Significant Involvement of Herpesvirus Entry Mediator in Human Esophageal Squamous Cell Carcinoma

A running title: HVEM in Esophageal Cancer

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Abstract

BACKGROUND: Herpesvirus entry mediator (HVEM) is known to regulate immune response and to be expressed in several human malignancies. However, the precise role of HVEM in human cancer biology remains unknown. This study aimed to clarify clinical importance of HVEM in human esophageal squamous cell carcinoma (ESCC) and also its *in vivo* functions. **METHODS:** We evaluated HVEM expression in 103 patients with ESCC to explore its clinical relevance and prognostic value. The functions of HVEM in tumors were analyzed *in vitro* and *in vivo* by using siRNA silencing technique. **RESULTS:** HVEM expression was significantly correlated with depth of the invasion and lymph node metastasis. Furthermore it was inversely correlated with tumor-infiltrating $CD4^+$, $CD8^+$, and $CD45RO^+$ lymphocytes. Importantly, the HVEM status was identified as an independent prognostic marker. HVEM gene silencing significantly inhibited cancer cell proliferation *in vitro* and cancer growth *in vivo*. This antitumor effect was associated with the reduced cell proliferation activity. The effect was also correlated with induction of $CD8^+$ cells and upregulation of local immune response. **CONCLUSIONS:** HVEM plays critical roles in tumor progression and evasion of host antitumor immune responses possibly through direct and indirect mechanisms. Therefore, HVEM may be a promising therapeutic target for human esophageal cancer.

KEYWORDS: esophageal squamous cell carcinoma; herpesvirus entry mediator (HVEM); prognosis; immunotherapy

INTRODUCTION

Esophageal cancer is one of the most difficult gastrointestinal malignancies to treat and cure.[1,2] Patients often experience distant metastasis or local recurrence even after curative resection.[1] Although multimodality approaches based on surgery combined with preoperative chemotherapy and/or radiotherapy has been attempted, efficacy of these treatments is limited, and the overall survival remains poor.[3,4] Therefore, novel strategies against esophageal cancer need to be developed and established to improve patients' prognosis.

Herpesvirus entry mediator (HVEM; TNFRSF14) is a member of the TNF receptor superfamily, which is expressed on several types of cells, including T cells, B cells, NK cells, dendritic cells, and myeloid cells, as well as non-lymphoid organs including lung, liver, and kidney.[5,6] HVEM is a ligand for the Ig superfamily members B and T lymphocyte attenuator (BTLA) and CD160, and is also a receptor for the TNF superfamily members LIGHT and LTα.[7-10] Ligation of HVEM by LIGHT promotes T cell proliferation and cytokine production by initiating activation of the prosurvival transcription factor NF-κB.[6,11] By sharp contrast, HVEM engagement of BTLA and CD160 activates inhibitory signaling in T cells, resulting in decreased T-cell proliferation and cytokine production.[9,10,12] Therefore, HVEM is known to display a dual functional activity for T cell activation depending on the ligands engaged. However, the inhibitory function of HVEM may be dominant over its co-stimulatory activity as demonstrated by enhanced activation of T cells in HVEM-deficient mice.[12] Furthermore,

HVEM-deficient mice have been shown to be more susceptible to concanavalin A-mediated T cell-dependent autoimmune hepatitis and myelin oligodendrocyte glycoprotein peptide-induced experimental autoimmune encephalomyelitis.[12] To date, many studies have focused on HVEM pathway in several diseases, such as autoimmune disease, infection, inflammatory bowel disease, and transplantation.[12-15] On the other hand, the role of HVEM in tumors remains largely unknown. HVEM expression has been identified in several human tumor cell lines including colon cancer, breast cancer, and T cell leukemia, and also in actual human malignancies such as melanoma and hematopoietic malignancies.[16-19] HVEM on tumor cells has been shown to inhibit cytokine production and proliferation of tumor antigen-specific $CD8^+$ T cells via BTLA *in vitro*.[17] In addition, several murine studies have shown that blockade of HVEM/BTLA pathway augments tumor antigen-specific immune responses and inhibits tumor growth.[20-22] Others have reported that LIGHT mediates tumor cell apoptosis via signaling through tumor-expressed HVEM, leading to suppression of tumor growth.[7,18] Thus, the functions and roles of HVEM in tumors seem complex.

In this study, we hypothesized that HVEM on tumor cells might have some roles in an intractable human malignancy, esophageal squamous cell carcinoma (ESCC). Therefore, we attempted to clarify its clinical importance in human ESCC. Furthermore, we investigated the biological roles of HVEM, using RNA interference method *in vitro* and *in vivo*.

MATERIALS AND METHODS

Patients

We examined 103 patients with esophageal cancer who underwent curative esophagectomy at Department of Surgery, Nara Medical University, between 1995 and 2008. Patients had received neither chemotherapy nor radiotherapy before the operation. All esophageal cancers evaluated in this study were pathologically diagnosed as squamous cell carcinoma. Tumors were classified according to the TNM staging system.[23] The median follow-up for all patients was 25.7 months. Written informed consent was obtained from all patients according to our institutional guidelines.

Immunohistochemistry

Sections were stained using a DAKO EnVision system (DAKO Cytomation, Kyoto, Japan) as previously described.[24] As primary antibodies, anti-HVEM antibody (94804, R&D systems, Minneapolis, MN, USA), anti-CD4 antibody (4B12, DAKO), anti-CD8 antibody (C8/144B; DAKO), and anti-CD45RO antibody (UCHL1; DAKO), and anti-FoxP3 antibody (ab22510; Abcam, Tokyo, Japan) were employed. Sections were incubated with primary antibodies overnight at 4°C. For the staining of mouse tissue, anti-Ki67 antibody (SP6, Spring Bioscience, Fremont, CA, USA), anti-CD4 antibody (Novus Biologicals, Littleton, CO, USA) and anti-CD8 antibody (Novus Biologicals) were used and incubated overnight at 4°C. The

staining was performed using the VectaStain ABC kit (Vector Laboratories). Immunohistochemistry for HVEM was evaluated by authorized pathologists who had no knowledge of the patients' clinical status. At least 1,000 tumor cells were scored, and percentage of tumor cells showing positive staining was calculated. To count each T cell subset, three randomly selected areas were counted, and an average number was scored.

Animal and Cell Line

Female BALB/c mice (5-week-old) were obtained from CLEA JAPAN (Tokyo, Japan). All experiments were conducted under a protocol approved by our institutional review board. The human ESCC cell lines, TE-1 and TE-6, and a murine colon adenocarcinoma cell line, Colon 26, were obtained from RIKEN BioResource Center.

Extraction of Total RNAs and Real-time Reverse Transcriptase PCR Analysis

Total RNA was isolated using RNAspin Mini (GE Healthcare, UK, Ltd.), and cDNA was synthesized using a ReverTra Ace qPCR RT Kit (TOYOBO). For real-time reverse transcriptase PCR analysis, cDNA was amplified in TaqMan Fast Universal PCR Master Mix (Applied Biosystems) with gene-specific primers and probes on the StepOnePlus Real-Time PCR System (Applied Biosystems). The expression level of the housekeeping gene, β_2-microglobulin was measured as an internal reference.

