γδ T (6), and CD8 T lymphocytes (7, 8) with cytolytic effector activity. In circulating cells, the highest expression of CD160 RNA is identified in peripheral blood CD56dimCD16+ NK cells greater than CDB T cells (9). CD160 signals upon engagement of the widely expressed molecules herpes virus entry mediator (HVEM) and/or HLA-C (10–12). The engagement of CD160 by soluble HVEM (HVEM conjugated to the Fc portion of IgG1) or HVEM expressed on the cell surface was shown to activate NK cells (10). Genetic deficiency of CD160 in mice specifically impairs NK cell production of IFN-γ, which is an essential component of the innate response to control tumor growth (13).

HVEM is a member of the TNFR superfamily and is expressed on many immune cells, including NK cells, T and B cells, monocytes, and neutrophils (14–18). HVEM is an immune regulatory molecule (15, 18) that signals bidirectionally both as a receptor and a ligand. HVEM interacts with three cell surface molecules, CD160, LIGHT (homologous to lymphotoxins [LT], shows inducible expression, Research, the National Institute of Allergy and Infectious Diseases, and the International AIDS Society. These contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health or other funding sources.

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Abbreviations used in this article: 7-AAD, 7-amino-actinomycin D; ADCC, Ab-dependent cellular cytotoxicity; BTLA, B and T lymphocyte attenuator; DC, dendritic cell; IgG1, human IgG1; HVEM, herpes virus entry mediator; LIGHT, homologous to lymphotoxins, shows inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes; LT, lymphotoxin; MFI, mean fluorescence intensity; rhCD16a, recombinant human CD16a; SSC, side scatter.

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and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes), and B and T lymphocyte attenuator (BTLA) and in humans with LT-α or TNF-β (14–18). HVEM generates bidirectional signals, and recent literature provides evidence of signaling induced by interaction between HVEM and CD160, LIGHT, BTLA, or LT-α in different immune cells (7, 15, 19–25). The extracellular domain of HVEM was fused to the Fc portion of human IgG1 (hIgG1) in previous studies to produce a soluble protein used to detect HVEM ligands or alternatively to specifically activate BTLA or CD160 receptors (10, 26, 27). Because hIgG1 Fc binds to Fc receptor expressed on innate cells, including NK cells, HVEM-Fc fusion proteins may engage receptors for both the HVEM domain and the Fc domain. Fc fusion proteins have been widely used to interrogate the activities of cell surface proteins or soluble molecules and are widely used in immunotherapies such as etanercept, alefacept, and abatacept. The Fc domain of these fusion proteins may contribute to biological activities unrelated to the fusion partner and which can be removed through mutation of the Fc domain. To determine how HVEM engagement of NK cells may specifically function to activate NK cells in the absence of Fc receptor binding, we generated fusion proteins constructed of the extracellular domain of HVEM conjugated to a mutant hIgG1 Fc that does not bind to Fc receptor (HVEM-[Fc*]) (28).

LIGHT, a member of the TNF ligand superfamily, is mainly expressed on T cells, monocytes, NK cells, and immature dendritic cells (DC) (29) and binds to HVEM and LTβR, two membrane receptors (30). LIGHT–HVEM interactions are thought to regulate a variety of immune responses, for example, costimulation of T cell proliferation, polarization of CD4 T cells into Th1 cells and associated cytokine production (31), inducing DC maturation (31), stimulating Ig production in B cells (32), and activating NK cells (33). This interaction enhances phagocytosis of monocytes and neutrophils and contributes to antibacterial activity via production of ROS, NO, other proinflammatory cytokines, and direct bactericidal activity (33). In contrast, engagement of HVEM with BTLA on T cells inhibits anti-TCR–induced activation and cytokine secretion (34).

The expression of CD160, LIGHT, and BTLA varies greatly between different cell types, activation, and differentiation states (14, 18, 34, 35). NK cells mainly express CD160 and little to no expression of LIGHT or BTLA (14, 18, 34, 35). In contrast, monocytes, bone marrow–derived DC, and other immune cells express mainly LIGHT and little to no expression of BTLA or CD160 (14, 18, 34, 35). Thus, dynamic regulation of these receptors especially on NK cells and cells interacting with NK cells via HVEM signaling provide a potential mechanism for control of activating and inhibitory signals depending on cellular context.

NK cells interact with other immune cells for optimal cytokine production, cytotoxicity, and control of virally infected or tumor cells (36–47). There are several reports showing that monocytes, DC, and macrophages interact with NK cells and cooperate in the innate immune response for protection against pathogens (36–47). However, the role of HVEM-CD160 interactions in the activation of NK cells by other accessory cells expressing HVEM, its ligands, or Fc receptors is not known. In this study, we investigated the role of soluble Fc-disabled HVEM-(Fc*)–induced activation of NK cells and regulation of NK-mediated effector responses.

Materials and Methods
Cells
Fresh human blood was collected from healthy donors giving written informed consent at Case Western Reserve University, and the Institutional Internal Review Board approved all handling. PBMCs were isolated by Lymphoprep (STEMCELL Technologies) density gradient centrifugation as per manufacturer’s description from fresh human blood and fresh leukocyte reduction filters obtained from Red Cross, Cleveland, and cryopreserved where indicated. NK cells were isolated from PBMCs by negative selection using the NK Cell Enrichment Kit (STEMCELL Technologies). Monocyte isolation was performed by positive selection using CD14 microbeads and MACS columns (Miltenyi Biotec). HVEM-expressing CHO cells (HVEM-CHO) were obtained from K. G. Koukos. CD160-CCHO and HVEM-CCHO expressing human FcγRIIa (CD16) plus modified Eagle’s minimal essential medium (1:1) supplemented with 10% FBS, 1% glutamine, and 1% penicillin and streptomycin.

