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## Epithelial HVEM maintains intraepithelial T cell survival and contributes to host protection

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### Abstract

Intraepithelial T cells (IETs) are in close contact with intestinal epithelial cells and the underlying basement membrane, and they detect invasive pathogens. How intestinal epithelial cells and basement membrane influence IETs survival and function, at steady state or following infection, is unclear. The herpes virus entry mediator (HVEM), a member of the TNF receptor superfamily, is constitutively expressed by intestinal epithelial cells and is important for protection from pathogenic bacteria. Here, we showed that at steady-state LIGHT, an HVEM ligand, binding to epithelial HVEM promoted the survival of small intestine IETs. RNA-seq and addition of HVEM ligands to epithelial organoids indicated that HVEM increased epithelial synthesis of basement membrane proteins, including collagen IV, which bound to  $\beta_1$  integrins expressed by IETs. Therefore, we proposed IETs survival depended on  $\beta_1$  integrin binding to collagen IV, and showed  $\beta_1$  integrin-collagen IV interactions supported IETs survival *in vitro*. Moreover, the absence of  $\beta_1$  integrin expression by T lymphocytes decreased TCR  $\alpha\beta^+$  IETs *in vivo*. Intravital microscopy showed that the patrolling movement of IETs was reduced without epithelial HVEM. As likely consequences of decreased number and movement, protective responses to *Salmonella enterica* were reduced in mice lacking either epithelial HVEM, HVEM ligands, or  $\beta_1$  integrins. Therefore, tissue-resident IETs, at steady state and following infection, depended on HVEM expressed by epithelial cells for the synthesis of collagen IV by epithelial cells. Collagen IV engaged  $\beta_1$  integrins on IETs that were important for their maintenance and ultimately for the protective function of IETs in mucosal immunity.

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**Competing interests:** MK and TFC have filed a patent on uses of HVEM with selective binding to ligands.

**One sentence summary:**

Epithelial HVEM maintains intraepithelial T cell survival and function and supports host defense.

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**INTRODUCTION**

Intestinal intraepithelial lymphocytes (IELs) are one of the largest population of lymphocytes in the body. They are found above the basement membrane within the intestinal epithelium, and they interact extensively with intestinal epithelial cells (IEC) by actively patrolling the basement membrane and by migration into the lateral intercellular space (1–4). IEL are believed to be crucial for maintenance of the intestinal barrier integrity, wound repair, and protection from pathogens (5). They include innate lymphoid cells (ILCs)(6) but are mostly T lymphocytes, referred to as intraepithelial T cells (IETs).

Based on development and antigen-recognition, IETs are divided into two groups termed “induced” IETs (also known as type a IETs) and “natural” IETs (also known as type b IETs) (5, 7, 8). Induced IETs express  $\alpha\beta$  TCRs and one of the TCR co-receptors that promotes TCR signaling, CD4 or CD8 $\alpha\beta$ . Induced IETs are tissue resident memory cells derived from antigen-primed conventional or mainstream CD4<sup>+</sup> or CD8 $\alpha\beta$ <sup>+</sup> T cells (9, 10). By contrast, natural or type b IETs express either  $\alpha\beta$  TCRs or  $\gamma\delta$  TCRs, but lack the expression of the TCR co-receptors. Natural IETs in mice are approximately 50% of the total population and they can be either CD4<sup>-</sup>, CD8 $\alpha$ <sup>-</sup> double negative (DN), but often express CD8 $\alpha\alpha$  homodimers, which lack TCR co-receptor function (11). TCR $\alpha\beta$ <sup>+</sup> precursors of natural IETs undergo an alternative, self-antigen-based or agonist thymic selection and maturation process (12–14) and they have a distinct set of specificities compared to TCR $\alpha\beta$ <sup>+</sup> CD8 $\alpha\beta$  lymphocytes (15–17). The functions of natural IETs have not been fully characterized, however, TCR $\gamma\delta$ <sup>+</sup> IETs are implicated in host defense and repair of damaged epithelium by various mechanisms (18–22).

The factors important for the maintenance of IETs are not completely defined, but the aryl hydrocarbon receptor (AHR), T-bet and MyD88 are implicated for both TCR $\alpha\beta$ <sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup> IET populations (23–25). G protein-coupled receptor 18 (GPR18) positively regulates TCR $\gamma\delta$ <sup>+</sup> IETs (3) while GPR55 is a negative regulator of these  $\gamma\delta$  cells (4). IL-15 contributes to maintenance of IETs, providing an example of an epithelial cell-derived influence (26–28). Epithelial expression of the thymus leukemia antigen, a nonclassical class I molecule, shapes the population of induced TCR $\alpha\beta$ <sup>+</sup> CD8 $\alpha\beta$ <sup>+</sup> IETs via induction of co-expression of CD8 $\alpha\alpha$  homodimers, which interact with the thymus leukemia antigen (29) and promote the survival of these cells (30). The homing and maintenance of intestinal IETs in the epithelium depends on expression of  $\beta_7$  integrins, including  $\alpha_4\beta_7$ , which binds MAdCAM-1 and the  $\alpha_E\beta_7$  integrin, which interacts with E-cadherin expressed by IEC (31–33).

HVEM is a member of the tumor necrosis factor receptor superfamily (TNFRSF14) expressed by intestinal epithelial cells, lymphocytes and other cell types. It is important for the regulation of barrier or mucosal immunity in mice in the context of infection or inflammation in a variety of contexts (34–41). HVEM binds to several types of proteins. One

of these is LIGHT (homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for binding to herpesvirus entry mediator, a receptor expressed on T lymphocytes) or TNFSF14 (42, 43), which engages HVEM in a trimeric form (44). It also binds immunoglobulin superfamily (IgSF) proteins including B and T lymphocyte attenuator (BTLA or CD272), CD160 and SALM5(45). In binding these proteins, HVEM engages in bidirectional signaling, also serving as a ligand for IgSF receptor signaling (45).

Here, we showed that HVEM expressed by small intestine (SI) epithelial cells was involved in the homeostasis of natural IETs, the patrolling function of IETs at steady-state, and the response to pathogenic bacteria. Ligand binding to epithelial HVEM at steady state stimulated the synthesis of extracellular membrane proteins, a key one being collagen IV. Collagen IV affected cell survival by binding to  $\beta_1$  integrins expressed by the IETs. Analysis of gene deficient mice showed that this homeostatic circuit, including epithelial HVEM, HVEM ligands and  $\beta_1$  integrins, was involved in protective responses to a bacterial pathogen. Therefore, the data reveal how epithelial responses influencing the basement membrane, a structural element of tissue, are integrated to regulate tissue resident T cells in the intestine at steady-state and following infection.

## RESULTS

### HVEM is involved in maintaining IETs

Because of the engagement of HVEM to CD160 expressed on IETs is involved in the protection of mice from oral infection with *Citrobacter rodentium*(38), we determined if HVEM also is involved in the regulation of IETs in the intestine at steady state. We used flow cytometry to examine the total CD45<sup>+</sup> cell number from mice with a germline deletion of the *Hvem* (*Tnfrsf14*) gene. Total CD45<sup>+</sup> cells were significantly reduced throughout the SI, but not in the cecum and colon (Fig. 1A). We also observed a decrease in CD45<sup>+</sup> cells throughout the SI epithelium in *Hvem*<sup>-/-</sup> mice with confocal microscopy (Figs. 1B, 1C). Most of the CD45<sup>+</sup> cells in the epithelium were T lymphocytes, including TCR $\gamma\delta$ <sup>+</sup> cells and TCR $\alpha\beta$ <sup>+</sup> cells, with a smaller population TCR negative cells (Fig. 1E) that are mostly ILC1 (6). We gated on the IETs subsets as shown in Fig. S1A. Considering the different subsets, TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs exhibited the most pronounced reduction in cell frequency (Fig. 1D) and cell number (Fig. 1E). Natural  $\gamma\delta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> and induced TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> were also decreased (Fig. 1E). Furthermore, induced TCR $\alpha\beta$ <sup>+</sup>CD4<sup>+</sup> IETs, including cells that co-express CD8 $\alpha\alpha$ <sup>+</sup> and which contain CD4<sup>+</sup> T cells that acquire the function of cytotoxic T lymphocytes (CTLs) (46, 47), were also decreased (Fig. 1E). These data indicated that HVEM was involved in the accumulation of the three most numerically prevalent IET populations in the intestine at steady state.

### Epithelial HVEM maintains IETs

Considering the importance of HVEM for TCR $\alpha\beta$ <sup>+</sup> CD8 $\alpha\beta$ <sup>+</sup> memory and resident memory T cell formation (34, 36), we tested for a T cell intrinsic effect of HVEM expression. Because of the striking reduction in TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs, initially we focused on this population and analyzed the number of CD4<sup>-</sup>, CD8 $\alpha$ <sup>-</sup> double-negative (DN) TCR $\alpha\beta$ <sup>+</sup> (DN TCR $\alpha\beta$ <sup>+</sup>) thymocytes, which are the precursors of TCR $\alpha\beta$ <sup>+</sup> CD8 $\alpha\alpha$ <sup>+</sup> IETs (5, 16, 48).

We found no difference between wild type and *Hvem*<sup>-/-</sup> mice (Fig. S1B). We transferred these precursor cells, NK1.1<sup>-</sup>TCR $\gamma\delta$ <sup>-</sup>TCR $\alpha\beta$ <sup>+</sup> thymocytes, into *Rag1*<sup>-/-</sup> mice. A 1:1 co-transfer of donor thymocytes from *Hvem*<sup>-/-</sup> mice (CD45.2) and congenic, wildtype CD45.1<sup>+</sup> C57BL/6 mice was performed (Fig. S1C). There was no defect in the ability of the *Hvem*<sup>-/-</sup> donor thymocytes to give rise to TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs. In fact, the *Hvem*<sup>-/-</sup> donor cells were more highly represented at four weeks after transfer but the populations were equivalent at six weeks (Fig. S1C). We also analyzed mice with a T cell-specific deletion of the *Hvem* gene driven by CD4-Cre. These *Cd4-cre*  $\times$  *Hvem*<sup>fl/fl</sup> (*Hvem*<sup>CD4</sup>) mice showed no difference in the numbers of the most prevalent IET subsets in the proximal SI (Fig. S1D), although the TCR $\alpha\beta$ <sup>+</sup> CD4<sup>+</sup> IETs, whether CD8 $\alpha\alpha$ <sup>+</sup> or not, were reduced. Considering these results, an intrinsic or T cell role for HVEM expression was not the major influence in determining the size of the total IET population.

As IECs have direct contact with resident IETs, we wanted to know the role of HVEM at steady-state in IECs. HVEM was predominantly on the basolateral surface of epithelial cells (Fig. S2A), an optimal position for contact with immune cells, and it was expressed throughout the intestine epithelium, although at a higher amount in IEC from the proximal SI compared to the middle portion (Fig. S2B). Interestingly, HVEM expression by IEC was not increased during infection (38) nor was it diminished in IEC from germ-free mice (Fig. S2C). Also, HVEM was expressed in proximal small intestinal organoid cultures (Fig. S2D, S2E). Therefore, constitutive HVEM expression by IEC was unaffected by acute infection or the absence of either the microbiota or IEL.

To test if there is a role for epithelial cell-expressed HVEM, we crossed *Villin-cre* mice with *Hvem*<sup>fl/fl</sup> mice to generate mice with HVEM expression ablated only in epithelial cells (*Hvem*<sup>IEC</sup> mice, Fig. S2F, S2G). Flow cytometric analyses indicated that *Hvem*<sup>IEC</sup> mice had significantly decreased CD45<sup>+</sup> cell numbers in the proximal and middle small intestine compared to *Hvem*<sup>fl/fl</sup> controls (Fig. 2A). The proximal SI has the greatest number of IETs, and when this segment was analyzed in more detail, TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> were most decreased, similar to *Hvem*<sup>-/-</sup> mice, but the TCR $\gamma\delta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs and TCR $\alpha\beta$ <sup>+</sup>CD4<sup>+</sup> IETs also were reduced (Fig. 2B, 2C). These trends were partially replicated in the middle SI but less so in the distal SI (Fig. S2H). The effect on TCR $\alpha\beta$ <sup>+</sup>CD4<sup>+</sup>CD8 $\alpha$ <sup>+</sup> CTL suggests there could have been HVEM contributions to this population that are both T cell-intrinsic and epithelial cell dependent. TCR $\alpha\beta$ <sup>+</sup> CD8 $\alpha\beta$ <sup>+</sup> IETs were not reduced in *Hvem*<sup>IEC</sup> mice although they were reduced in *Hvem*<sup>-/-</sup> mice. The complexities regarding induced IET subsets may reflect their history, which unlike natural IETs, require priming in lymph nodes and many of the cell types they encounter during priming (5, 10) and while circulating express HVEM and HVEM ligands. Regardless, the effects were specific to the SI, as no IET populations were diminished in the colon of *Hvem*<sup>IEC</sup> mice (Fig. S2H).

