

Expression of anti-HVEM single-chain antibody on tumor cells induces tumor-specific immunity with long-term memory

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Abstract Genetic engineering of tumor cells to express immune-stimulatory molecules, including cytokines and co-stimulatory ligands, is a promising approach to generate highly efficient cancer vaccines. The co-signaling molecule, LIGHT, is particularly well suited for use in vaccine development as it delivers a potent co-stimulatory signal through the Herpes virus entry mediator (HVEM) receptor on T cells and facilitates tumor-specific T cell immunity. However, because LIGHT binds two additional receptors, lymphotoxin β receptor and Decoy receptor 3, there are

significant concerns that tumor-associated LIGHT results in both unexpected adverse events and interference with the ability of the vaccine to enhance antitumor immunity. In order to overcome these problems, we generated tumor cells expressing the single-chain variable fragment (scFv) of anti-HVEM agonistic mAb on the cell surface. Tumor cells expressing anti-HVEM scFv induce a potent proliferation and cytokine production of co-cultured T cells. Inoculation of anti-HVEM scFv-expressing tumor results in a spontaneous tumor regression in CD4+ and CD8+ T cell-dependent fashion, associated with the induction of tumor-specific long-term memory. Stimulation of HVEM and 4-1BB co-stimulatory signals by anti-HVEM scFv-expressing tumor vaccine combined with anti-4-1BB mAb shows synergistic effects which achieve regression of pre-established tumor and T cell memory specific to parental tumor. Taken in concert, our data suggest that genetic engineering of tumor cells to selectively potentiate the HVEM signaling pathway is a promising antitumor vaccine therapy.

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Introduction

Herpes virus entry mediator (HVEM), also known as TNFR superfamily 14 (TNFRSF14), is a type I transmembrane protein expressed on most hematopoietic cells including B cells, T cells, NK cells, dendritic cells (DC), and myeloid cells, as well as non-lymphoid organs including lung, liver, and kidney [1]. To date, five molecules have been reported to interact with HVEM; glycoprotein D (gD) of herpes simplex virus origin, trimeric lymphotoxin alpha (LT α), LIGHT (homologous to lymphotoxins, exhibits inducible

expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed on T lymphocytes), B and T lymphocyte attenuator (BTLA), and CD160 [2–6]. Upon ligation, immune signals are delivered to HVEM and/or HVEM can induce signals through its “ligands,” employing a mechanism termed reverse signaling [7]. Signaling through HVEM recruits TNF receptor-associated factor (TRAF) 1, 2, 3, and 5, inducing activation of NF- κ B and JNK/AP-1 pathways [8]. HVEM signal mediates potent T cell co-stimulatory effects, which enhances proliferation, Th1-type cytokine production, and survival of T cells [9–11]. Consistent with these effects, upregulation of HVEM signal facilitates tumor-reactive T cell activation and leads to tumor regression as well as protective antitumor immunity [9, 12–14].

In order to adapt HVEM-mediated cancer immunotherapy for clinical use, two potential issues need to be overcome. First, HVEM is constitutively expressed on almost all naïve T cells at high levels [15]. Therefore, systemic administration of HVEM-stimulating reagents has the potential to induce non-specific T cell activation. This issue has been addressed by selective expression of HVEM-stimulating molecules on tumor cells or in the tumor microenvironment, predominantly restricting HVEM signals to tumor-reactive T cells [9, 12–14]. The second challenge is to signal selectively through HVEM, without altering other receptor/ligand interactions. For example, previous studies have exclusively applied LIGHT over-expression as a means to stimulate HVEM [9, 12–14, 16]. However, the potential of LIGHT to bind and stimulate HVEM may be neutralized by Decoy receptor 3 (DcR3), another LIGHT receptor highly expressed in various human malignancies [17–19]. In addition, LIGHT binds lymphotoxin β receptor (LT β R) besides HVEM and mediates various immunologic and non-immunologic functions including chemokine production, apoptosis, lymphoid structure formation/maintenance, liver inflammation/oncogenesis, and lipid homeostasis [20–24]. While these broad functions associated with LT β R signal could be advantageous for some aspects of antitumor immunity, they may also induce unexpected adverse effects. Thus, although activation of the HVEM co-stimulatory pathway is a promising strategy to potentiate antitumor T cell immunity, modification of current approaches is required prior to successful clinical implementation.

As a novel and improved approach to overcome potential drawbacks associated with current LIGHT/HVEM-mediated cancer immunotherapy, we designed a single-chain variable fragment (scFv) of an anti-HVEM monoclonal antibody (mAb) which selectively triggers HVEM signals. In this study, we generated tumor cells expressing anti-HVEM scFv on their cell surface and assessed their potential to induce antitumor T cell immunity and stimulate tumor-

specific memory responses. Our results indicate that anti-HVEM scFv-expressing tumor cells are a promising tumor vaccine that induces potent antitumor immunity and subsequent memory immunity specific to parental tumor.

Materials and methods

Mice

Female C57BL/6 (B6) and DBA/2 mice were purchased from the National Cancer Institute (Frederick, MD). Transgenic mice expressing TCR specific to tumor antigen P1A were originally generated by Dr. Yang Liu (University of Michigan, Ann Arbor, MI) and backcrossed with DBA/2 mice for more than 10 generations [25]. All mice were maintained under specific pathogen-free conditions and were used at 6–10 weeks of age. All animal experiments were conducted in accordance with the protocols approved by the Animal Care and Use Committee at the University of Maryland Baltimore.

Cell lines and reagents

P815 mouse mastocytoma (DBA/2, H-2^d) and L1210 lymphoma (DBA/2, H-2^d) cell lines were originally obtained from ATCC (Manassas, VA) and maintained in RPMI 1640 complete medium. Hybridoma producing anti-mouse HVEM agonistic mAb clone HM3.30 was established in our lab by immunizing Armenian hamsters with mouse HVEM protein. Anti-4-1BB mAb clone 2A was prepared as previously described [26]. Control hamster IgG and rat IgG were purchased from Rockland Immunochemicals (Gilbertsville, PA). Phycoerythrin (PE)-conjugated anti-mouse IgG Ab and ELISA kits to measure IFN- γ and IL-2 in the culture supernatants were purchased from eBioscience (San Diego, CA).

