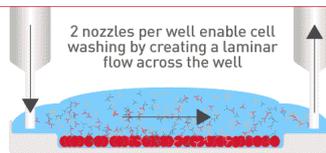


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Pathogenic Function of Herpesvirus Entry Mediator in Experimental Autoimmune Uveitis by Induction of Th1- and Th17-Type T Cell Responses

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Herpesvirus entry mediator (HVEM), a member of the TNFR superfamily, serves as a unique molecular switch to mediate both stimulatory and inhibitory cosignals, depending on its functions as a receptor or ligand interacting with multiple binding partners. In this study, we explored the cosignaling functions of HVEM in experimental autoimmune uveitis (EAU), a mouse model resembling human autoimmune uveitis conditions such as ocular sarcoidosis and Behcet disease. Our studies revealed that EAU severity significantly decreased in HVEM-knockout mice compared with wild-type mice, suggesting that stimulatory cosignals from the HVEM receptor are predominant in EAU. Further studies elucidated that the HVEM cosignal plays an important role in the induction of both Th1- and Th17-type pathogenic T cells in EAU, including differentiation of IL-17-producing $\alpha\beta^+\gamma\delta^-$ conventional CD4⁺ T cells. Mice lacking lymphotoxin-like, inducible expression, competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes (LIGHT), B- and T-lymphocyte attenuator (BTLA) or both LIGHT and BTLA are also less susceptible to EAU, indicating that LIGHT–HVEM and BTLA–HVEM interactions, two major molecular pathways mediating HVEM functions, are both important in determining EAU pathogenesis. Finally, blocking HVEM cosignals by antagonistic anti-HVEM Abs ameliorated EAU. Taken together, our studies revealed a novel function of the HVEM cosignaling molecule and its ligands in EAU pathogenesis through the induction of Th1- and Th17-type T cell responses and suggested that HVEM-related molecular pathways can be therapeutic targets in autoimmune uveitis. *The Journal of Immunology*, 2016, 196: 000–000.

Uveitis is a sight-threatening intraocular inflammatory disease that is estimated to account for ~10% of blindness, affecting 17–52 out of 100,000 people per year in the United States (1, 2). The socioeconomic impact of uveitis is highly significant, as uveitis commonly affects the working age population (3). Based on its etiology, uveitis can be classified into two categories: uveitis related to infection and uveitis that is not. Noninfectious uveitis is frequently associated with autoimmune diseases, including Behcet disease, Vogt-Koyanagi-Harada syndrome, systemic lupus erythematosus, sarcoidosis, autoimmune hepatitis,

and multiple sclerosis (4). Corticosteroids are usually among the first choices to treat patients with autoimmune-type noninfectious uveitis, because of their effectiveness in controlling inflammation. However, some patients are resistant to or become refractory to the treatment, and more importantly, long-term use of corticosteroids is often associated with severe adverse effects (5, 6). Thus, more effective and specific therapies with less toxicity for autoimmune uveitis are urgently needed.

Experimental autoimmune uveitis (EAU) is the most commonly used animal model that recapitulates human autoimmune uveitis. EAU can be induced by immunizing an animal with retinal proteins, such as interphotoreceptor-binding protein (IRBP) or retinal soluble Ag, by adoptive transfer of T cells specific to these Ags or by infusion of mature dendritic cells (DCs) pulsed with these Ag (7, 8). Based on previous studies, both Th1 and Th17 cells play pivotal roles in the development of EAU; however, these two cell populations appear to mediate distinct pathogenic responses, either independently or cooperatively (9–12). In EAU triggered by immunization with IRBP in CFA, blocking IL-17, but not IFN- γ , prevents disease development (10, 11), indicating a predominant role for Th17 cells in this model. In contrast, EAU caused by infusion of IRBP-pulsed DCs or transfer of IRBP-specific T cells is dependent of Th1 cells, as attenuation of IFN- γ by neutralizing Abs or gene knockout in recipient mice ameliorates disease progression (8, 10). Thus, the major phenotypes of effector T cells are likely affected by the conditions in which pathogenic responses of EAU are induced (13). Because of the heterogenic nature of the disease, no single model of EAU covers all aspects of human uveitis. However, elucidation of pathogenic mechanisms using animal models gives us an opportunity to develop rational therapies for human uveitis.

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Abbreviations used in this article: BM, bone marrow; BTLA, B- and T-lymphocyte attenuator; DC, dendritic cell; DKO, double knockout; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; HVEM, herpesvirus entry mediator; IRBP, interphotoreceptor-binding protein; KO, knockout; LIGHT, lymphotoxin-like, inducible expression, competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes; LN, lymph node; Treg, regulatory T; WT, wild-type.

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Cosignaling molecules play a crucial role in fine-tuning T cell-mediated immune responses as well as determining T cell fate (14). Herpesvirus entry mediator (HVEM), a molecule belonging to the TNFR superfamily, has a unique cosignaling function, as it provides positive or negative cosignals in T cells according to distinct cellular conditions (15). For instance, HVEM, as a receptor on T cells, transmits stimulatory cosignals when it binds with its ligands, lymphotoxin-like, inducible expression, competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes (LIGHT) or B- and T-lymphocyte attenuator (BTLA) (16–19). In contrast, HVEM also serves as a ligand to deliver inhibitory cosignals to BTLA or CD160 receptors on T cells (17, 20, 21). Although it remains unclear how HVEM regulates its bidirectional and opposing cosignaling functions, types of Ag and pathogenic conditions seem to play important roles. For instance, HVEM signaling elicits potent stimulatory cosignals in models of graft-versus-host disease and allograft rejection (18, 22). Thus, severity of these diseases is attenuated by knockout of the HVEM gene or by treatment with anti-HVEM-neutralizing mAbs. However, deficiency or blocking of HVEM exacerbates severity of ConA-induced hepatitis, experimental autoimmune encephalomyelitis (EAE), and collagen-induced arthritis (23, 24), indicating inhibitory functions of HVEM in these autoimmune diseases. Thus far, it remains unexplored whether HVEM plays a stimulatory or inhibitory role in the pathogenesis of uveitis and, if any, how it regulates the pathology.