To confirm that the homeostasis of IET populations in the small intestine was affected by epithelial HVEM expression, we performed *in situ* immunofluorescence (IF) multi-color staining in frozen sections from the proximal small intestine of *Hvem*<sup>IEC</sup> and control mice. To identify TCR $\gamma\delta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs, we used fluorochrome-coupled antibodies to TCR $\delta$ , CD8 $\alpha$ , CD8 $\beta$  and to detect TCR $\gamma\delta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs (Fig. S3A). Similarly, to identify TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs, we used fluorochrome-coupled antibodies to TCR $\beta$ , CD8 $\alpha$ ,

CD8 $\beta$  (Fig. S3B). Although this method did not distinguish TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs from TCR $\alpha\beta$ <sup>+</sup>CD4<sup>+</sup>CD8 $\alpha$ <sup>+</sup> CTL, the CD4<sup>+</sup> CTL were less frequent (Figs. 1E, 2C, S2H). After imaging by confocal microscopy, the IET subsets were quantified (Figs. 2D–2F). The total villus area in the proximal SI was comparable between *Hvem*<sup>fl/fl</sup> and *Hvem*<sup>IEC</sup> mice (Fig. 2F). Similar to the flow cytometry results, *Hvem*<sup>IEC</sup> mice had a decrease of total CD8 $\alpha\alpha$ <sup>+</sup> IETs, including individual populations of TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> plus TCR $\alpha\beta$ <sup>+</sup>CD4<sup>+</sup>CD8 $\alpha$ <sup>+</sup> CTL and TCR $\gamma\delta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs, but no decrease in CD8 $\beta$ <sup>+</sup> cells in the proximal small intestine (Figs. 2D–2F). In summary, loss of epithelial HVEM expression led to decreases in natural IET subsets.

### Epithelial HVEM is required for IETs survival

Among the possible mechanism(s) for the decreased number of IETs in *Hvem*<sup>IEC</sup> mice, we tested for components involved in homing to the intestine or the maintenance of the cells in the epithelial site, for IETs proliferation and for IETs survival. Flow cytometric analysis indicated that the expression of the integrin  $\beta_7$  chain and the integrin  $\alpha_E$  were not affected in IETs from *Hvem*<sup>IEC</sup> mice (figs. S4A, S4B). The expression of mRNA for the *Cdh1* gene encoding E-cadherin in *Hvem*<sup>IEC</sup> IEC was normal as well (fig. S4C).

To address if IETs proliferation was responsible, Ki-67 staining and EdU incorporation assays were performed. Although the trend suggested increased Ki-67, the IET from *Hvem*<sup>IEC</sup> mice were not significantly different from the controls (Fig. S4D). Similarly, after six days of continuous EdU labeling, the percent of labeled IETs was not different in *Hvem*<sup>IEC</sup> mice (Fig. 3A). When the EdU label was chased or removed for 14 days, this led to a decreased percentage in EdU<sup>+</sup> in the two main natural IET subsets compared to control mice (Fig. 3A). These data suggested there was a survival defect in the TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs in *Hvem*<sup>IEC</sup> mice. Compared with control mice, cells from *Hvem*<sup>IEC</sup> mice exhibited higher Annexin V<sup>+</sup> labeling in both the TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IET subsets when analyzed *ex vivo* (Fig. 3B, Fig S4E). They also expressed greater amounts of the pro-apoptotic Bcl2 family proteins Bim and Bax, while even the TCR $\alpha\beta$ <sup>+</sup> CD8 $\alpha\beta$ <sup>+</sup> IETs expressed more Bax (Figs. 3C, 3D), suggesting there could be a small effect on these cells. To confirm that the absence of epithelial HVEM affected IETs survival, we analyzed mice with a TCR $\alpha\beta$  cell-specific overexpression of the pro-survival Bcl-2 family gene *Bclxl* (*Bcl2l1*) driven by the *Lck* proximal promoter (*Lck*<sup>Pr</sup>-*Bcl-xL*<sup>Tg</sup>) crossed to either *Hvem*<sup>fl/fl</sup> or *Hvem*<sup>IEC</sup> mice. *Hvem*<sup>IEC</sup> mice expressing the *Bclxl* transgene had a normal number of TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs. The increase in TCR $\gamma\delta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs was not significant (Fig. 3E), but the proximal *Lck* promoter does not work efficiently in TCR $\gamma\delta$ <sup>+</sup> cells (49). To gain further insight into the effects of epithelial HVEM on TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs, we performed RNA-seq analysis on the most affected population, TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs sorted from SI of *Hvem*<sup>IEC</sup> and co-housed littermate *Hvem*<sup>fl/fl</sup> mice. The top 50 differentially expressed genes included 19 genes that have been reported to affect cell cycle, cell survival or cell growth (Figs. 3F, 3G). Among those genes reduced in IETs from *Hvem*<sup>IEC</sup> mice were autophagy and beclin 1 regulator 1 (*Ambr1*), which promotes cell survival under stress conditions by inducing autophagy (50), cullin associated and neddylation dissociated gene 1 (*Cand1*), with reduced expression associated with apoptosis in cancer cells (51) and also, there was decreased expression of

some genes in the NF- $\kappa$ B pathway. Overall, the data were consistent with the hypothesis that epithelial HVEM affected the survival of the natural IETs, including TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs.

### HVEM signaling increases basement membrane synthesis

To interpret how epithelial HVEM affects CD8 $\alpha\alpha$ <sup>+</sup> IETs, especially IETs survival, we performed RNA-seq analysis on IEC sorted from proximal SI of *Hvem*<sup>IEC</sup> and co-housed littermate *Hvem*<sup>fl/fl</sup> mice. By gene set enrichment analysis, we observed a significant down-regulation of a number of genes associated with the extracellular matrix in *Hvem*<sup>IEC</sup> mice (fig. S5A). Down-regulated genes in IEC from *Hvem*<sup>IEC</sup> mice revealed significant enrichment in genes associated with the proteinaceous extracellular matrix according to gene set enrichment analysis (Fig. 4A). In agreement with this, analysis of differentially expressed genes also identified extracellular matrix, basement membrane and collagen, all categories decreased in the absence of epithelial HVEM expression (figs. S5B, S5C). These results suggested a possible role for epithelial HVEM in supporting IETs survival and migration in the epithelium through stimulation of production of extracellular matrix proteins.

Collagen IV is a major component of basement membranes and there is evidence that IETs bind to collagen (52, 53). Collagen IV composed of the  $\alpha$ 1 and  $\alpha$ 2 chains is present in nearly all basement membranes (54, 55). To further test if epithelial HVEM regulated the production of collagen IV *in vivo*, we carried out quantitative real-time PCR (qPCR) and showed reduced expression of *Col4a1* and *Col4a2*, mRNA, encoding the collagen  $\alpha$ 1 (IV) and  $\alpha$ 2 (IV) chains, respectively, by IEC from *Hvem*<sup>IEC</sup> mice (Fig. 4B). *Hvem*<sup>IEC</sup> IEC also exhibited reduced collagen  $\alpha$ 1 (IV) protein expression by Western blot (Fig. 4C).

To address if HVEM signaling induced *Col4a1* mRNA expression in IEC, intestinal organoid cultures from *Hvem*<sup>IEC</sup> and control mice were stimulated *in vitro* with HVEM ligands, LIGHT or CD160. *Col4a1* mRNA expression was increased by either HVEM binding partner in the wild type organoid culture, but not in *Hvem*<sup>IEC</sup> organoids (Fig. 4D). Together, these data indicated that epithelial HVEM signaled to stimulate increased synthesis of mRNA for collagen IV.

To investigate the role of collagen IV and other basement membrane proteins, such as laminin and fibronectin, in promoting IETs survival, we carried out *in vitro* cultures. Sorted IET populations were expanded briefly in IL-15 and cultured on plates coated with basement membrane proteins, and cell death was monitored by flow cytometry. We gated on the two natural IET populations, TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> IETs, as well as induced TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> IETs. When cultured on collagen-coated wells, each of these three cell subsets had decreased cell death compared to IETs cultured on either laminin, fibronectin or BSA (Figs. 4E, 4F).

### Collagen-binding integrins increase IET survival

Integrins containing the  $\beta$ <sub>1</sub> chain are known to bind collagen IV, especially  $\alpha$ <sub>1</sub> $\beta$ <sub>1</sub> integrin, with  $\alpha$ <sub>2</sub> $\beta$ <sub>1</sub> integrin binding to a lesser extent (52). The three prevalent IET populations, TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup>, TCR $\gamma\delta$ <sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> and TCR $\alpha\beta$ <sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> IETs expressed relatively high



amounts of integrin  $\alpha_1$  and  $\beta_1$ , in agreement with a previous report (56), although expression was lower by TCR $\alpha\beta^+$ CD4 $^+$  IETs (Fig. 5A). To investigate the role of integrins in IETs survival *in vitro*, we blocked  $\alpha_1\beta_1$  integrin binding to collagen IV with a  $\beta_1$ -specific antibody. This led to increased death of all three of the most prevalent IET populations (Fig. 5B). Cell death was also increased using the peptide disintegrin (inhibitor), Obtustatin (Fig. 5B). These data suggested that collagen IV might have influenced IETs survival in part through binding to  $\alpha_1\beta_1$  and perhaps other collagen-binding  $\beta_1$  integrins.

To determine if there was a role for T cell-expressed integrins in CD8 $\alpha\alpha^+$  IETs survival *in vivo*, we generated *Itgb1<sup>prLck</sup>* mice by crossing *Lck<sup>pr</sup>-Cre* mice with *Integrin b1<sup>fl/fl</sup>* (*Itgb<sup>fl/fl</sup>*) mice. Flow cytometric analyses indicated that *Itgb1<sup>prLck</sup>* mice had no difference in total CD45 $^+$  cell numbers throughout the SI compared to *Itgb1<sup>fl/fl</sup>* controls (Fig. 5C). However, the TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$  and TCR $\alpha\beta^+$ CD8 $\alpha\beta^+$  IETs in the proximal SI of *Itgb1<sup>prLck</sup>* mice were decreased (Figs. 5D–5E), in agreement with the greater activity of the *Lck<sup>pr</sup>-Cre* in TCR $\alpha\beta$  cells (49). TCR $\alpha\beta^+$ CD4 $^+$  IETs were not affected, consistent with their reduced expression of  $\beta_1$  integrins. In line with the *in vitro* survival results, TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$  IETs expressed greater amounts of the pro-apoptotic Bcl2 family proteins Bim and Bax, while TCR $\alpha\beta^+$  CD8 $\alpha\beta^+$  expressed higher Bax in *Itgb1<sup>prLck</sup>* mice (Figs. 5F–5G). Therefore, the selective effect in the *Itgb1<sup>prLck</sup>* mice was consistent with the hypothesis that collagen IV promoted IETs survival, in part through  $\alpha_1\beta_1$  integrin binding.

### LIGHT expression is required for IETs

We tested for the effect of the loss of HVEM binding partners expressed by IETs on their survival. CD160, important for anti-bacterial responses in the intestine (38, 57, 58), was expressed most prominently in the distal small intestine (Fig. S6A). In contrast, BTLA protein could not be detected on any IET subset besides intraepithelial ILC (Fig. S6B). We could not examine protein expression of LIGHT in IETs due to the absence of a reliable antibody for detecting mouse LIGHT. TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$  IETs expressed RNA encoding LIGHT and CD160, but not BTLA (Fig. S6C). Whole body deletion of *Cd160* had no effect on IETs number (Fig. S6D–S6E), in agreement with a previous report (57) and the expression of the pro-apoptotic Bcl2 family proteins Bim and Bax was not increased (Fig. S6F). In contrast, by flow cytometric analysis, whole body deletion of the gene encoding LIGHT (*Tnfrsf14*) led to significantly decreased total IETs in the proximal and middle SI (Fig. 6A). In the proximal SI, the mice had significant reductions in the two natural IET populations. The percentage of induced TCR $\alpha\beta^+$ CD8 $\alpha\beta^+$  IETs actually was increased (Fig. 6B), because these cells were less affected by LIGHT deficiency, but the number of TCR $\alpha\beta^+$ CD8 $\alpha\beta^+$  IETs also was reduced (Fig. 6C). These findings were corroborated by fluorescent microscopy-based *in situ* analysis (Fig. 6D–6F). The three predominant populations of IETs expressed higher Bax in mice lacking LIGHT protein (*LIGHT<sup>-/-</sup>*) (Fig. 6G), consistent with the possibility that decreased survival contributed to the decreased IET subpopulations. Therefore, although HVEM has multiple binding partners, LIGHT is important for survival of IETs at steady-state.



### HVEM affects IET patrolling

The top 50 differentially expressed genes in IETs from wild type and *Hvem*<sup>IEC</sup> mice also contained eight genes that influence cell migration (Figs. 3F, 3G). Included in these genes with decreased expression in *Hvem*<sup>IEC</sup> mice were clathrin heavy chain (*Cltc*), which among its different functions influences lymphocyte migration by inducing actin accumulation at the cellular leading edge (59), N- $\alpha$ -acetyltransferase 20 (*Naa20*), which acetylates some terminal methionine amino acids and affects the actomyosin fibers needed for cell migration (60), and CAS1 domain containing protein 1 (*Casd1*), which catalyzes the 9-O-acetylation of sialic acids and which is required for cell migration in the nervous system (61).