P815 cells expressing anti-HVEM scFv or control scFv

Expression vectors encoding anti-HVEM scFv or control scFv cDNA were constructed as follows. First, immunoglobulin heavy chain variable region (V_H) and light chain variable region (V_L) genes were cloned from mRNA isolated from hybridoma producing anti-HVEM mAb HM3.30 using the methods previously reported with minor modifications [27]. Anti-HVEM scFv cDNA was constructed by assembling sequences of V_L , (Gly₄-Ser)₂ linker, V_H , human IgG₁ constant region, and GPI anchor of human decay-accelerating factor (DAF). Control scFv was constructed by replacing V_H CDR3 sequence of anti-HVEM scFv with the corresponding sequence of an irrelevant hamster mAb against anti-fluorescein mAb [28]. The scFv cDNA

constructs were inserted into the pLIB retroviral expression vector (Clontech Laboratories, Inc. Mountain View, CA), and the produced retrovirus were used for transduction of P815. The cells expressing scFv were identified as human IgG Fc-positive cells and sorted by FACS Aria (BD Biosciences, San Jose, CA) to establish stable clones by limiting dilution. Binding of mouse HVEM-mouse Ig fusion protein with anti-HVEM scFv but not control scFv was assured by flow cytometry using LSR-II (BD Biosciences) and FlowJo software (Tree Star, Inc. Ashland OR).

T cell proliferation and cytokine production assay

T cells were isolated from spleen and lymph node (LN) cells of naïve B6 or P1A TCR transgenic mice by MACS cell separation method using CD90.2 MicroBeads (Miltenyi Biotec Inc. Auburn CA). Purity of CD3+ T cells was consistently >95%. Purified T cells were co-cultured with P815 expressing anti-HVEM scFv or control scFv, which had been irradiated 100 Gy prior to the culture. In case of co-culture employing P1A TCR transgenic T cells, the number of T cells was titrated so as to make T cell/tumor ratios of 5, 10, and 20. After 2–4 days of co-culture, proliferation and cytokine production in the supernatants were measured by ³H-thymidine incorporation and ELISA kits specific to IFN- γ and IL-2, respectively. ³H-thymidine was included during the last 18 h of the culture, and the incorporation was measured by Microbeta Trilux (PerkinElmer Health Sciences, Shelton, CT). In some experiments, tumor-draining LN cells (5×10^5 cells/well) were used as responding T cells in the co-culture with 100 Gy-irradiated wild-type P815 (4×10^4 cells/well) to assess IFN- γ production in culture supernatants.

Cytolytic T lymphocytes assay

Cytolytic activity of tumor-reactive T cells was examined as previously described [9]. Briefly, spleen cells or tumor-draining LN cells harvested from tumor-rejected or naïve DBA/2 mice were co-cultured with 100 Gy-irradiated wild-type P815 cells. After 4 days, cytolytic activity of the culture cells was measured by a standard 4 h ⁵¹Cr-release assay against P815 and L1210 target cells.

In vivo tumor growth and survival assay

Naïve DBA/2 mice were injected subcutaneously (s.c.) with 1×10^5 P815 expressing anti-HVEM scFv or control scFv in lateral flank. Mortality and tumor size was monitored twice a week. In some experiments, mice received intraperitoneal (i.p.) injection of 250 μ g anti-CD4 (GK1.5) or anti-CD8 (53-6.72) mAb 3 days before tumor inoculation followed by weekly injections for 5 weeks. To assess

antitumor T cell memory, DBA/2 mice which had rejected anti-HVEM scFv-expressing P815 for more than 2 months were re-challenged s.c. with 1×10^5 parental P815 cells or irrelevant L1210 in the right and left flanks, respectively. As a control, naïve DBA/2 mice were also inoculated P815 and L1210, and the tumor growth was monitored. In models to treat pre-established tumors, DBA/2 mice were first inoculated s.c. with 1×10^5 wild-type P815 in the right flank on day 0. On day 5, the mice were injected with 1×10^5 anti-HVEM scFv- or control scFv-expressing P815 in the left flank, and further treated i.p. with 150 μ g anti-4-1BB mAb or control rat IgG on day 10 and 15. In some groups, scFv-expressing tumor cells were exposed to 10 Gy irradiation prior to injection.

Immunohistochemistry

Tumor tissues were harvested 14 days after s.c. inoculation of 1×10^5 P815 expressing anti-HVEM scFv or control scFv. Tissue sections were stained with hematoxylin and eosin (H&E) or anti-CD4 or anti-CD8 mAb for immunohistochemistry using Vectastain Elite ABC kits (Vector laboratories Inc., Burlingame, CA). Tissue images were acquired by a Nikon Eclipse E600 fluorescence microscope (Nikon Instruments Inc.) equipped with a SPOT digital camera and imaging software.

Statistical analysis

Two-tailed student's *t* test was used to compare two groups. For survival data, Kaplan–Meier survival curves were prepared, and statistical differences were analyzed using the logrank (Mantel-Cox) test. *P* values <0.05 were considered significant.

Results

Expression of anti-HVEM scFv on tumor cells augments proliferation and cytokine production of tumor-reactive T cells

We generated mAbs against mouse HVEM and selected a clone HM3.30 which co-stimulated potent T cell proliferation when immobilized on culture plates together with anti-CD3 mAb (Supplementary Figure 1). In order to generate scFv of HM3.30, the DNA sequences of immunoglobulin V_L and V_H were cloned, connected by artificial linker, and further fused with human IgG₁ constant region and decay-accelerating factor (DAF)-derived GPI anchor sequence, so that this construct is presented on cell surface (Fig. 1a). We also generated control scFv in which V_H was replaced with an irrelevant sequence. Following transfection, anti-HVEM

scFv bound HVEM-Ig protein whereas control scFv did not (Fig. 1b), confirming that anti-HVEM scFv was both successfully expressed on the cell surface and retained its HVEM binding capacity. Anti-HVEM scFv expressed on tumor cells did not bind with LT β R (Supplementary Figure 2). In addition, soluble LIGHT did not interfere with the interaction between anti-HVEM scFv and HVEM, suggesting that specific binding of anti-HVEM scFv to HVEM is not disturbed by endogenous molecular interaction. Next, to assess co-stimulatory function, scFv-expressing P815 (H-2^d) were irradiated and co-cultured with T cells isolated from B6 mice (H-2^b). In this allogeneic response, T cells cultured with anti-HVEM scFv-expressing P815 showed an enhanced proliferation compared with those cultured with control scFv-expressing P815, with cluster formation of blast cells (Fig. 1c, d). In addition, IFN- γ and IL-2 production was significantly elevated when T cells were cultured with anti-HVEM scFv-expressing P815 (Fig. 1e). In order to determine the specificity of this response, T cells isolated from P1A TCR transgenic mice were co-cultured with scFv-expressing P815, which express P1A as an endogenous tumor Ag. Both proliferation and IFN- γ production significantly increased when P1A T cells were cultured with anti-HVEM scFv-expressing P815 compared with control scFv-expressing P815 (Fig. 1f). Taken together, these results indicate that tumor cells expressing the anti-HVEM scFv stimulate potent antigen-specific T cell responses in vitro.