In this study, we elucidated the regulatory role of HVEM cosignaling in autoimmune uveitis using an IRBP-induced EAU model. Our studies revealed that HVEM stimulates the onset and progression of EAU by enhancing Th1- and Th17-type T cell responses. Interactions of LIGHT–HVEM and BTLA–HVEM were both found to be functionally important for HVEM-mediated stimulatory effects, and blocking these interactions by anti-HVEM mAbs attenuated EAU severity. This study is the first, to our knowledge, to demonstrate pathogenic functions of HVEM cosignaling in EAU and the potential for this molecule to serve as a therapeutic target in autoimmune uveitis.

Materials and Methods

Mice, Abs, and reagents

C57BL/6 (B6, H-2^b) mice were purchased from Japan SLC (Shizuoka, Japan). B6-background HVEM-knockout (KO), BTLA-KO, and LIGHT-KO mice were generated as previously described (17–19). Mice deficient in both BTLA and LIGHT genes were generated by crossing BTLA-KO and LIGHT-KO mice in our facility. Age- and sex-matched 6–12 wk-old mice were used for all experiments. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of Yamaguchi University and performed in compliance with the Yamaguchi University Animal Care and Use guidelines.

Anti-mouse HVEM mAb (clone HM3.30; hamster IgG) and anti-mouse HVEM antagonistic mAb (clone LBH1; hamster IgG) were generated in our laboratory as previously described (18, 25). Fluorochrome-conjugated Abs for flow cytometric analysis were purchased from BD Biosciences (San Diego, CA), eBioscience (San Diego, CA), or BioLegend (San Diego, CA).

Induction and evaluation of IRBP-immunized EAU model

EAU was induced by s.c. immunization in one hind food pad and the inguinal region with 100 μ g human IRBP amino acid (Scrum, Tokyo, Japan) 1–20 (GPTHLFQPSLVLDMAKVLDD) in CFA supplemented with 300 μ g *Mycobacterium tuberculosis* strain H37RA (Difco Laboratories, Detroit, MI). Each mouse also received an i.p. injection of *Bordetella pertussis* toxin (100 ng/mouse; Sigma-Aldrich, St. Louis, MO) concurrent with IRBP immunization. In some experiments, the mice were also treated with i.p. injections of anti-HVEM antagonistic mAb (LBH1) or control hamster IgG at 250 μ g/mouse on days 0, 3, 6, and 9.

Following immunization, the EAU clinical score was assessed by funduscopy in a blinded manner, based on the extent of inflammation and tissue

damage as follows: score 0, no signs of inflammation; score 1, focal vasculitis or spotted soft exudate (<5 spots); score 2, linear vasculitis in half of the retina or spotted soft exudate in half of the retina; score 3, linear vasculitis over half of the retina or spotted soft exudate over half of the retina; score 4, retinal hemorrhage along with vasculitis or severe exudate along with vasculitis; and score 5, exudative retinal detachment or subretinal (or vitreous) hemorrhage.

EAU severity was also histopathologically assessed 21 d after immunization. Freshly enucleated eyes were fixed in 4% paraformaldehyde and then embedded in paraffin. Sections were cut and stained with H&E. Disease severity was graded as previously reported (26, 27) on a scale of 0–4 as follows: score 0, no signs of uveitis; score 0.5, focal non-granulomatous and monocytic infiltrates in the choroid, ciliary body, and retina; score 1, retinal perivascular infiltration and monocytic infiltration in the vitreous; score 2, granuloma formation in the uvea and retina, the presence of occluded retinal vasculitis, along with photoreceptor folds, serous detachment, and loss of photoreceptors; and scores 3 and 4, formation of Dalen-Fuchs nodules (granuloma at the level of the retinal pigmented epithelium) and the development of subretinal neovascularization according to the number and size of lesions.

Induction of EAU by adoptive transfer of IRBP-specific T cells

EAU was induced in wild-type (WT) B6 mice by immunization with 250 μ g IRBP peptide in CFA containing 500 μ g *M. tuberculosis* and concurrent administration of 200 ng *Bordetella pertussis* toxin. Fifteen days later, T cells were isolated from cervical, axillary, and inguinal lymph nodes (LNs) of the immunized mice by MACS MicroBead mouse pan-T cell

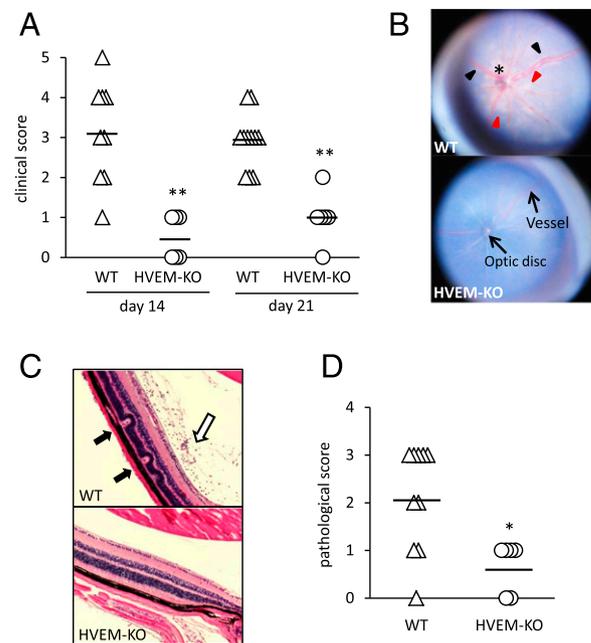


FIGURE 1. Decreased severity of EAU in HVEM-KO mice. **(A)** Clinical scores of EAU in WT (open triangles) and HVEM-KO (open circles) mice were examined by funduscopy on days 14 and 21 after immunization with IRBP peptide. Individual clinical scores for eyes are shown by each symbol. The average of scores is shown as a horizontal bar in each group. **(B)** Fundus images from WT (clinical score 4) and HVEM-KO mice (clinical score 0) on day 16 after IRBP immunization are shown. Black arrowheads indicate enlarged retinal blood vessels. Red arrowheads indicate retinal or choroidal infiltrates. The asterisk indicates inflammation with blurred optic disc margins. Histopathology in posterior segments of the eye in IRBP-immunized WT and HVEM-KO mice was examined **(C)**, and EAU pathological scores **(D)** were determined on day 21. **(C)** Representative pictures of WT (pathological score 2) and HVEM-KO (pathological score 0) mice are shown. The open white arrow indicates inflammatory cell infiltration in the vitreous body. Black arrows indicate retinal folds. H&E staining, original magnification $\times 200$. **(D)** Individual pathological scores for eyes are shown by each symbol. Data are representative of three independent experiments with similar results. * $p < 0.05$, ** $p < 0.01$.