Reagents and mAbs
Soluble Fc-disabled HVEM-(Fc*) (product ID: HVEM-Fc0, SKU#: FCL0096K; available at: https://www.gnbiophio.com/index.php/products/1050/11/recombinant-proteins-enzymes-human-hvem-ecd-extracellular-domain-protein-fc-fusion-adsc-k0-mutant-recombinant-detail) was custom-synthesized by G&P Biosciences, Santa Clara, CA, and demonstrated having binding activity to recombinant BTLA and LIGHT protein and also to inhibit LIGHT-mediated signaling activities including cytotoxicity in L-929 mouse fibroblast. Soluble-CD160 and HVEM-His were purchased from SinoBiological, Beijing, China. HVEM-Fc was purchased from G&P Biosciences and PeproTech (Rocky Hill, NJ). The purity of fusion proteins was determined by SDS-PAGE western blots: HVEM-his expressed in HEK293 cells showed by endotoxin level <0.1 endotoxin unit/μg protein; HVEM-Fc >95% pure, endotoxin level <0.1 endotoxin unit/μg protein; HVEM-his >90% pure, endotoxin level <1 endotoxin unit/μg protein; CD160-his >95% pure, endotoxin level <1 endotoxin unit/μg protein. Alefacept was a generous gift from K. D. Cooper, Case Western Reserve University, Cleveland, OH. Anti-CD3–PE, AF700 or allopolyocyanin-Cy7, anti-CD160–BV785 or PE-Cy7, anti-CD16–allophycocyanin (SinoBiological, Beijing, China). HVEM-Fc was purchased from G&P Biosciences, Santa Clara, CA, and demonstrated having binding activity to recombinant BTLA and LIGHT protein and also to inhibit LIGHT-mediated signaling activities including cytotoxicity in L-929 mouse fibroblast. Soluble-CD160 and HVEM-His were purchased from SinoBiological, Beijing, China. HVEM-Fc was purchased from G&P Biosciences and PeproTech (Rocky Hill, NJ).

Immunophenotyping of cells by flow cytometry
PBMCs within the live gate (7-AAD–negative population) were analyzed for expression of HVEM and its receptors on CD56dim NK cells (CD14+/CD19−/CD56dim) and CD56bright NK cells (CD14+/CD19−/CD56bright), and CD14 and CD16 monocytes (CD14+side scatter [SSC]gly/h) by flow cytometry (BD LSRII), and data were analyzed by Flowjo (version 10.1; Tree Star).

Cytokine expression analysis
Supernatants from PBMC, NK cells, monocytes, and NK cell and monocyte coculture were analyzed for IFN-γ by ELISA (capture Ab [clone M700A] and biotin-conjugated secondary Ab [clone M701B] from Thermo Fisher Scientific) and were also analyzed by 65-plex LumineX array (Eve Technologies).

Assessment of Fc binding to CD16 and HVEM binding to CD160
To assess the binding of the Fc stalk of HVEM-Fc and HVEM-(Fc*) to CD16, beads conjugated with anti-His mAb (Miltenyi Biotec, Auburn,
CA) were treated by His-tag–conjugated recombinant human CD16a (rhCD16a) at 4°C for 2 h. Prebound rhCD16a beads (0.3 × 10^7) were incubated with hlgG1 (10 μg/ml), HVEM-Fc (10 μg/ml; PeproTech), HVEM-Fc (10 μg/ml; G&P Biosciences), or HVEM-(Fc*) (10 μg/ml; G&P Biosciences) on ice for 45 min. Then, beads were stained with anti-HVEM–PE mAb (1 μg/ml) at 4°C for 30 min and were analyzed by flow cytometry.

To assess the binding capacity of HVEM-Fc and HVEM-(Fc*) to CD160, CHO cells expressing CD160 (CD160-CHO cells) were incubated and stained with mouse anti-human HVEM–PerCP-Cy5.5 (2 μg/ml) and goat anti-hIgG Fc secondary Ab on ice for 45 min. Then, cells were washed on ice for 45 min. After washing, cells were incubated with PE-conjugated goat anti-hlgG1 Fc secondary Ab on ice for 45 min. Then, cells were washed and stained with mouse anti-human HVEM–PE mAb (1 μg/ml) at room temperature for 15 min. Cells were analyzed by flow cytometry.

**NK cell cytotoxicity assay**

Frozen PBMCs from healthy controls were thawed and cultured overnight in RPMI 1640 supplemented with 10% FBS, 1% glutamine, and 1% penicillin and streptomycin. PBMCs were then incubated with or without 1 μg/ml HVEM-(Fc*) for 44–44 h. Activation marker CD69 on NK cells was measured after 44 h by flow cytometry. PBMCs were further cocultured with prelabeled K562 cells at an E:T ratio of 20:1 in the presence of anti-CD107α–allophycocyanin-Cy7 for 5 h. K562 cells were stained with 7AAD and annexin–V–PE to quantify dead cells (7AAD+annexin–V+) by flow cytometry.