Tumor cells expressing anti-HVEM scFv spontaneously regress in a CD4+ and CD8+ T cell-dependent fashion

We next examined the potential of anti-HVEM scFv-expressing tumor cells to activate immune responses in vivo. To this end, P815 expressing anti-HVEM scFv or control scFv were inoculated s.c. into syngeneic DBA/2 mice, and tumor growth and mouse survival were assessed. Whereas control scFv-expressing P815 grew progressively and eventually killed all mice during 20–60 days, 80% of mice inoculated with anti-HVEM scFv-expressing P815 rejected tumor and survived over 70 days (Fig. 2a, b, $P < 0.001$ between the groups in survival). In addition, tumor-draining LN cells from the mice inoculated with anti-HVEM scFv-expressing P815 exhibited significantly enhanced antitumor cytolytic activity and IFN- γ production when re-stimulated in vitro with P815 tumor, compared with those from mice inoculated with control scFv-expressing P815 (Fig. 2c, d). These results indicate that anti-HVEM scFv stimulates T cell activation in vivo and induces potent antitumor immunity leading to tumor regression.

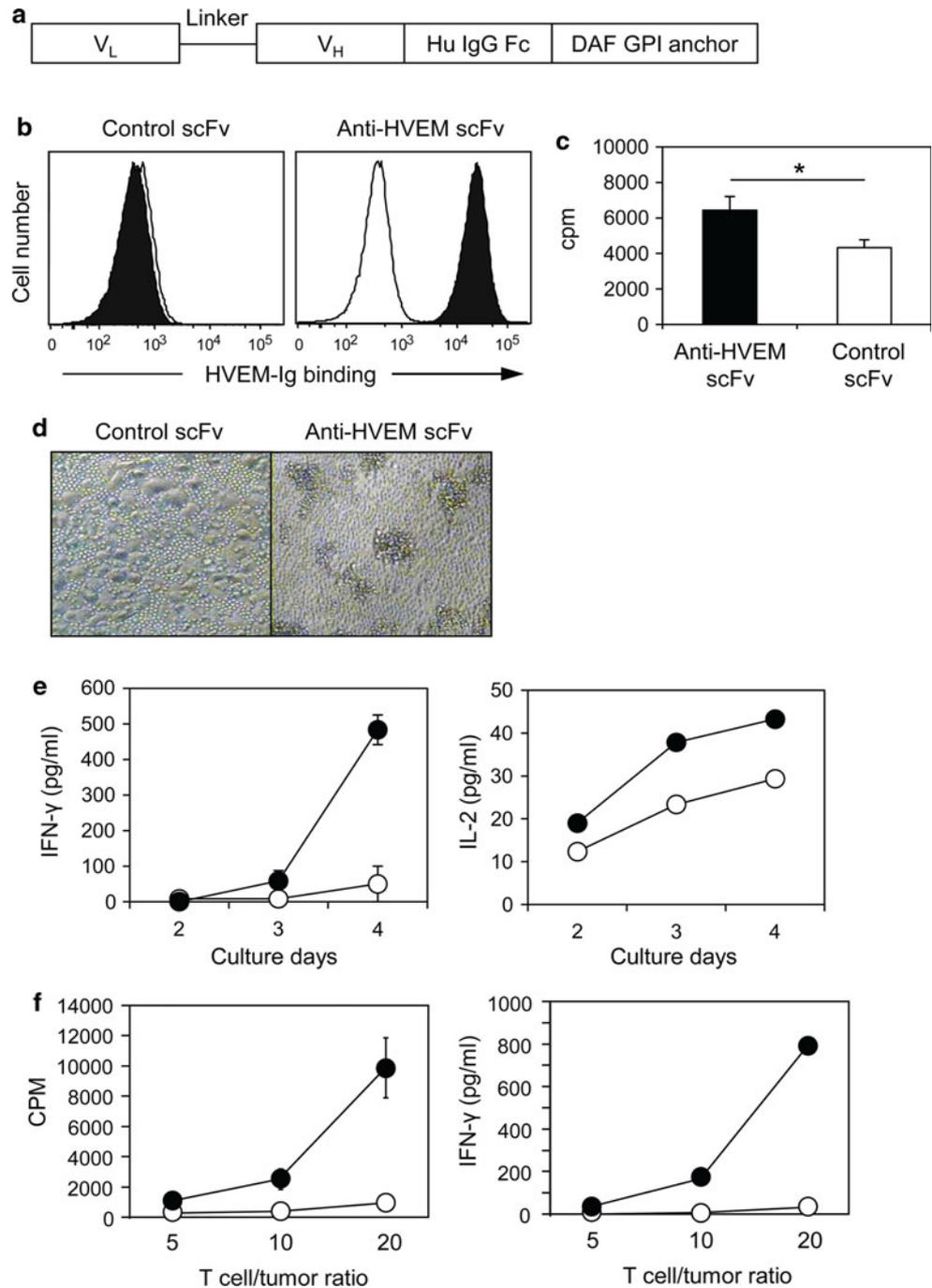
We next examined immune responses at the local site of tumor. P815 expressing control scFv or anti-HVEM

scFv were inoculated into DBA/2 mice, from which tumor tissues were harvested 14 days later and examined by H&E and immunohistochemical staining. Infiltration of lymphocytes detected inside and surrounding area of anti-HVEM-expressing P815 tumor was more vigorous than that in control scFv-expressing P815 (Fig. 3). Massive lymphocyte infiltration consisted of both CD4+ and CD8+ T cells. Therefore, we next addressed whether CD4+ T cells, CD8+ T cells, or both are required for the regression of anti-HVEM scFv-expressing tumors. Consistent with Fig. 2a, control scFv-expressing P815 grew in 100% of recipient mice while anti-HVEM scFv-expressing P815 underwent spontaneous rejection in 75% of mice (Fig. 4a, b). When CD4+ T cells were depleted, anti-HVEM scFv-expressing P815 grew progressively and eventually killed 100% of recipient mice (Fig. 4c). Similarly, outgrowth of anti-HVEM scFv-expressing P815 and 100% mortality was observed in CD8+ T cell-depleted recipients (Fig. 4d). Thus, regression of anti-HVEM scFv-expressing tumors is both CD4+ and CD8+ T cell dependent.

