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Immunity to infection

Research Article

LCMV induced downregulation of HVEM on antiviral T cells is critical for an efficient effector response

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T-cell responses against tumors and pathogens are critically shaped by cosignaling molecules providing a second signal. Interaction of herpes virus entry mediator (HVEM, CD270, TNFRSF14) with multiple ligands has been proposed to promote or inhibit T-cell responses and inflammation, dependent on the context. In this study, we show that absence of HVEM did neither affect generation of effector nor maintenance of memory antiviral T cells and accordingly viral clearance upon acute and chronic lymphocytic choriomeningitis virus (LCMV) infection, due to potent HVEM downregulation during infection. Notably, overexpression of HVEM on virus-specific CD8$^+$ T cells resulted in a reduction of effector cells, whereas numbers of memory cells were increased. Overall, this study indicates that downregulation of HVEM driven by LCMV infection ensures an efficient acute response at the price of impaired formation of T-cell memory.

Keywords: HVEM · BTLA · T-cell costimulation · LCMV · memory T cells

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

The protective immune responses against viral infections are characterized by the activation, expansion, and differentiation of CD8$^+$ T cells. These effector cells promote pathogen clearance through production of cytotoxic mediators and proinflammatory cytokines. While the majority of effector CD8$^+$ T cells are short-lived, a fraction survive as self-renewing memory T cells to maintain long-term immunity and a rapid secondary immune response upon reinfection (reviewed in [1]). The fate of T cells is regulated through costimulatory or coinhibitory ligand-receptor interaction that can either subsequently promote or inihibit T-cell responses. TNF family of receptors (TNFR) and members of Ig superfamilies represent cosignaling molecules that are critical to determine T-cell function during an immune response. Some of the major costimulatory molecules include CD28, CD27, 4-1BB, and OX-40 that promote survival, expansion, and/or long-term maintenance of antigen-specific T cells [2–5]. On the contrary, coinhibitory molecules, including CTLA-4 and PD-1, interfere with T-cell expansion and effector responses [6–9].

Herpes virus entry mediator (HVEM) is a member of the TNFR superfamily that was originally identified as the main target receptor for HSV to enter host cells via the viral gp D [10]. HVEM has been described to have diverse functions, engaging both positive or negative signals depending on the interacting ligand. HVEM engagement with TNF family ligand LIGHT (TNFSF14, CD258) and lymphotoxin alpha (LT-α) has been reported to induce a costimulatory signal [11], promoting T-cell proliferation in vitro [12, 13] and development of effector and memory T cells in several inflammatory disease models [14–17]. On the contrary, HVEM

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signaling induced by engagement of Ig superfamily members B and T lymphocyte attenuator (BTLA) and CD160 has been shown to induce proinflammatory signal by triggering NFkB and STAT3 activation in epithelial cells [18, 19] with CD160 reported to deliver an essential innate signal through HVEM required for protection against pathogenic intestinal and pulmonary bacterial infection [19]. However, when HVEM serves as a ligand for CD160 and BTLA receptors, this interaction delivers a negative signal to T cells [20–22]. Indeed, BTLA engagement on T cells by HVEM on intestinal epithelial cells is required to inhibit the development of pathological T-cell responses in a T-cell transfer-induced colitis model [23]. Concurrently, however, HVEM signaling in T cells by interaction with BTLA ligand on APCs delivers a positive signal, resulting in expansion and/or survival of T cells [18, 24, 25]. Moreover, HVEM expression on B cells has been shown to restrain T-cell help by interaction with BTLA and thereby inhibit GC B-cell proliferation [26].

Lymphocytic choriomeningitis virus (LCMV) infection model has served to study many cosignaling molecule receptor-ligand interactions in T-cell activation. In this study, we used both acute and chronic LCMV to investigate the role of HVEM during T-cell priming, expansion, contraction, memory, and exhaustion. We found that HVEM was potently downregulated during the effector phase on virus-specific T cells and remained dampened for at least 4 weeks after viral clearance. Accordingly, HVEM-deficiency did not affect the generation of antiviral effector $CD8^+$ T cells and the maintenance of memory cells after acute infection. It also did not play a role in T-cell exhaustion upon chronic infection. Conversely, sustaining HVEM by overexpression on virus-specific $CD8^+$ T cells, reduced the number of effector T cells, although cytokine responses at the single-cell level remained intact. Generation of memory T cells was, however, increased upon HVEM overexpression. Overall, these data indicate that downregulation of HVEM on virus-specific $CD8^+$ T cells ensures effector cell accumulation, whereas memory formation is impaired by decreased expression of the molecule.

Results

In vitro expansion and cytokine responses of $CD8^+$ T cells are independent of HVEM

HVEM ligand on APCs has been shown to deliver a negative signal by interaction with CD160 and BTLA receptors on T cells [20–22, 27, 28]. To study the role of HVEM as a ligand on professional APCs in activation, differentiation and proliferation of T cells, we cocultured HVEM-deficient and -sufficient BM-derived DCs (BMDCs) together with LCMV-gp_{33-41}-specific $CD8^+$ TCR transgenic (P14) T cells in serial dilutions of gp_{33-41} peptide. We determined the expression of $CD25^+$ (to define early activation), $IFN\gamma^+$, and Ki-67^+ $CD8^+$ T cells, and total number of expanded $CD8^+$ T cells at indicated time points (Supporting information Fig. S1A-D). No differences were found comparing cultures containing $HVEM^{-/-}$ and WT BMDCs. The requirement of cosignaling for T-cell priming and expansion depends substantially on the TCR-peptide affinity [29–31]. To address this possibility, we performed cocultures with $HVEM^{-/-}$ BMDCs and gp_{33}-specific T cells in the presence of gp_{33-41} peptide and low-affinity altered peptide ligands (APL), A4Y and V4Y. However, no differences were found in IFN-γ production (Supporting information Fig. S1E) and T-cell expansion (Supporting information Fig. S1F), indicating that T-cell activation, proliferation, and effector differentiation are neither positively nor negatively influenced by prevention of BTLA and CD160 signaling in T cells through HVEM ligand on DCs.