isolation kit (Miltenyi Biotec, Auburn, CA) and then in vitro restimulated with 10 $\mu\text{g}/\text{ml}$ IRBP peptide in the presence of 30 Gy-irradiated WT B6 spleen cells. After 4 d, cultured cells were harvested, and 5×10^6 CD4⁺ T cells were transferred i.v. into sex- and age-matched WT or HVEM-KO B6 recipient mice, which were exposed to sublethal irradiation (4 Gy) using an x-ray irradiator (MBR-1505R2; Hitachi Medico, Tokyo, Japan) prior to the injection. Subsequently, EAU clinical scores were determined by funduscopy up to 5 wk after the cell transfer.

IRBP-specific T cell proliferation and cytokine/chemokine production

Cervical and axillary LNs were harvested from the mice that had been immunized with IRBP peptide to induce EAU as described above. T cells were isolated and incubated at 3×10^5 cells/well in 96-well flat-bottom tissue-culture plates in the presence of titrated doses of IRBP peptide and 30 Gy-irradiated WT B6 spleen cells (3×10^5 cells/well). Proliferation of the cells was assessed by [³H]thymidine incorporation during the last 18 h of a 3-d culture.

To assess the production of cytokines and chemokines from IRBP-specific T cells, T cells were isolated as described above and then were restimulated in vitro with 10 $\mu\text{g}/\text{ml}$ IRBP peptide. After 48 h, the culture supernatants were harvested and analyzed using Bio-Plex Pro Mouse 23-plex and Th17 8-plex assay kits according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

The levels of cytokines and chemokines in ocular fluids were also measured. First, eyes were enucleated from WT or HVEM-KO mice 14 d after EAU induction, and conjunctival tissue was removed. The remaining eye tissues were homogenized using a Biomasher II (Nippi, Tokyo, Japan), and the supernatants were collected and analyzed using a Bio-Plex Pro Mouse 23-plex kit (Bio-Rad) as described above.

Intracellular cytokine staining of IRBP-specific Th17-type T cells

WT or HVEM-KO mice were immunized with IRBP peptide for EAU induction as described above. After 9 d, T cells were isolated from cervical, axillary, and inguinal LNs and cultured at 3×10^6 cells/well in 24-well flat-bottom plates in the presence of 20 $\mu\text{g}/\text{ml}$ IRBP peptide and irradiated B6

spleen cells (3×10^6 cells/well), together with 20 ng/ml rIL-23 (R&D Systems, Minneapolis, MN). After 4 d, Cell Stimulation Cocktail plus protein transport inhibitors (eBioscience) were added to the cell culture, and cells were incubated for another 4 h. Then, the cells were harvested and stained for CD4 and $\gamma\delta$ TCR markers, and treated with Fixation/Permeabilization Buffer (eBioscience) according to the manufacturer's instructions, followed by intracellular cytokine staining with mAbs to mouse IL-17A or IFN- γ . Staining of cell-surface markers and intracellular cytokines was measured by an EC800 Flow Cytometry Analyzer (Sony Biotechnology, Tokyo, Japan) and analyzed using FlowJo software (Tree Star, CA).