Preparation of Cell Lysates and Western Blot Analysis

We resolved the cell lysates in SDS-polyacrylamide gels and transferred them onto polyvinylidene difluoride membranes (Millipore, Ltd.). Anti-human HVEM antibody (94804; R&D systems) and anti-mouse HVEM antibody (R&D systems) were employed. The membranes were incubated with the indicated primary antibody overnight at 4°C, and then incubated with horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology). We detected peroxidase activity on X-ray films using an enhanced chemiluminescence detection system.

siRNA Transfection of HVEM

For transfection analyses, TE-1 and TE-6 cells were transfected either with control RNA or with 80 nmol/L of siRNA of HVEM. Colon 26 cells were also transfected either with control RNA or with 20 nmol/L of siRNA of HVEM. Transfections were carried out using the Lipofectamine system (Invitrogen). The human HVEM siRNA duplexes, generated with 3'-dTdT overhangs and prepared by QIAGEN, were chosen against the DNA target sequence as follows: 5'-CACCTACATTGCCCACCTCAA-3'. For the mouse HVEM, the DNA target sequence was as follows: 5'-CTGTATGTGCTGACTGCCTAA-3'.

Cell Viability Assay and Cell Cycle Analysis

Cell viability was determined using the Cell-titer 96 aqueous one solution cell proliferation assay kit (Promega Corporation, Madison, WI, USA). The absorbance at 490 nm was recorded. Analysis of the cell cycle was performed using the CycleTEST™ PLUS DNA Reagent Kit (BD Biosciences). The cellular DNA content of at least 20,000 cells was analyzed using FACSCalibur instrument (Becton-Dickinson, Franklin Lakes, NJ) and the proportion of cells in the different phase of the cell cycle was determined using the CellQuest™ software (Becton Dickinson).

Animal Experimental Protocol

In *in vivo* model, Colon 26 cells were subcutaneously inoculated in the lower flank region of mice. Treatment was started 3 days after tumor implantation when a small palpable lump was evident. As described previously,[25] we locally injected either control RNA or HVEM siRNA with AteloGene Local Use (Koken Co.) twice a week for 2 weeks. The tumor volume was calculated according to the following formula: $V = A \times B^2/2$ (mm^3), where A is the largest diameter (mm) and B is the smallest diameter (mm).

Statistical Analysis

Categorical variables were presented as numbers and percentages, and groups were compared

using the chi-square or Fisher's exact test. Continuous variables were expressed as means and standard errors, and were compared using the *t* test. The survival curves were calculated by the Kaplan-Meier method and were analyzed by the log-rank test. A multivariate survival analysis was performed using the Cox proportional hazard model. A P value < 0.05 was considered statistically significant.

RESULTS

HVEM Expression in Human ESCC

We first compared the relative expression of HVEM between ESCC tissues and non-cancer tissues using available frozen tissues. Real-time PCR analysis showed that ESCC tissues expressed much higher levels of HVEM mRNA than non-cancer tissues ($P < .001$; Fig. 1A). Furthermore, the HVEM expression of cancer tissues was consistently higher than that of non-cancer tissues in each individual esophageal cancer patient (Fig. 1A). We next examined the HVEM expression in actual ESCC tissues by immunohistochemistry. Positive staining for HVEM was seen both on the cell membrane and in the cytoplasm of cancer cells in 91 out of 103 patients (88.3%, Fig. 1B). The mean percentage of HVEM-positive tumor cells was 42.8%. In non-cancer tissues, some mononuclear cells were also positive for HVEM.

Clinicopathological Significance of HVEM Expression in Human ESCC

To further investigate the clinical relevance of HVEM expression, all specimens were classified into two groups according to percentage of HVEM-positive tumor cells as follows: 42 tumors (40.8%) with high expression (≥ 50% of HVEM-positive tumor cells) and 61 tumors (59.2%) with low expression (< 50% of HVEM-positive tumor cells; Fig. 1B). We then evaluated the correlation of the HVEM status with various clinicopathological findings (Table 1). The tumors with high HVEM expression were significantly larger in size than the tumors with low HVEM expression ($P < .001$). Furthermore, the tumors with high HVEM expression had a significantly deeper invasion of the wall and more common lymph node metastasis ($P < .001$). Thus, our data suggested that tumor-expressing HVEM might be involved in cancer progression in human ESCC.

Prognostic Importance of HVEM Expression

We then investigated the prognostic value of HVEM expression. The 5-year survival rate was significantly lower in patients with the HVEM-high tumor than in patients with the HVEM-low tumor (18.3% vs 49.6%, $P < .001$; Fig. 1C). Furthermore, the multivariate analysis showed that the HVEM status as well as tumor status, nodal metastasis and distant metastasis was identified as an independent prognostic factor ($P = .041$; Table 2). Taken together, HVEM on tumor cells might play a critical role and also be a promising potential therapeutic target for ESCC.

Inverse Correlation between HVEM Expression and TILs

Since HVEM/BTLA/CD160 pathways are known to inhibit T cell function, we evaluated the tumor-infiltrating lymphocytes (TILs) by immunohistochemistry to clarify the correlation of the HVEM status with TILs. HVEM expression levels were inversely correlated with the number of $CD4^+$ (P = .02) and $CD8^+$ lymphocytes (P = .004; Fig. 1D). There was also a significant inverse correlation between HVEM expression and $CD45RO^+$ lymphocytes (P = .049). By contrast, no significant correlation with $FoxP3^+$ lymphocytes was observed (data not shown). Data indicated that HVEM on tumor cells might inhibit T cell infiltrations into ESCC tissues.

HVEM Silencing Inhibits The Proliferation of Esophageal Cancer Cells in Vitro

To further investigate the precise functions of HVEM under tumor environments, we next investigated the roles of HVEM expression in ESCC *in vitro*. We used human ESCC cell lines, TE-1 and TE-6, and examined the effects of HVEM down-regulation using siRNA knockdown approach. At 72 hours post-transfection, siRNA knockdown significantly reduced HVEM expressions compared with control (Fig. 2A and 2B). We then examined its role in the regulation of cancer cell proliferation by MTS assay. Cell proliferation was significantly

suppressed by HVEM gene silencing in both cells (Fig. 2C). Thus, tumor-expressing HVEM might directly play an important role in ESCC proliferation.