HVEM is dispensable for $CD8^+$ T-cell responses during acute and chronic LCMV infection

We next assessed the quantity and quality of virus-specific $CD8^+$ T-cell response during the course of acute LCMV WE infection in $HVEM^{-/-}$ mice. Surprisingly, absence of HVEM did not affect the expansion, contraction, and long-term maintenance of virus-specific $gp33^+$ and total $CD8^+$ T cells (Fig. 1A and D; gating strategy Supporting information Fig. S2). Killer cell lectin-like receptor G-1 (KLRG-1) together with IL-7R-α (CD127) can serve as markers to predict the fate of T cell after antigen clearance [32, 33]. While most of KLRG-1^+ $CD127^-$ short-lived effector cell (SLEC) die after pathogen control, KLRG-1^- $CD127^+$ memory precursor effector cells (MPEC) survive and give rise to long-lasting memory cells. Frequencies of virus-specific SLECs were slightly reduced, whereas MPECs had increased tendency in the blood and spleen of $HVEM^{-/-}$ mice at 8 (days post infection (dpi; Fig. 1B and C).

Different memory subsets can be distinguished on $CD127^+$ T cells by L-selectin (CD62L) expression [34–37]. $CD62L^-$ $CD127^+$ effector memory T cells patrol peripheral tissues and mediate protection against local infection by rapid production of cytokines, whereas $CD62L^+$ $CD127^+$ central memory T cells reside mostly in lymphoid tissues and proliferate extensively upon antigen re-encounter. The frequency of those memory subsets was not influenced by the absence of HVEM over the course of infection (Fig. 1E). Furthermore, development of IFN-γ-producing $CD8^+$ T cells in spleen and lung was unchanged (Fig. 1F).

To address a potential involvement of HVEM during chronic viral infection, we infected mice with high-dose LCMV docile or LCMV clone-13 (Cl13), and monitored T-cell responses over time in the blood, spleen, and liver at 16 weeks p.i. Expansion, contraction, and exhaustion (Fig. 2A-I) of virus-specific, total $CD8^+$ and virus-specific PD-1^+ T cells as well as IFN-γ and TNF-α production (Fig. 2J and K) were comparable in $HVEM^{-/-}$ and WT mice. Moreover, $CD4^+$ T-cell effector responses revealed no difference between $HVEM^{-/-}$ and WT mice (data not shown). Accordingly, virus titers in the blood (Fig. 2L) and various organs (Fig. 2M) remained unaffected in the absence of HVEM.

Overall, our data indicate that cosignaling molecule HVEM is dispensable for $CD8^+$ T-cell response against acute and chronic LCMV infections.