Where described, cytotoxicity assays were performed using purified NK cells (purity ≥95%) and monocytes (purity ≥95%) in cocultures. Briefly, NK cells were purified from thawed overnight-cultured PBMC by negative selection. Monocytes were purified by CD14-positive selection from the same donor. Purified NK cells (0.3 × 10^6), purified monocytes (0.3 × 10^6), or purified NK cells plus purified monocytes coculture at a ratio of 1:1 (0.3 × 10^6 + 0.3 × 10^6) was performed in the presence or absence of HVEM-(Fc*) (1 μg/ml) for 44 h. After 44 h, cells were then stained for activation markers and analyzed by flow cytometry. NK cells, monocytes, or NK cells plus monocyte mixture were further cocultured with labeled K562 cells at a ratio of NK cells to K562 cells of 1.5:1 for 5 h in the presence of anti-CD107α mAb–allophycocyanin-Cy7 or its isotype control Ab. The cocultured cells were then stained with 7AAD and annexin–V–PE to quantitate dead cells (7AADannexin–V+) by flow cytometry. For transwell cultures (96-well plate), NK cells (0.3 × 10^5 cells per well) were seeded into the lower chamber, and monocytes (0.3 × 10^5 cells per well) were seeded in the upper chamber or both NK cells plus monocyte mixture (1:1) were seeded in the lower chamber and were cultured in the absence or presence of HVEM-(Fc*) for 44 h. Activation markers and cytotoxicity were measured identically to above in nontranswell cultures.

We performed reverse Ab-dependent cellular cytotoxicity (ADCC) assay using the well-characterized P815 mouse mastocytoma cell line that abundantly expresses mouse CD16 (mouse Fc receptor) as a target cell to evaluate the activity of HVEM-(Fc*)–induced activation of NK cells. This is a well-studied model, similar to K562 killing assays for spontaneous killing, to ascertain reverse ADCC assay with a defined system. A mouse anti-human CD16 monoclonal is used to bridge human NK cell effectors with mouse P815 mastocytoma targets resulting in reverse ADCC assay (49). Briefly, the NK cells were pretreated with either HVEM-Fc, HVEM-(Fc*), or IgG1 for 16 h. Cells were washed to remove the unbound stimuli (HVEM-Fc, HVEM-[Fc*], or IgG1) and then cocultured with P815 target cells in the presence of mouse anti-human CD16 Ab (containing a murine Fc stalk) or its isotype control Ab to evaluate the effect of HVEM-(Fc*) on NK cell reverse ADCC assay.

**Statistical analysis**

Student paired t test was performed to determine the difference between two groups unless otherwise indicated. All tests were considered statistically significant at p < 0.05.

**Results**

**Soluble HVEM-(Fc*) activates NK cells and induces IFN-γ production and cytotoxicity independent of Fc binding**

HVEM-Fc fusion proteins have been used to study the interaction between HVEM and its ligands on NK cells and immune responses (10, 48, 50–52). We sought to determine how a soluble HVEM-Fc fusion may function independent of Fc receptor interactions through mutation of residues required for Fc receptor binding (28, 53, 54). The entire HVEM extracellular domain was conjugated to a loss-of-function Fc stalk (Fc*) of hlgG1 (HVEM-[Fc*]) that has been previously shown to lack Fc receptor functionality (Fig. 1A). Six amino acids were mutated at position E233P, L234V, L235A, ΔG236, A327G, A330S on the Fc* stalk of hlgG1 as described (28). The binding capacity of the Fc* stalk of HVEM-(Fc*) was tested using rhCD16a-conjugated beads by flow cytometry. HVEM-(Fc*) had significantly reduced mean fluorescence intensity (MFI) (1267 ± 421) compared with HVEM-Fc MFI (67266 ± 5232) (Fig. 1B). These data show that the loss-of-function Fc* stalk of HVEM-(Fc*) does not bind efficiently to rhCD16a-conjugated beads compared with HVEM-Fc containing the wild type Fc stalk. The binding capacity of HVEM-Fc and HVEM-(Fc*) to CD160 was tested using CD160-expressing CHO cells. As shown in Fig. 1C, both HVEM-Fc and HVEM-(Fc*) had comparable MFI (7814 ± 587 and 6900 ± 469, respectively) and percentage of HVEM* cells (93 ± 3% versus 88 ± 4%). Taken together, these data demonstrate that HVEM binding to CD160 is comparable for both HVEM-Fc and HVEM-(Fc*), but binding to the Fc receptor of HVEM-(Fc*) is reduced 50-fold compared with HVEM-Fc. As an additional control, we used HVEM-His (which does not contain an Fc binding stalk) to determine HVEM ligand function uncoupled from Fc–Fc receptor effects in subsequent experiments.

To evaluate the effect of HVEM on general activation of NK cells, we cultured purified NK cells with HVEM-(Fc*) and compared its effect with HVEM-Fc–induced responses. The percentage of NK cells expressing CD69 that were treated with HVEM-(Fc*) was similar to the percentage of NK cells that were treated with hlgG1 (Fig. 2A). In contrast, HVEM-Fc treatment increased 3.4-fold and 4-fold percentage of NK cells expressing CD69 compared with HVEM-(Fc*) and IgG1 treatment, respectively (Fig. 2A). Despite absent percentage of CD69 induction on NK cells by HVEM-(Fc*), this Fc-disabled HVEM was a more potent inducer of IFN-γ in the presence of IL-2 from NK cells than HVEM-Fc (Fig. 2B) but not in the absence of IL-2 (Supplemental Fig. 1A). These data suggested that HVEM-induced NK cell production of IFN-γ required IL-2 priming.