HVEM Silencing Induces The Cell Cycle Arrest

To reveal the underlying mechanisms in the inhibition of cell proliferation observed by HVEM knockdown, we analyzed cell cycle profiles. The cell cycle analysis showed a significant increase in the S and the G_2/M cell population in both TE-1 and TE-6 cells treated with HVEM siRNA compared to control (TE-1: control RNA vs HVEM siRNA, S; 13 ± 0.3% vs 15.4 ± 0.1%, $P = .001$, G_2/M; 22.1 ± 0.4% vs 28.9 ± 0.2%, $P < .001$, TE-6: control RNA vs HVEM siRNA, S; 12.9 ± 0.4% vs 15.2 ± 0.3%, $P = .013$, G_2/M; 22.1 ± 0.9% vs 29.7 ± 0.8%, $P = .003$; Fig. 2D). Furthermore, the percentage of apoptotic cells was measured by Annexin V/PI double staining. There was no significant difference between cells treated with HVEM siRNA or control RNA (data not shown). These data suggest that HVEM silencing might induce the cell cycle arrest but not apoptosis, leading to inhibition of cancer cell proliferation *in vitro*.

HVEM Silencing Inhibits Tumor Growth in Vivo

Next, we were intrigued with the function of HVEM under physiological conditions. We employed a murine colon cancer cell line, Colon 26, for *in vivo* analysis, since no murine

esophageal cancer cell line was available. First, we examined the *in vitro* effect of HVEM silencing in Colon 26. Similarly to human ESCC cells, siRNA knockdown significantly reduced HVEM expression (Fig. 3A). Furthermore, HVEM silencing significantly inhibited cell proliferation *in vitro* (Fig. 3B). In contrast to human ESCC cells, HVEM silencing induced a significant increase in the G_1 cell population (control RNA vs HVEM siRNA, 39.7 ± 1.3% vs 58.8 ± 0.1%, $P < .001$; Fig. 3C). We then evaluated the *in vivo* effect of HVEM silencing. Colon 26 cells were subcutaneously inoculated on syngeneic BALB/c mice and treated with control or HVEM siRNA. HVEM expressions were successfully down-regulated by *in vivo* HVEM siRNA transfection (Fig. 3D and 3E). Interestingly, HVEM down-regulation significantly inhibited tumor growth (Fig. 3F). Furthermore, the percentage of the necrotic area was significantly higher in tumors treated with HVEM siRNA than control (35.2 ± 10% vs 10.2 ± 3.1%, $P = .038$; Fig. 3G and 3H). In addition, we examined the proliferation activity of tumor cells by Ki67 staining. Percentage of Ki67-positive cells was significantly decreased in tumors treated with HVEM siRNA compared to control (34.5 ± 0.2% vs 64.8 ± 2.7%, $P < .001$; Fig. 4A).

HVEM Silencing Enhances $CD8^+$ Lymphocyte Recruitment and Local Immunity

Finally, we evaluated TILs by immunohistochemistry in this *in vivo* model. As a result, $CD8^+$ but not $CD4^+$ lymphocytes infiltrating into the surrounding area of the tumor were

significantly more abundant in tumors treated with HVEM siRNA than control (Fig. 4B). Then, we analyzed local immune status in tumors. The expression levels of IFN-γ and IL-2 were significantly higher in tumors treated with HVEM siRNA (Fig. 4C). Data indicated that HVEM blockade not only directly reduced cancer cell proliferation, but also promoted $CD8^+$ infiltration into tumors and enhanced local immune response, thereby resulting in the inhibition of tumor growth *in vivo*.

DISCUSSION

Tumors evade immune surveillance by expressing several ligands that engage inhibitory T-cell receptors and dampen T-cell functions within the tumor microenvironment.[26, 27] PD-L1/PD-1 pathway is known to be one of major negative regulatory pathways in tumor immunity. We and others have reported that tumor-expressing PD-L1 is correlated with adverse clinicopathological features and has an independent prognostic value in several human cancers, including esophageal cancer.[26, 28-30] Furthermore, targeting this pathway is currently under investigation in clinical trials.[31,32] However, the clinical efficacy seems to be limited. Therefore, there is still need to explore other novel therapeutic target. In this study, we have addressed the clinical significance and functional role of a recently discovered immunoinhibitory ligand HVEM in esophageal cancer.

There are relatively few studies on HVEM in cancer biology. Derré et al have shown that HVEM on melanoma cells inhibited IFN-γ production and proliferation of tumor-specific CD8$^+$ T cells via BTLA *in vitro*, suggesting that inhibitory interactions of HVEM-BTLA may play a role for evasion of host antitumor immunity.[17] To date, however, the role of HVEM in actual human cancer remains largely unknown. In this study, we first confirmed the overexpression of HVEM in human ESCC tissues. We further found that the tumor with higher HVEM expression had more advanced features. Importantly, the multivariate analysis identified the tumor-expressing HVEM status as an independent prognostic factor. Then, we analyzed the correlation of the HVEM status with TILs. It is widely recognized that TILs play some roles in inhibiting tumor progression and recurrence, and have prognostic significance in several human malignancies, including esophageal cancer.[33,34] More recently, our and other studies have shown that CD45RO$^+$ for memory T cell may be a better prognostic marker in human esophageal cancer.[24,35] As a result, we found that HVEM expression levels were inversely correlated not only with tumor-infiltrating CD4$^+$ and CD8$^+$ T cells but also CD45RO$^+$ memory T cells. Interestingly, recent studies have suggested that HVEM regulates the generation and maintenance of memory T cells.[36,37] Taken together, HVEM on tumor cells may play a critical role in evasion of host antitumor immune responses and contribute to tumor progression. Therefore, these data further emphasized that HVEM could be a promising target for novel cancer therapy against human ESCC.

Besides the immunological roles of HVEM, it functions as either ligand or receptor in diverse physiological and pathological processes. Recent studies have demonstrated that BTLA, CD160, and glycoprotein D function as activating ligands for HVEM, promoting NF-κB activation and cell survival via HVEM in lymphoid and epithelial cells.[15,38] However, to our knowledge, there is no study to address the direct effect of HVEM in cancer cell survival. We examined the biological mechanisms of HVEM on tumor cells by using siRNA method, and found several important observations. First, cell proliferation was significantly inhibited by HVEM gene silencing in human ESCC cells and murine colon cancer cell. Second, HVEM silencing induced the cell cycle arrest but not apoptosis *in vitro*. While HVEM silencing induced the G_2/M arrest in human ESCC cells, it mediated the G_1 arrest in a murine colon cancer cell. These differences may be due to p53 status in these cells. p53 is a key regulator of both G_1/S phase and G_2/M phase.[39] Both human ESCC cells retain mutated p53, and Colon 26 cell has wild-type p53.[40] Thus, HVEM silencing may induce differential cell cycle alterations, depending on p53 status. However, underlying molecular mechanisms of HVEM silencing-mediated cell cycle arrest are still unclear. Further fundamental studies are therefore needed. Nevertheless, our data indicated that HVEM silencing suppresses cell proliferation through the induction of the cell cycle arrest. Taken together, our data indicates that HVEM might be directly involved in cancer cell proliferation.