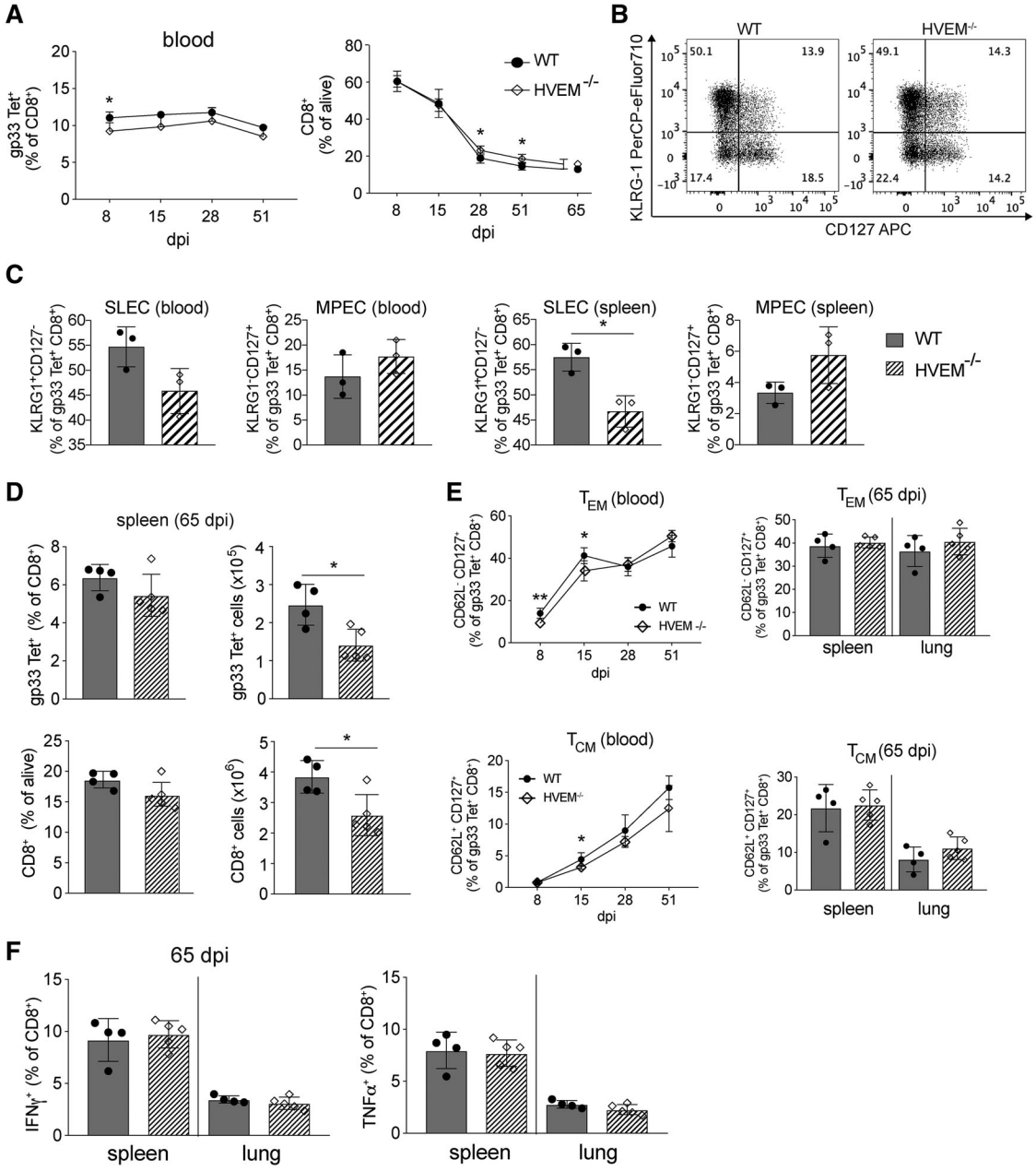


Figure 1. Absence of HVEM does not affect antiviral T-cell responses during acute LCMV infection. WT and HVEM$^{-/-}$ mice were infected with 2000 pfu LCMV WE. (A) Frequency of gp33-tetramer-specific (left) and total CD8$^+$ T cells (right) in the blood at indicated dpi. (B) Representative FACS plots of KLRG-1 and CD127 expression on gp33-specific CD8$^+$ T cells from the blood at 8 dpi. (C) Frequency of KRLG-1$^+$ CD127$^-$ SLEC and KLRG-1$^-$ CD127$^+$ MPEC in the blood and in the spleen at 8 dpi. (D) Frequency and total number of gp33-specific (upper panel) and overall CD8$^+$ T cells (lower) in the spleen at 65 dpi. (E) Frequency of effector memory (T_{EM}) and central memory (T_{CM}) virus-specific CD8$^+$ T cells in blood and organs and at days indicated. (F) Frequency of IFN-γ and TNF-α producing CD8$^+$ T cells from the spleen and the lung of infected animals 65 dpi after in vitro restimulation with gp33 peptide. Errors bars indicate mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$ (two-tailed Student *t* test). Data are representative of two independent experiments with at least four mice per group (A, B and D to F), and two experiments with three mice per group (C).

HVEM is downregulated on virus-specific CD8$^+$ and CD4$^+$ T cells during acute LCMV infection

To understand the kinetics of HVEM surface expression on virus-specific CD4$^+$ and CD8$^+$ T cells over the course of an acute LCMV infection, we adoptively transferred congenically marked CD4$^+$ and CD8$^+$ T cells from Smarta-1 and P14 transgenic mice that express LCMV- gp_{61-80} and gp_{33-41} respectively, to C57BL/6 recipients and monitored HVEM surface levels by flow cytometry during the course of infection. HVEM expression was detected on the surface of naïve CD8$^+$ T cells (Fig. 3A) and became potently downregulated on effector CD8$^+$ T cells. Surprisingly, this low

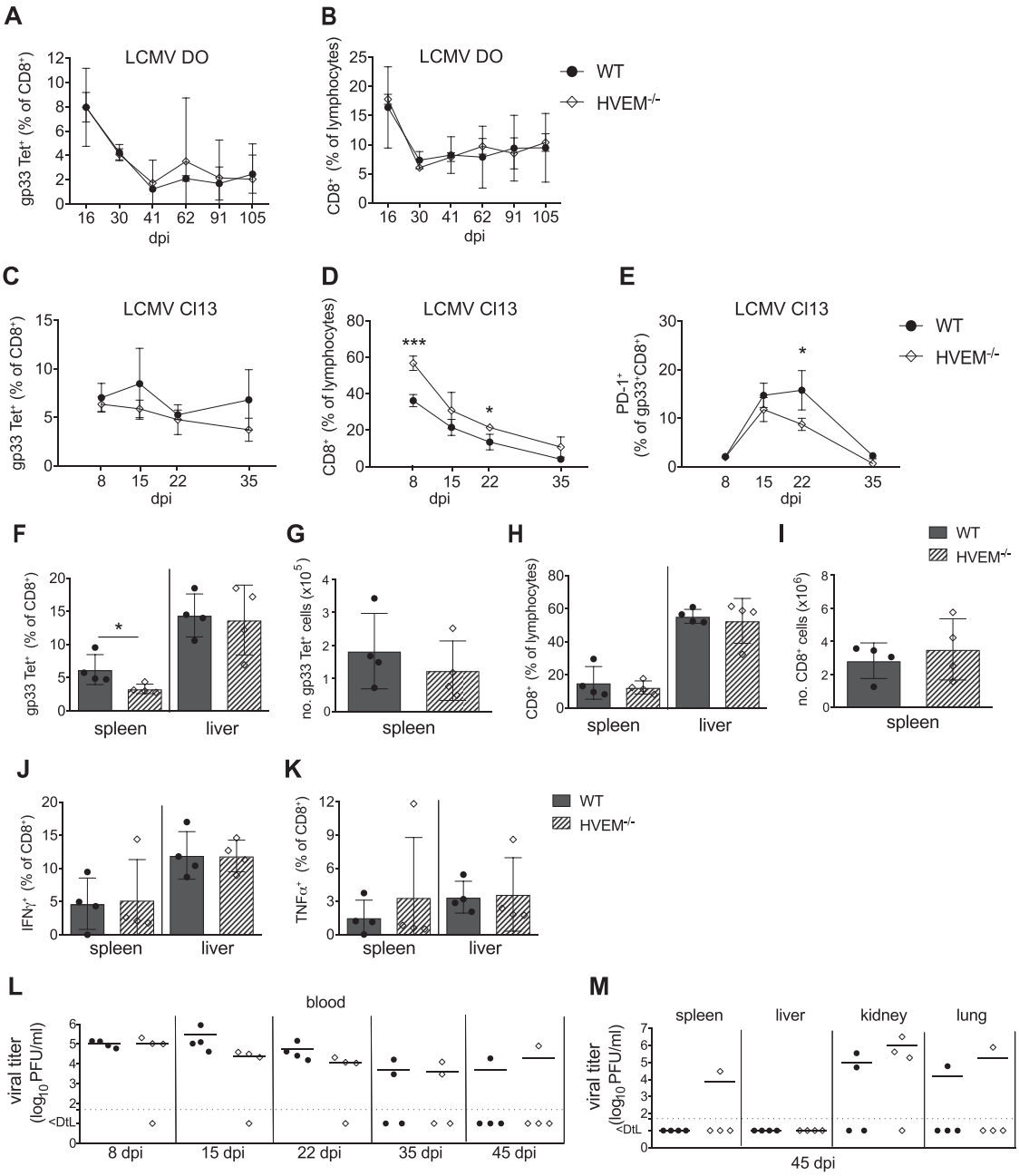


Figure 2. Absence of HVEM does not affect antiviral T-cell responses during chronic LCMV infection. WT and HVEM$^{-/-}$ mice were infected with 2×10^6 pfu LCMV docile (A,B) or clone 13 (C-M) i.v. Frequency (A-E, F, H) and total number (G, I) of gp33-Tetramer$^+$ (A, C, F), overall CD8$^+$ T cells (B, D, H), and virus-specific PD-1$^+$ cells in blood at indicated days (A-E), spleen and liver at 16 weeks p.i. (F-I). (J, K) Frequency of IFNγ- (J) and TNFα- (K) producing CD8$^+$ T cells 16 weeks p.i. after in vitro restimulation with gp33 peptide. Viral titers in the blood (L) and in indicated organs at 16 weeks p.i. (M). Dotted lines indicate detection limit (DtL). Errors bars indicate mean ± SD. $^*p < 0.05$, $^{***}p < 0.001$ (two-tailed Student *t* test). Data are representative of two independent experiments with four mice per group.

expression on memory CD8$^+$ T cells retained for at least 4 weeks p.i. (Fig. 3B). Similarly, HVEM expression on naïve Smarta CD4$^+$ T cells (Fig. 3A) was suppressed in both effector and memory phases (Fig. 3B). HVEM was also expressed on splenic B cells in naïve mice (Fig. 3A). However, in contrast to T cells, it is slightly upregulated at the peak of LCMV infection and then gradually declines at 4 weeks p.i. (Fig. 3B).