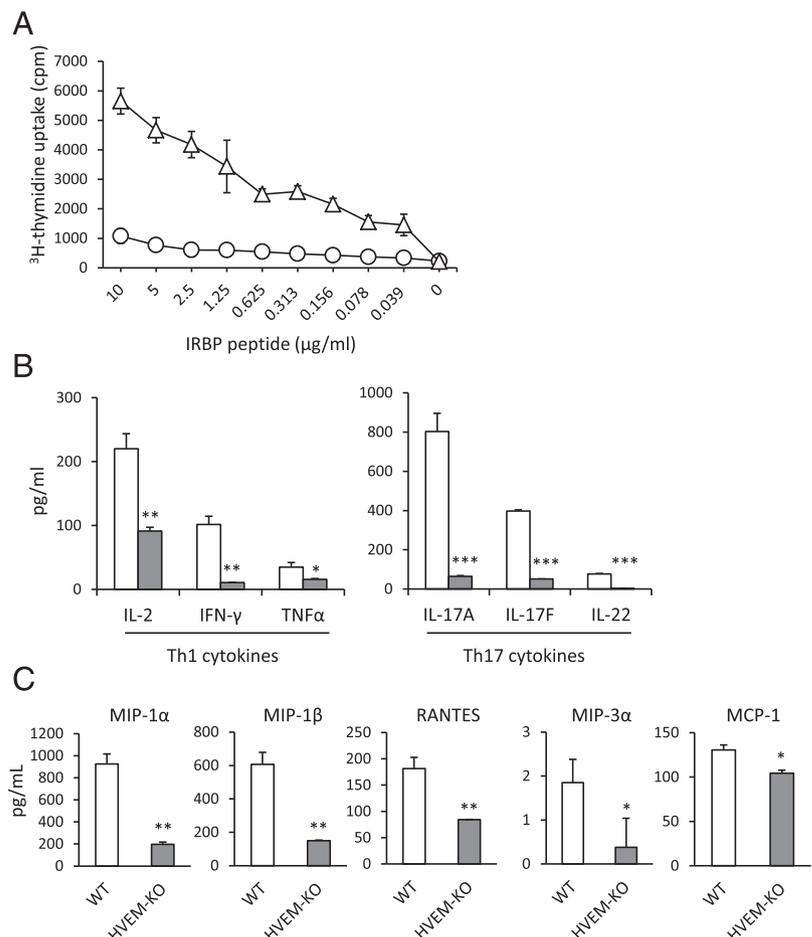
Western blot analysis

The retina and uvea, consisting of the iris, ciliary body, and choroid, were carefully dissected from enucleated eyeballs of naive or EAU-induced WT or HVEM-KO mice 21 d after immunization. As positive and negative controls of HVEM-expressing tissues, thymuses harvested from naive WT or HVEM KO mice, respectively, were used. Tissues were homogenized and solubilized in lysis buffer containing protease inhibitors and Triton X-100 (Bio-Rad). The protein concentrations of cell extracts were measured by BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Proteins (20 μg each) were dissolved in Laemmli sample buffer (Bio-Rad), separated by SDS-PAGE electrophoresis using 10% polyacrylamide gels, and transferred onto nitrocellulose membranes. Membranes were then blocked with 5% nonfat milk and incubated with 1 $\mu\text{g}/\text{ml}$ anti-HVEM mAb (clone HM3.30), followed by HRP-conjugated goat anti-hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). β -Actin was also assessed in each sample to confirm equal loading amounts. After Ab staining, the membranes were washed, and the proteins were detected by ECL Prime Western blotting Detection Reagent (GE Healthcare Japan, Tokyo, Japan) according to the manufacturer's instructions. The chemiluminescence signal was then visualized by exposure to x-ray film.

Statistical analysis

Unpaired, two-tailed Student *t* test was used for parametric data such as cytokine and proliferation data, and Mann-Whitney *U* test was used for

FIGURE 2. Decreased IRBP-specific T cell responses in HVEM-KO mice. T cells isolated from LNs of WT or HVEM-KO mice 21 d after IRBP immunization were restimulated in vitro with IRBP peptide in the presence of irradiated spleen cells from WT mice. **(A)** After 3 d, T cell proliferation in WT (open triangles) and HVEM-KO (open circles) mice in response to the indicated doses of IRBP peptide were measured by [³H]thymidine incorporation assay. After 48 h, culture supernatants from WT (open bars) and HVEM-KO (filled bars) T cells were harvested and examined for the concentration of cytokines **(B)** and chemokines **(C)** by Bio-Plex assay. The results, representative of three independent experiments with similar results, are presented as the mean \pm SD of triplicate samples. **p* < 0.05, ***p* < 0.001, ****p* < 0.0001.



nonparametric data such as EAU scores. The results are expressed as the mean \pm SD. Differences were considered significant with p values <0.05 .

Results

Biological role of the HVEM cosignaling molecule in exacerbating EAU

To investigate whether HVEM-mediated cosignaling induces stimulatory or inhibitory effects in EAU, we first immunized WT or HVEM-KO B6 mice with IRBP peptide and determined clinical scores of EAU by funduscopy examination. The onset and progression of uveitis was significantly lessened in HVEM-KO mice compared with WT mice on days 14 and 21 post-immunization (Fig. 1A). Fundus images of WT mice showed severe inflammation with blurring of the optic disc margins, retinal vasculitis, and inflammatory infiltrates, whereas modest funduscopy changes with less inflammation were observed in HVEM-KO mice (Fig. 1B). Consistent with these data, histopathological analysis of WT mice indicated severe ocular injury and inflammation associated with retinal folding and vitreous cellular infiltrates, whereas such changes were infrequently observed in HVEM-KO mice (Fig. 1C). Pathology scores from HVEM-KO mice were significantly lower than those observed in WT mice (Fig. 1D). Collectively, these results indicate that

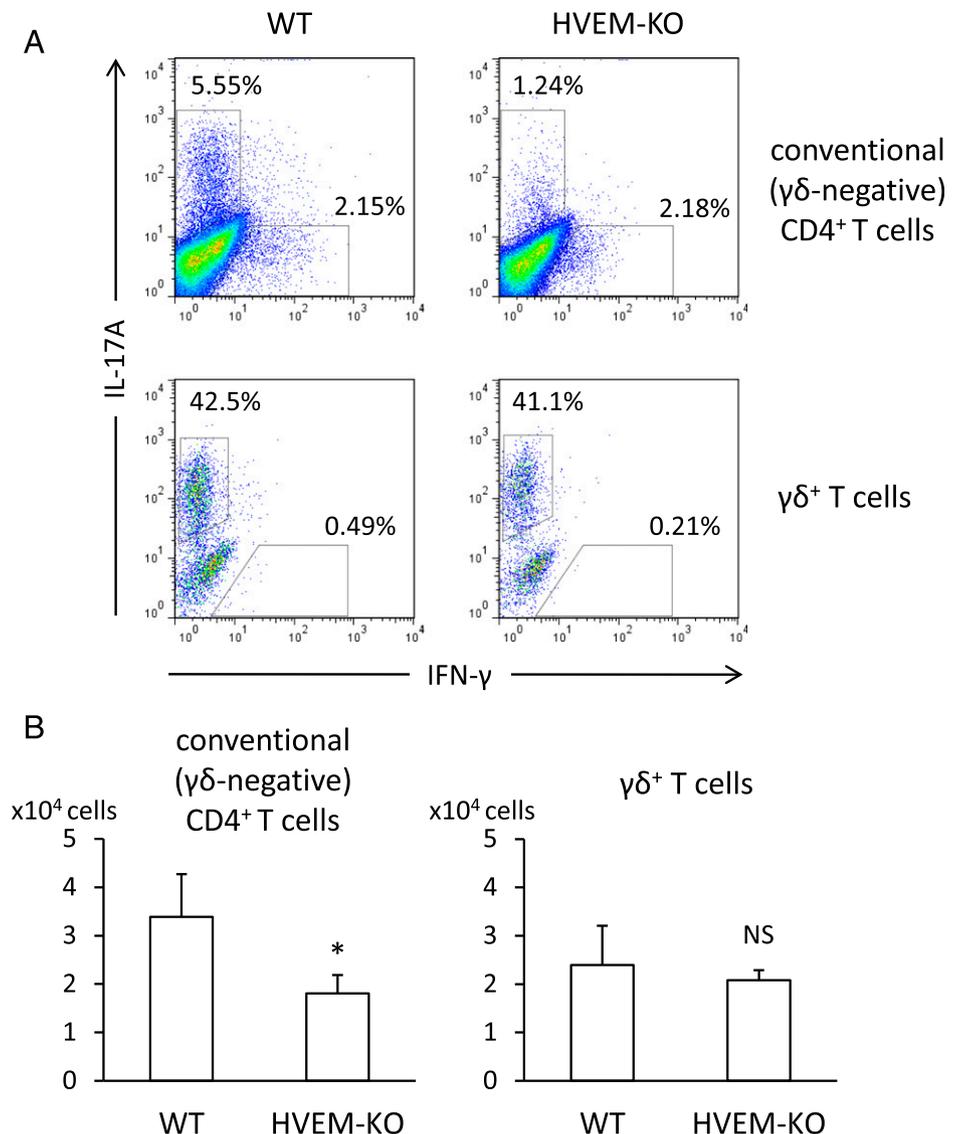
HVEM-mediated cosignaling has a stimulatory function in EAU pathogenesis.