To further examine if epithelial HVEM deficiency affected IETs migration, we used intravital imaging to track lymphocytes labeled with a CD8 $\alpha$ -specific mAb in the SI epithelium of live mice (1, 3). We used confocal reflection microscopy to provide tissue context for the CD8 $\alpha$ -labeled cells. With this modality, we were able to observe the epithelial layer, underlying blood capillaries, and lamina propria extending below (Fig. S7). In some cases, we used an EpCAM-specific antibody to label the epithelial cells. Our analysis was constrained to the cells in the epithelial layer. The average coverage of the villus area by migrating CD8 $\alpha$  labeled cells was greatly reduced in *Hvem*<sup>IEC</sup> mice (Figs. 7A–7B). This decrease in coverage reflected not only the decreased cell number described above, but also decreased average movement. The mean speed, track length, and track displacement length of CD8 $\alpha$  mAb-labeled IETs were all reduced in *Hvem*<sup>IEC</sup> mice (Figs. 7C–7F, movies S1 and S2). Most IETs, including CD8 $\alpha$  $\alpha$ <sup>+</sup>, CD8 $\alpha$  $\beta$ <sup>+</sup> and CD4<sup>+</sup>CD8 $\alpha$ <sup>+</sup> IETs, express CD8 $\alpha$  (10). Therefore, the intravital microscopy data suggested that epithelial HVEM expression was required for the patrolling behavior of most IETs, including natural and induced subsets.

### Epithelial HVEM is involved in host defense against bacteria

We have thus far established a role for epithelial HVEM in regulating IETs homeostasis and migration at steady state, and therefore we interrogated if there were correlated effects on host defense. TCR $\gamma$  $\delta$ <sup>+</sup> IETs play a critical role in preventing invasion of *Salmonella enterica* serovar typhimurium (*S. typhimurium*), which causes gastroenteritis in humans and other mammals, including mice (62). IETs migration among epithelial cells in contact with *S. typhimurium* is essential for T cell surveillance and immediate host defense (2, 63). To determine if epithelial HVEM expression contributed to host protection, mice were orally infected with *S. typhimurium* and analyzed. *Hvem*<sup>IEC</sup> mice had a decreased survival rate (Fig. 8A), rapid weight loss (Fig. 8B), and increased bacterial burden in the spleen, liver, small intestine, and colon (Fig. 8C) after oral *S. typhimurium* administration. After *S. typhimurium* infection, *Cd160*<sup>-/-</sup> mice had rapid weight loss (Fig. S8A) and increased bacterial colonies in the spleen and liver (Fig. S8B). LIGHT-deficient *Tnfrsf14*<sup>-/-</sup> mice also had rapid weight loss (Fig. S8C) and a trend toward increased colonies in the spleen and liver that did not reach statistical significance (Fig. S8D). *Btla*<sup>-/-</sup> mice, however, exhibited no differences in body weight, and bacterial burden in the spleen and liver (Fig. S8E–S8F). Therefore, the data suggested that epithelial HVEM expression, probably with interactions with both CD160 and LIGHT, contributed to host defense against *S. typhimurium* infection.

The proposed mechanism for HVEM affecting the survival of natural IETs is indirect, through stimulation of synthesis of  $\beta_1$  integrin ligand. To investigate if the same mechanism might be relevant for the anti-bacterial response, we infected *Itgb1<sup>prLck</sup>* mice with *S. typhimurium*. Although weight loss was not affected, the deficiency of  $\beta_1$  integrin expression only in TCR $\alpha\beta^+$  cells led to an increased bacterial burden in the spleen and liver (Fig, 8D). These data suggest that the interaction between  $\beta_1$  integrins expressed by IETs and the basement membrane, a mechanism that contributed to the maintenance of natural IETs, might also contribute to protection from mucosal infection.

## DISCUSSION

The mechanisms by which IEC regulate IET homeostasis in the intestine have not been fully elucidated, although they include IL-15 (26–28) and epithelial expression of the thymus leukemia antigen (30). Here, we provided evidence that HVEM, a TNFR superfamily member that is constitutively expressed by IEC, was highly involved in regulating different aspects of IETs behavior, including the survival of natural IETs, the migration of most small intestine IETs, and in the protective response to an intestinal bacterial infection that requires IET responses. We propose that some of these HVEM-mediated influences at steady state, especially natural IET subsets survival, were due to LIGHT-mediated stimulation of HVEM, that caused the increased synthesis of basement membrane proteins that interacted with  $\beta_1$  integrins expressed by IETs. In contrast, we propose that the interaction of HVEM with CD160 might have contributed by triggering the protective response of IETs in the context of an infection.

While present throughout the SI, the effects of epithelial HVEM deficiency on IETs survival were most prominent in the proximal segment, where the majority of these cells are found. They were not uniform across different IET populations, with TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$  IETs most severely reduced, although TCR $\gamma\delta^+$  IETs, also were consistently diminished. HVEM effects on CD4 $^+$  IETs, which were less thoroughly analyzed, involved expression by both T cells and epithelial cells. In contrast, TCR $\alpha\beta^+$  CD8 $\alpha\beta^+$  IETs, were not decreased in *Hvem<sup>IEC</sup>* mice, although they had increased pro-apoptotic Bax protein expression in *Hvem<sup>IEC</sup>* mice, they expressed  $\beta_1$  integrins, and  $\beta_1$  integrin-collagen IV interactions affected the survival of this subset *in vitro*. Additionally, germ line deficiency of the gene encoding LIGHT caused decreased TCR $\alpha\beta^+$  CD8 $\alpha\beta^+$  IETs. A reduction in TCR $\alpha\beta^+$  CD8 $\alpha\beta^+$  IETs also was observed in mice with deficiency for  $\beta_1$  integrin subunit in TCR $\alpha\beta^+$  T cells, and TCR $\alpha\beta^+$  CD8 $\alpha\beta^+$  IETs also had increased Bax expression in this strain. There could have been effects of the LIGHT or  $\beta_1$  integrin gene knockouts outside the epithelium, however, consistent with the decreased TCR $\alpha\beta^+$  CD8 $\alpha\beta^+$  IETs in whole body knockouts of HVEM, that were not observed in *Hvem<sup>IEC</sup>* mice. Despite this caveat, collectively the *in vivo* and *in vitro* data suggested that the HVEM-dependent mechanism for IETs homeostasis was compensated for or overridden by other survival mechanisms in TCR $\alpha\beta^+$  CD8 $\alpha\beta^+$  IETs. There are precedents for signals that selectively influence IET subsets (23, 26–28, 64, 65), and therefore, variation of the effects in *Hvem<sup>IEC</sup>* mice on the survival of different IET populations was not surprising.

Our data showed there was decreased extracellular matrix protein synthesis by epithelial cells from *Hvem*<sup>IEC</sup> mice at steady-state. Furthermore, signaling by HVEM ligands *in vitro* increased *Col4a1* and *Col4a2* mRNA synthesis in intestinal organoid cultures, providing direct evidence that HVEM signaling in IEC could increase basement membrane protein synthesis. There are previous reports that collagen IV promotes survival and proliferation of different types of cancer cells (66, 67). Human and mouse IETs bind collagen (52, 56), and we showed IETs cultured on collagen IV coated microwells had increased survival. Therefore, the findings here provide a link between HVEM signals, extracellular matrix synthesis and IETs survival, although there could have been other effects of epithelial HVEM expression that promoted the accumulation of natural IET populations.

Various integrins containing a  $\beta_1$  subunit bind to extracellular membrane components, including different types of collagen and fibronectin (68). For example, integrin  $\alpha_1\beta_1$  preferentially binds collagen type IV, while  $\alpha_2\beta_1$  preferentially binds to collagen type I (52, 68, 69). Human IETs bind collagen VI through integrin  $\alpha_1\beta_1$  (52), and we confirmed that mouse IETs express collagen-binding integrins. The positive survival effect of IETs culture on collagen IV coated microwells could be reversed by blocking integrin binding. Moreover, mice with a deficiency for *Itgb1* in TCR  $\alpha\beta^+$  cells had reduced TCR $\alpha\beta^+$  IETs. We note that previous *in vivo* analyses of the role of  $\alpha_1\beta_1$  for IETs accumulation provides conflicting results. In agreement with our findings, mice with a full body deletion of the gene encoding the integrin  $\alpha_1$  have reduced total IET (53). In contrast to the findings here, in bone marrow chimeras in which the donor cells have a poly I:C induced deletion of *Itgb1*, normal IET numbers are present (56). The gene deletion is not complete in these mice, however, with approximately 50% of the IET retaining integrin  $\beta_1$  expression. Also consistent with the data here, Very Late Activation Antigen 1 (VLA-1), or  $\alpha_1\beta_1$  integrin, is important for the retention of memory CD8<sup>+</sup> T cells in the lung after influenza virus infection (70). Integrins activate downstream signaling pathways that affect survival and other processes, and although the signaling mechanism operating downstream of  $\alpha_1\beta_1$  engagement of collagen in mouse IETs has not been defined, in human peripheral blood T cells  $\alpha_2\beta_1$  interaction with collagen increased resistance to apoptosis through the Erk pathway (71).

IETs are migratory cells located above the basement membrane, which they patrol extensively (1). Movement and survival of IETs may be linked, as both were negatively regulated in TCR $\gamma\delta^+$  IETs by GPR55 (4). Furthermore, collagen IV promotes migration of tumor cells and endothelial cells (72, 73). RNA-Seq analyses confirmed that the expression of genes related to migration and survival were reduced in TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$  IETs in the absence of epithelial HVEM. Consistent with this, in *Hvem*<sup>IEC</sup> mice IETs speed, track length and displacement were decreased. Combined with fewer cells, this led to a greatly decreased area of the villi covered. Because these effects were evident when CD8 $\alpha^+$  IETs were labeled, this indicated epithelial HVEM deficiency had a broad effect on migration of all or most IET subsets. Therefore, a plausible hypothesis is that epithelial HVEM deficiency affected not only the survival of natural IETs but also patrolling of IETs through altered basement membrane synthesis and the action of  $\beta_1$  integrins, although other HVEM-dependent effects on IETs migration have not been excluded.

IETs movement and surveillance likely are important for protecting the epithelium from injury or infection. TCR $\gamma\delta^+$  IETs movement in the small intestine is dynamically regulated by MyD88-dependent signaling by IEC (2). Furthermore, exposure to invasive *Salmonella* bacteria enhanced the movement of TCR $\gamma\delta^+$  IETs between the IEC in a “flossing” movement, and movement of  $\gamma\delta$  IEL to epithelial cells in contact with pathogen is important for immediate host defense from *Salmonella* infection (62). Epithelial deficiency of HVEM led to increased susceptibility to *S. typhimurium* infection, which may be related to reduced IETs migration as well as decreased cell number. Although for survival in the proximal small intestine LIGHT was the key HVEM ligand, following infection CD160 deficiency had an equal or even greater influence than LIGHT deficiency, consistent with the importance of CD160 during other mucosal infections(38, 57, 58). The role of CD160 during infection might be due in part to the signaling capacity of CD160, which might trigger the protective response of the IETs, as it has been reported to do for NK cells (74).

According to previous results (62), TCR $\gamma\delta^+$  IETs could have been responding to *Salmonella*, but the TCR $\alpha\beta^+$  IETs might have contributed as well, consistent with the increased bacterial colonies in *Itgb1<sup>prLck</sup>* mice, in which TCR $\alpha\beta^+$  cells were affected. We don't know which population(s) were relevant, but CD4<sup>+</sup> T cells are important for host defense against this microbe (75).

HVEM is a multi-functional protein that influences inflammation and the response to acute infections in the intestinal mucosae (34, 37, 38, 41). Here, our results showed that the interaction of LIGHT with epithelial HVEM influences the survival of natural IET subsets at steady-state, in part by promoting synthesis of  $\beta_1$  integrins ligands, whereas HVEM interaction with CD160 might exert an influence in enhancing the protective capacity of the responding IETs. Therefore, our data indicate that overall HVEM expression by IEC has a role in regulating mucosal immunity at steady state and following oral infection.

## MATERIALS AND METHODS

### Study design

HVEM had previously been shown to affect mucosal immune responses, and the goal of this study was to determine how it affected intraepithelial T cells (IETs) in the small intestine at steady state and after infection. We determined that although HVEM is expressed by many cell types, epithelial HVEM was critical for IETs. Using mice with intestine epithelial cell-specific deletion of *Hvem*, flow cytometry and in vitro cultures, we showed that epithelial HVEM influenced IETs survival, migration in the epithelium, and the host response to *Salmonella typhimurium* infection. RNA-seq of HVEM deficient and wildtype epithelial cells implicated the synthesis of basement membrane proteins as a key function reduced when HVEM was missing, and we confirmed this in organoid cultures. We showed with in vitro cultures that collagen IV, a basement membrane component dependent on HVEM expression, affected IETs survival.  $\beta_1$  integrins expressed by IETs bind collagen IV and these integrins mediated a survival benefit in vitro by binding collagen IV and in vivo. Mice deficient for epithelial HVEM, HVEM ligands or  $\beta_1$  integrins, were more susceptible to *S. typhimurium*, indicating the mechanisms governing steady state survival and the response to acute infection were similar. Experiments were done at least two times with at least

four mice in each experimental group. Observational studies, such as intravital microscopy analysis, immunofluorescence analysis, or bacterial infection, were carried by an individual blinded as to the experimental conditions or a third party not involved in the study.