Normal antibody responses to LCMV in HVEM-deficient mice

While antibody responses play a minor role during the control of acute LCMV WE infections, B cells and late development of neutralizing antibodies are critical for long-term LCMV control [38–40]. HVEM has recently been identified to regulate T-follicular helper (Tfh) cells and antibody responses [26], and

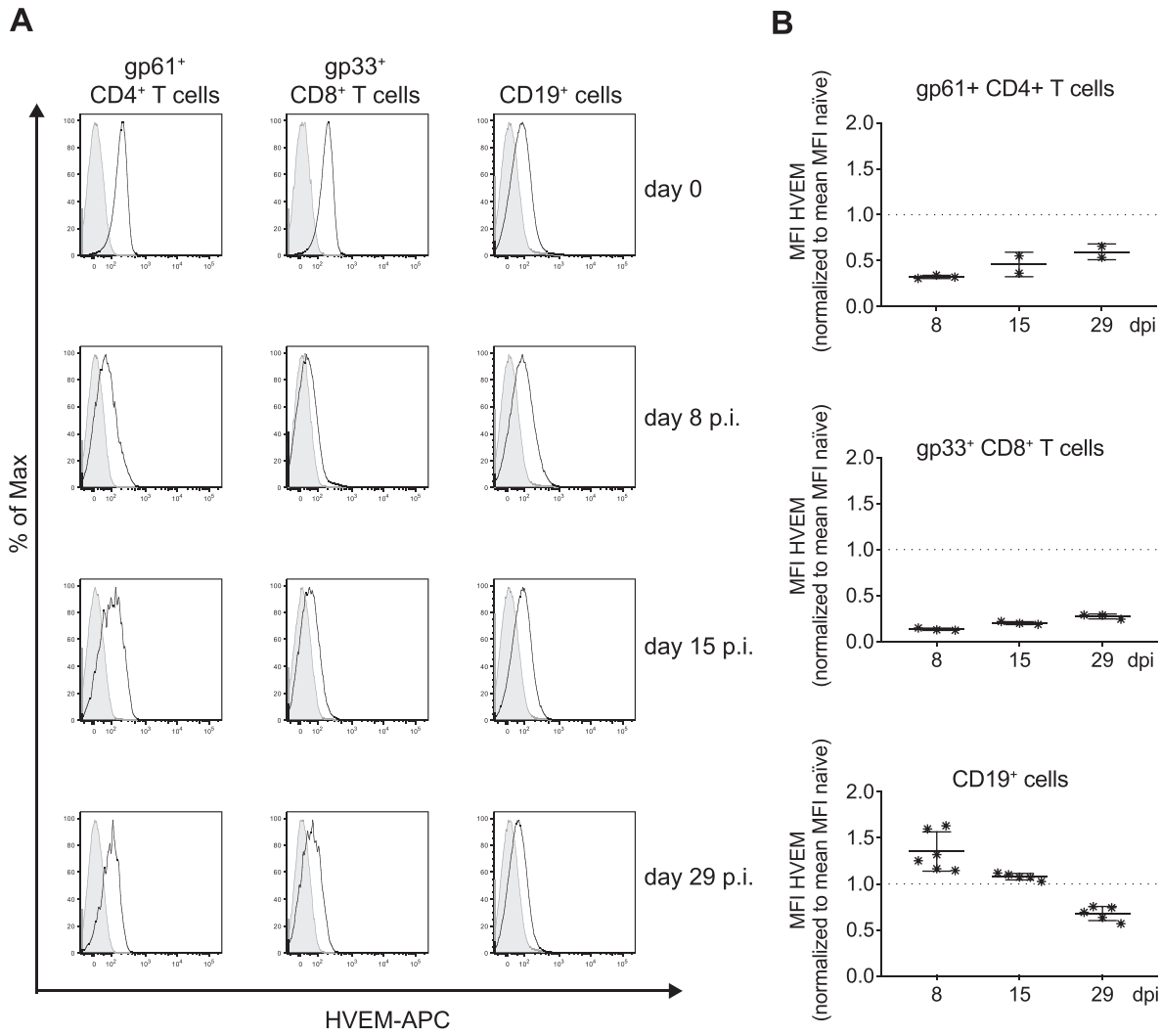


Figure 3. Downregulation of HVEM expression on antiviral T cells during infection. C57BL/6 mice (CD45.2) (n = 6) were infected with LCMV WE i.v. and adoptively transferred with gp61-specific $CD4^+$ T cells or gp33-specific $CD8^+$ T cells from Smarta1 or P14 TCR transgenic mice (both CD45.1) on the following day (n=3 each). HVEM expression was monitored by flow cytometry at days indicated. (A) Histograms show HVEM expression on $CD4^+$ Smarta1, $CD8^+$ P14 and B cells (black lines). Endogenous $CD4^+$, $CD8^+$, and B cells from $HVEM^{-/-}$ mice served as controls (grey shaded). (B) Data from histograms were normalized to the mean MFI of HVEM from the respective naïve cell populations (dotted line). From all values, the MFI of the respective population in $HVEM^{-/-}$ mice was subtracted before normalization. Errors bars indicate mean ± SD. $^*p < 0.05$, $^{***}p < 0.001$ (two-tailed Student *t* test). Data are representative of three independent experiments with three to six samples per time point.

in line with this, we found maintained HVEM expression on B cells after LCMV infection contrary to T cells. We, therefore, investigated the requirement of HVEM for long-term antibody responses to LCMV Cl13. Notably, we did not observe a difference in LCMV-specific IgG2a and IgG2b antibody responses in WT and HVEM-deficient mice between 1 and 3.5 months after infection (Fig. 4A and B).