Additionally, we observed increased NK cell death with HVEM-Fc versus HVEM-(Fc*) and hlgG1. This is likely attributable to NK fratricide via Fc–Fc receptor binding (Fig. 2C) resulting in the observed reduction in CD16 (Fc receptor) expression on NK cells after HVEM-Fc treatment (Fig. 2D). Furthermore, we suspect that apparent IFN-γ secretion induced by CD16 and/or CD160 ligation of HVEM-Fc would be attenuated as compared with HVEM-Fc* (Fig. 2B) secondary to HVEM-Fc–induced fratricide (i.e., HVEM-Fc–Fc receptor cross-linking likely occurs before these NK cells fully produced and/or released IFN-γ from HVEM-Fc engagement [Fig. 2C]).

In contrast, HVEM-(Fc*) poorly engaged CD16 (Fig. 1B) and did not cause a reduction in CD16 expression (Fig. 2D) nor increased NK cell death (Fig. 2C). Thus, HVEM-(Fc*) treatment of NK cells significantly preserved NK cell viability and CD16 expression compared with HVEM-Fc and hlgG1 treatment (Fig. 2D).

Given the somewhat unexpected findings of increased IFN-γ production and decreased NK cell death with HVEM-(Fc*) versus HVEM-Fc, we then measured NK cell-induced cytotoxicity of K562 target cells. HVEM-(Fc*) enhanced NK cell–induced cytosis of K562 cells (Fig. 2E). Surprisingly, HVEM-Fc caused significantly reduced spontaneous killing of K562 cells by NK cells (Fig. 2E) compared with HVEM-(Fc*) and hlgG1. We also tested the effect of HVEM-(Fc*)–activated NK cells in a classic reverse ADCC assay using the well-characterized P815 mouse mastocytoma cell line that abundantly expresses mouse CD16 (mouse Fc receptor)
HVEM-(Fc*) drives broad NK cytokine production that can be further augmented by IL-2 more so than other NK cell–stimulating cytokines

Previously, it was reported that CD160 signaling induces NK production of IFN-γ, IL-6, IL-8, TNF-α, MIP-1β, and lower amounts of IL-4 and IL-10 (12, 55). To more comprehensively evaluate the effects of soluble HVEM on cytokine production in a relatively unbiased manner, we performed 65-plex cytokine protein array analysis using the Luminex platform. We repeated this analysis in the presence of media ± the best characterized NK-activating cytokines (IL-2, IFN-β, IL-12, and IL-15) to more fully capture the stimulating potential of soluble HVEM given that IFN-γ production required the presence of IL-2 (Fig. 2B, Supplemental Fig. 1A). We reasoned that other NK-activating cytokines might be required for the production of additional cytokines similarly to what has been observed for NK cell IFN-γ production. HVEM-(Fc*)–stimulated NK cells produced significant amounts of IL-4 and IL-10 (12, 55). To more comprehensively evaluate the effects of soluble HVEM on cytokine production in a relatively unbiased manner, we performed 65-plex cytokine protein array analysis using the Luminex platform. We repeated this analysis in the presence of media ± the best characterized NK-activating cytokines (IL-2, IFN-β, IL-12, and IL-15) to more fully capture the stimulating potential of soluble HVEM given that IFN-γ production required the presence of IL-2 (Fig. 2B, Supplemental Fig. 1A). We reasoned that other NK-activating cytokines might be required for the production of additional cytokines similarly to what has been observed for NK cell IFN-γ production. HVEM-(Fc*)–stimulated NK cells produced significant amounts of GM-CSF, GRO-α, MIP-1α, TNF-α, I-309, IL-1α, IL-1β, IL-6, IL-8, and IL-10 without IL-2 priming compared with hlgG1.
IL-2 priming upregulates LIGHT and BTLA but not CD160 expression on CD56<sup>bright</sup> NK cells

NK cells are divided into two major subtypes: CD56<sup>dim</sup> and CD56<sup>bright</sup>. CD160 is mostly expressed on CD56<sup>dim</sup> NK cells, which make up the vast majority of circulating NK cells. In contrast, LIGHT and BTLA are expressed at low levels on both CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells (10, 35). IL-2 priming enhances HVEM-(Fc<sup>*</sup>)-stimulated cytokine production and might enhance it by increasing expression of NK cell HVEM receptors. Therefore, we determined whether IL-2 priming enhanced CD160, LIGHT, and/or BTLA expression on bulk NK cells (Fig. 4, Supplemental Fig. 2). IL-2 priming of purified bulk NK cells did not change CD160 expression on either CD56<sup>dim</sup> or CD56<sup>bright</sup> subsets of NK cells (Fig. 4C). We confirmed IL-2 activation of purified bulk NK cells by demonstrating upregulation of CD69 expression on both subsets of NK cells (Fig. 4D). Interestingly, HVEM-His treatment of purified bulk NK cells reduced cell surface expression of LIGHT on CD56<sup>bright</sup> NK cells in the presence of IL-2 (Fig. 4E) but did not change BTLA expression on CD56<sup>bright</sup> NK cells either in the presence and absence of IL-2 (Fig. 4F). These data suggest that IL-2 priming distinctly enhanced the cell surface expression of HVEM receptors on different NK cell subsets and may enhance NK cell function differentially by HVEM activation.