Administration of anti-HVEM scFv-expressing tumor cells stimulates tumor-specific long-term T cell memory

One of the most important features of cancer immunotherapy is to develop long-term immunological memory, which is vital for successful prevention of tumor recurrence. In order to evaluate whether injection of anti-HVEM scFv-expressing tumor induces memory responses, spleen cells from the mice which had rejected anti-HVEM scFv-expressing P815 for over 2 months were harvested and co-cultured with irradiated P815 tumor cells. After 4 days, cytolytic activity against parental P815 or L1210, a control target derived from syngeneic DBA/2 mice, was examined. Spleen cells from mice that had rejected anti-HVEM scFv-expressing P815 developed potent cytolytic activity specific to P815 but not L1210 (Fig. 5a). Next, in order to assess a potential to resist tumor recurrence in vivo, the mice which had rejected anti-HVEM scFv-expressing P815 for more than 2 months were re-challenged with parental P815 and control L1210 in the right and left flank, respectively. As a control, the same number of tumors was inoculated into naïve DBA/2 mice. While both P815 and L1210 grew progressively in naïve hosts, all mice that had rejected anti-HVEM scFv-expressing P815 were resistant to re-challenge with P815 but not L1210 (Fig. 5b). Taken in concert, these findings indicate that administration of anti-HVEM scFv-expressing tumor develops long-term tumor-specific memory, rendering the vaccinated animals resistant to recurrence.

Fig. 1 Structure, expression, and function of anti-HVEM scFv on P815 tumor. **a** Construct of anti-HVEM scFv is schematically shown. **b** P815 expressing anti-HVEM scFv or control scFv were stained with 2 μ g mouse HVEM-mouse Ig fusion protein followed by PE-conjugated anti-mouse Ig Ab and analyzed by flow cytometry (*filled histogram*). Background staining level with secondary Ab alone is also shown (*open histogram*). **c–e** T cells isolated from naïve B6 mice were cultured at 2.5×10^6 cells/ml with irradiated P815 cells expressing anti-HVEM scFv or control scFv (2×10^5 cells/ml). **c** After 2 days, T cell proliferation was assessed by ^3H -thymidine incorporation assay. * $P < 0.001$. **d** Pictures of T cell blast formation were taken under the observation by microscopy on day 3. **e** The culture supernatants were harvested 2–4 days later from the group of anti-HVEM scFv (*filled circle*) and control scFv (*open circle*), and the concentration of IFN- γ and IL-2 was measured by ELISA. **f** P1A-specific T cells isolated from P1A TCR transgenic mice were cultured at 1, 2, or 4×10^6 cells/ml together with 2×10^5 cells/ml irradiated P815 cells expressing anti-HVEM scFv (*filled circle*) or control scFv (*open circle*). After 2 days, T cell proliferation activity (*left panel*) and IFN- γ level in culture supernatants (*right panel*) were measured by ^3H -thymidine incorporation assay and ELISA, respectively. Experiments were repeated at least three times, and the representative data are shown as mean \pm SEM

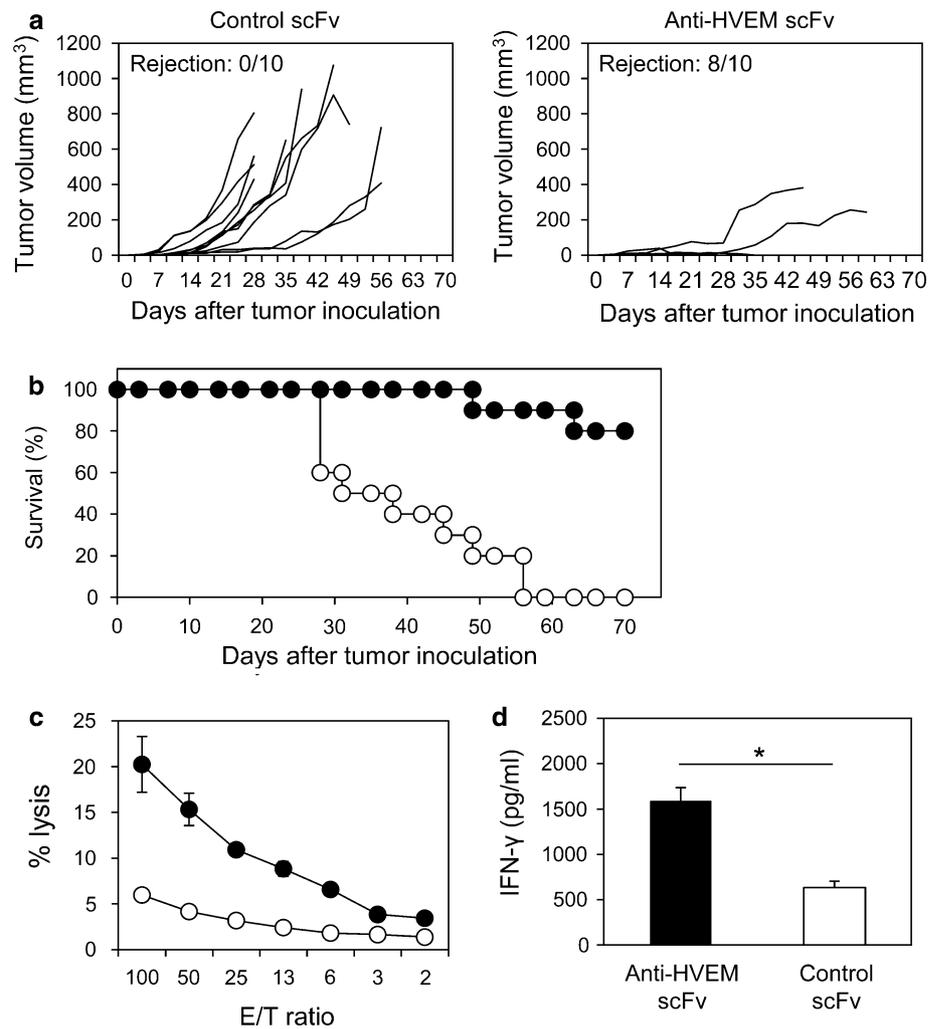


Anti-HVEM scFv-expressing tumor vaccines combined with 4-1BB stimulation mediate the regression of established tumors