Reduced expansion but increased memory formation of virus-specific $CD8^+$ T cells overexpressing HVEM

Our data show that surface expression of HVEM on naïve $CD8^+$ T cells is downregulated on antiviral effector $CD8^+$ T cells and stays low on memory cells, which may explain why complete absence of the molecule does not significantly alter T-cell responses. Thus, we were wondering whether downregulation of the molecule on antiviral $CD8^+$ T cells during infection of WT mice has a consequence for effector and memory responses. To study this possibility, we generated a bicistronic retroviral vector encoding HVEM with IRES-linked GFP ($P14^{rvHVEM\text{-}GFP}$) and control expressing GFP alone ($P14^{rvGFP}$). Gp33-specific $CD8^+$ T cells harvested from CD45.1 P14 transgenics were transduced with either HVEM or control vector, GFP-expressing cells were sorted and adoptively transferred to C57BL/6 recipients (CD45.2) prior to acute LCMV WE infection.

Analysis of GFP expression in control $P14^{rvGFP}$ and $P14^{rvHVEM\text{-}GFP}$ cells by flow cytometry revealed a gradual decrease

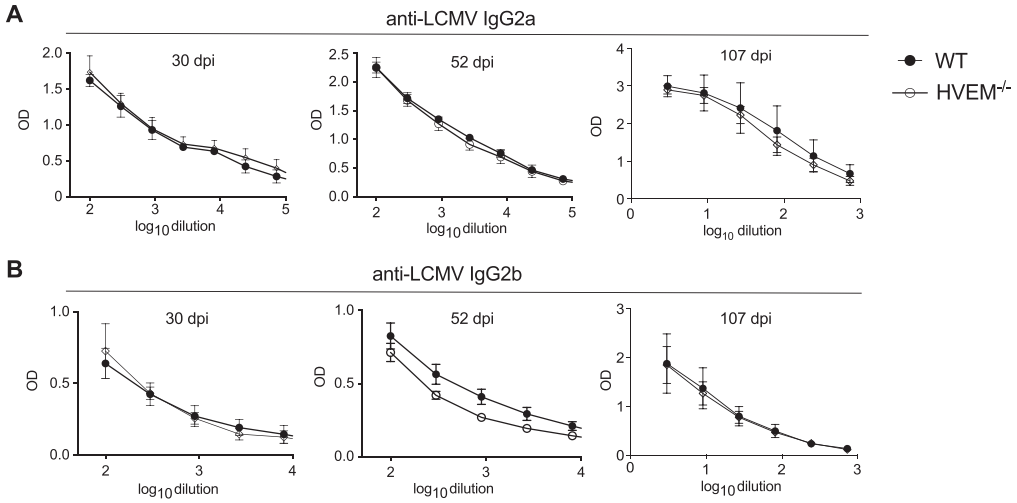


Figure 4. Antibody response is independent of HVEM. (A, B) WT and HVEM$^{-/-}$ mice were infected with 1×10^6 pfu LCMV Cl13. Virus-specific IgG2a (A) and IgG2b (B) antibody levels in the serum were measured by ELISA at indicated time points post infection. Errors bars indicate mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$ (two-tailed Student *t* test). Data are representative of two independent experiments with six mice (day 30) and four mice (day 52 and 107) per group.

in expression levels between days 7 and 28 dpi (Fig. 5A), which might be explained by silencing of the integration sites of the retrovirus in transduced P14 cells. Interestingly, overexpression of HVEM potently inhibited expansion of P14$^{rvHVEM\text{-}GFP}$ cells in the spleen (Fig. 5B upper) and the liver (Fig. 5C) with a bias of MPEC over SLEC in the acute phase of LCMV infection (Fig. 5E). In contrast, maintenance of P14$^{rvHVEM\text{-}GFP}$ cells was increased when compared to P14^{rvGFP} controls (Fig. 5B bottom and C) at 28 dpi. Importantly, IFN-γ and TNF-α responses remained unaffected in HVEM overexpressing gp33$^+$ CD8$^+$ T cells in the acute and memory phases of the response.