Finally, we investigated the functional role of HVEM in tumor *in vivo* under physiological condition. As a result, HVEM blockade induced by local injection of siRNA significantly inhibited tumor growth of Colon 26 in syngeneic immunocompetent mice. We also found that HVEM blockade significantly inhibited the proliferation of tumor cells *in vivo*. Thus, HVEM blockade had a direct antitumor effect on tumor cells *in vivo*. We also found that HVEM blockade significantly induced the infiltration of $CD8^+$ TILs. Furthermore, IFN-γ and IL-2 were significantly upregulated in tumors treated with HVEM siRNA. Several recent studies showed that blockade of HVEM/BTLA pathways using soluble BTLA or the vaccine fused to glycoprotein D enhanced tumor-reactive T-cell activation and led to tumor regression or tumor growth inhibition.[20-22] In addition, the blockade of HVEM/BTLA interactions increased levels of IFN-γ and IL-2 in the tumor microenvironments.[21] Our data may further corroborate these previous findings. Thus, HVEM blockade might have an indirect antitumor effect induced by the inhibition of T cell negative pathway. These direct and indirect effects of HVEM blockade on tumor are not mutually exclusive and may be synergistic.

In conclusion, we have shown for the first time that higher HVEM expression is correlated with advanced features of human cancer and fewer TILs, and that HVEM is an independent prognostic marker in human ESCC. Furthermore, HVEM contributes to cancer cell proliferation and impairs antitumor immune responses. Importantly, HVEM blockade has

a significant antitumor effect under physiological condition. Therefore, this study may provide the rationale of developing a novel cancer therapy targeting HVEM for human malignancy.

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FIGURE LEGEND

Figure 1. Clinical significance of herpesvirus entry mediator (HVEM) expression in human esophageal squamous cell carcinoma. (A) The HVEM expression in cancer tissue was significantly higher than that in non-cancer tissue (left panel). HVEM expression in cancer tissue was consistently higher than that in non-cancer tissue of individual patient (right panel). (B) Representative case of low and high expression of HVEM. (C) Patients with HVEM-high tumors had significantly poorer postoperative survival compared with HVEM-low tumors. (D) The number of tumor-infiltrating $CD4^+$, $CD8^+$, and $CD45RO^+$ lymphocytes was significantly fewer in HVEM-high tumors compared with HVEM-low tumors. A single asterisk indicates $P < .05$; double asterisks, $P < .01$; triple asterisks, $P < .001$.

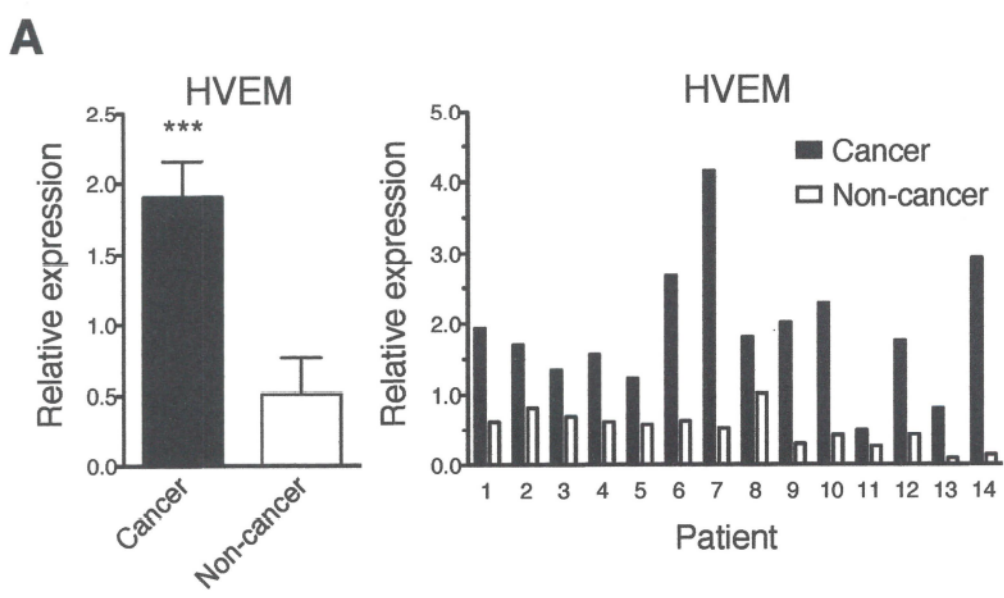
Figure 2. Downregulation of herpesvirus entry mediator (HVEM) by siRNA inhibits cell proliferation and induces the cell cycle arrest in human esophageal carcinoma cells. (A) TE-1 and TE-6 cells were transfected with control RNA or HVEM siRNA. The HVEM expression was evaluated by quantitative real-time PCR. It was strongly reduced in both cell lines, when transfected with HVEM siRNA for up to 72 hours. $n = 3 - 5$. (B) The protein expression of HVEM was effectively suppressed in both cell lines as determined by immunoblotting analysis. (C) Cell proliferation was

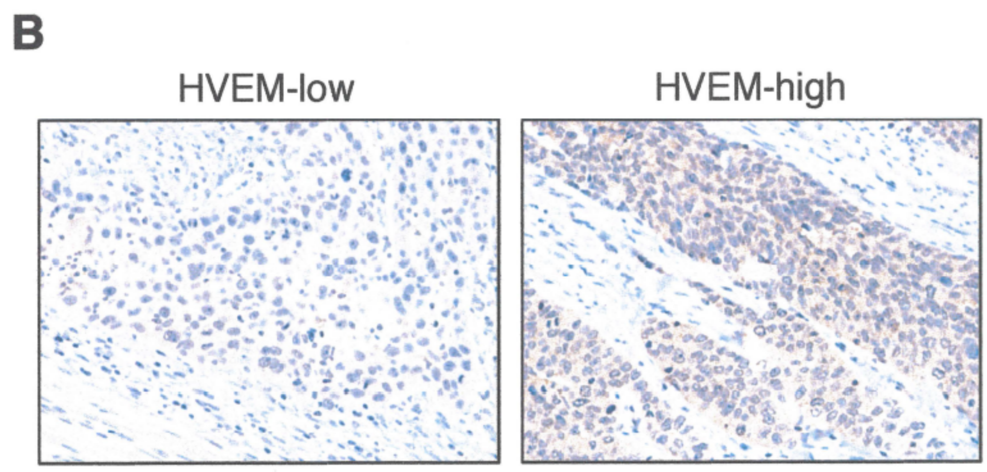
significantly inhibited in cells treated with HVEM siRNA as determined by MTS assay after 72 hours incubation ($n = 7$ of each group). (D) Cell cycle analysis indicated that HVEM silencing significantly increased the proportion of cells at S phase and G_2/M phase in both cells compared with controls ($n = 3$ of each group). A single asterisk indicates $P < .05$; double asterisks, $P < .01$; triple asterisks, $P < .001$.