In order to apply anti-HVEM scFv-expressing tumor as an effective tumor vaccine in clinical settings, it is crucial to demonstrate the potential of this approach to induce regression of pre-established tumor. To this end, DBA/2 mice were first inoculated s.c. with wild-type P815 tumor in right flank and 5 days later treated with anti-HVEM or control

scFv-expressing P815 s.c. in left flank, followed by anti-4-1BB mAb or control Ab treatments on day 10 and 15. We selected anti-4-1BB mAb as a reagent to combine with anti-HVEM scFv for two reasons. First, 4-1BB co-stimulation with anti-4-1BB mAb has been demonstrated by multiple investigators, including us, to induce potent antitumor effects through T cell-dependent mechanisms [26]. Second, 4-1BB is inducibly expressed on activated T cells, while HVEM is constitutively expressed on naïve T cells, suggesting that stimulation of HVEM in the early phase of activation

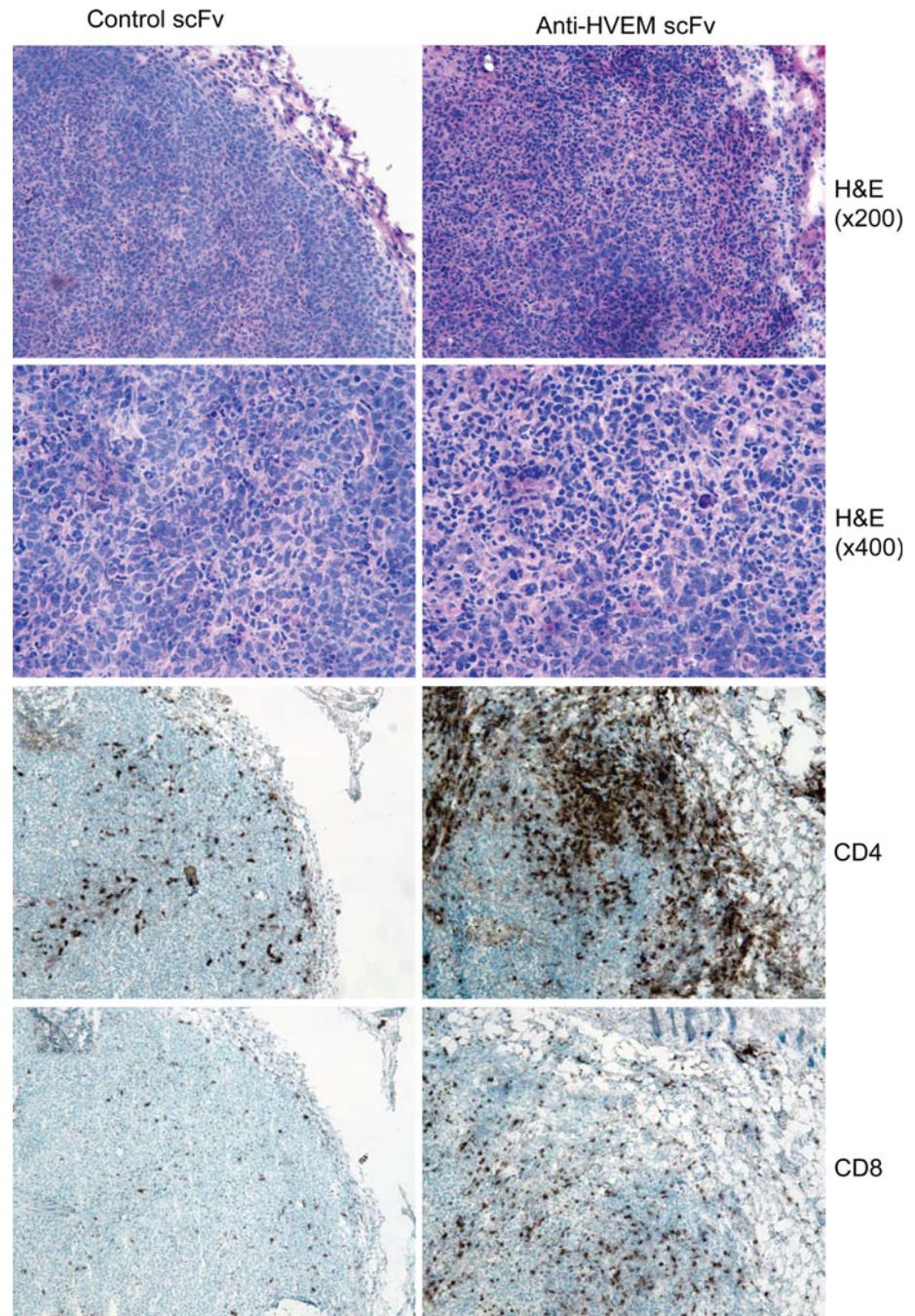
Fig. 2 Induction of in vivo anti-tumor immunity and tumor regression by anti-HVEM scFv-expressing P815. **a** and **b** DBA/2 mice ($n = 10$) were injected s.c. with 1×10^5 P815 expressing anti-HVEM scFv (filled circle) or control scFv (open circle). Tumor growth of individual mice (**a**) and survival of the cohort (**b**) were assessed. $P < 0.001$ between (filled circle) versus (open circle). **c** and **d** DBA/2 mice were injected s.c. with 1×10^6 P815 expressing anti-HVEM scFv (filled circle) or control scFv (open circle). Seven days later, tumor-draining LN cells were harvested and co-cultured with irradiated wild-type P815 cells. **c** After 4 days, cytolytic activity against P815 was examined by 4 h ^{51}Cr -release assay at the indicated Effector/Target (E/T) ratios. **d** Culture supernatants were harvested on day 3, and the levels of IFN- γ were assessed by ELISA. $*P < 0.001$. Experiments were repeated at least 3 times, and the representative data are shown as mean \pm SEM



(day 5) in combination with 4-1BB stimulation in late phase (day 10 and 15) may be synergistic. Our results indicated that vaccination of anti-HVEM scFv-expressing P815 alone conferred little survival benefit in pre-established P815 tumor (Fig. 6a), while this insufficient effect was not due to a loss of HVEM expression on T cells in the mice bearing established tumor (Supplementary Figure 3). Treatment with anti-4-1BB mAb alone slightly prolonged the survival, but all mice were eventually killed by tumor within 90 days. In contrast, in keeping with our hypothesis, when anti-HVEM scFv-expressing P815 tumor vaccine and anti-4-1BB mAb treatment were combined, the survival was significantly prolonged ($P = 0.0021$ compared with anti-HVEM scFv alone, $P = 0.0256$ compared with anti-4-1BB mAb alone) and 60% of mice survived more than 140 days. Furthermore, the mice which rejected pre-established P815 tumor by anti-HVEM scFv-expressing P815 vaccination and anti-4-1BB mAb treatment developed potent cytolytic activity specific to P815 but not control L1210 (Fig. 6b).