HVEM cosignaling accelerates IRBP-specific T cell responses

To explore the mechanisms underlying this less severe EAU observed in HVEM-KO mice, we next compared the proliferative responses of IRBP-specific T cells in WT and HVEM-KO mice. T cells isolated from LNs of WT or HVEM-KO mice, which had been immunized with IRBP peptide, were restimulated in vitro with IRBP peptide in the presence of feeder cells. HVEM-KO T cells showed significantly lower proliferation in response to IRBP peptide than did WT T cells (Fig. 2A). We further examined the levels of cytokines and chemokines produced by IRBP-specific T cells. Both Th1- and Th17-related cytokines, as well as various chemokines important for T cell and microphage migration, were significantly decreased in the culture supernatants of HVEM-KO T cells compared with WT T cells (Fig. 2B, 2C, Supplemental Fig. 1). These results suggest that HVEM cosignaling exacerbates EAU by biasing IRBP-specific T cell activation toward Th1/Th17-type and inflammatory responses.

Because there are several reports suggesting the functions of HVEM in regulatory T (Treg) cells (28–30), we considered the possibility that decreased EAU severity and IRBP-specific T cell responses in HVEM-KO mice might be related to dysregulation of

FIGURE 3. Impaired differentiation of IRBP-specific Th17 cells in conventional CD4⁺ T cells in HVEM-KO mice. Nine days after IRBP immunization in WT and HVEM-KO mice, T cells were isolated from LNs and restimulated in vitro with IRBP peptide in the presence of IL-23 and WT B6 syngeneic irradiated spleen cells for 4 d. **(A)** Expression of IL-17A and IFN- γ in conventional ($\gamma\delta$ -negative) CD4⁺ T cells and $\gamma\delta$ ⁺ T cells was analyzed by intracellular staining with a flow cytometer. Representative data from two independent experiments with similar results are shown. **(B)** The absolute numbers of IL-17A-positive conventional ($\gamma\delta$ -negative) CD4⁺ T cells and $\gamma\delta$ ⁺ T cells in WT or HVEM-KO mice were determined by flow cytometry. The results are shown as the mean \pm SD of pooled data from two independent experiments. * $p < 0.05$.



Treg cells. In order to address, we examined the frequency of Treg cells in our EAU model. The percentages of CD4-positive, Foxp3-positive Treg cells were comparable in WT and HVEM-KO mice with or without IRBP immunization (Supplemental Fig. 2), suggesting a negligible role for Treg cells in the regulatory effects of HVEM in EAU.

HVEM plays a crucial role in Th17 differentiation of CD4⁺ T cells in EAU

Our previous studies demonstrated that HVEM-mediated stimulatory cosignaling promotes Th1-type T cell responses (17–19). Although a potential role for HVEM in Th17-mediated antibacterial immune responses in mucosal tissues has been suggested (31, 32), its functions in autoimmune-related Th17 responses remain largely unexplored. In this regard, the current study found that Th17-type cytokine production was decreased in IRBP-specific HVEM-KO T cells (Fig. 2B). Because it has been reported that Th17-type cytokines are produced by conventional ($\alpha\beta^+\gamma\delta^-$) CD4⁺ T cells as well as $\gamma\delta^+$ T cells in EAU (33, 34), we next examined whether differentiation of Th17 cells from IRBP-specific conventional and/or $\gamma\delta^+$ T cells was impaired in HVEM-KO mice. The number of IL-17–positive conventional CD4⁺ T cells induced by IRBP restimulation in the presence of IL-23 was significantly decreased in HVEM-KO T cells compared with WT T cells (Fig. 3A, 3B). In contrast, induction of IL-17–positive $\gamma\delta^+$ T cells under the same conditions was comparable between HVEM-KO and WT T cells. Thus, these results suggest that HVEM cosignaling is important for the differentiation of Th17 cells from conventional CD4⁺ T cells, but not $\gamma\delta^+$ T cells, in the EAU model.

HVEM on nonhematopoietic cells is dispensable for EAU pathogenesis

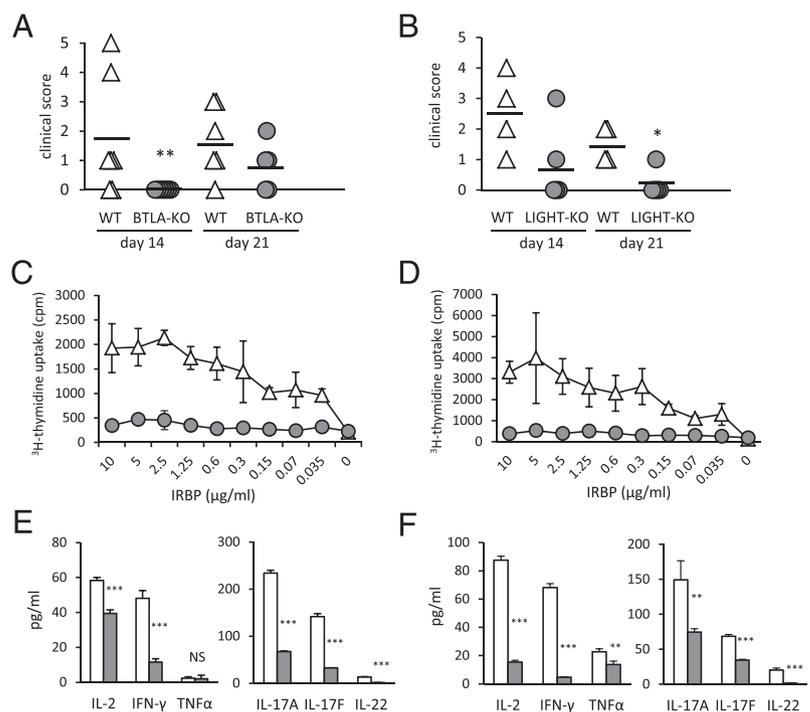
HVEM is widely expressed on the surfaces of hematopoietic cells as well as nonhematopoietic cells (35). In addition, it has been reported that HVEM on nonhematopoietic cells, including mucosal epithelial cells, promotes antipathogen inflammation related to a Th17-type response (31, 32, 36). Although the cornea and