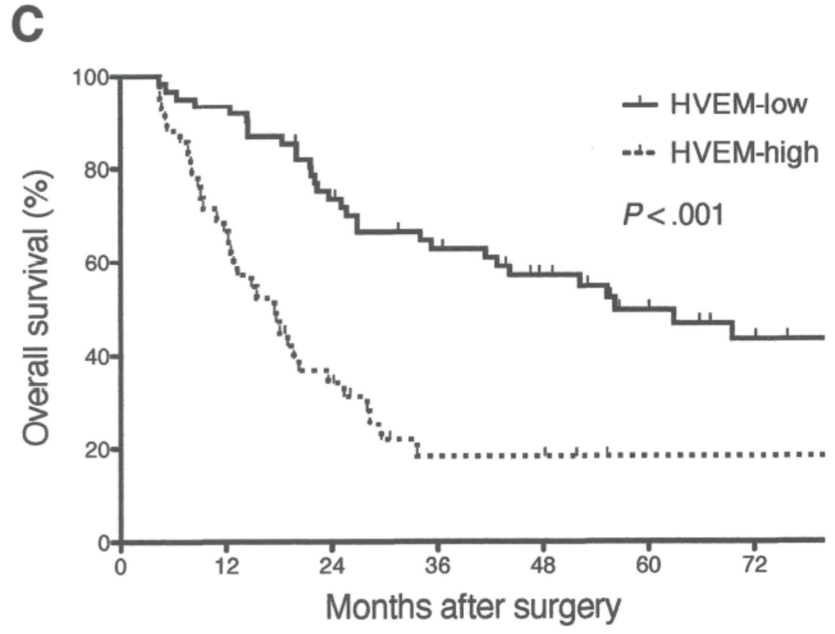
Figure 3. Herpesvirus entry mediator (HVEM) silencing inhibits tumor growth *in vivo.* (A) Colon 26 cells were transfected with control RNA or HVEM siRNA. The mRNA HVEM expression was significantly reduced, when transfected with HVEM siRNA for up to 72 hours ($n = 3$ of each group; left panel). The protein level was also effectively suppressed as determined by immunoblotting analysis (right panel). (B) Cell proliferation was significantly inhibited in Colon 26 treated with HVEM siRNA as determined by MTS assay after 72 hours incubation ($n = 7$ of each group). (C) Cell cycle analysis indicated that HVEM silencing significantly increased the proportion of cells at G_1 phase in Colon 26 ($n = 3$ of each group). (D) BALB/c mice were subcutaneously inoculated with 1×10^6 Colon 26 cells. Mice were then locally injected either control RNA ($n = 6$) or HVEM siRNA ($n = 6$) on day 3, 6, 10, and 13. Immunohistochemical staining showed that a significant decrease in HVEM expression was evident in tumors obtained from HVEM siRNA-treated mice. (E) The

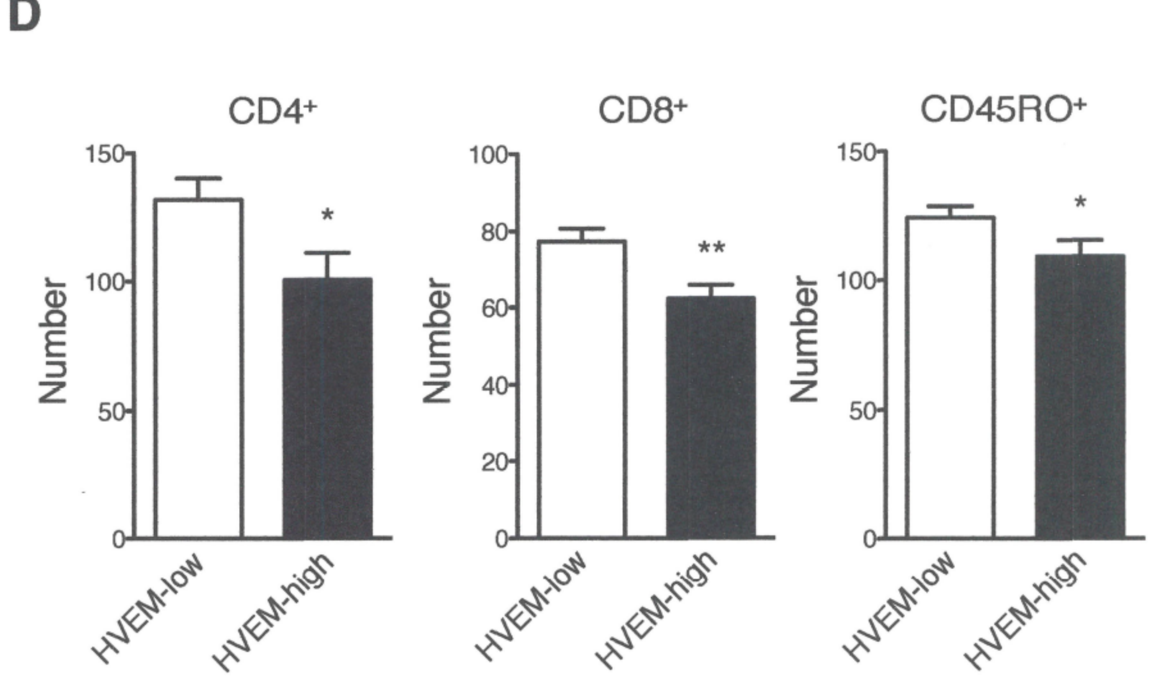
mRNA HVEM expression was reduced in tumors from HVEM siRNA-treated mice ($n = 6$ of each group). (F) Tumor growth was inhibited by HVEM blockade *in vivo.* (G and H) Histological analysis indicated that there was more extensive necrotic area in tumors obtained from HVEM siRNA-treated mice. A single asterisk indicates $P < .05$; double asterisks, $P < .01$; triple asterisks, $P < .001$.

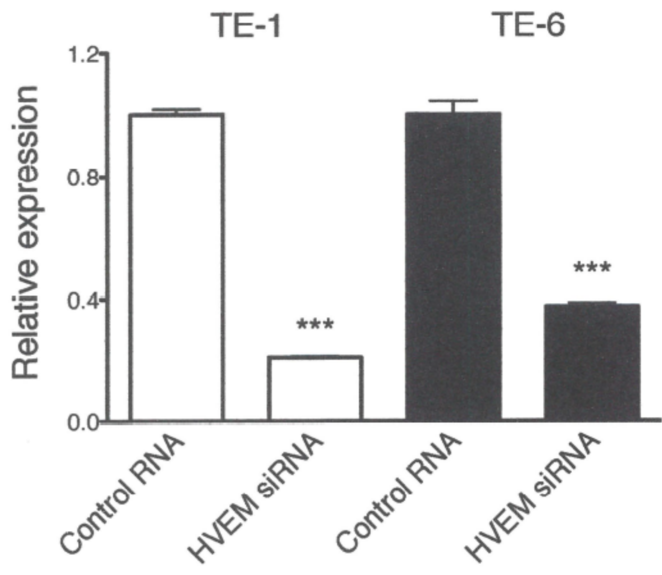
Figure 4. Inhibition of herpesvirus entry mediator (HVEM) reduces the proliferation activity of tumor cells and enhanced local immune responses *in vivo.* (A) The representative micrographs of immunihistochemical staining for cell proliferation (Ki67) in tumor tissues. A significant decrease in Ki67 staining was evident in tumors from HVEM siRNA-treated mice when compared with controls. (B) The representative micrographs of immunihistochemical staining for $CD4^+$ and $CD8^+$ lymphocytes in tumors. $CD8^+$ lymphocytes in tumors treated with HVEM siRNA were more abundant. (C) The IFN-γ and IL-2 mRNA levels was significantly increased in tumors obtained from HVEM siRNA-treated mice. Data were obtained from 5-6 mice of each group. A single asterisk indicates $P < .05$; double asterisks, $P < .001$.