FIGURE 2. HVEM-(Fc<sup>*</sup>) activates NK cells and induces IFN-γ production and cytotoxicity. (A–F) Purified NK cells from PBMC of healthy controls (n = 9) were cultured with 20 μg/ml hlgG, HVEM-(Fc<sup>*</sup>), or HVEM-Fc for the presence of IL-2 (100 IU/ml) for 16 h. After 16 h, cells were examined by flow cytometry for expression of (A) CD69, (B) IFN-γ was quantitated from supernatants via ELISA, (C) viability was measured via 7-AAD staining, (D) and CD16 cell surface expression was examined on CD56<sup>dim</sup>CD16<sup>+</sup> NK cells. (E and F) To evaluate (E) spontaneous killing and (F) reverse ADCC activity, NK cells cultured with 20 μg/ml hlgG, HVEM-(Fc<sup>*</sup>), or HVEM-Fc for 16 h were further cocultured with target K562 cells or P815 cells, respectively, prelabeled with fluorescent dye at effector E:T ratio of 1:1 and 0.5:1 for 5 h. For reverse ADCC assays, each coculture was treated with 10 μg/ml mouse IgG1 or anti-CD16 mAb. Dead target cells were gated as 7-AAD<sup>+</sup> cells. (G–J) PBMC from healthy controls (n = 9 except [H] where n = 5) were cultured with PBS or HVEM-(Fc<sup>*</sup>) (1 μg/ml) for 44 h. Then, cell surface (G) CD69 expression on NK cells and (H) IFN-γ secretion in culture supernatants were determined via flow cytometry and ELISA, respectively. Next, (I and J) PBMC were cultured with PBS or HVEM-(Fc<sup>*</sup>) for 44 h and were washed, counted, and further cocultured with prelabeled K562 cells with fluorescent dye at an E:T ratio of 20:1 for 5 h in the presence of GolgiStop and anti-CD107a Ab. CD69 expression on NK cells and (J) dead K562 target cells (7-AAD<sup>+</sup>annexin-V<sup>+</sup> cells) using flow cytometry. *p < 0.05, **p < 0.01, ***p < 0.001.

(3A), whereas HVEM-(Fc<sup>*</sup>)-induced IL-5 and IL-13, similar to IFN-γ, required IL-2 priming of NK cells (Fig. 3B, 3C). During infection, type I IFN, IL-12, and IL-15 production promotes NK cell effector function (56, 57). When we evaluated the effect of these cytokines in 65-plex Luminex-based assays, we found that only IFN-γ (Fig. 3D) production was significantly augmented by IFN-β, IL-12, and IL-15 versus media alone and that HVEM-(Fc<sup>*</sup>)-induced production of other cytokines (e.g., IL-10, GM-CSF, IFN-α, GM-SCF, GRO-α, MIP-1α, and IL-1β) that, to our knowledge, were not previously published, and that were elaborately directed by soluble HVEM treatment and did not require the presence of IL-2 or other NK-activating cytokines. Moreover, we identified that IL-5 and IL-13 could be induced by HVEM-(Fc<sup>*</sup>) in the presence of IL-2, similarly to IFN-γ. These data highlight the potential therapeutic application of HVEM-(Fc<sup>*</sup>).

IL-2 priming upregulates LIGHT and BTLA but not CD160 expression on CD56<sup>bright</sup> NK cells

NK cells are divided into two major subtypes: CD56<sup>dim</sup> and CD56<sup>bright</sup>. CD160 is mostly expressed on CD56<sup>dim</sup> NK cells, which make up the...
Because CD160 expression was not enhanced by soluble HVEM treatment of NK cells and BTLA was identified as an inhibitory receptor (10), we tested whether HVEM activates NK cells via LIGHT engagement. Human CD56 dim NK cells only express CD160. However, both CD56 bright and CD56 dim NK cells express LIGHT at low levels (Fig. 4). To test the potential sole effect of HVEM via LIGHT, we made use of the KHYG1 NK cell line, which we discovered only expressed LIGHT and not BTLA (Fig. 5A). The surface expression of CD160 on KHYG1 was similar to background isotype control (Fig. 5A). HVEM-His