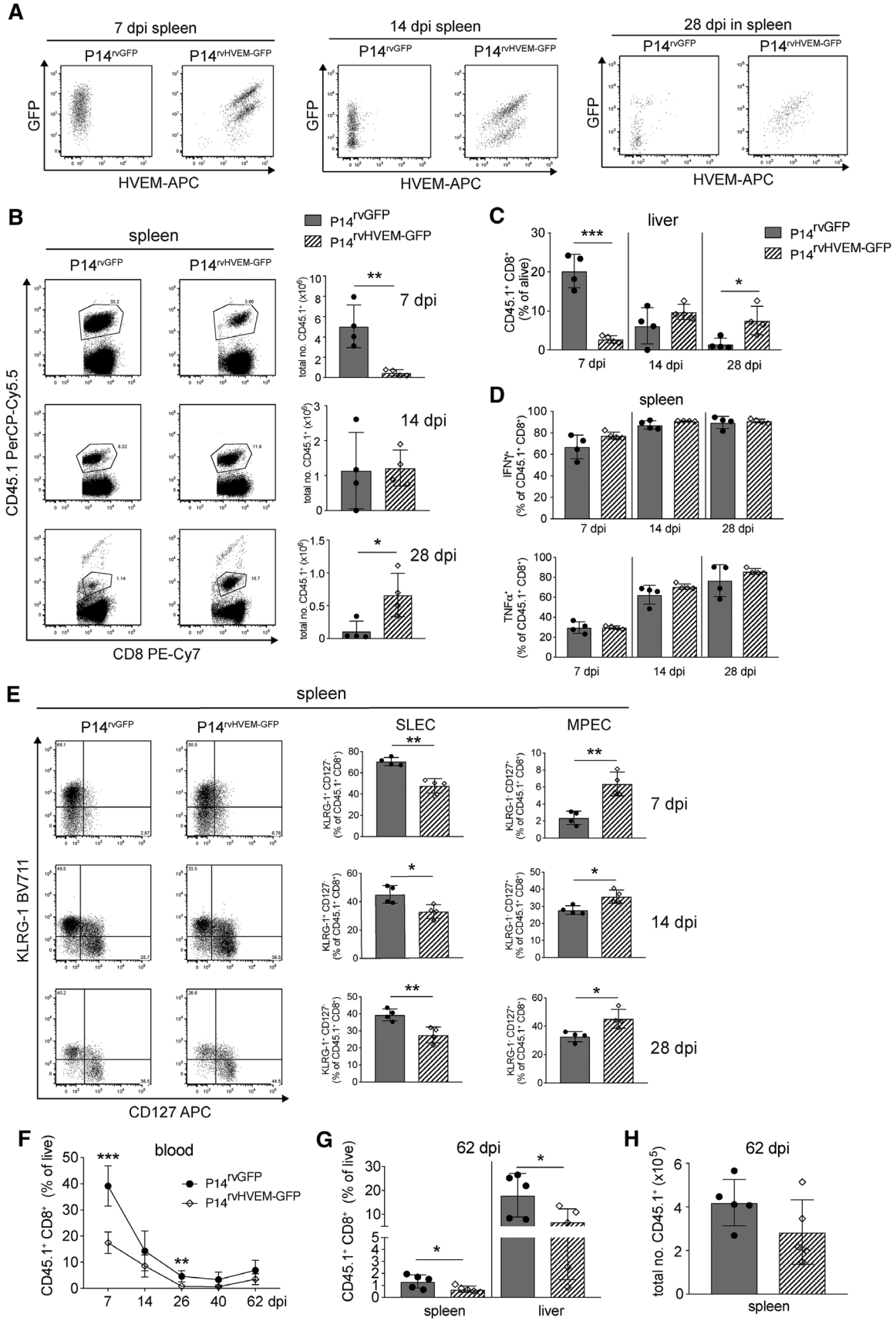
Next, we studied the effects of HVEM overexpression during chronic LCMV Cl13 infection. To avoid the participation of endogenous CD8$^+$ T cells, we transferred P14$^{rvHVEM\text{-}GFP}$ or control P14^{rvGFP} cells into OT-I recipients prior to infection. Similar to acute infection, forced expression of HVEM resulted in a dramatically reduced number of virus-specific CD8$^+$ T cells at the peak of the expansion phase, which remained low over the course of chronic infection (Fig. 5F). Similarly, frequencies (Fig. 5G) and numbers (Fig. 5H) of HVEM-GFP transgenic P14 cells in the spleen and the liver of OT-I mice 62 dpi were reduced.

Thus, overexpression of HVEM on virus-specific CD8$^+$ T cells results in reduced numbers of effector cells but increased formation of memory cells during an acute LCMV infection.

Discussion

HVEM has been shown to serve as a receptor on T cells, delivering a costimulatory signal and promoting T-cell responses by interaction with LIGHT and LT-α. Alternatively, HVEM can serve as a ligand to receptors on T cells, including BTLA and CD160, and deliver a coinhibitory signal. In our study, we found that HVEM is dispensable for the antiviral CD8$^+$ T-cell response and viral clearance during acute and chronic LCMV infections. Furthermore, we did not observe any differences in the effector response of virus-specific CD8$^+$ T cells and viral clearance comparing HVEM-deficient and WT control mice. We also found that HVEM expression on DCs is dispensable for efficient T-cell priming, expansion, and cytokine responses (Supporting information Fig. S1), indicating that BLTA and CD160 engagement on T cells by HVEM on DCs does not affect T-cell responses. In contrast to our findings, triggering of BTLA and CD160 on T cells with HVEM-Ig or nonprofessional APCs (i.e., CHO cells) transfected with HVEM (and various combinations of MHC class II and B7.1) has been shown to inhibit T-cell proliferation and responses [21, 27]. These data indicate that engagement of BTLA and CD160 on T cells with HVEM on nonprofessional APCs in peripheral tissues delivers a negative signal that promotes T-cell tolerance, while HVEM-BTLA interactions have a limited role in the priming of T cells by professional APCs that are armed with a variety of costimulatory and coinhibitory molecules, overruling a putative negative signal mediated by HVEM-BTLA interactions.

Interestingly, while HVEM-deficient mice showed impaired accumulation of effector T cells following vaccinia virus infection [24], our data demonstrate that HVEM is dispensable for efficient expansion, contraction, and maintenance of CD8$^+$ T cells during acute and chronic LCMV infection. The requirement of CD28 costimulation has been shown to depend on the type of infection; whereas CD28-deficient mice mount only a slightly impaired cytotoxic T-cell response to LCMV [41, 42], CD8$^+$ T cell-mediated killing is highly dependent on CD28 costimulation upon infection with vesicular stomatitis virus (VSV) [42]. Comparison of CD8$^+$ T-cell responses in mice infected with VSV strains that differ in virulence showed the need for CD28 costimulation during an infection that induces a weak signal 1, due to lower viral burden or shorter exposure to virus [42]. In addition, the cytokines required for mounting CD8$^+$ T-cell response may influence the



need for HVEM costimulation. Whereas the expansion of CD8$^+$ T cells during an LCMV infection critically depends on type I IFNs [43], the CD8$^+$ T-cell response during VSV infection is independent of cytokines acting as signal 3 [44]. HVEM engagement has also been shown to promote a generation of MPECs during respiratory VSV infection [45], which we did not observe in LCMV infection. Taken together, the requirement of HVEM costimulation (as a signal 2) for mounting efficient antiviral CD8$^+$ T-cell response during VSV but not LCMV infection may be caused by differences in the strength of signal 1 and 3.

A likely explanation for normal T-cell responses in HVEM-deficient mice is the potent downregulation of HVEM on virus-specific CD4$^+$ and CD8$^+$ T cell during both effector and contraction phases. Surprisingly, even 4 weeks after antigen clearance, HVEM expression did not return to baseline levels of naïve T cells. Retroviral overexpression of HVEM on virus-specific CD8$^+$ T cells strongly reduced the numbers of effector cells at the peak of the acute response to LCMV, suggesting that downregulation of HVEM ensures efficient expansion of virus-specific CD8$^+$ T cells. The reduction may be due to impaired proliferation and/or increased cell death. However, engagement of HVEM on anti-CD3/anti-CD28 activated T cells by BTLA-Fc has been shown to increase survival and proliferation in vitro [18]. These opposing results may be reconciled by proposing that HVEM on T cells in vivo acts as ligand for BTLA or CD160 on adjacent APCs, triggering an inhibitory pathway that limits survival of T-cell noncell autonomously.