To further examine clinical applicability of this approach, anti-HVEM scFv-expressing P815 cells were exposed to irradiation prior to usage as vaccine. Sixty percent of the mice treated with irradiated tumor vaccine combined with anti-4-1BB mAb survived >140 days (Fig. 6c) and developed P815-specific memory cytolytic T lymphocyte (CTL) responses (Fig. 6d). In irradiated tumor vaccine model, combination therapy achieved a significantly prolonged survival compared with anti-HVEM scFv-expressing tumor cells alone ($P < 0.001$) and showed a trend of better survival compared with anti-4-1BB mAb alone although it was not statistically significant ($P = 0.395$). These results collectively indicate that pre-conditioning of the vaccine cells with irradiation does not diminish their ability to induce antitumor immunity. Taken together, vaccination of anti-HVEM scFv-expressing tumor cells combined with anti-4-1BB mAb induces potent antitumor immunity, which both mediates regression of pre-established tumor and results in tumor-specific T cell memory.

Fig. 3 Histological analysis of P815 tumor expressing anti-HVEM scFv. DBA/2 mice were injected s.c. with 1×10^5 P815 expressing anti-HVEM scFv or control scFv. After 14 days, tumor tissues were harvested and examined for histological analysis of H&E staining (*top panels*: $\times 200$, *second panels*: $\times 400$), anti-CD4 mAb (*third panels*: $\times 100$) and anti-CD8 mAb (*bottom panels*: $\times 100$). In immunohistochemistry, positive cells are shown as *brownish* staining. Representative pictures of the sections are shown



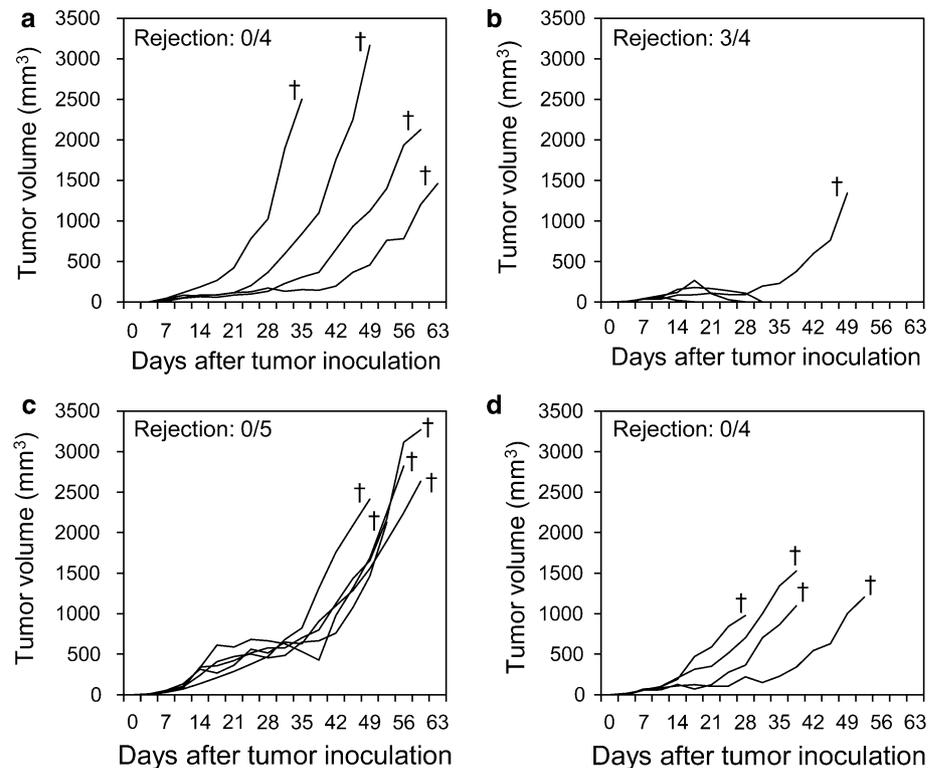
Discussion

In this study, we developed the genetically modified tumor cells which deliver a potent co-stimulatory signal selectively through HVEM. Vaccination with these engineered cells induces T cell infiltration at the tumor site, increased cytokine production, and tumor-reactive CTL, which renders mice resistant to tumor growth. Generation of antitumor immunity is dependent on both CD4+ and CD8+ T cells and leads to the generation of long-term tumor-specific CTL. Vaccination of anti-HVEM scFv-expressing

tumor combined with anti-4-1BB mAb treatment significantly prolongs the survival of mice with pre-established tumors. These data demonstrate, for the first time, that tumor cells expressing agonistic scFv against HVEM mediate potent antitumor vaccine effects by inducing tumor-specific immune responses.

Vaccination of LIGHT-expressing tumor cells or injection of LIGHT-encoding vectors in tumor tissue elicits potent antitumor immunity leading to tumor regression in mouse models [9, 12–14]. It has been speculated that the antitumor effects of LIGHT are mediated by not only

Fig. 4 Crucial role of CD4⁺ and CD8⁺ T cells in the regression of anti-HVEM scFv-expressing P815. DBA/2 mice were inoculated s.c. with 1×10^5 P815 expressing control scFv (a) or anti-HVEM scFv (b–d). Groups of mice inoculated with anti-HVEM scFv-expressing P815 were injected i.p. with anti-CD4 mAb (c) or anti-CD8 mAb (d) for depletion of CD4⁺ or CD8⁺ T cells. Tumor growth of individual mice was assessed. *Dagger*: death due to tumor