FIGURE 3. HVEM-(Fc*)-induced cytokines in NK cells. NK cells purified from PBMC of healthy controls (n = 5) were cultured in wells precoated with 20 μg/ml hlgG1 or HVEM-(Fc*) in the presence of (A) media only versus (B–F) media, IL-2 (100 IU/ml), IFN-β (50 IU/ml), IL-12 (1 ng/ml), or IL-15 (5 ng/ml) for 16 h. Cell-free supernatants were collected and analyzed for 65 cytokines via Luminex-based multiplex assay. (A–F) show the cytokines whose concentrations were significantly changed by HVEM-(Fc*) treatment compared with hlgG. (E) and (F) show the HVEM-(Fc*)-induced cytokines minus hlgG-induced cytokines. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 4. IL-2 priming upregulates LIGHT and BTLA expression on CD56 bright NK cells, and cotreatment with HVEM-His downregulates expression of LIGHT and CD160 on NK cells. (A–D) Purified NK cells from healthy donor PBMC (n = 6) were incubated with IL-2 (100 IU/ml) for 18 h, and then cells were analyzed for (A) LIGHT, (B) BTLA, (C) CD160, or (D) CD69 expression on CD56 bright versus CD56 dim NK cells using mAbs (clones T5-39, J168-540, 688327, and FN-5, respectively) by flow cytometry. The gating strategy for assessment of LIGHT, BTLA, CD160, and CD69 expression on CD56 bright NK cells and CD56 dim NK cells is shown in Supplemental Fig. 2. (E–G) Purified NK cells from healthy donor PBMC (n = 6) were incubated with media or plate-bound HVEM-His (20 μg/ml, 20 μl/well) in the presence or absence of IL-2 (100 IU/ml) for 18 h, and then cells were analyzed for (E) LIGHT or (F) BTLA expression on CD56 bright NK cells and (G) CD160 expression on CD56 dim NK cells using mAbs (clones T5-39, J168-540, 688327, and FN-5, respectively) by flow cytometry. *p < 0.05, ***p < 0.001.
treatment of KHYG1 NK caused significant production of IFN-γ (Fig. 5B). Given this finding, we evaluated HVEM-induced activation via LIGHT on purified NK cells by incubating them with HVEM-(Fc*) in the presence of anti-CD160 blocking Ab (clone 688327; R&D Systems). We found that HVEM-(Fc*) caused significant production of IFN-γ from NK cells after blocking of CD160 receptor (Fig. 5C). We hypothesize that CD160 blockade–mediated enhancement of IFN-γ production from HVEM-(Fc*)–treated NK cells resulted from more HVEM-(Fc*) binding of other HVEM ligands, such as LIGHT. The IL-2–pretreated NK cells express high levels of CD160 (the majority is localized on the CD56dim subset and the minority is localized on the CD56hi subset) and low levels of LIGHT and BTLA, which are mostly localized on the CD56hi subset. In the physiological condition (absence of anti-CD160 blocking Ab), CD160 will compete with LIGHT, BTLA, or other unknown ligand(s) on the NK cells to bind with plate-bound HVEM-(Fc*). Therefore most IFN-γ is likely released from NK cells by ligation of HVEM and CD160. Conversely, in the presence of anti-CD160 blocking Ab, the other HVEM ligands, such as LIGHT, will have a greater likelihood to bind with HVEM-(Fc*), and this binding might elicit greater IFN-γ production than CD160-HVEM binding. These data support the notion that HVEM may activate NK cells via LIGHT in addition to CD160.

HVEM-(Fc*) promotes activation of monocytes

Several reports have demonstrated that monocytes and macrophages regulate NK cell effector function(s) (36, 38–41, 43–46). Monocytes also express receptors of HVEM (18, 34). Thus, we determined the level of expression of all three HVEM receptors on monocytes as a readily available cell type that may explain why PBMC cultures showed different results than isolated NK cells with respect to soluble HVEM treatment (Fig. 2). We found that monocytes expressed LIGHT and BTLA and there was no CD160 expression (Fig. 6). Among monocytes, NK cells, and DC, LIGHT expression was greatest on monocytes. Next, we tested whether HVEM-(Fc*) activated monocytes similarly to NK cells. Activation of monocytes was measured by surface expression of CD80, CD83, CD86, and CD40 (Fig. 7). Expression of activation markers CD80, CD83, and CD40 but not CD86 was enhanced by HVEM-(Fc*) treatment of both purified monocytes (Fig. 7A) and flow-gated monocytes in PBMC cultures (Fig. 7B). Consistent with Schwarz et al. (58) who also found upregulation of CD80, CD83, and CD40 but not CD86 was enhanced by HVEM-(Fc*) treatment of both purified monocytes (Fig. 7A) and flow-gated monocytes in PBMC cultures (Fig. 7B), we found regulatory monocytes in PBMC cultures (Supplemental Fig. 3), confirming that soluble HVEM could directly activate monocytes in addition to NK cells.

**HVEM-(Fc*) promotes cross-talk between NK cells and monocytes**

Next, we reasoned that because monocytes can modulate NK activity, were activated by HVEM-(Fc*), and NK cell activation was different in PBMC cultures versus enriched NK cells incubated with HVEM-(Fc*), that monocytes may play a role in HVEM-(Fc*)–induced NK activation and subsequent effector functions. Thus, we compared purified NK cells cultured with and without purified monocytes in the presence or absence of HVEM-(Fc*). Activation of NK cells was quantified via cell surface CD69 expression, degranulation via CD107a, cytotoxicity to K562 target cells, and IFN-γ production.
When purified NK cells were cocultured with purified monocytes in the presence of HVEM-(Fc*), there was significant enhancement of CD69 (Fig. 8A) and CD56 (Fig. 8B) cell surface expression on the NK cells. There was also enhanced NK cell degranulation (increased cell surface expression of CD107a) and enhanced cytotoxicity toward K562 cells when NK cells were cocultured with monocytes in the presence of HVEM-(Fc*) (Fig. 8C, 8D) compared with NK cells cultured with HVEM-(Fc*). These data demonstrate that monocytes can augment HVEM-(Fc*)–induced NK cell activation and effector function.

To test whether monocytes augment NK cell effector function via cell–cell contact or soluble mediators, NK cells were cocultured with monocytes in the presence or absence of HVEM-(Fc*) in a transwell tissue culture plate. Monocytes or NK cells were either cultured separately in the upper or lower chambers respectively separated by transmembrane or cultured together in the bottom chamber in a transwell culture plate. Transwell separation of purified NK cells and monocytes resulted in a slightly lower basal expression of CD69 (Fig. 9A); however, this was not statistically significant. Otherwise, HVEM-(Fc*)–induced activation of monocytes in transwell cocultures was assessed by cell surface expression of CD80, CD83, and CD86 (Supplemental Fig. 4). Similar to the HVEM-(Fc*)–treated purified monocytes (Fig. 7), there was significant enhancement of CD80, CD83, and CD40 but not CD86 (Supplemental Fig. 4) expression on monocytes in the transwell cocultures. Taken together, these transwell experiments indicated that HVEM-(Fc*) activation of NK via NK–monocyte cross-talk required cell–cell contact mechanism(s) for maximal enhancement of IFN-γ production and potentially mild enhancement of spontaneous killing.