In contrast to preventing accumulation of effector cells, the forced retrovirus-driven preservation of HVEM expression on antiviral CD8$^+$ T cells had a positive outcome for the generation or maintenance of memory cells, which is consistent with previous results, suggesting that HVEM expression during the contraction phase protects effector cells in a cell autonomous manner from apoptosis due to cell intrinsic prosurvival signals [24, 25].

Interestingly, while we did not observe a significant difference in MPEC frequency between LCMV-infected WT and HVEM-deficient mice, HVEM overexpression on virus-specific CD8$^+$ T cells increased the frequency of MPECs at the peak of effector response to LCMV. Thus, forced retrovirus-driven maintenance of HVEM surface expression promotes MPEC formation in the acute phase of LCMV infection.

While HVEM expression on B cells was recently shown to restrain GC B cells and antibody responses cell extrinsically [26], LCMV-specific antibody responses remained largely unaffected in HVEM-deficient mice for at least 3 months after infection, implying that HVEM does not restrict the function of Tfh cells. Notably, in contrast to T cells, HVEM expression was maintained on splenic B cells, including GC B cells, during infection.

In summary, our data show that LCMV infection potently downregulates expression of HVEM on T cells, which is beneficial for the efficient generation of virus-specific effector CD8$^+$ T cells but limits the formation of memory cells after viral clearance.

Material and Methods

Mice

All mouse strains used in this study, including HVEM$^{-/-}$ mice [46] (kindly provided by Kenneth M. Murphy [Washington University School of Medicine, St. Louis, USA]), Smarta1 (CD45.1$^+$) [47], P14 (CD45.1$^+$) [48] and OT-I (CD45.2$^+$) [49] transgenics, and C57BL/6 mice were housed in individually ventilated cages under specific pathogen-free conditions at ETH Phenomics Center (EPIC, Zurich, Switzerland). Groups of age- and sex-matched mice were infected at 6 to 12 weeks of age. Mice were euthanized with CO_2. All experiments were performed according to institutional guidelines and Swiss animal protection law (Swiss Animal Protection Ordinance [TschV and TschG] Switzerland) and had been approved by the animal ethics committee (Kantonales Veterinärsamt Zürich, permissions 113/2012 and ZH135/15). The manuscript does not contain any human studies.

Virus and infection

Mice were infected i.v. with indicated doses of LCMV strains WE, Cl13, or docile. All viral strains were originally provided by Rolf Zinkernagel (University of Zurich, Zurich, Switzerland), and propagated on L-929, BHK-21, or MDCK cells, respectively.

Retroviral expression of HVEM

Hvem gene was PCR amplified from mouse tissue cDNA, followed by cloning into the Moloney murine leukemia-based retroviral

Figure 5. Reduced expansion but increased memory formation of HVEM overexpressing virus-specific CD8$^+$ T cells. (A-E) WT mice were transferred i.v. with 1×10^4 congenically marked CD45.1$^+$ gp33-specific CD8$^+$ T cells (P14) that were transduced with either HVEM-GFP or control GFP retrovirus (n = 4/group). Mice were infected with 2000 pfu LCMV WE on the following day. (A) Representative FACS plots pregated on CD45.1$^+$ CD8$^+$ cells showing expression of HVEM and GFP of transferred P14 cells that were isolated from the spleen of WT hosts at indicated days. (B) Representative FACS plots pregated on live cells (left panel) and total number (right panel) of HVEM overexpressing or control transferred CD45.1$^+$ P14 cells in the spleen of WT hosts at 7, 14, and 28 dpi. (C) Frequency of transferred cells in the liver at 7, 14, and 28 dpi. (D) Frequency of IFNγ- (upper) and TNFα- (lower) producing transferred P14 cells in the spleen after in vitro restimulation with gp33 peptide. (E) Representative FACS plots gated on CD45.1$^+$ CD8$^+$ cells showing KLRG-1 and CD127 expression and bar graph showing the frequency of SLEC and MPEC overexpressing HVEM or control P14 cells in the spleen at indicated dpi. (F-H) CD45.1$^+$ gp33-specific CD8$^+$ T cells (1×10^4 P14 cells) transfected with either HVEM-GFP or control GFP retrovirus were transferred into OT-I mice (n = 5/group), which were infected the next day with 1×10^6 pfu LCMV Cl13. (F) Frequency of HVEM overexpressing or control CD45.1$^+$ P14 cells in the blood of OT-I hosts over time. Frequency of retrovirally transfected P14 cells in the spleen and liver (G) and total numbers in the spleen (H) of host mice 62 dpi. Errors bars indicate mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed Student *t* test). Data are representative of two independent experiments with at least four mice per group.

expression vector pMYs-IRES-GFP upstream of IRES. Retroviruses were produced in Phoenix-eco packaging cell line, kindly provided by Garry Nolan (Stanford University, California, USA), by transfection of cells with pMYs-HVEM-IRES-GFP (rvHVEM-GFP) or pMYs-IRES-GFP vector (rvGFP) as a control.

Generation of HVEM overexpressing CD8$^+$ T cells

Retrovirus transduced CD8$^+$ T cells were generated as described [50]. One day after transduction, overexpressing GFP$^+$ cells were FACS sorted with BD FACS Aria III (BD Bioscience) and adoptively transferred into WT animals.

Adoptive T-cell transfer

HVEM expression on virus-specific T cells during LCMV infection was assessed by transfer of 1×10^4 MACS-sorted splenic CD8$^+$ T cells from P14 TCR-transgenic mice or 5×10^5 MACS-sorted splenic CD4$^+$ T cells from Smarta1 TCR-transgenic mice into LCMV WE (2000 pfu)-infected CD45.2$^+$ WT mice (1 d.pi.). Kinetics of overexpressing virus-specific CD8$^+$ T cells during an acute infection were examined by adoptive transfer of 1×10^4 retrovirally transduced CD8$^+$ T cells into naïve CD45.2$^+$ WT mice, which were infected with 2000 pfu LCMV WE the next day. To study the effector response of overexpressing CD8$^+$ T cells during chronic infection, 1×10^4 retrovirally transduced CD8$^+$ T cells were transferred into CD45.2$^+$ OT-I mice, which were infected with 1×10^6 pfu LCMV Cl13 the next day.