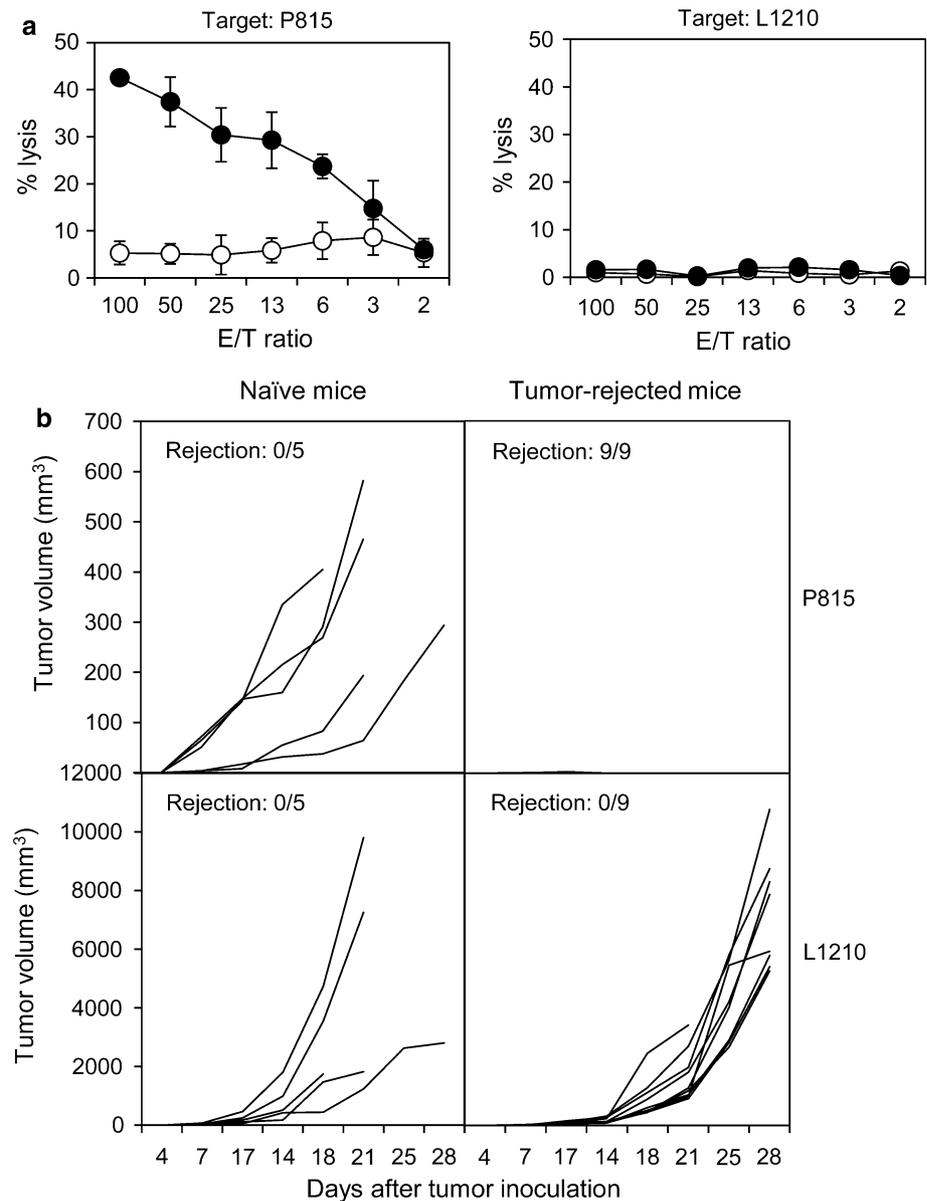


through HVEM co-stimulatory signaling in T cells but also through LT β R signals in tumor stromal cells which induce chemokine production and subsequent recruitment of immune cells at the tumor site [16]. However, the role of LT β R remains controversial because recent studies found that stimulation of HVEM alone is sufficient to upregulate chemokine gene expression [29]. In this report, we demonstrate that selective stimulation of HVEM signal by the scFv of an anti-HVEM agonistic Ab expressed on tumor cells induces massive infiltration of T lymphocytes in tumor sites and mediates tumor regression associated with tumor-specific long-term immunologic memory. From the translational perspective, anti-HVEM scFv stimulation appears superior to LIGHT when used as an antitumor vaccine for at least three reasons. First, LIGHT binding to HVEM is neutralized by DcR3, which is identified in human but not in mouse as a soluble LIGHT receptor expressed at high levels in various tumors [17–19]. Second, LIGHT is cleaved by matrix metalloproteinase and converts to a soluble form which generates a ternary complex with HVEM and BTLA, and enhances BTLA-mediated inhibition [15]. Although deletion of 4 amino acids at a potential recognition site of metalloproteinase renders LIGHT partly resistant to cleavage [12], we find that a substantial amount of soluble LIGHT is still detected even in the deletion mutant (data not shown). Third, LT β R stimulation by LIGHT may result in unexpected adverse effects such as liver and intestinal toxicity, cancer progression, and

dysregulated lipid metabolism due to broad biological functions of LT β R signal [22–24, 30]. Application of anti-HVEM scFv overcomes these drawbacks associated with LIGHT as a means to induce T cell co-stimulation.

Vaccination of anti-HVEM scFv-expressing tumor cells induces massive infiltration of CD4⁺ T helper cells, as well as CD8⁺ CTL, at the tumor site. In addition, depletion of CD4⁺ T cells hampers tumor regression, indicating a crucial role of CD4⁺ T cells in mediating the observed antitumor effects. HVEM signals in CD4⁺ T cells likely stimulate antitumor immunity via several mechanisms. First, HVEM signals enhance the priming of CD4⁺ T cells, which secondarily promotes tumor-reactive CTL due to CD40L/CD40-mediated DC activation and/or production of helper cytokines [31, 32]. Second, CD4⁺ T cells activated by HVEM co-signaling may differentiate into cytotoxic CD4⁺ Th1 cells and execute direct killing of tumor cells [33, 34]. Third, as indicated in recent studies, HVEM signaling promotes the persistence of large pools of memory CD4⁺ T cells [35]. These mechanisms are not mutually exclusive and may function cooperatively to stimulate tumor-reactive CD4⁺ T cells. Besides T lymphocytes, HVEM signals positively regulate the functions of NK cells, DC, and myeloid cells. In NK cells, HVEM signal induces proliferation and IFN- γ production, which in turn promote antitumor activity of CTL [36]. HVEM signal in DC induces their maturation, by which Ag presentation potential as well as the expression of adhesion and co-stimulatory molecules on DC is

Fig. 5 Induction of tumor-specific protective immunity following regression of anti-HVEM scFv-expressing P815. DBA/2 mice were inoculated s.c. with 1×10^5 P815 expressing anti-HVEM scFv. The mice which had rejected tumor and survived more than 2 months were designated as tumor-rejected mice. **a** Spleen cells harvested from tumor-rejected mice (filled circle) or control naïve DBA/2 mouse (open circle) were co-culture with irradiated wild-type P815 cells. Four days later, cytolytic activity of the cultured cells was examined by 4 h ^{51}Cr -release assay against P815 or control L1210 at the indicated E/T ratios. Data are shown as mean \pm SEM. **b** Tumor-rejected mice or naïve DBA/2 mice were inoculated s.c. with 1×10^5 P815 and L1210 at the right and left flank, respectively. Tumor growth of individual mice was assessed



upregulated [37]. In macrophages and granulocytes, HVEM signal stimulates productions of cytokines, chemokines, nitric oxide, and reactive oxygen species [38]. These broad immune-regulatory functions of HVEM may also contribute to tumor rejection. Furthermore, in case of HVEM-positive hematologic malignancies, HVEM signaling triggered by anti-HVEM scFv could upregulate caspase activities and pro-apoptotic protein Bax in tumor cells [29]. Accelerated tumor cell death promotes the release of tumor Ag and subsequent Ag presentation by APC, which potentiates antitumor T cell responses. Collectively, anti-HVEM scFv expression on tumor cells triggers multifaceted immune responses which orchestrate to accomplish potent antitumor vaccine effects.