## Mice

*Hvem*<sup>-/-</sup>, which is *Hvem*<sup>flox-neo/flox-neo</sup> (*Hvem*<sup>fn/fn</sup>) mice, *Hvem*<sup>fl/fl</sup> mice, *Btla*<sup>-/-</sup>, which is *Btla*<sup>flox-neo/flox-neo</sup> (*Btla*<sup>fn/fn</sup>) mice, *Btla*<sup>fl/fl</sup>, which is a control for *Btla*<sup>-/-</sup> and LIGHT gene deficient (*Tnfsf14*<sup>-/-</sup>, listed as *LIGHT*<sup>-/-</sup> in the figures) mice were bred and described previously (37). These mice were generated by us except *Tnfsf14*<sup>-/-</sup> mice were a gift from Dr. Klaus Pfeffer (Heinrich Heine University, Dusseldorf, Germany). *Cd160*<sup>-/-</sup> mice were provided by Dr. Yang-Xin Fu (UT Southwestern, TX) (74). *Cd4-Cre* (STOCK Tg(Cd4-cre)1Cwi/BfluJ; Stock No: 017336), *Villin-Cre* (B6.Cg-Tg(Vil1-cre)997Gum/J, Stock No: 004586), *Lck*<sup>pr</sup>-Cre (B6.Cg-Tg(Lck-cre)548Jxm/J, Stock No:003802), *Itgb1*<sup>fl/fl</sup> (B6;129-*Itgb1*<sup>tm1Efu</sup>/J, Stock No: 004605) and *Lck*<sup>pr</sup>-*Bcl-xL*<sup>Tg</sup> mice (B6.Cg-Tg(LCKprBCL2L1)12Sjk/J; Stock No: 013738) were all purchased from The Jackson Laboratory. *Lck*<sup>pr</sup>-*Bcl-xL*<sup>Tg</sup> mice express the pro-survival Bcl2 family gene *Bclxl* (*Bcl2l1*) under the control of the mouse *Lck* proximal promoter (*Lck*<sup>pr</sup> Cre). *Hvem*<sup>fl/fl</sup> mice were bred to *Villin-cre* mice to generate conditional knockout mice, *Hvem*<sup>IEC</sup>. *Integrin b1*<sup>fl/fl</sup> mice were bred to *Lck*<sup>pr</sup> Cre mice to generate conditional knockout mice, *Itgb1*<sup>pLck</sup> mice. All Cre mouse strains were maintained on the C57BL/6 background or were backcrossed to C57BL/6 for at least for 6 generations. 15 to 17-week-old male mice on the C57BL/6 genetic background were used in this study. In most experiments, groups of co-housed control and gene knockout mice were analyzed to minimize the effect of housing conditions on experimental variation. In a few experiments, as indicated in the figure legends, co-housed mice that were not littermates were analyzed. For tissue or cell analyses, tissues were collected and used for immunofluorescence analysis and intraepithelial lymphocyte preparation. Mice were bred and housed under specific pathogen-free conditions in the vivarium of the La Jolla Institute for Immunology (LJI). All procedures were approved by the LJI Animal Care and Use Committee.

## Germ-free mouse husbandry

Germ-free mice were bred and housed in the animal facility of POSTECH Biotech Center. Germ-free C57BL/6 (B6) mice were kindly provided originally by Drs. Andrew Macpherson (Bern Univ., Switzerland) and David Artis (Univ. Pennsylvania, USA) and maintained in sterile flexible film isolators (Class Biological Clean Ltd., USA). The sterility of germ-free mice was checked regularly by the absence of bacterial colonies in the culture experiment using their fecal pellets. Germ free C57BL/6 mice maintained in the animal facility of POSTECH Biotech Center in accordance with institutional ethical guideline and the protocols approved by the Institutional Animal Care and Use Committees (IACUC) of the POSTECH.

## Isolation of IEL

Intestines were collected from mice, and were divided into five parts, including proximal SI, middle SI, and distal SI, approximately one-third each, as well as cecum and colon. Peyer's patches were carefully removed, and tissues were cut open longitudinally, briefly

washed, and cut into 1.5 cm pieces. The tissue pieces were incubated in 20 mL of RPMI (5% FBS, 25 mM HEPES and 1 mM DTT) in a shaker at 200 rpm, 37°C, for 20 min, followed by incubated in 20 mL HBSS (25 mM HEPES and 20 mM EDTA) in a shaker at 200 rpm, 37°C, for 30 min. After each incubation, the cell suspension was filtered through a metal mesh and the supernatant was saved for IEL preparation. The flow-through cell suspension was spun down. The cell pellets were then re-suspended in 40% Percoll solution and overlaid above 80% Percoll solution carefully, followed by centrifugation at 2000 rpm, 25°C for 20 min without the brake. IEL were collected from the interface, washed once and re-suspended in the complete RPMI-1640 medium. These purified cells constituted the epithelial cell fraction enriched for IEL. The cells were used immediately for cell counting and staining.

### Flow cytometry

Flow cytometry analysis was performed on an LSRII instrument (BD Biosciences). The data were analyzed by using FlowJo software (Tree Star). Absolute cell counts were obtained by using CountBright™ Absolute Counting Beads (Life Technologies). The following mAbs were used: TCR $\delta$  (eBioscience, eBioGL3), TCR $\beta$  (BioLegend, H57–597), CD4 (eBioscience, RM4–5), CD8 $\alpha$  (BD Biosciences, 53–6.7), CD8 $\beta$  (eBioscience, eBioH35–17.2), CD45 (BioLegend, 30-F11), CD45.1 (BioLegend, A20), CD45.2 (Thermo Fisher Scientific, 104), HVEM (BioLegend, HMHV-1B18), integrin  $\beta$ 7 (BD Biosciences, M293), integrin  $\alpha$ E (Thermo Fisher Scientific, 2E7), Bax (Thermo Fisher Scientific, 6A7), Bim (Bio-Rad AbD Serotec, AHP933), CD49a (BD Biosciences, Ha31/8), CD29 (BioLegend, HM $\beta$ 1–1), Ki-67 (Thermo Fisher Scientific, SP6), EdU (Thermo Fisher Scientific), Annexin V (BD Biosciences), SYTOX™ AADvanced™ Dead Cell Stain Kit (Thermo Fisher Scientific). All representative FACS gating not included in the main or supplemental figures is present in supplemental data file S2. Staining methods are further shown in data file S2.

### Cryosection immunofluorescence

SI tissue was collected, opened longitudinally and the luminal contents washed in 20 mL RPMI in a 50 mL tube by inverting 20 times. The tissues were placed on foil with O.C.T. for the Swiss-roll technique. Once the entire intestine length was rolled up, the intestine Swiss roll was transferred into a tissue mold and was frozen on a thermo-conductive platform (ThermalTray, Biocision) pre-cooled with dry ice, and then placed into –80°C freezer. Frozen sections were cut to a 10  $\mu$ m thickness. After drying at RT for 1 hr, frozen sections were fixed in pre-cooled ethanol at –20 °C for 10 min followed by pre-cooled acetone at –20 °C for 10 min. The fixed sections were used for immunofluorescence (IF) staining or kept in –80°C freezer for later use. Primary antibodies were diluted in PBST (PBS, 0.5% BSA, 0.1% Tween-20). Antibodies used were: CD45 (BioLegend, clone 30-F11), EpCAM (BioLegend, clone G8.8), TCR $\delta$  (BioLegend, clone GL3), TCR $\beta$  (BioLegend, clone H57–597), CD8 $\alpha$  (BD, clone 53–6.7), CD8 $\beta$  (BD, clone H35–17.2). In Figs. 1B–1C, fluorescence imaging was performed on an FV10i confocal microscope (Olympus). Areas from Pro-SI, Mid-SI or Dis-DI were outlined and CD45<sup>+</sup> IEL per villus were counted manually. In Figs. 2D–2F, fluorescence imaging was performed on Zeiss LSM 780 or LSM 880 confocal microscopes (Zeiss). Data analysis was performed using ZEN software (Zeiss); areas of each villus from Pro-SI was outlined and measured and IET subsets of each villus were counted



manually. The number of IET subsets was normalized to the area ( $\text{mm}^2$ ) in each villus. Fiji software (National Institutes of Health) were used for data for representative images.

### Intravital microscopy

To track  $\text{CD8}\alpha^+$  IEL in the epithelium, mice were injected with 15–20  $\mu\text{g}$  of anti- $\text{CD8}\alpha$ -AF488 (eBioscience, clone 53–6.7) or anti- $\text{CD8}\alpha$ -AF647 (BD Biosciences, clone 53–6.7) with/without EpCAM-AF647 (BioLegend, clone G8.8) by retro-orbital injection 4 hr before imaging. After anesthetization, mice were positioned on a WPI ATC2000 heating pad ventral side up and kept at  $37^\circ\text{C}$ . The distal duodenum was exposed and opened along the antimesenteric border. The mucosal surface was hydrated with PBS and placed against a coverslip fitted onto a suction ring (76). All intravital imaging was done using  $25\times 0.95\text{NA}$  water immersion objective on the Leica SP5 upright confocal microscope with a resonant scanner and acquired using Leica Application Suite software. The sample was excited with 488 nm and 633 nm lasers using 488/543/633 triple dichroic mirror. The internal detectors were set to collect AF488 fluorescence at 494–563 nm, reflection image at 619–651 nm, and AF647 fluorescence at 651–722 nm. Images were acquired by taking Z-stacks encompassing the epithelium and the upper layers of the lamina propria at 2.8  $\mu\text{m}$  step size every 10 sec. Each XY plane spans  $365 \times 365 \mu\text{m}^2$ . To calculate cell coverage, the data was median filtered in Fiji (77). Then, the sections from the top of the villi encompassing nearly exclusively epithelium were Z projected. A maximum intensity time projection was performed and coverage was calculated as the area with intensity values over a threshold, demonstrating it was visited by a cell. Additionally, a rainbow time projection was generated to visualize cell movement. Individual cells in Z stacks were tracked in Imaris (Bitplane) semi-automatically using the spots function. Cell movement metrics (mean speed, track length, track displacement length) for each  $\text{CD8}\alpha^+$  lymphocyte cell were calculated with Imaris and plotted using Prism (version 8, GraphPad). Spider graphs of individual cell paths, each beginning at the origin, were plotted using Matlab (Mathworks, 2021A) using the positions output from Imaris.

### Small intestinal organoid culture

SI organoids were derived from *Hvem*<sup>-/-</sup> or littermate *Hvem*<sup>+/+</sup> mice. The proximal SI was cut into 5-mm segments and incubated in  $4^\circ\text{C}$  2 mM EDTA in PBS for 5 min and washed by pipetting. The segments were incubated in 2 mM EDTA in PBS for 30 min at  $4^\circ\text{C}$ , and crypts were isolated by pipetting with cold HBSS. Dissociated crypts were passed through a 70- $\mu\text{m}$  cell strainer and pelleted by centrifuge at 600 rpm for 3 min at  $4^\circ\text{C}$ . The crypts were resuspended in Advanced DMEM/F12 medium (Thermo Fisher Scientific); the number of crypts was counted, and they were resuspended in Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning). The crypts were plated in a 24-well plate with organoid growth medium supplemented with 100  $\mu\text{g}/\text{ml}$  penicillin, 100 U/ml streptomycin, 2 mM Glutamax, 1 $\times$ N-2 supplement, 1 $\times$ B27 supplement, 10 mM HEPES (Thermo Fisher Scientific), 1 mM N-acetylcysteine (Sigma-Aldrich), 100 ng/ml recombinant mouse Noggin (Peprotech), 50 ng/ml recombinant mouse EGF (BioLegend), 500 ng/ml recombinant human R-spondin 1 (Peprotech). Media were changed every 2 days.

### Quantitative Real-time PCR

Total RNA extraction from IECs was performed with RNeasy Kit (Qiagen), according to the manufacturer's instructions. cDNA synthesis was performed by using iScript Advanced cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR reactions were performed with SYBR Green I Master Kit and LightCycler 480 system (Roche). mRNA levels of *Col4a1* were normalized to the housekeeping gene *Actb* or *Rpl32*. The primers were synthesized by the Integrated DNA Technologies. Primers used were:

Col4a1-forward 5'- TCCGGGAGAGATTGGTTTCC-3'

Col4a1-reverse 5'- CTGGCCTATAAGCCCTGGT-3'

Actb- reverse 5'- GATCTGGCACCACACCTTCT-3'

Actb - reverse 5'- GGGGTGTTGAAGGTCTCAAA-3'

Rpl32- reverse 5'- TTCCTGGTCCACAATGTCAA-3'

Rpl32 - reverse 5'- GGCTTTTCGGTTCTTAGAGGA-3'

### RNA-seq library preparation

RNA-seq libraries were prepared from sorted proximal SI IEC or from sorted SI TCR $\alpha$  $\beta$ <sup>+</sup>CD8 $\alpha$  $\alpha$ <sup>+</sup> IET by the Sequencing Core at La Jolla Institute for Immunology using the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (TaKaRa)(78). Libraries were sequenced on an Illumina HiSeq 2500, generating 50 bp single-end reads.