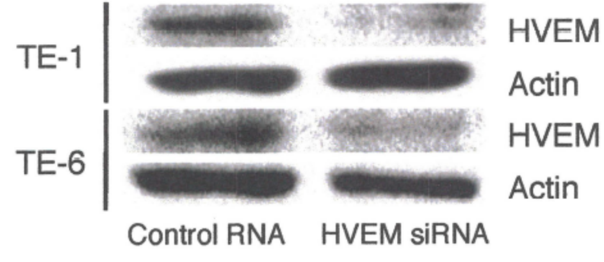

A
HVEM
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
***
Cancer
Non-cancer
HVEM
Relative expression
5.0
4.0
3.0
2.0
1.0
0.0
Cancer
Non-cancer
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Patient

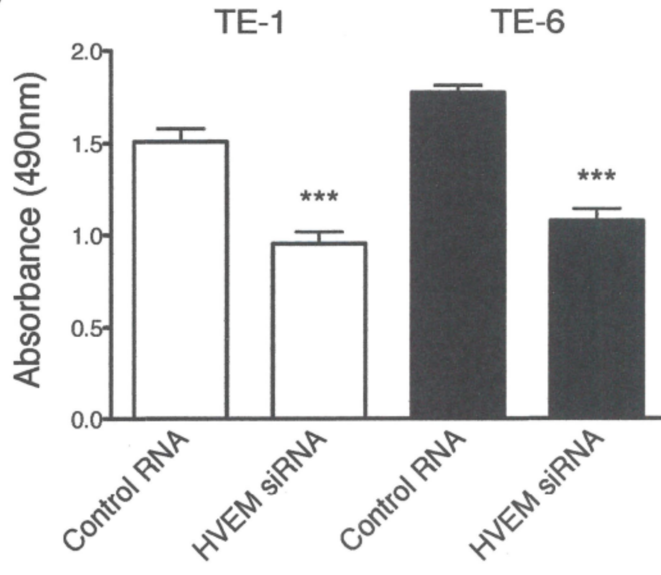

B
HVEM-low
HVEM-high

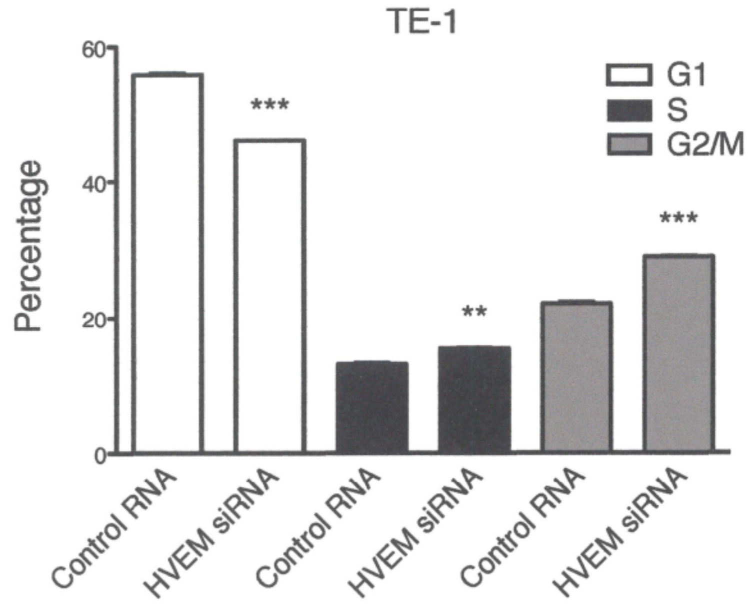

C
Overall survival (%)
100
80
60
40
20
0
0 12 24 36 48 60 72
Months after surgery
HVEM-low
HVEM-high
P < .001

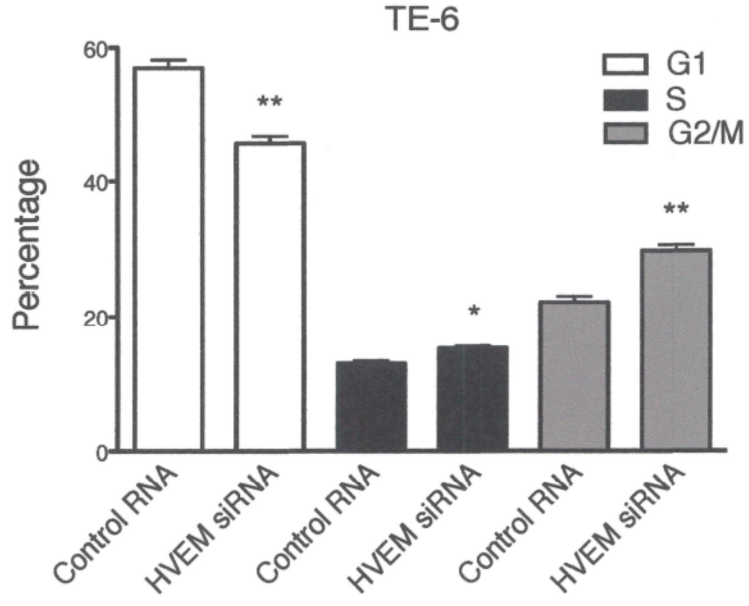

D
CD4+
Number
150
100
50
0
*
HVEM-low
HVEM-high
CD8+
Number
100
80
60
40
20
0
**
HVEM-low
HVEM-high
CD45RO+
Number
150
100
50
0
*
HVEM-low
HVEM-high


A
TE-1
TE-6
Relative expression
1.2
0.8
0.4
0.0
***
***
Control RNA
HVEM siRNA
Control RNA
HVEM siRNA