Combination of our anti-HVEM scFv-expressing tumor cell vaccine and anti-4-1BB mAb injection demonstrated a potent therapeutic effect against pre-established tumor, which led to complete tumor regression in 60% of mice. The synergy of these therapies can be explained by the expression kinetics of the co-stimulatory receptors as below. First, circulating tumor-reactive T cells show naïve phenotype which express high-level HVEM but not 4-1BB. When these T cells encounter anti-HVEM scFv-expressing tumor cells, signals from TCR and HVEM induce T cell activation. Activated T cells then inducibly express 4-1BB while downregulating HVEM expression [15], in which anti-4-1BB mAb has a predominant effect to deliver co-stimulatory signal. At this stage, T cells undergo full activation and

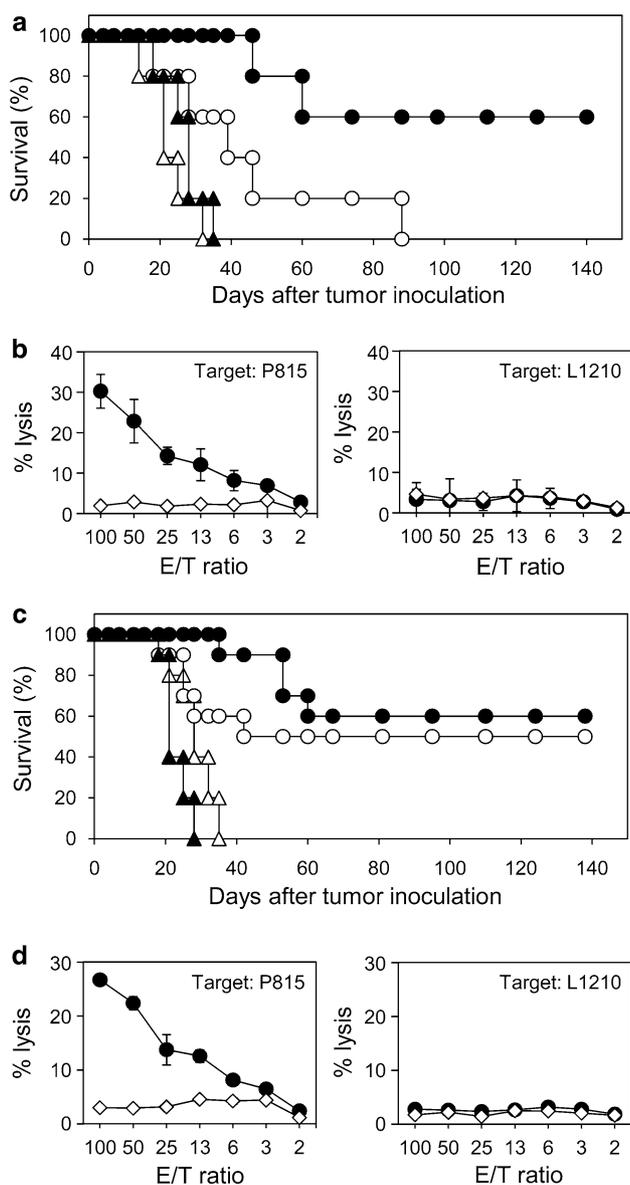


Fig. 6 Treatment of pre-established tumor by vaccination of anti-HVEM scFv-expressing P815 in combination with anti-4-1BB mAb injections. **a** and **b** Naïve DBA/2 mice were inoculated s.c. with 1×10^5 wild-type P815 at the right flank on day 0. On day 5, mice were injected s.c. with 1×10^5 P815-expressing anti-HVEM scFv (filled triangle and filled circle) or control scFv (open triangle and open circle) at the left flank. The mice were then injected i.p. with 150 μ g anti-4-1BB mAb (open circle and filled circle) or control rat IgG (open triangle and filled triangle) on day 10 and 15. **a** Survival of the mice was assessed. $P = 0.0256$ between anti-4-1BB mAb alone (open circle) and anti-HVEM scFv-expressing P815 plus anti-4-1BB mAb injection (filled circle), $P = 0.0021$ between anti-HVEM scFv-expressing P815 alone (filled triangle) and anti-HVEM scFv-expressing P815 plus anti-4-1BB mAb injection (filled circle). **b** Spleen cells were harvested from the mice which had rejected pre-established P815 by vaccination with anti-HVEM scFv-expressing P815 plus anti-4-1BB mAb treatment (filled circle). As control, spleen cells of naïve DBA/2 mice were also prepared (open diamond). These cells were co-cultured in vitro with irradiated wild-type P815 cells. After 4 days, CTL activity against P815 or L1210 was examined by 4 h ^{51}Cr -release assay at the indicated E/T ratios. **c** and **d** Naïve DBA/2 mice were inoculated s.c. with 1×10^5 wild-type P815 and treated with 1×10^6 irradiated anti-HVEM scFv- or control scFv-expressing P815 vaccine in combination with anti-4-1BB mAb or control rat IgG injections, in the schedule same to (a). Survival of mice (c) and the CTL activity of spleen cells from the tumor-rejected mice (d) were examined as (a) and (b), except exposing tumor cells to irradiation prior to usage as vaccine. $P < 0.001$ between (filled circle) versus (filled triangle), and $P = 0.395$ between (filled circle) and (open circle). Data are shown as mean \pm SEM

execute their effector functions to eliminate tumor cells. Thereafter, T cells differentiate into memory phenotype, in which both HVEM and 4-1BB retain high expression so that signals to these receptors synergistically promote maintenance of memory T cell population [35, 39]. Thus, combined stimulation of HVEM and 4-1BB, two important co-stimulatory receptors holding regulatory functions in naïve, effector, and memory T cells, is a promising approach in cancer immunotherapy.

In summary, we demonstrate that tumor cells genetically engineered to express scFv of agonistic anti-HVEM Ab induce potent antitumor immunity in CD4+ and CD8+ T cell-dependent fashion. Combination of this approach with anti-4-1BB mAb further demonstrated therapeutic effects in pre-established tumors. Thus, current studies open new avenues in cancer vaccine targeting HVEM co-stimulatory

signal. Combination with other approaches including immune adjuvants and blockade of immune check points is anticipated to develop more efficient cancer immunotherapies.

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