**Discussion**

NK cells play a key role in the protection from pathogens and cancer. NK cell–mediated effector function depends on the type and number of receptor/ligand interactions occurring between NK cells and other cells. HVEM augments NK effector function by NK–monocyte cross-talk.
and their targets. In this context, it has been shown that CD160 drives NK cell activation, cytokine production, and cytotoxicity via engagement with HLA-C (12) and HVEM (10, 16, 18, 48). In this study, we have shown that HVEM conjugated to the wild type sequence of Fc (HVEM-Fc) activates NK cells; however, the Fc portion can cause interaction and activation of other Fc receptor–expressing immune cells via Fc–Fc receptor ligation in addition to HVEM (Figs. 1, 2). This is notable given this form of HVEM-Fc is the reagent readily available “off the shelf” from multiple vendors including BioLegend, R&D Systems, Sigma-Aldrich, SBH Sciences, AB Biosciences, BPS Bioscience, Enzo, PromoCell, Tonbo Biosciences, GenScript, G&P Biosciences, and others. We designed and evaluated a soluble Fc-disabled HVEM to interrogate the specific function of HVEM-mediated NK cell responses and provide an alternative reagent for potential therapeutic application. To do this, we fused the HVEM extracellular domain to a mutant Fc stalk that poorly binds the Fc receptor HVEM-(Fc*). HVEM-(Fc*) binding to CD160 on primary NK cells resulted in increased inflammatory cytokine expression, degranulation (enhanced CD107a expression), and cytolysis by NK cells as compared with wild type HVEM-Fc.

We have also shown for the first time, to our knowledge, that HVEM induces a broader array of cytokines than previously demonstrated, which occurred in both IL-2–dependent and –independent manners. Previously, it has been reported that engaging CD160 via its agonist monoclonal anti-CD160 Ab clone CL1R2 (CL1R2 Ab) or HLA-C binding on peripheral blood NK cells drove a large amount of IFN-γ, IL-6, and TNF-α (12) as well as IL-8 and MIP-1β but marginal amounts of IL-4 or IL-10 (55). The same authors reported their unpublished data that IL-1β, IL-5, IL-7, IL-12, IL-13, IL-17, G-CSF, and MCP-1 production was not detectable by NK cells via CL1R2 Ab or HLA-C (55). We have shown that HVEM-(Fc*) treatment of NK cells can drive proinflammatory cytokines including GM-CSF, GRO-α, TNF-α, I-309, IL-1α, IL-1β, IL-6, IL-8, and IL-10 that did not require IL-2 priming, and IFN-γ, IL-5, and IL-13 required IL-2 priming. We did not uncover any significant additional synergy of HVEM-(Fc*) with type 1 IFN (IFN-β), IL-12, or IL-15 beyond

FIGURE 8. HVEM-(Fc*) promotes cross-talk between monocytes and NK cells, enhancing activation and cytotoxicity of NK cells. (A–D) Purified NK cells from healthy donor PBMC (n = 6) were cocultured without purified monocytes (MN) or with purified monocytes (NK cells + MN) at a ratio of 1:1 in the presence of media or HVEM-(Fc*) (1 μg/ml) for 44 h. (A and B) After 44 h, NK cells were analyzed for cell surface (A) CD69 and (B) CD56 expression by flow cytometry. After 44 h of HVEM-(Fc*) treatment of NK cells and NK cells + MN cocultures, prelabeled K562 target cells were added at an E:T ratio of 1:5:1 in the presence of anti-CD107a Ab for 5 h. HVEM-(Fc*) treatment–enhanced NK cell cytotoxicity was evaluated as (C) percentage of dead 7-ADD*annexin-V− K562 cells (HVEM-induced minus media-induced), and (D) NK cell degranulation was estimated by cell surface CD107a expression (HVEM-induced minus media-induced) for both NK cells alone and the mixture of NK cells + MN as the effector cells. *p < 0.05, **p < 0.01.

FIGURE 9. HVEM-(Fc*)–induced IFN-γ production and cytotoxicity of NK cells is enhanced by monocyte-NK cell–cell contact. (A–D) NK cells and monocytes were purified from PBMC by negative and positive selection, respectively. The purified NK cells and monocytes were either cocultured in a flat-bottom well or separately cultured in a transwell in the presence or absence of HVEM-(Fc*) (1 μg/ml) for 44 h. After 44 h, cell surface (A) CD69 expression on NK cells was measured by flow cytometry, and (B) IFN-γ production in cell-free supernatants was quantified via ELISA. (C and D) Cells were treated as above with HVEM-(Fc*) for 44 h and subsequently cocultured with prelabeled K562 cells in the presence of anti-CD107a Ab for 5 h. (C) Dead 7AAD*annexin-V− K562 cells were quantified, and (D) NK cell CD107a expression was quantified, all by flow cytometry. *p < 0.05, **p < 0.01.
what we observed for IL-2 in 65-plex Luminex-based cytokine quantitation. It is plausible that previous reports of undetectable levels of several cytokines reported is due to nonpriming of NK cells with IL-2.