palpebral conjunctiva are known to express HVEM (37, 38), it remains unknown whether HVEM is expressed in the retina and uvea (i.e., target tissues in EAU), and if so, whether HVEM in these tissues plays any role in the pathogenesis of EAU. To address this question, we first examined HVEM expression in eye tissues, including the retina and uvea, and found positive staining by Western blot analysis, even though the level of expression was much lower than in the thymus (Supplemental Fig. 3A). Interestingly, HVEM expression levels in eye tissues increased with EAU severity (Supplemental Fig. 3B), suggesting its possible contribution to EAU progression. For further clarification, we next transferred IRBP-specific effector T cells, which were generated from LN T cells of IRBP-immunized WT mice by in vitro restimulation with IRBP peptide, into sublethally irradiated WT or HVEM-KO mice. In this model, the severity of EAU was comparable between WT and HVEM-KO recipient mice (Supplemental Fig. 3C), suggesting a negligible role for HVEM on nonhematopoietic cells in the T cell effector phase of EAU. Next, to assess the role of HVEM in nonhematopoietic cells in the priming phase, we generated bone marrow (BM) chimeric mice by reconstituting lethally irradiated HVEM-KO or WT recipient mice with WT donor BM cells and then examined EAU susceptibility. Clinical scores were comparable between the WT and HVEM-KO recipient chimeric mice (Supplemental Fig. 3D). In contrast, EAU clinical scores of WT recipient mice reconstituted with HVEM-KO BM cells were significantly lower than those of WT mice reconstituted WT BM cells (Supplemental Fig. 3E). Taken together, these findings indicate that HVEM expressed on hematopoietic cells, but not nonhematopoietic cells, plays a critical role in both priming and effector phases of EAU.

Importance of BTLA and LIGHT ligands in HVEM-mediated stimulatory effects in EAU

Among the physiological ligands of HVEM, BTLA and LIGHT have been demonstrated to provide stimulatory cosignals in T cells via the HVEM receptor (16–19). To explore the potential role of these ligands in HVEM-mediated EAU, we immunized BTLA-KO and LIGHT-KO mice with IRBP peptide and assessed the severity

FIGURE 4. Decreased EAU severity and IRBP-specific T cell responses in BTLA-KO and LIGHT-KO mice. Clinical scores of EAU in BTLA-KO (**A**) and LIGHT-KO (**B**) mice were determined by funduscopy on days 14 and 21 after immunization with IRBP peptide (filled circles). As a control, WT mice immunized with IRBP peptide (open triangles) were also examined. The average of scores is shown as a horizontal bar in each group. T cells were isolated from LNs of WT [open triangles in (**C**) and (**D**)], BTLA-KO [filled circles in (**C**), or LIGHT-KO [filled circles in (**D**)] mice 21 d after IRBP peptide immunization and restimulated in vitro with the indicated doses of IRBP peptide in the presence of irradiated WT spleen cells. After 3 d, proliferation was assessed by [³H]thymidine incorporation assay. (**E** and **F**) T cells isolated from WT, BTLA-KO, and LIGHT-KO mice were restimulated in vitro as described in (**C**) and (**D**). After 48 h, culture supernatants of BTLA-KO [filled bars in (**E**)], LIGHT-KO [filled bars in (**F**)], and WT T cells [open bars in (**E**) and (**F**)] were harvested and examined for the concentration of cytokines by Bio-Plex assay (Bio-Rad). The results are shown as the mean \pm SD of triplicate samples. The experiment was performed twice with similar results, and one representative datum is shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.



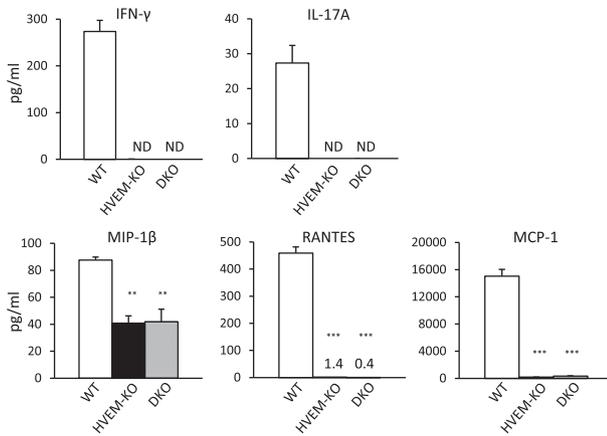


FIGURE 5. Suppression of inflammatory responses in target organs of HVEM-KO and DKO mice. Ocular fluids were harvested from the eyes of WT (open bars), HVEM-KO (filled bar), and DKO (gray bar) mice 14 d after EAU induction and the concentration of cytokines and chemokines was measured by Bio-Plex assay (Bio-Rad). The results are shown as the mean \pm SD of triplicate samples. ** $p < 0.01$, *** $p < 0.001$.

of EAU as well as IRBP-specific T cell responses. We found that both BTLA-KO and LIGHT-KO mice exhibited decreased EAU severity compared with WT mice (Fig. 4A, 4B), suggesting that both BTLA and LIGHT serve as ligands for HVEM stimulatory cosignaling in EAU. We also found that proliferation (Fig. 4C, 4D) and Th1/Th17-type cytokine production (Fig. 4E, 4F) from IRBP-specific T cells were significantly decreased in both BTLA-KO and LIGHT-KO mice compared with WT mice.