Generation of BMDCs

BMDCs were obtained by differentiating BM cells isolated from the hind legs of WT or HVEM$^{-/-}$ mice in vitro for 7 days with 20 ng/mL GM-CSF (Peprotech). Cells were frozen in liquid nitrogen until use.

Coculture

Frozen BMDCs were thawed and cultured overnight. On the next day, cells were counted and seeded at a concentration of 4×10^4 cells in 96-well plates. Gp33 or its APLs A4Y or V4Y (Mimotopes, Victoria, Australia) were added at indicated concentrations. MACS-sorted 5×10^4 splenic P14 transgenic CD8$^+$ T cells were added and cocultured for 1, 2, or 3 days. All T-cell assays comply to the guidance provided by MIATA.

LCMV ELISA

For coating of ELISA plates, LCMV Cl13-enriched medium (FBS-free) was added to wells, irradiated with 900,000 μj/cm^2 with a Spectrolinker XL-1500 UV cross-linker and incubated overnight at 4°C. ELISAs were performed according to standard protocols using alkaline phosphatase (AP)-conjugated anti-IgG2a and anti-IgG2b antibodies (Southern Biotech). AP *p*-nitrophenyl phosphate (Sigma-Aldrich) was added to wells and the absorbance was measured at 405 nm.

Determination of virus titer

Viral titers were determined on MC57 cells as previously described [51]. Detection limit (DtL) correlates to 50 plaques per organ or milliliter blood. For samples with a PFU below the DtL, the value was arbitrarily set as 10 pfu.

Cell preparation

Blood samples were collected in Heparin (Liquemin, 25 000 U.I./5 mL) and subsequently treated with Ammonium-Chloride-Potassium (ACK) lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA dissolved in ddH_2O) to remove erythrocytes. Single-cell suspension of the spleen was obtained by smashing organs through a 70 μm cell strainer followed by lysis of erythrocytes. Liver was minced and smashed through a 70 μm cell strainer. After centrifugation, the cell pellet was resuspended in PBS and centrifuged at 20*g* for 5 min at room temperature to exclude hepatocytes. The upper layer was transferred into a new tube, underlayed with lympholyte-M solution (Cedarlane), and centrifuged at 836g for 20 min without breaks. Before FACS staining, spleen and liver cells were filtrated through a 35 μm cell strainer. Mice were perfused with PBS prior to lung harvest. Organ was minced with a gentle MACS (Miltenyi Biotec) and digested with 600 U/mL Collagenase IV (Worthington) and 200 U/mL DNase I (Sigma-Aldrich) for 30 min at 37°C. Tissue was homogenized with gentle MACS and passed through a 70 μm cell strainer. RBCs were removed by ACK and single-cell suspension was filtered through a 35 μm cell strainer.

Reagents

$Gp_{33\text{-}41}$ peptide (KAVYNFATM) and $gp_{61\text{-}80}$ (GLNGPDIYKGVYQFKSVEFD) peptides were purchased from Mimotopes. PE-conjugated gp33-MHC class I tetramer (H-2Db/gp33-41) was kindly provided by the NIH tetramer core facility. For exclusion of dead cells, efluor780 (eBioscience) or ZombieAqua (BioLegend) was used. Antibodies used were as follows: PD-1-FITC (J43; Thermo Fisher Scientific), HVEM-APC (LH1; eBioscience), CD19-APC-Cy7 (6D5; BioLegend), CD4-PE (RM4-5; BD Bioscience), CD4-PerCP-Cy5.5 (RM4-5; BioLegend), CD8a-FITC (53-6.7, eBioscience), CD8a-PE-Cy7 (53-6.7; BioLegend), CD8a-APC-Cy7 (53-6.7; BioLegend), CD8a-PE (53-6.7; BD Bioscience), IFNγ-APC (XMG1.2; BioLegend), CD127-APC (SB/199; BioLegend), CD25-PE (PC61; eBioscience), Ki-67-PE

(16A8; BioLegend), KLRG-1-PerCP-eFluor710 (2F1; eBioscience), CD62L-BV421 (MEL-14; BioLegend), CD45.1-PerCP-Cy5.5 (A20; BioLegend), TNFα-PE (MP6-XT22; BD Bioscience), and TNFα-FITC (MP6-XT22; BioLegend).

Flow cytometry

Prior to surface staining with antibodies, Fc-γ receptors were blocked by incubating single-cell suspension with anti-CD16/CD32 mAb (2.4G2). Staining of gp33 tetramer$^+$ CD8$^+$ T cells was performed at room temperature for 15 min in the dark. Before acquisition, samples were fixed with 4% formalin at room temperature for 5 min. Ki-67 staining was performed at −20°C in 70% ethanol diluted at ratio 1:10 in PBS.

For analysis of cytokine production, spleen and liver cells were stimulated with 2 μg/mL monensin (Sigma-Aldrich), 1 μM gp33, and 1 μM gp61 peptide for 3-4 h at 37°C. Intracellular staining was performed for 20 min at 4°C with antibodies diluted in permeabilization buffer (PBS + 0.5% heat-inactivated FBS + 0.5% saponin).

Data were acquired on a BD FACSCanto II or LSRFortessa (BD Bioscience) and analyzed with FlowJo software version 9.9 (TreeStar; Oregon, USA).

All flow data adhere to the guidelines [52].

Statistics

Data are shown as average ± SD. For statistical analysis, two-tailed Student *t* test was performed using Prism 7 software (GraphPad software, CA, USA). $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

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Author's contribution: P.D. designed and performed most of the experiments, and analyzed and interpreted data. I.S., I.I., and M.M. performed specific experiments. J.K. provided conceptual input. M.M. analyzed and interpreted data. M.K. conceived, designed, and supervised the research program. P.D., M.M., and M.K. wrote the manuscript.

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Abbreviations: **AP**: alkaline phosphatase · **BTLA**: B and T lymphocyte attenuator · **BMDCs**: BM-derived DCs · **Cl13**: clone-13 · **dpi**: days post infection · **DtL**: detection limit · **HVEM**: Herpes virus entry mediator · **KLRG-1**: Killer cell lectin-like receptor G-1 · **LCMV**: Lymphocytic choriomeningitis virus · **LT-α**: lymphotoxin alpha · **MPEC**: memory precursor effector cells · **SLEC**: short-lived effector cells · **TNFR**: TNF family of receptors · **Tfh**: T-follicular helper · **VSV**: vesicular stomatitis virus

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