The expression of HVEM ligands on NK cells can be regulated by stimulation with cytokines and tumor cells (59, 60). For example, long-term stimulation of NK cells with high concentration of IL-2 or IL-15 downregulates the CD160-GPI isoform expression and simultaneously upregulates the CD160 transmembrane (CD160-TM) isoform expression (60). The data of Giustiniati et al. (60) suggests complex CD160 signaling specific to NK cells. Specifically, the GPI isoform lacks any intracellular domain. In contrast, the CD160-TM possesses an intracellular domain that induces phosphorytrosine-dependent Erk activation signaling via Src family kinase p56^ck. These data suggest that cytokines can modulate the expression of CD160 isoforms via chronic stimulation. Similarly, stimulation of NK cells with K562 tumor cells and IL-2 or IL-15 upregulates LIGHT expression, another HVEM-interacting receptor implicated in the enhancement of antimicrobial responses (59). Together, these data support the notion that stimulated NK cells become uniquely receptive to HVEM engagement. Secretion of cytokines via HVEM-activated NK cells (novel members identified in this paper) might further regulate the cascade of events leading to a specific and efficient recruitment of these receptors for their respective signaling pathways and the function(s) of NK cells and other immune cells. In our study, we found IL-2 induced expression of LIGHT and BTLA only on the CD56^bright NK cells (Fig. 4A, 4B). CD56^bright NK cells are mainly cytokine producers, more so found in lymphoid organs and considered to be precursors of CD56^dim NK cells, the latter mainly perform cytotoxicity in addition to cytokine production. Holmes et al. (59) have shown that tumor-activated NK cells enhanced the expression of LIGHT in human NK cells and are linked to the initiation of adaptive immunity via LIGHT-mediated NK–DC cross-talk. In this context, the modulation of LIGHT and BTLA expression on CD56^bright NK cells could regulate NK cell activity and subsequently participate in the shaping of adaptive immune responses. Overall, these findings support a mechanism for directing specific NK action within the HVEM-LIGHT-BTLA–CD160–LT^B signaling network. This work serves as a basis for better understanding and ultimately adjusting immune responses for therapeutic benefit. When pursuing this application, it will be important to test in future studies additional potential mechanisms that might govern NK cell activation via the HVEM-LIGHT-BTLA–CD160–LT^B signaling network including potentially counterregulatory mechanisms.

Interestingly, costimulation of bulk NK cells with HVEM-(Fc^*) and IL-2 downregulates expression of CD160 and LIGHT on CD56^dim and CD56^bright NK cells, respectively (Fig 4E, 4G). These data suggest that IL-2 priming modulates the expression of HVEM receptors distinctly on the two subsets of NK cells and cross-talk may occur between both cell types, resulting in a potentially additional layer of complexity in HVEM-stimulated NK responses.

We reasoned that the Fc portion of HVEM-Fc found in commercially available reagents could contribute to effects beyond HVEM receptor engagement on innate immune cells (i.e., via Fc receptor engagement). Recent literature suggests that NK cells also interact with other immune cells (e.g., monocytes, macrophages, or DC) for optimal cytokine production, cytotoxicity, and control of virally infected or tumor cells. It has been shown that both TLR ligands and pathogens can promote cross-talk between monocytes/ macrophages and NK cells via cell–cell contact and/or cytokines (36). We found that HVEM-(Fc^*) activated monocytes and enhanced expression of activation markers CD40, CD80, and CD83 but not CD86 (Fig. 7). Our finding is consistent with previous reports demonstrating that LPS, similar to HVEM-(Fc^*), enhanced the expression of activation markers CD40, CD80, and CD83 but not the expression of CD86 (58, 61). Recent human and mouse studies have shown that CD80 and CD86 have differential roles in different disease states (62–65). However, the significance of CD80 upregulation and CD86 downregulation on monocytes for subsequent immune responses is unknown.

We have also shown that HVEM-(Fc^*)-induced NK cell cytotoxicity was enhanced when NK cells were cocultured with monocytes and is reduced when NK cells were separated by monocytes in a transwell coculture system. Likewise, HVEM-(Fc^*)–induced IFN-γ production in NK cell plus monocyte cocultures was significantly reduced when monocytes were separated by NK cells in transwells. Our data show that HVEM-(Fc^*) promotes cross-talk between NK cells and monocytes in vitro that requires cell–cell contact for maximal enhancement of IFN-γ secretion and optimum killing of K562 target cells.

In this study, we have reported for the first time, to our knowledge, a reagent HVEM-(Fc^*) (which negligibly binds to the Fc receptor) activates monocytes and promotes cross-talk between NK cells and monocytes largely via cell–cell contact. Based on the expression levels of HVEM receptors on monocytes, we propose that it is most likely that HVEM-(Fc^*) engages LIGHT for the activation of monocytes (versus CD160 on NK cells), which subsequently may provide for enhanced overall NK function.

In conclusion, we report in this study that HVEM-(Fc^*) activates NK cells and monocytes and promotes NK cell and monocyte cross-talk for enhanced activation of NK cells and NK cell cytotoxicity. We have also shown that the Fc portion of HVEM-Fc but not HVEM-(Fc^*) can interfere with augmenting NK function by reducing CD16 expression on NK cells and NK cell cytotoxicity via Fc–Fc receptor engagement. Our work supports the potential use of soluble HVEM as a therapeutic agent by eliminating the potentially counteracting effects of the Fc portion of conventional HVEM-Fc fusion proteins, consistent with Boice et al. (22).

Disclosures

The authors have no financial conflicts of interest.

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