To confirm the roles of BTLA and LIGHT as functional ligands of HVEM stimulatory cosignaling in EAU, we generated BTLA and LIGHT-double KO (DKO) mice and examined their susceptibility to IRBP-induced EAU. Clinical scores of DKO mice were significantly decreased compared with WT mice (Supplemental Fig. 4A). Pathology scores as well as IRBP-specific T cell proliferation and cytokine production were also decreased in DKO mice (Supplemental Fig. 4B–D). These results further support the

importance of BTLA–HVEM and LIGHT–HVEM pathways in EAU pathogenesis.

Next, to examine immune responses at the site of inflammatory, the levels of cytokines and chemokines in the ocular fluids of EAU mice were measured. We found that the levels of IFN- γ , IL-17A, and inflammatory chemokines were virtually undetectable in situ, with the exception of significantly reduced levels of MIP-1 β , in HVEM-KO and DKO mice (Fig. 5). This result indicates that BTLA–HVEM and LIGHT–HVEM cosignaling pathways play a crucial role in inducing inflammatory responses in the target organs of EAU and further supports our finding that HVEM-KO mice are less susceptible to EAU than WT mice.

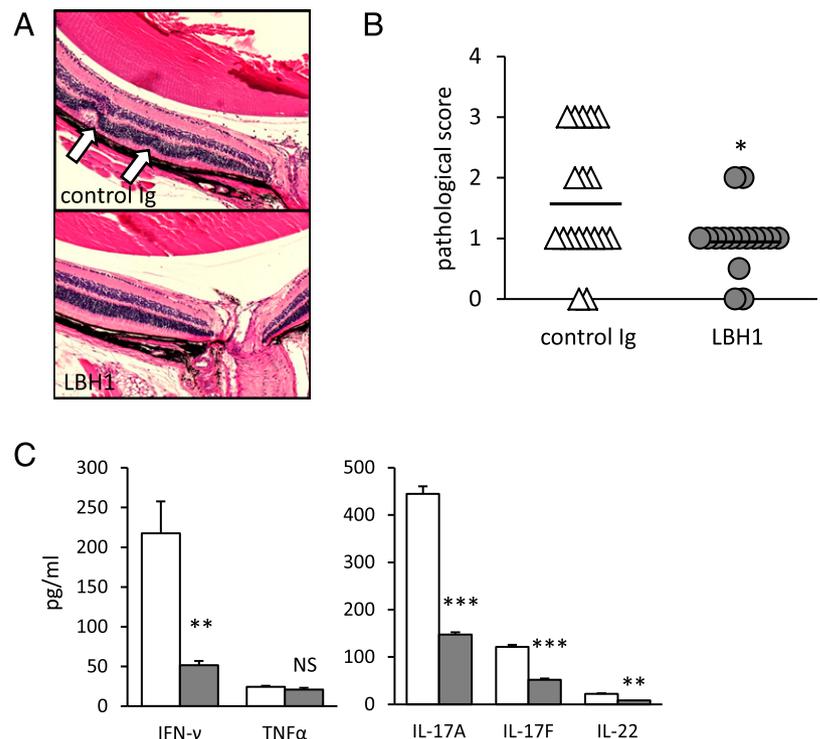
Amelioration of EAU by treatment with antagonistic anti-HVEM mAb

We previously developed an anti-HVEM mAb clone LBH1 that has antagonistic but not agonistic ability (18, 25). Our data indicated that LBH1 mAb abrogated not only LIGHT–HVEM interaction (18, 25) but also BTLA–HVEM interaction (data not shown). Thus, we next examined whether LBH1 treatment ameliorated EAU by interfering with HVEM stimulatory cosignals. Inflammatory responses in the retinas and uveas of IRBP-immunized WT mice decreased with LBH1 treatment (Fig. 6A). Consistent with this observation, pathology scores from LBH1-treated mice were significantly lower than those from control Ig-treated mice (Fig. 6B). Production of Th1- and Th17-related cytokines by IRBP-specific T cells was also significantly decreased with LBH1 treatment (Fig. 6C). Collectively, these findings suggest the potential for the HVEM cosignaling molecule and its ligands to serve as therapeutic targets in autoimmune uveitis.

Discussion

In this study, we explored the immunological functions of the HVEM cosignaling molecule in an IRBP-induced EAU model and revealed its pathogenic functions of upregulating Th1- and Th17-type IRBP-specific CD4⁺ T cells. BTLA and LIGHT, two endogenous ligands known to deliver HVEM stimulatory cosignals

FIGURE 6. Amelioration of EAU by treatment with anti-HVEM antagonistic mAb. WT mice were immunized with IRBP peptide to induce EAU and then treated with either control hamster IgG or an anti-HVEM antagonistic mAb, LBH1, on days 0, 3, 6, and 9. On day 21, histopathology of the posterior segments was examined (A), and EAU pathological scores (B) of the eyes were determined. (A) Representative pictures of IRBP-immunized mice treated with control IgG (pathological score 2) and LBH1 (pathological score 0) are shown. Arrows indicate retinal folds. H&E staining, original magnification $\times 200$. (B) Individual pathological scores for eyes are shown by each symbol. The average of scores is shown as a horizontal bar in each group. The experiment was performed twice with similar results, and the pathological scores shown are from pooled data. On day 21, T cells were isolated from the mice treated with either control hamster IgG (open bars) or LBH1 (filled bars) and then restimulated in vitro with IRBP peptide in the presence of irradiated WT spleen cells. After 48 h, culture supernatants were harvested and examined for the concentration of cytokines by Bio-Plex assay (Bio-Rad). The results are shown as the mean \pm SD of triplicate samples. Representative data from two similar independent experiments are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



to T cells, were both found to be important for this effect. We also demonstrated that treatment with anti-HVEM antagonistic mAb ameliorated EAU. Thus, this study is the first, to our knowledge, to elucidate the role of HVEM in the induction of pathogenic Th17 cells in an autoimmune disease and to propose the HVEM-related pathway as a possible novel therapeutic target in autoimmune uveitis cases.

