Adoptively Transferred Tumor-Specific T Cells Stimulated \textit{Ex vivo} Using Herpes Simplex Virus Amplicons Encoding 4-1BBL Persist in the Host and Show Antitumor Activity \textit{In vivo}

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Abstract

4-1BB is a T-cell costimulatory receptor which binds its ligand 4-1BBL, resulting in prolonged T cell survival. We studied the antitumor effects of adoptively transferred tumor-specific T cells expanded \textit{ex vivo} using tumors transduced with herpes simplex virus (HSV) amplicons expressing 4-1BBL as a direct source of antigen and costimulation. We constructed HSV amplicons encoding either the 4-1BBL (HSV.4-1BBL) or B7.1 (HSV.B7.1) costimulatory ligands. Lewis lung carcinoma cells expressing ovalbumin (LLC/OVA) were transduced with HSV.4-1BBL, HSV.B7.1, or control HSV amplicons and used to stimulate GFP+ OVA-specific CD8+ T cells (OT-1/GFP) \textit{ex vivo}. Naive or \textit{ex vivo} stimulated OT-1/GFP cells were adoptively transferred into LLC/OVA tumor-bearing mice. Higher percentages of OT-1/GFP cells were seen in the peripheral blood, spleen, and tumor bed of the HSV.4-1BBL–stimulated OT-1/GFP group compared with all other experimental groups. OT-1 cells identified within the tumor bed and draining lymph nodes of the HSV.4-1BBL–stimulated OT-1 group showed enhanced bromodeoxyuridine (BrdUrd) incorporation, suggesting ongoing expansion \textit{in vivo}. Mice receiving HSV.4-1BBL–stimulated OT-1/GFP had significantly decreased tumor volumes compared with untreated mice \((P < 0.001)\) or to mice receiving naive OT-1/GFP \((P < 0.0001)\). Transfer of HSV.B7.1–stimulated OT-1/GFP did not protect mice from tumor. Mice that received HSV.4-1BBL–stimulated OT-1/GFP exhibited increased cytolytic activity against LLC/OVA and higher percentages of Ly-6C+ OT-1/GFP in the spleen and tumor bed compared with controls. Tumor-specific T cells stimulated \textit{ex vivo} using tumor transduced with HSV.4-1BBL expand \textit{in vivo} following adoptive transfer, resulting in tumor eradication and the generation of tumor-specific CD44+Ly-6C-CD62L- effecter memory T cells. [Cancer Res 2007;67(20):10027–37]

Introduction

Adoptive transfer of autologous tumor-specific T cells is a promising approach to cancer immunotherapy. T cells isolated from tumor-infiltrating lymphocytes, tumor-draining lymph nodes (TDLN), or peripheral blood contain tumor-specific cells, which can be expanded \textit{ex vivo} and transferred into the host. Responses have been observed in metastatic melanoma (1), renal cell carcinoma (2), and glioma (3), among other cancers, when treated with \textit{ex vivo} expanded CTLs. Immune ablation may further augment responses seen with adoptive T-cell transfer (1). Efficacy of T-cell adoptive transfer may be improved through optimization of \textit{in vitro} expansion, improved characterization of effector populations, and/or by enhancing the function and survival of transferred CTLs to facilitate establishment of immunologic memory.

4-1BB (CD137, ILA, TNFRSF9) is a type I transmembrane glycoprotein belonging to the tumor necrosis factor receptor superfamily (4, 5). 4-1BB expression is observed in a range of myeloid and lymphoid cells, including CD4+ and CD8+ T cells, intraepithelial lymphocytes, natural killer cells, monocytes, and dendritic cells (5, 6). In contrast to CD28 expression on naive T cells, 4-1BB is induced on T cells following activation. CD28 seems to relay an initial costimulatory signal followed by 4-1BB signaling, which serves to further shape the T-cell response.

4-1BB ligation induces cytokine secretion, especially IFN-\(\gamma\), enhances proliferation and survival of T cells in \textit{vivo} and \textit{in vitro} (5, 7–12), and plays a crucial role in the generation and expansion of effector and memory CTLs (8, 13). Administration of agonistic anti–4-1BB monoclonal antibody (mAb) enhanced antitumor responses in the poorly immunogenic Ag104A sarcoma model (14) and improved antitumor effects seen with adoptive transfer of CD8+ T cells in several tumor models (15–17). 4-1BB–mediated antitumor effects have been ascribed to the prevention of programmed cell death, leading to the accumulation of antitumor effecter cells (15, 18).

Previous experiments have also shown the effectiveness of 4-1BBL gene transduction for antitumor immunity. Melero et al. (19) first transduced 4-1BL into P815 mastocytoma using a retroviral vector and inoculated mice, which then developed a strong CTL response and long-term immunity against wild-type tumor. In the A20 lymphoma (20) and NRS1 squamous cell carcinoma (21) models, 4-1BBL expression also reduced tumor growth. Combined adenoviral 4-1BBL and interleukin-12 (IL-12) gene transfer conferred protection against hepatic metastases induced by a poorly immunogenic MCA26 colon carcinoma line (22).