### RNA-seq analysis

The single-end reads that passed Illumina filters were filtered for reads aligning to tRNA, rRNA, adapter sequences, and spike-in controls. The reads were then aligned to mm10 reference genome using TopHat (v 1.4.1) (79). DUST scores were calculated with PRINSEQ Lite (v 0.20.3) (80) and low-complexity reads (DUST > 4) were removed from the BAM files. The alignment results were parsed via the SAMtools (81) to generate SAM files. Read counts to each genomic feature were obtained with the htseq-count program (v 0.7.1) (82) using the "union" option. After removing absent features (zero counts in all samples), the raw counts were then imported to R/Bioconductor package DESeq2 (v 1.6.3) (83) to identify differentially expressed genes among samples. P-values for differential expression were calculated using the Wald test for differences between the base means of two conditions. These P-values were then adjusted for multiple test correction using the Benjamini-Hochberg algorithm (84). Principal Component Analysis (PCA) was performed using the 'pcomp' function in R. The sequences used in this article have been submitted to the Gene Expression Omnibus under accession number GSE 206183. Gene set enrichment analysis was done using the "GseaPreranked" method with "classic" scoring scheme. All the GO gene sets were downloaded from MSigDB in GMT format. Rank files for each DE comparison of interest were generated by assigning a rank of  $-\log_{10}(\text{pValue})$  to genes with  $\log_2\text{FoldChange}$  greater than zero and a rank of  $\log_{10}(\text{pValue})$  to genes with  $\log_2\text{FoldChange}$  less than zero. GO analysis of DEGs was performed with ToppGene (<http://toppgene.cchmc.org>) (85). FDR (Benjamini-Hochberg) < 0.05 was considered statistically

significant. Heatmap was performed with Morpheus (<https://software.broadinstitute.org/morpheus/>). For classical pathway, Ingenuity Pathway Analysis (Qiagen) was used.

### Western-blot analysis

Harvested IECs were lysed in triton lysis buffer (137 mM NaCl, 20 mM Tris base at pH 7.4, 10% glycerol and 1% Triton X-100) supplemented with a protease and phosphatase inhibitor mixture (Roche) for 20min on ice, and centrifuged at 15,000 rpm for 10 min at 4°C. 10 µg of denatured proteins were loaded onto a Mini-PROTEAN Precast Gel (Bio-Rad) and transferred onto a PVDF membrane (Thermo Fisher Scientific). The membranes were blocked by 5% skim milk in TBST (Tris-buffered saline /0.1% Tween 20, Bio-Rad) and then incubated with antibodies to collagen a1 (IV) (Origene) and Villin (Abcam). Immunoreactive bands were detected by chemiluminescence (ECL solution, Santa Cruz).

### CD8α<sup>+</sup> IEL culture

For the isolation of IEL, CD8α<sup>+</sup> cells were enriched from the SI IEL preparation by negative and positive selection with the iMag cell separation system (BD Biosciences) according to the manufacturer's instructions. Briefly, single-cell IEL suspension was incubated with staining buffer (PBS containing 2% FBS with 2 mM EDTA) containing a mixture of biotin-conjugated mAbs against CD4 (RM4-5; BioLegend), CD19 (6D5; BioLegend), CD326 (G8.8; BioLegend), and TER-119 (TER-119; BioLegend) in a 5-ml round tube (BD Biosciences) for 30 min at 4°C. Cells were washed with staining buffer and incubated with Streptavidin Particle Plus-DM (BD Biosciences) for 30 min at 4°C. The tube was placed on the Cell Separation Magnet at room temperature for 8 min. The supernatant was carefully aspirated off as a negative fraction. The negative fraction was incubated with biotin-conjugated anti-CD8α (53-6.7; BioLegend) in staining buffer for 30 min at 4°C, washed with staining buffer, and incubated with Streptavidin Particle Plus-DM in a 5-ml round tube for 30 min at 4°C. The tube was placed on the Cell Separation Magnet at room temperature for 8 min. The supernatant was carefully removed and CD8α<sup>+</sup> positive fraction was washed and resuspended with complete medium. CD8α<sup>+</sup> IEL were expanded in complete medium containing 50 ng/ml recombinant mouse IL-15/IL-15R complex (Thermo Fisher Scientific) on a tissue culture plate at the concentration of 5×10<sup>5</sup> cells/ml for 3 days. CD8α<sup>+</sup> IEL were then cultured on a 96-well protein high binding plate (Corning) coated with 100 µg/ml bovine serum albumin (BSA, Thermo Fisher Scientific), type IV collagen (Corning), Laminin (Corning) or Fibronectin (Abcam). For monitoring cell death, isolated SI CD8α<sup>+</sup> IET (EpCAM<sup>-</sup>CD4<sup>-</sup>CD19<sup>-</sup>TER119<sup>-</sup>CD8α<sup>+</sup>) from wild type mice were labeled with CellTrace™ Violet (CTV, Thermo Fisher Scientific), and then labeled cells were incubated in the presence of recombinant IL-15/15Rα (50ng/mL) in the presence of absence of anti-integrin α1 (10µg/mL, BD Biosciences) or Integrin α1β1 inhibitor Obtusatin (10nM, R&D Systems) in the coated culture plate. After 3 days of culture, cells were stained with antibodies and 7-aminoactinomycin D (7AAD, BD Biosciences) for viability assessment of the different CD8α<sup>+</sup> IET subsets when analyzed by flow cytometry. % cell death in proliferating cells is calculated as: % cell death in proliferating cells = (CTV<sup>-</sup>7AAD<sup>+</sup>) / (CTV<sup>-</sup>7AAD<sup>-</sup>) + (CTV<sup>-</sup>7AAD<sup>+</sup>) × 100.

## Bacterial infection

*Salmonella enterica serovar Typhimurium* strain IR715 (*S. typhimurium*), which is a naturally nalidixic acid resistant derivative of ATCC 14028, was obtained from Dr. M. Raffatellu (UCSD) (86) and used in the infection studies. Bacteria were aerobically grown overnight at 37°C in Luria-Bertani broth, then diluted 1:20 and subcultured for 4 hr in fresh medium. Bacteria were washed twice in ice-cold phosphate-buffered saline (PBS) and then suspended in cold PBS ( $1 \times 10^7$  colony forming units [CFU]/100  $\mu$ l). Mice were pretreated with 20 mg streptomycin 24 h prior to infection with  $1 \times 10^7$  CFU *S. typhimurium* by oral gavage in a total volume of 100 $\mu$ l PBS (87). Recipients were analyzed for weight loss until day 3 or 4 p.i. At the terminal endpoint, tissues were collected for CFU assays and histopathology analysis. For CFU assays, several tissues including spleen, liver, small intestine and colon were weighed, homogenized in sterile PBS, serially diluted and plated on LB agar plates supplemented with 200 $\mu$ g/mL nalidixic acid. Histopathology analysis of liver samples was performed on zinc formalin (Medical Chemical Corporation)-fixed tissue after hematoxylin and eosin (H&E) stain.

## Statistical analysis

Details concerning the statistical analysis methods are provided in each figure legend. Briefly, all data were analyzed using GraphPad Prism 8 software and were shown as mean and the standard error of the mean (SEM). Statistical significance was determined by unpaired t-test for cell numbers, % of cell proliferation, cell survival and cell death and Mann-Whitney test for intravital microscopy and infection experiments, Log-rank test for survival curves, 2 way ANOVA with Bonferroni's multiple comparison test for cell death. Statistical significance is indicated by \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; ns, not significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data and materials availability:

All data needed to evaluate the conclusions in the paper are present in the main text or the Supplementary Materials. All reasonable requests for materials including, mouse strains, will be available upon request to Mitchell Kronenberg (mitch@lji.org). Mouse strain # 030862 with a floxed allele of the gene encoding HVEM (B6;SJL-*Tnfrsf14<sup>tm1.1Kro</sup>/J*) has

been deposited at the Jackson lab. RNA-seq data are available from GEO under accession code GSE206183.

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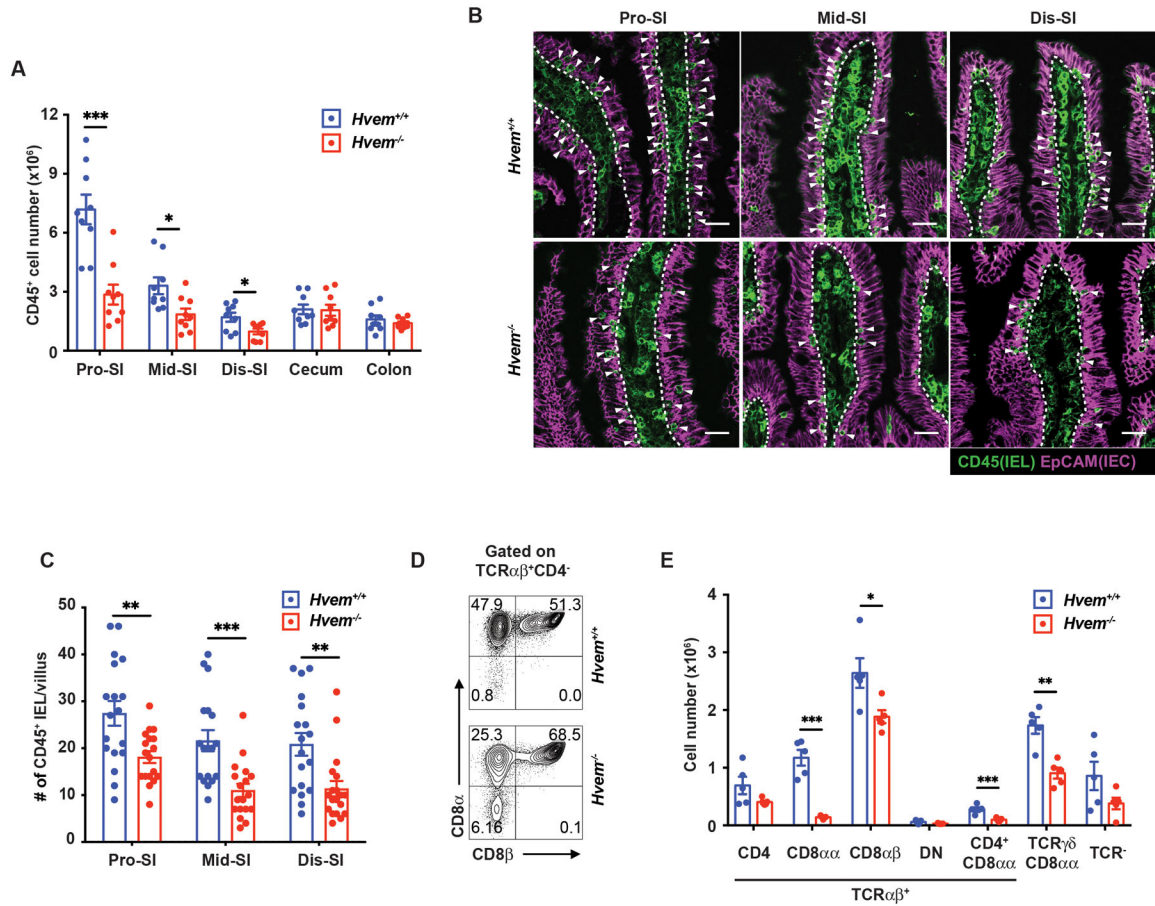