It is well established that T cell cosignaling molecules play a crucial role in the pathogenesis of various autoimmune diseases. Previous studies have indicated that overactivation of stimulatory cosignals and/or dysfunction of inhibitory cosignals can lead to the onset and progression of autoimmune disease. Accordingly, regulation of cosignals can be used in the treatment of autoimmune diseases, and clinical drugs (e.g., CTLA4-Ig for psoriasis and rheumatoid arthritis) have been developed based on this principle. In EAU, ICOS expression has been reported to be upregulated, and attenuation of ICOS function has been shown to reduce the disease severity (39, 40). The therapeutic potential of CTLA4-Ig in EAU was also demonstrated (41). Nevertheless, no studies have been reported, to the best of our knowledge, that examine whether HVEM cosignaling has any pathogenic functions in EAU and, if any, whether regulation of these functions can be beneficial in the treatment of EAU. This study demonstrated the stimulatory role of HVEM in EAU through the induction of Th1- and Th17-type T cell responses and suggested its potential as a therapeutic target. This finding is not necessarily consistent with previous studies, because HVEM has been reported to mediate suppressive effects, serving as a ligand to deliver BTLA inhibitory cosignals, in autoimmune models including EAE, hepatitis, and rheumatoid arthritis (23, 24). In order to exclude the possibility that HVEM-KO mice in our animal facility exhibited variable phenotypes for undetermined reasons such as microbiomes, we examined EAE severity in HVEM-KO mice in our facility. We found that EAE was exacerbated in HVEM-KO mice concomitantly with increased IFN- γ and IL-17 production by MOG-specific T cells (Supplemental Fig. 5), as has been reported by other laboratories (23). Thus, these results suggest a possibility that HVEM deficiency differently affects the generation of pathogenic T cells in response to the uveitogenic and encephalomyelitogenic peptides, although the detailed mechanisms remain unclear. It is also possible that the distinct functions of HVEM in these autoimmune models could be associated with the expression patterns of HVEM and its counterreceptors on immune cells that mediate the pathogenic effects. For instance, costimulatory signaling by HVEM could become predominant when it binds LIGHT and BTLA in T cell–T cell interactions (17, 18). In addition, ligation of HVEM on macrophages is known to affect functions of these cells (25, 42). Thus, it is possible that HVEM mediates stimulatory effects via T cells and/or macrophages, which are both pathogenic to EAU. Further experiments using conditional KO mice are needed to address this hypothesis.

Induction of Th1-type T cell responses by HVEM cosignaling has been documented in multiple studies by others and us (19, 43). However, only a few studies have reported HVEM functions in the development of Th17-type responses, in which HVEM signaling has been suggested to promote NF- κ B-inducing kinase-dependent STAT3 activation and subsequent IL-17 production (44). In the current study using an EAU model, we revealed that HVEM induces both Th1 and Th17 T cell responses against the same Ag *in vivo*. Interestingly, recent reports have demonstrated that HVEM expressed on epithelial cells contributes to Th17 induction and regulates antipathogen immune responses in mucosal tissues (31, 32). In this regard, whereas HVEM expression was detected in the eye tissue, the current study indicates a negligible role for HVEM on nonhematopoietic cells in the pathogenesis of EAU. Further studies will be necessary to elucidate the intrinsic mo-

lecular mechanisms behind HVEM regulation of Th1 and Th17 responses in EAU.

Previous reports have suggested that IL-17 produced by $\gamma\delta^+$ T cells activates IRBP-specific $\alpha\beta^+$ CD4⁺ T cells, which leads to Th17 induction and EAU progression (33, 34). In contrast, our results suggest a direct effect of HVEM signaling in IRBP-specific $\alpha\beta^+$ CD4⁺ T cells, without affecting IL-17-producing $\gamma\delta^+$ T cells. This finding, however, does not necessarily exclude the pathogenic role of IL-17-producing $\gamma\delta^+$ T cells in our EAU model. Our results also suggest that HVEM cosignaling increases EAU severity irrespective of Treg cells. Treg cells have been reported to contribute to resolution and remission of EAU and to mediate therapeutic effects (45, 46). In addition, a potential role for HVEM in the development and function of Treg cells has also been described (28–30). Nevertheless, our findings indicate that HVEM cosignaling regulates EAU via its effects on conventional CD4⁺ T cells.

Our study indicates that BTLA and LIGHT, functional ligands of HVEM, are both responsible for EAU pathogenesis. Although any functional differences between BTLA–HVEM and LIGHT–HVEM pathways in EAU remain unexplored, the current study provides intriguing data to show that BTLA deficiency decreased EAU severity in the early phase of EAU, whereas LIGHT deficiency mainly affected the late phase of EAU (Fig. 4A, 4B). These results might suggest differential roles for BTLA and LIGHT in EAU, perhaps due to the distinct spatiotemporal expression of these molecules on immune cells. For instance, in B6 mice, BTLA is broadly expressed on B cells, T cells, DCs, macrophages, and NK cells, whereas LIGHT expression is rather limited, appearing on immature DCs and activated T cells (16, 19, 47). Thus, it is possible that HVEM signaling is triggered by BTLA on inflammatory immune cells in the early phase of EAU, whereas LIGHT, expressed on the infiltrating T cells, interacts with HVEM in the later phase. Further studies are necessary to address this possibility.

Recent clinical trials of secukinumab, a fully human anti-IL-17A Ab, indicate its efficacy and safety for the treatment of chronic and active noninfectious uveitis that requires corticosteroid-sparing immunosuppressive therapy (48). In addition, mAb therapies for noninfectious uveitis to attenuate various inflammatory cytokines, including TNF- α , IL-1 β , and IL-6, have been actively investigated in clinical trials (49). It should be noted that ablation of HVEM signaling simultaneously reduces the production of all of these inflammatory cytokines, as shown in this study. Thus, the current study underscores the pathogenic functions of HVEM and its importance as a promising target for the treatment of autoimmune uveitis.

Disclosures

The authors have no financial conflicts of interest.

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