We used herpes simplex virus I (HSV) amplicons, replication-defective viral particles, for gene transfer of 4-1BBL because of their broad cellular tropism, large transgene capacity, and ability to induce high levels of gene expression. By triggering an innate response, HSV amplicons may facilitate a more vigorous adaptive
response. We have noted the strong activation of several toll-like receptors (TLR), induction of cytokines, and NKG2D-ligand expression following transduction with HSV ampicrons in macrophage cell lines and human chronic lymphocytic leukemia (CLL; ref. 23). Because HSV ampicrons can readily transduce primary tumor cells, we reasoned that HSV ampicrons encoding 4-1BBL could facilitate direct antigen presentation by tumor cells to expand tumor-specific effectors for adoptive transfer. We hypothesized that HSV.4-1BBL-expanded CD8+ T cells would show desirable effector properties, including in vivo expansion and therapeutic efficacy, as well as potentially confer a memory response.

We used HSV.4-1BBL ampicrons to transduce tumor for purposes of activating and expanding tumor-specific CD8+ OT-1 cells in vitro and studied the behavior of adoptively transferred ex vivo expanded cells in LLC/OVA tumor-bearing mice. Our studies show that HSV.4-1BBL has the potential to induce significant expansion of CTLs in vitro and in vivo, and that the adoptive transfer of expanded T cells may result in a reduction of tumor growth in vivo as well as the persistence of CD4+Ly-6C+CD62L+ tumor-specific T cells with memory characteristics.

**Materials and Methods**

**Animals and cells.** C57BL/6 and Thy1.1 (C57BL/6.PL-Thy1.1/Cy) mice were obtained from The Jackson Laboratory. Thy1.1 mice (obtained courtesy of M. Bevan, Howard Hughes Medical Institute, University of Washington, Seattle, WA) express a transgenic TCR that is specific for OVA257-264 (SIINFEKL) peptide bound to H-2Kb. GFP+ mice (24) were bred with OT-1 mice to generate OT-1/GFP T cells expressing green fluorescent protein (GFP). Mice were maintained in pathogen-free facilities at the University of Miami, and procedures were done in agreement with the Institutional Animal Care and Use Committee per NIH guidelines.

Lewis lung carcinoma (LLC) cells stably transfected with ovalbumin (LLC/OVA; ref. 25) were grown in Iscove's modified Dulbecco medium plus 10% fetal bovine serum (FBS), penicillin (50 units/mL), streptomycin (50 μg/mL), and 1 mg/mL geneticin.CTL assay.

**Splenocytes were incubated with mitomycin C–treated LLC/OVA at a 10:1 ratio for 6 days with recombinant mIL-2 (10–20 units/mL) in R-10 media and then plated in 96-well round-bottom plates at the indicated effector:target ratios. LLC/OVA and LLC targets were labeled with 51Cr and separated from the tumor using anti-CD8a magnetic beads before adoptive transfer.**

**Intracellular staining and flow cytometry.** Cells were stained on the surface with fluorochrome-conjugated anti-CD4 and anti-CD8a antibodies in PBS at 4°C for 20 min. Cells were then washed with PBS, fixed using Cytofix/Cytoperm buffer (BD PharMingen) for 20 min at 4°C, and washed thrice in RPMI 1640 plus 10% FBS, penicillin, streptomycin, and 50 μM/1-2 ME (R-10). Freshly isolated OT-1/GFP cells were then plated with mitomycin C–treated LLC/OVA at a 3:2 ratio in 24-well plates. Each well contained 2.4 × 10^5 OT-1/GFP cells plus 1.6 × 10^6 tumor cells in 2 mL R-10. R-10 media (0.5–1 mL) was added to each well after 2 days. Cells were harvested on the third day and separated from the tumor using anti-CD8a magnetic beads before adoptive transfer.

**Detection of GFP+ cells in frozen tissue sections.** Spleens were frozen in OCT compound (Sakura Finetek) with dry ice and stored at −80°C until sectioning. Tissues were sectioned 6 μm thick and adhered onto Superfrost Plus slides (VWR). Slides were kept cold to prevent diffusion of GFP and exposed to a closed-lid container to 37°C formaldehyde vapor at −20°C for 24 h, as first described by Jockusch et al. (31). Tissues were outlined with an ImmEdge pen (Vector Laboratories), washed with PBS, and counter-stained with 1 μg/mL Hoechst 33342 (Sigma) for 15 min at 37°C. After washing, slides were mounted with Prolong Gold anti-fade reagent (Molecular Probes/Invitrogen). Sections were viewed using a Leica DMIRB Inverted Microscope, and images were captured with MetaMorph Imaging System (Molecular Devices Corporation).