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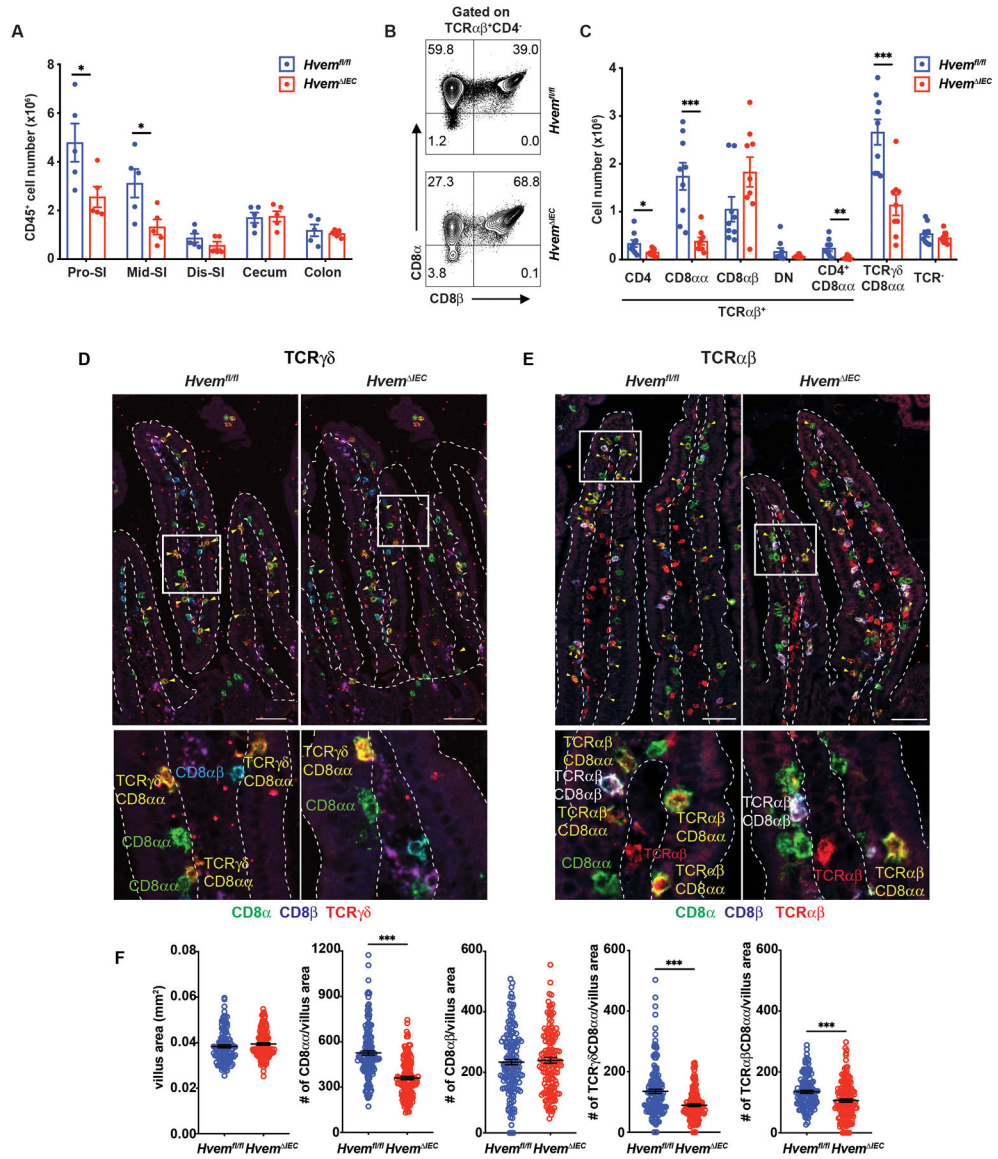
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**Fig. 1. HVEM is important for maintaining IET.**

(A) Total IEL numbers by flow cytometry in proximal SI (Pro-SI), middle SI (Mid-SI), distal SI (Dis-SI), cecum and colon from *Hvem*<sup>+/+</sup> (n=9) and *Hvem*<sup>-/-</sup> (n=9) mice. (B) Representative immunofluorescence staining of CD45<sup>+</sup> cells from the SI in *Hvem*<sup>+/+</sup> and *Hvem*<sup>-/-</sup> mice. White arrowheads indicate CD45<sup>+</sup> intraepithelial cells (IEL) in the epithelium. Dashed white lines indicate the interface between the epithelium and lamina propria. Scale bars, 25μm. (C) Quantification of CD45<sup>+</sup> IEL in villi from *Hvem*<sup>+/+</sup> (n=4) and *Hvem*<sup>-/-</sup> (n=4) mice. (D) Representative plots of TCRαβ<sup>+</sup>CD8αα<sup>+</sup> and TCRαβ<sup>+</sup>CD8αβ<sup>+</sup> IET in TCRαβ<sup>+</sup> IET from proximal SI in *Hvem*<sup>+/+</sup> and *Hvem*<sup>-/-</sup> mice. (E) Absolute numbers of the indicated subsets in total IEL from proximal SI in *Hvem*<sup>+/+</sup> (n=5) and *Hvem*<sup>-/-</sup> (n=5) mice. Statistical analysis was performed using an unpaired t-test (A, C, E). Statistical significance is indicated by \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001. Data in A, C, and E show means ± SEM. In A and E, each symbol represents a measurement from a single mouse. In C, each symbol represents cell numbers of CD45<sup>+</sup> IEL per a boundary approximating a villus. Data represent pooled results from at least two independent experiments with at least four mice per group in each experiment (A), compiled from four independent experiments (B, C) or representative results from one of at least two independent experiments with at least four mice in each experimental group (E). Groups of co-housed mice were analyzed.



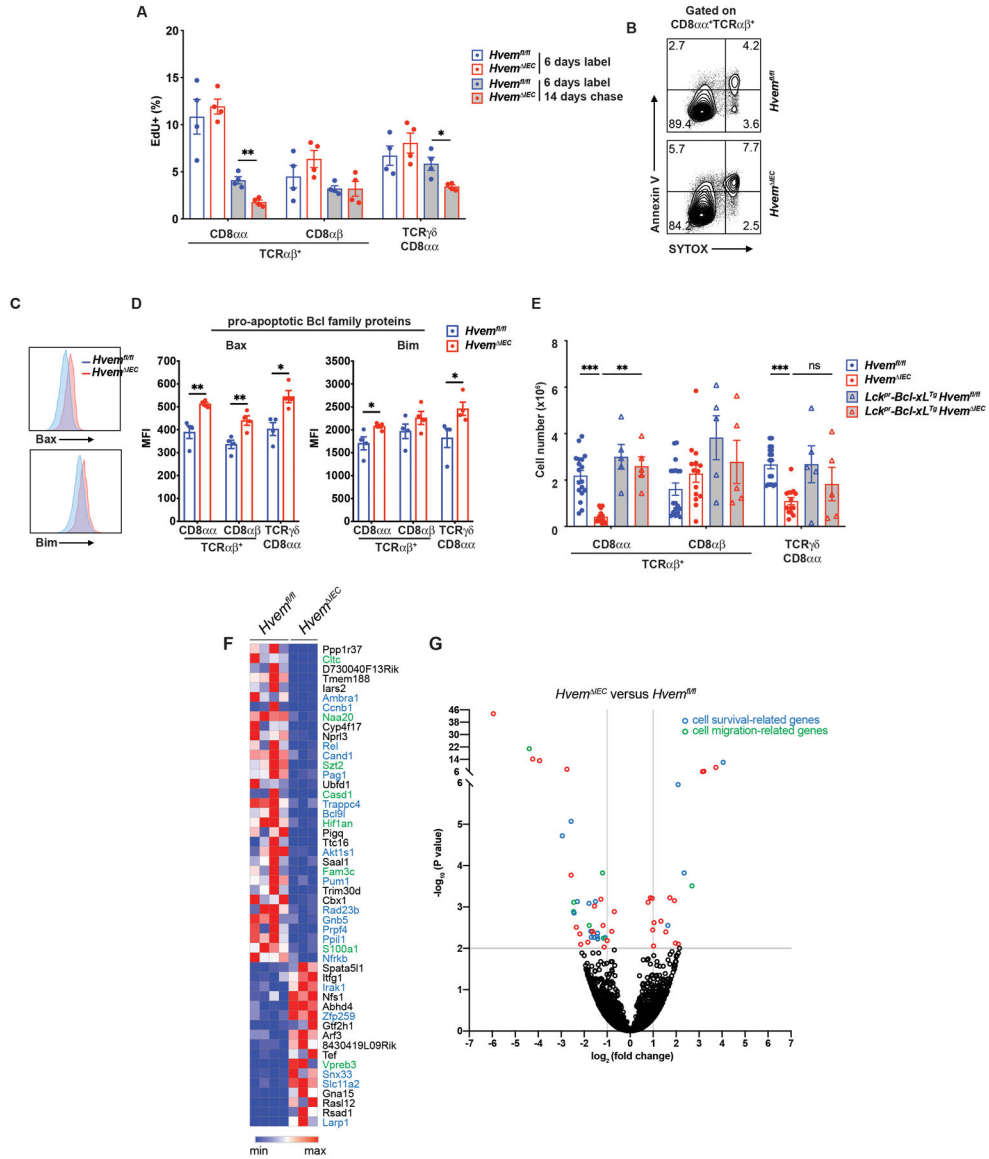


**Fig. 2. Epithelial HVEM maintains IETs.**

(A) Total IEL numbers in portions of the intestine from *Hvem<sup>fl/fl</sup>* (n=5) and *Hvem<sup>IEC</sup>* (n=5) mice. (B) Representative plots of TCRαβ<sup>+</sup>CD8αα<sup>+</sup> and TCRαβ<sup>+</sup>CD8αβ<sup>+</sup> in TCRαβ<sup>+</sup> IET from proximal SI in *Hvem<sup>fl/fl</sup>* and *Hvem<sup>IEC</sup>* mice. (C) Absolute numbers of indicated IET subsets in total IEL from proximal SI in *Hvem<sup>fl/fl</sup>* (n=9) and *Hvem<sup>IEC</sup>* (n=9) mice. (D-E) Representative immunofluorescence staining of TCRγδ IET (D) and TCRαβ IET (E) from proximal SI in *Hvem<sup>fl/fl</sup>* and *Hvem<sup>IEC</sup>* mice. Composite images in which the three channels were merged. Composite images depict expression of CD8α (green), CD8β (blue), and TCRδ (red, D) or TCRβ (red, E). Yellow arrowheads highlight TCRγδ<sup>+</sup>CD8αα<sup>+</sup> (D) and TCRαβ<sup>+</sup>CD8αβ<sup>+</sup> IET (E). Single-channel images are in Figure S3. Dashed white lines indicate the boundaries of the epithelium, approximately one villus in each case, used for the quantitation. Scale bars, 50μm. Two independent experiments were carried out yielding similar results. (F) Quantification of TCRγδ<sup>+</sup>CD8αα<sup>+</sup> and TCRαβ<sup>+</sup>CD8αβ<sup>+</sup> per



mm<sup>2</sup> from proximal SI in *Hvem<sup>fl/fl</sup>* (n=6) and *Hvem<sup>IEC</sup>* (n=7) mice. Statistical analysis was performed using unpaired t-test (A, C, F). \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001. Data in A, C, and F show means ± SEM. In A and C, each symbol represents a measurement from a single mouse. In F, each symbol represents a calculation to give the number per mm<sup>2</sup>. Data are representative results from one of at least two independent experiments with at least four mice in each experimental group (A) or pooled results from at least two independent experiments with at least four mice per group in each experiment (C, F). Groups of co-housed littermates were analyzed.



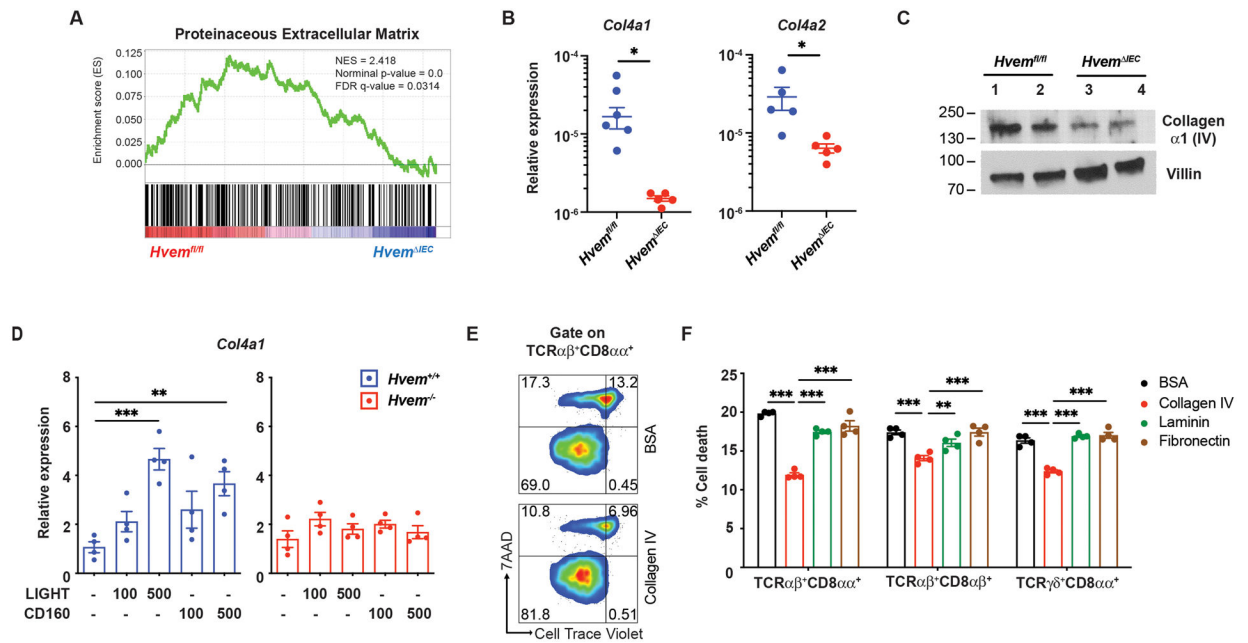
**Fig. 3. Epithelial HVEM affects CD8αα<sup>+</sup> IET survival.**

(A) Frequencies of EdU<sup>+</sup> cells from proximal SI IET of *Hvem<sup>fl/fl</sup>* (n=4) and *Hvem<sup>IEC</sup>* (n=4) mice. Mice were administered EdU once per day for 6 days. Cells were isolated from proximal SI and EdU<sup>+</sup> cells were measured at either day 6 or day 20. (B) Representative flow cytometry images detecting apoptotic cells in TCRαβ<sup>+</sup>CD8αα<sup>+</sup> IET from proximal SI in *Hvem<sup>fl/fl</sup>* and *Hvem<sup>IEC</sup>* mice.