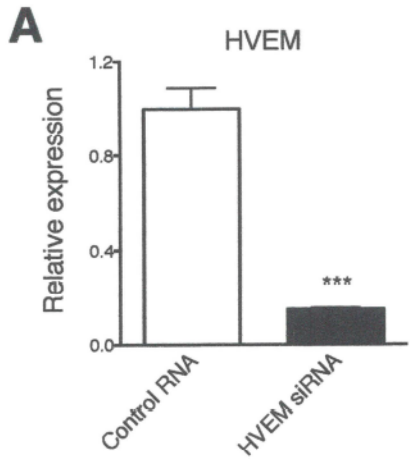

B
TE-1
TE-6
HVEM
Actin
HVEM
Actin
Control RNA
HVEM siRNA

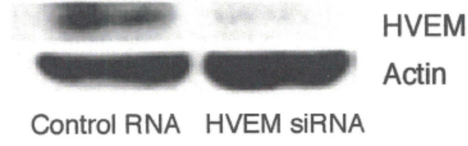

C
TE-1
TE-6
Absorbance (490nm)
2.0
1.5
1.0
0.5
0.0
***
***
Control RNA
HVEM siRNA
Control RNA
HVEM siRNA


D
TE-1
Percentage
60
40
20
0
G1
S
G2/M
***
**
***
Control RNA
HVEM siRNA
Control RNA
HVEM siRNA
Control RNA
HVEM siRNA

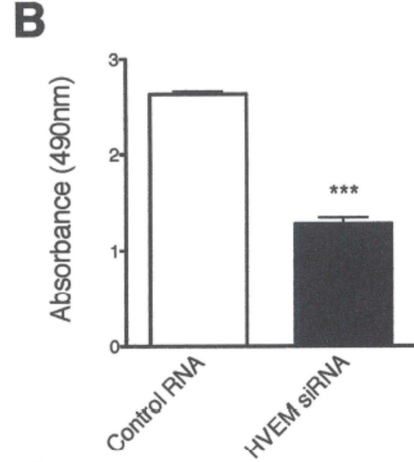
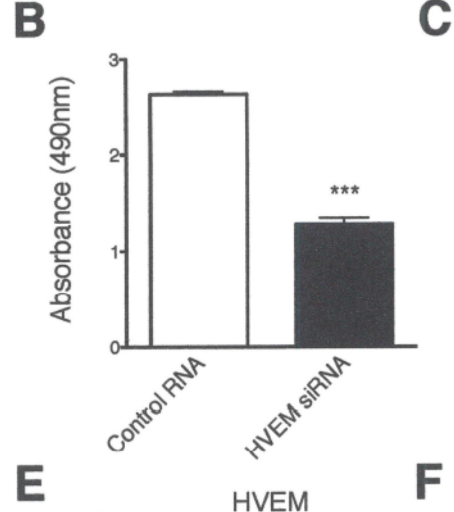

TE-6
Percentage
60
40
20
0
G1
S
G2/M
**
*
**
Control RNA
HVEM siRNA
Control RNA
HVEM siRNA
Control RNA
HVEM siRNA

A

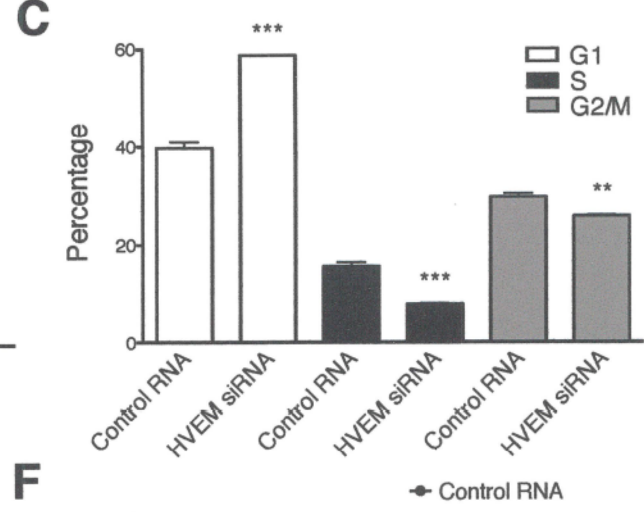




B

C

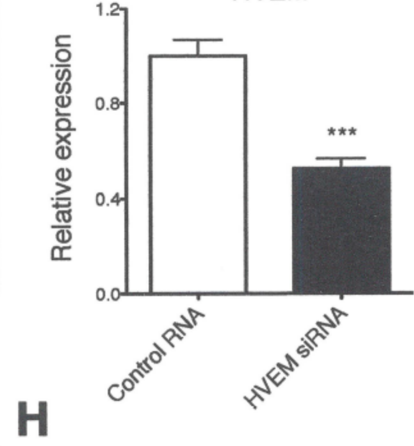


D

Control RNA

HVEM siRNA

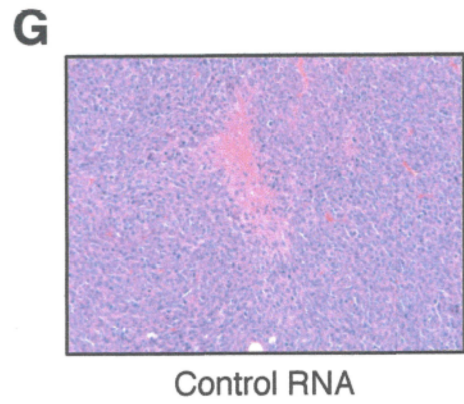
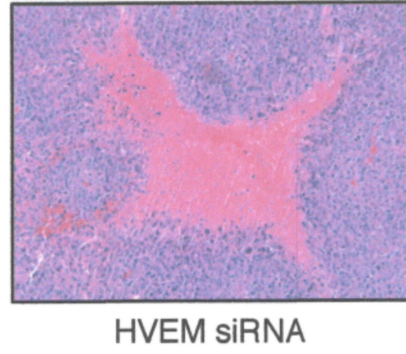
E



F

Control RNA
HVEM siRNA
Tumor volume (mm^3)
1500
1000
500
0
*
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Days after inocculation