(C, D) Representative flow cytometry showing expression of pro-apoptotic Bcl family proteins (Bax and Bim) on TCRαβ<sup>+</sup>CD8αα<sup>+</sup> IET from proximal SI of *Hvem<sup>fl/fl</sup>* (n=4) and *Hvem<sup>IEC</sup>* (n=4) mice (C) and compiled MFI from multiple individual mice (D).

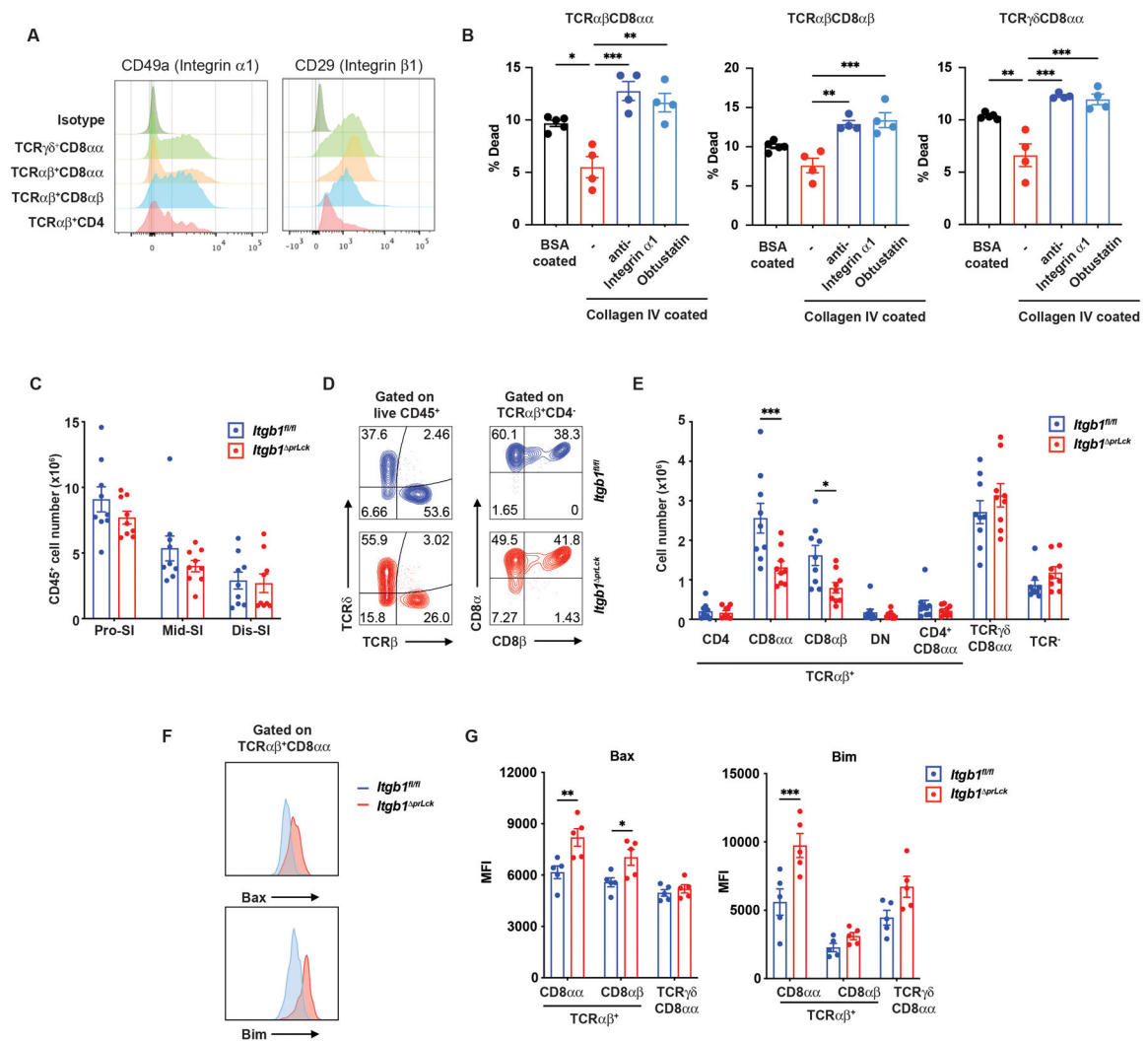
(E) Absolute cell number of TCRαβ<sup>+</sup>CD8αα<sup>+</sup>, TCRαβ<sup>+</sup>CD8αβ<sup>+</sup>, and TCRγδ<sup>+</sup>CD8αα<sup>+</sup> IET subsets from *Hvem<sup>fl/fl</sup>* (n=18), *Hvem<sup>IEC</sup>* (n=14), *Lck<sup>pr</sup>-Bcl-xL<sup>Tg</sup>Hvem<sup>fl/fl</sup>* (n=5) and *Lck<sup>pr</sup>-Bcl-xL<sup>Tg</sup>Hvem<sup>IEC</sup>* (n=5) mice. Statistical analysis was performed using an unpaired t-test (A, D) or 2 way ANOVA with Bonferroni's multiple comparison test (E). Statistical

significance is indicated by \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Data in A and C show means  $\pm$  SEM. In A, D and E, each symbol represents a measurement from a single mouse. Data are representative results of one of at least two independent experiments with at least four mice in each experimental group. **(F)** Sorted TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$  IET from SI of *Hvem<sup>fl/fl</sup>* and *Hvem<sup>IEC</sup>* mice were analyzed by RNA-seq. The top 50 most differentially expressed genes with respect to *P*-value. **(G)** Volcano plots showing mean  $\log_2$ -transformed fold change (x axis) and significance ( $-\log_{10}$  (adjusted P value)) of differentially expressed genes between the TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$  IET from the SI of *Hvem<sup>fl/fl</sup>* and *Hvem<sup>IEC</sup>* mice. In F and G, genes in blue font or blue symbol are associated with cell survival or proliferation and those designated with green are associated with cell migration. Groups of co-housed littermates (A-D) or cohoused mice (E) were analyzed.



**Fig. 4. HVEM signaling contributes to induction of basement membrane synthesis.**

(A) Gene set enrichment analysis (GSEA) of transcripts from isolated IEC (CD31<sup>-</sup>CD45<sup>-</sup>EpCAM<sup>+</sup>) showing downregulation of GO Proteinaceous Extracellular Matrix genes in *Hvem<sup>IEC</sup>* mice. (B) Gene expression of *Col4a1* and *Col4a2* mRNA in IEC from *Hvem<sup>fl/fl</sup>* (n=5) and *Hvem<sup>IEC</sup>* mice (n=5) by q-PCR. Data are normalized to the *Actb* housekeeping gene. (C) Expression of collagen  $\alpha 1$  (IV) in sorted IEC from *Hvem<sup>fl/fl</sup>* and *Hvem<sup>IEC</sup>* mice by Western blot. The expression level of villin is used as a control for the amount of protein lysates loaded. (D) Gene expression of *Col4a1* mRNA determined by q-PCR in cells from intestinal organoid cultures from proximal SI of *Hvem<sup>fl/fl</sup>* (n=5) and *Hvem<sup>IEC</sup>* mice (n=5). Isolated crypts from *Hvem<sup>+/+</sup>* and *Hvem<sup>-/-</sup>* mice were cultured with growth factors in the presence or absence of HVEM ligands, indicated concentrations in ng/ml, for 7 days. Data are normalized to *Rpl32* as the housekeeping gene. (E-F) Isolated SI IET subsets (CD4<sup>-</sup> from wild type mice were labeled with CellTrace<sup>TM</sup> Violet (CTV) and cultured on plates coated with the indicated mouse basement proteins or BSA control (n=4 per group). After 3 days, cells were stained with antibodies and 7AAD for monitoring cell death by flow cytometry. Representative plots (E) and calculated of % cell death (F) in IET subsets. Statistical analysis was performed using an unpaired t-test (B) or 2 way ANOVA with Bonferroni's multiple comparison test (D, F). Statistical significance is indicated by \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001. Data in B, D, and F show means  $\pm$  SEM. Each symbol represents a measurement from a single mouse (B), single organoid culture (D) or an individual well (E, F). Data are representative results from one of at least two independent experiments. Groups of co-housed littermates were analyzed.

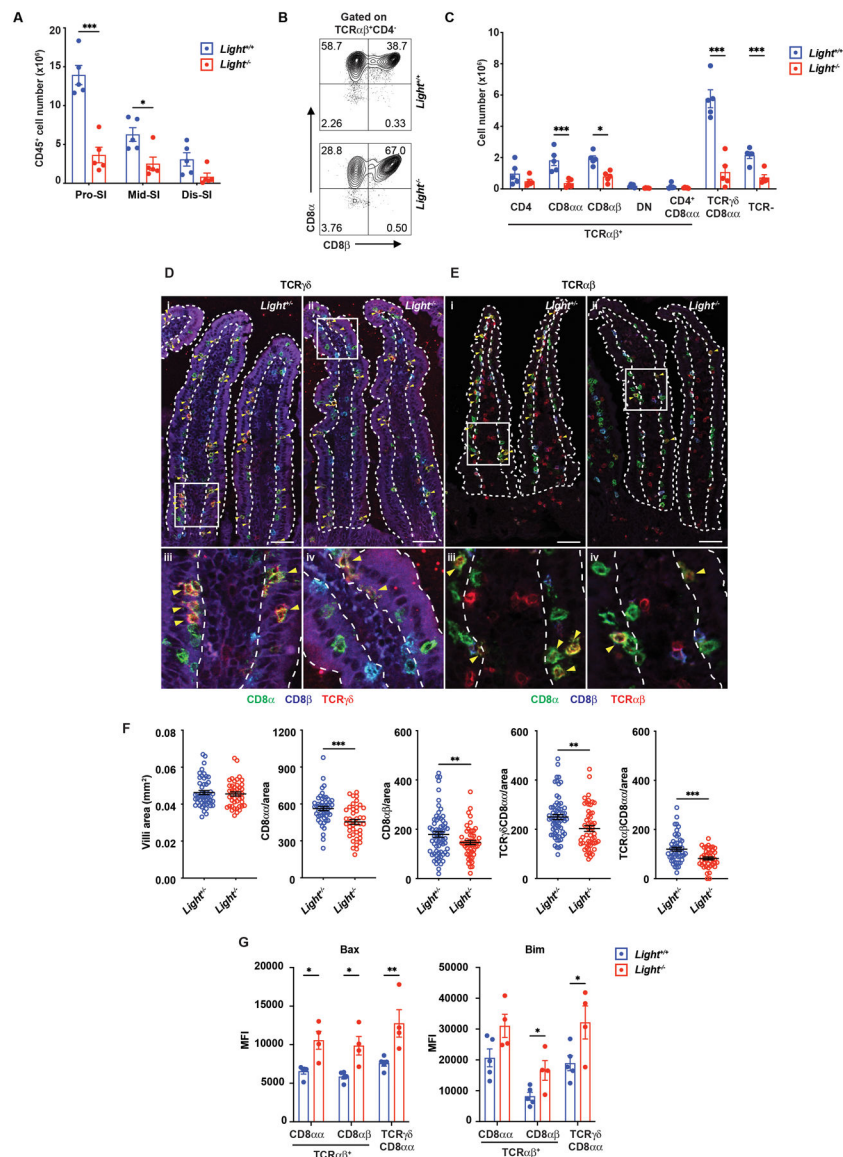


**Fig. 5. Collagen-binding integrins influence IET survival and host defense.**

(A) Expression of integrin  $\alpha 1$  and  $\beta 1$  subunits by IET populations from proximal SI of wild type mice by flow cytometry. (B) Isolated IET subsets from SI of wild type mice labeled with CellTrace™ Violet (CTV) and cultured on plates coated with BSA control (n=5) or collagen IV in the presence or absence of anti-integrin  $\alpha 1$  mAb or inhibitor Obtusatin (n=4 per group). After 3 days, cells were monitored for viability by flow cytometry as described in Materials and Methods. (C) Total IEL numbers in segments of SI from *Itgb1<sup>fl/fl</sup>* (n=9) and *Itgb1<sup>prLck</sup>* (n=9) mice. (D) Representative plots of TCR $\alpha\beta^+$  and TCR $\gamma\delta^+$  among CD45<sup>+</sup> IEL (left) and TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$  and TCR $\alpha\beta^+$ CD8 $\alpha\beta^+$  among TCR $\alpha\beta$  IET (right) from proximal SI in *Itgb1<sup>fl/fl</sup>* and *Itgb1<sup>prLck</sup>* mice. (E) Absolute numbers of indicated subsets in total IEL from proximal SI in *Itgb1<sup>fl/fl</sup>* (n=9) and *Itgb1<sup>prLck</sup>* (n=9) mice. (F, G) Representative flow cytometry shows expression of pro-apoptotic Bcl family proteins (Bax and Bim) on TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$  IET from proximal SI of *Itgb1<sup>fl/fl</sup>* (n=5) and *Itgb1<sup>prLck</sup>* (n=5) mice (F). The MFI on CD8 $\alpha^+$  IET from multiple individual mice was measured by flow cytometry (G). Statistical analysis was performed using 2 way ANOVA with Bonferroni's multiple comparison test (B) or unpaired t-test (C, E, G). Statistical

significance is indicated by \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Data show means  $\pm$  SEM. Each symbol represents a measurement from an individual well (B) or a single mouse (C, E, G). Data represent representative results from one of at least two independent experiments (B, G) or pooled results from at least two independent experiments (C, E). Groups of co-housed littermates were analyzed.

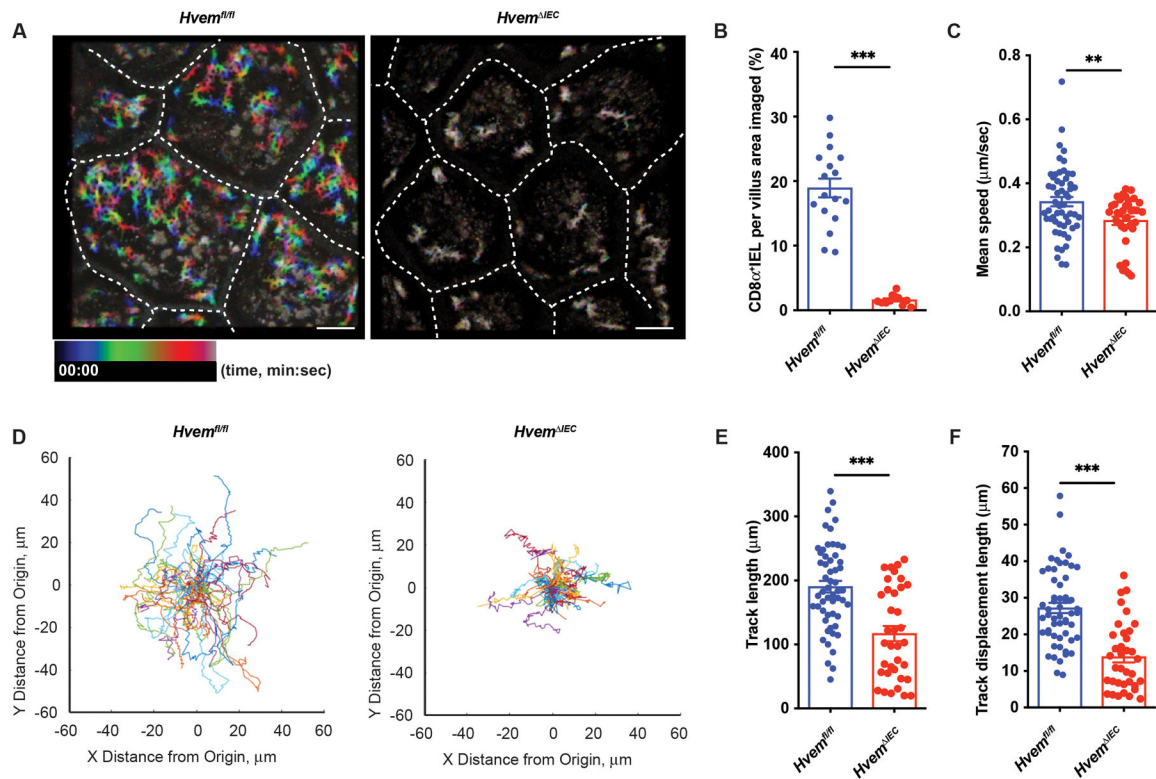




**Fig 6. LIGHT is required for IET homeostasis.**

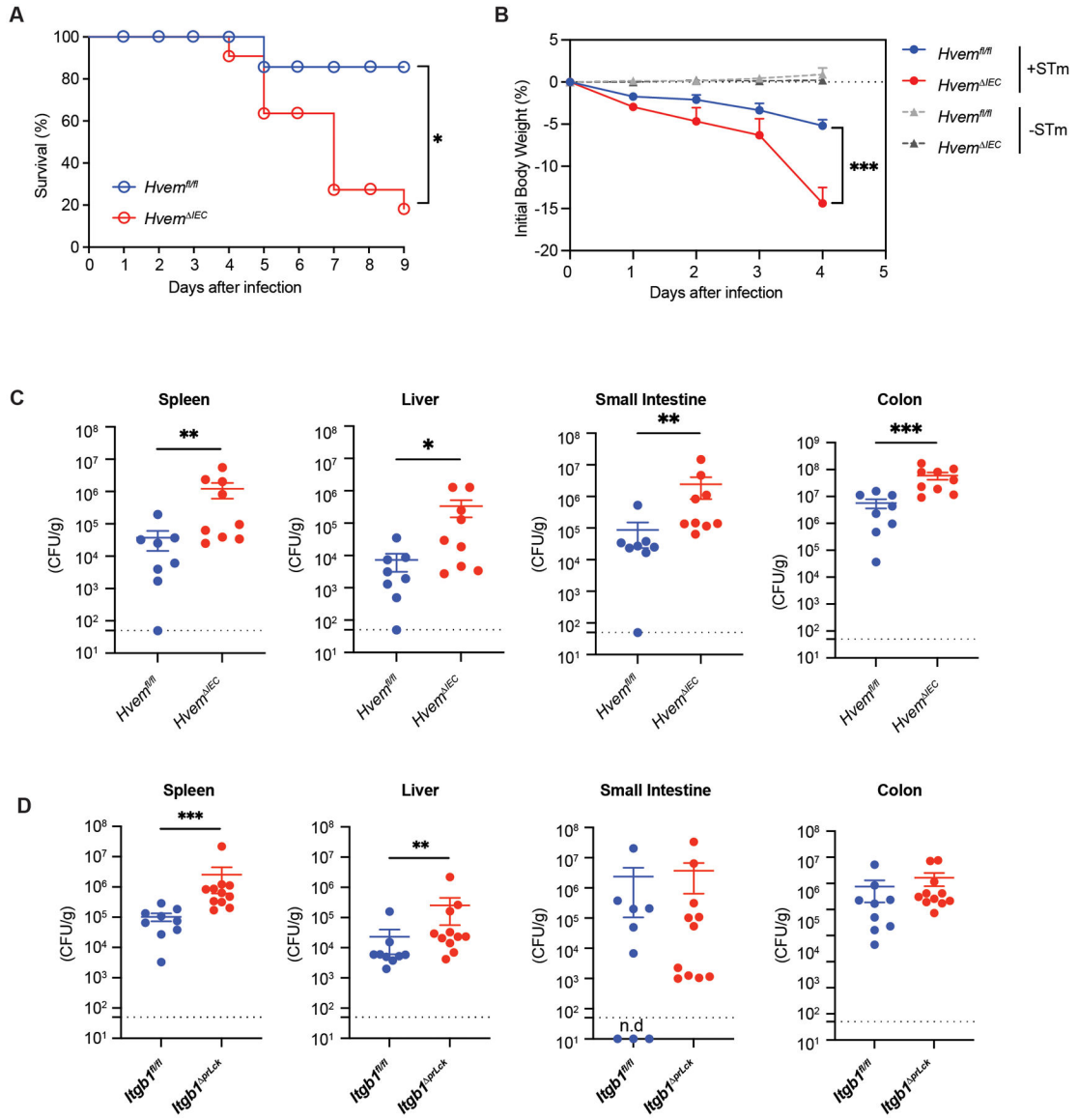
(A) Total IEL numbers in portions of the intestine from *Light*<sup>+/+</sup> (n=5) and *Light*<sup>-/-</sup> (n=5) mice. (B) Representative plots of TCRαβ<sup>+</sup>CD8αα<sup>+</sup> and TCRαβ<sup>+</sup>CD8αβ<sup>+</sup> in TCRαβ IET from proximal SI in *Light*<sup>+/+</sup> and *Light*<sup>-/-</sup> mice. (C) Absolute numbers of indicated IET subsets in total IEL from proximal SI in *Light*<sup>+/+</sup> (n=5) and *Light*<sup>-/-</sup> (n=5) mice. (D-E) Representative immunofluorescence staining of TCRγδ IET (D) and TCRαβ IET (E) from proximal SI in *Light*<sup>+/+</sup> and *Light*<sup>-/-</sup> mice. Composite images in which the three channels were merged. Composite images depict expression of CD8α (green), CD8β (blue), and TCRδ (red, D) or TCRβ (red, E). Yellow arrowheads highlight TCRγδ<sup>+</sup>CD8αα<sup>+</sup> (D) and TCRαβ<sup>+</sup>CD8αβ<sup>+</sup> IET (E). Dashed white lines indicate the boundaries of the epithelium, approximately one villus in each case, used for the quantitation. Scale bars, 50μm. Two independent experiments were carried out yielding similar results. (F) Quantification of TCRγδ<sup>+</sup>CD8αα<sup>+</sup> and TCRαβ<sup>+</sup>CD8αβ<sup>+</sup> per mm<sup>2</sup> from proximal SI in *Light*<sup>+/+</sup> (n=5) and

*Light*<sup>-/-</sup> (n=4) mice. (G) The MFI of Bax and Bim expression on CD8 $\alpha$ <sup>+</sup> IET from *Light*<sup>-/-</sup> (n=5) and *Light*<sup>+/+</sup> (n=4) mice. Statistical analysis was performed using unpaired t-test (A, C, F, G). Statistical significance is indicated by \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001. Data show means  $\pm$  SEM. In A, C and G, each symbol represents a measurement from a single mouse. In F, each symbol represents a calculation to give the number per mm<sup>2</sup>. Data are representative results from one of at least two independent experiments with at least four mice in each experimental group (A, C, G) or pooled results from at least two independent experiments with at least four mice per group in each experiment (F). (A-G) Groups of co-housed littermates were analyzed.



**Fig. 7. Epithelial HVEM influences patrolling by CD8 $\alpha^+$  IET.**

Mice were injected with anti-CD8 $\alpha$  before imaging of the epithelium by taking Z-stacks encompassing the epithelium and the upper layers of the lamina propria at a 2.8  $\mu\text{m}$  step size every 10 sec. Each XY plane spans 365  $\times$  365  $\mu\text{m}^2$ . Fiji was used to calculate the coverage area and generate representative images. Movement metrics (mean speed, track length, track displacement length, and paths) for each CD8 $\alpha^+$  lymphocyte were calculated with Imaris. (A) Rainbow plot time projection reveals the area covered by CD8 $\alpha^+$  IET during 10 min, starting from time 0 (00:00) to 10 mins (10:00). Rainbow color shows the tracks of moving CD8 $\alpha^+$  IET. Blue denotes starting position at the beginning of the recording it goes to green and then red, denotes ending position, as the time elapses. If the cell does not move, the superposition of colors blends to give a color closer to white. Scale bars, 50 $\mu\text{m}$ . (B-C) Quantification of the fraction of villus area covered by CD8 $\alpha^+$  cells (B), mean speed (C) in a 10 min recording (*Hvem<sup>fl/fl</sup>*, n=3; *Hvem<sup>IEC</sup>*, n=3). (D) Spider plots of tracks show the migratory path of each CD8 $\alpha^+$  IEL in the 10 min recording. Each cell path was translated to begin at (0,0) and randomly colored. (E-F) Quantification of track length (E), and the displacement between beginning and ending positions (F) in a 10 min recording (*Hvem<sup>fl/fl</sup>*, n=3; *Hvem<sup>IEC</sup>*, n=3). Statistical analysis was performed using unpaired t-test (B-C, E-F). Statistical significance is indicated by \*, p < 0.05; \*\*\*, p < 0.001. In B-C and E-F, bars show the mean and each symbol represents a measurement from a single cell. Dashed white lines (A) indicate the boundaries of each quantified villus. Data represent combined results of two independent experiments with at least three mice in each experimental group. Groups of co-housed littermates were analyzed.



**Fig. 8. Epithelial HVEM is required for host defense against *S. typhimurium* infection.** *Hvem*<sup>IEC</sup> mice and control *Hvem*<sup>fl/fl</sup> mice were infected orally with  $1 \times 10^7$  *S. typhimurium* colony-forming units (CFU)/mouse (A-C). (A) Survival curves (*Hvem*<sup>fl/fl</sup>, n=8; *Hvem*<sup>IEC</sup>, n=11). (B) Changes in body weight (% change from baseline) (infected *Hvem*<sup>fl/fl</sup>, n=8; infected *Hvem*<sup>IEC</sup>, n=9; uninfected *Hvem*<sup>fl/fl</sup>, n=11; uninfected *Hvem*<sup>IEC</sup>, n=8). (C) Bacterial burdens at day 4 p.i. (*Hvem*<sup>fl/fl</sup>, n=8; *Hvem*<sup>IEC</sup>, n=9). (D) *Itgb1*<sup>fl/fl</sup> (n=9) and *Itgb1*<sup>prLck</sup> (n=11) mice were infected orally with *S. typhimurium* and bacterial burdens measured at days 3–4 p.i. Dashed line represents the limit of detection (C, D). Statistical analysis was performed using Log-rank test (A), 2 way ANOVA with Bonferroni's multiple comparison test (B), or Mann-Whitney test (C, D). Statistical significance is indicated by \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001. Data shown are means  $\pm$  SEM (A). Bars show the mean, symbols represent individual mice. Data represent

pooled results from at least two independent experiments having at least three mice per group in each experiment. Groups of co-housed littermates were analyzed.

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