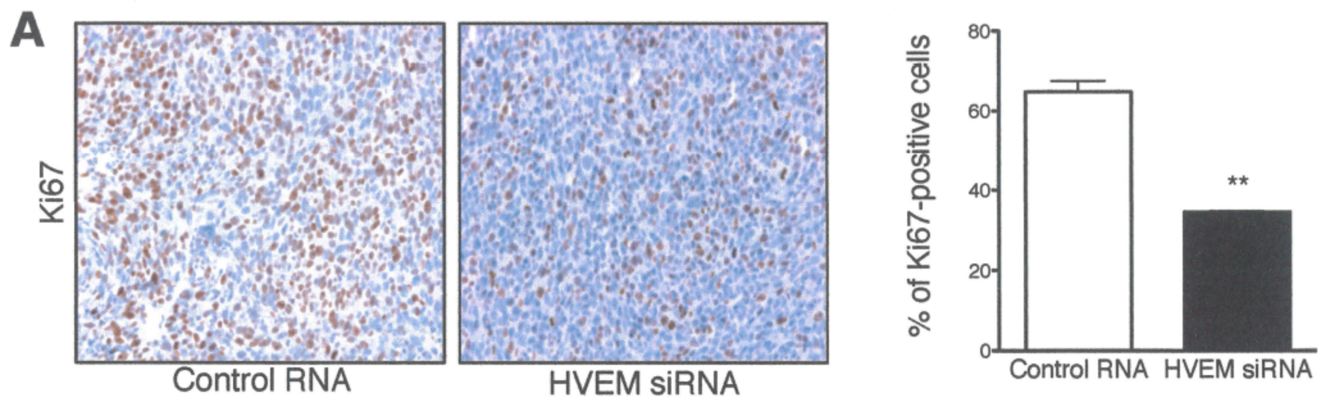
G

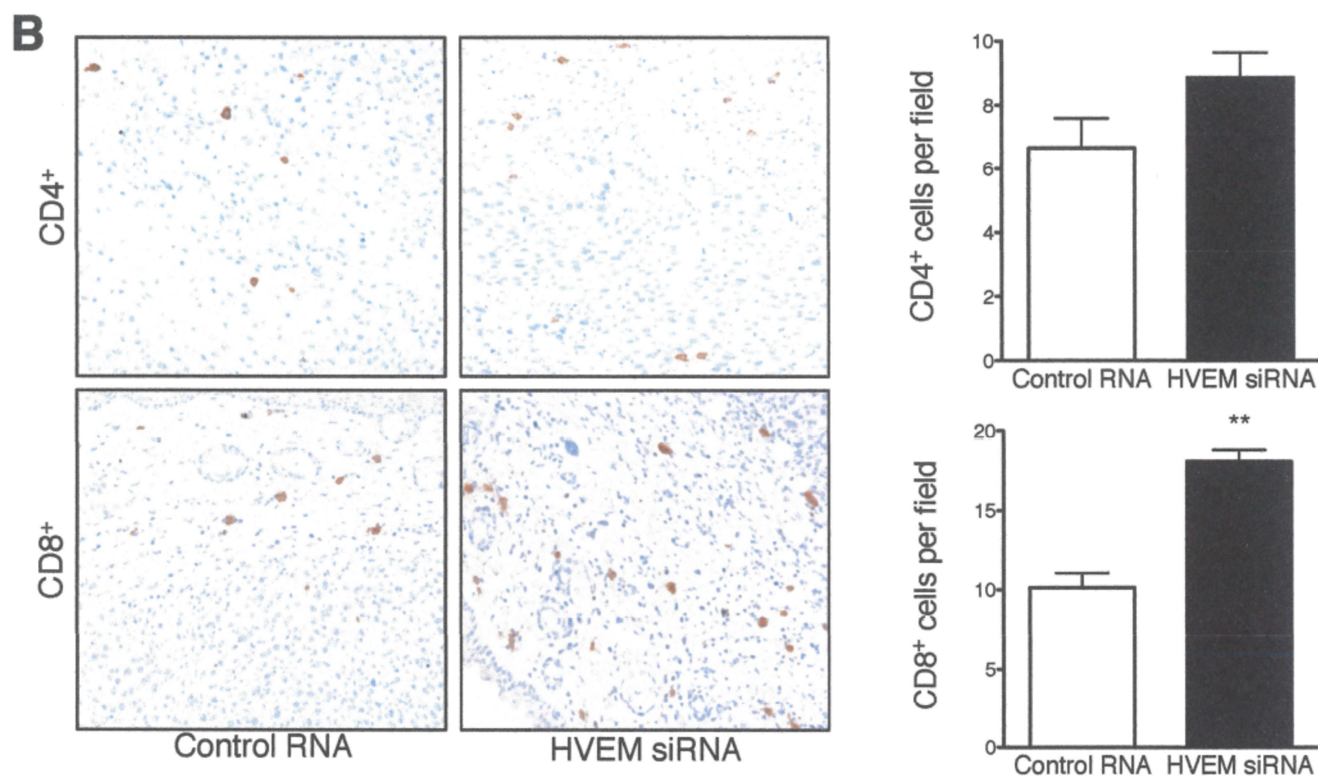



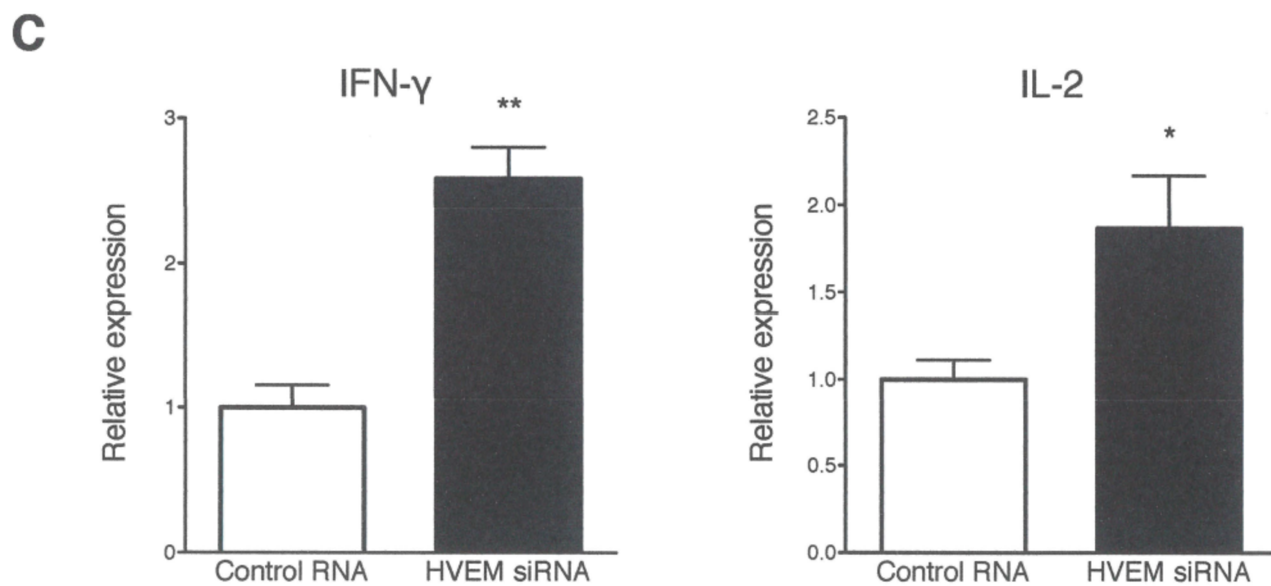
Control RNA

HVEM siRNA

H


A
Ki67
Control RNA
HVEM siRNA
% of Ki67-positive cells
80
60
40
20
0
**
Control RNA
HVEM siRNA


B
CD4+
CD8+
Control RNA
HVEM siRNA
CD4+ cells per field
10
8
6
4
2
0
Control RNA
HVEM siRNA
**
CD8+ cells per field
20
15
10
5
0
Control RNA
HVEM siRNA


C
IFN-γ
**
Relative expression
3
2
1
0
Control RNA
HVEM siRNA
IL-2
*
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
Control RNA
HVEM siRNA