**Tumor measurements and statistics.** Statistical analyses were done using Microsoft Excel, StatView, and SAS 9.1. Average cell counts were compared by Student’s t test. Tumor burden was calculated as the volume of a sphere with radius based on the average of two diameters, D1, and D2, measured by caliper [Volume = 4/3π(D1 + D2)/2]^2. Where possible, t tests were used to compare the average tumor volume in treatment groups at the end of the experiment. For experiments involving animal sacrifice, tumor growth was compared across groups by fitting a log-linear regression model, and differences in tumor-free mice across groups were compared by Fisher’s exact test.

**CTA assay.** Splenocytes were incubated with mitomycin C–treated LLC/OVA at a 1:1 ratio for 6 days with recombinant mIL-2 (10–20 units/mL) in R-10 media and then plated in 96-well round-bottom plates at the indicated effector:target ratios. LLC/OVA and LLC targets were labeled with 51Cr (150 μL/10^6 cells) and plated at 5 × 10^4 cells per well. Plates were incubated at 37°C for 8 h. Supernatant was collected and added to Ready Safe Liquid
Figure 1. HSV amplicon vector encoding 4-1BBL and proliferation of CD8+ OT-1 T cells in response to HSV amplicon-transduced tumor. A, the HSV-4-1BBL amplicon plasmid DNA is packaged into helper-virus–free viral stocks using a HSV genome-containing bacterial artificial chromosome (BAC), which has its cognate HSV packaging elements (pac sequences) deleted. Vhs, or the viral host shutdown gene, is expressed from a separate plasmid (pBS-vhs) to enhance viral titers. Baby hamster kidney (BHK) cell monolayers are transfected with all three vectors, and the HSV amplicon viral particles are later harvested, concentrated, and titered for use. B, LLC/OVA and LLC tumors were transduced with HSV amplicons encoding either human B7.1 (HSV.B7.1) or murine 4-1BBL (HSV.4-1BBL) as T-cell costimulatory ligands or -galactosidase (HSV.LacZ) as an HSV control at MOI = 1. After 2 d, the tumor cells were treated with 0.4 mg/mL mitomycin C and stained for B7.1 and 4-1BBL expression. Solid line, staining of untransduced tumor; dashed line, staining of HSV.LacZ-transduced tumor; filled histogram, staining of HSV.B7.1-or HSV.4-1BBL–transduced tumor. C, purified OT-1 T cells (10^5) were added to mitomycin-treated tumor cells (2 x 10^4) and incubated at 37°C. Thymidine was added 20 h before the plate was harvested following 3 d of coculture. Thymidine uptake of tumor cells alone was minimal. Columns, mean uptake, one of six separate experiments; bars, SD of triplicates. D, 10^5 total CD8+ T cells (OT-1 and/or normal CD8+ splenocytes) were incubated with or without HSV-4-1BBL–transduced LLC/OVA (5 x 10^4) for 3 or 5 d. Thymidine was added 19 h before harvest. Columns, mean uptake, one of two separate experiments; bars, SD of triplicates.
scintillation cocktail for aqueous samples (Beckman Coulter). Samples were counted on a LS 6500 multipurpose scintillation counter (Beckman Coulter). Percent lysis = (sample counts – spontaneous counts)/(maximum counts – spontaneous counts) × 100.

**In vivo bromodeoxyuridine labeling.** Three and six days following adoptive transfer of OT-1, Thy1.1+ mice were injected i.p. with 100 µL bromodeoxyuridine (BrdUrd; 1 mg APC BrdU Flow kit, BD PharMingen). On day 8, OT-1 numbers and incorporation of BrdUrd were assessed in the spleen, tumor, and draining and nondraining lymph nodes by staining with anti-CD8-PE, anti-Thy1.2-FITC, and anti-BrdUrd-APC per manufacturer's instructions.

**Results**

Tumors transduced by HSV.4-1BBL express murine 4-1BBL and can induce proliferation of CD8+ OT-1 T cells. Murine 4-1BBL cDNA was cloned into the HSV amplicon vector pHSVPrPUC and packaged into amplicons (HSV.4-1BBL) using a helper-virus–free packaging method (Fig. 1A; refs. 29, 30). Packaged virions contain only amplicon genomes, without the propagation of the helper virus (Fig. 1A). HSV.B7.1 and HSV.LacZ amplicons, which encode for human B7.1 and bacterial β-galactosidase, respectively, were also packaged using the helper-free method. Both mouse and human B7.1 can stimulate T-cell CD28 receptors of either species (24).

We tested the ability of the amplicons to transduce LLC/OVA or the parental LLC tumor cell line. LLC and LLC/OVA cells transduced with HSV.B7.1 or HSV.4-1BBL showed high levels of expression of B7.1 or 4-1BBL, respectively, by day 2 as shown by flow cytometric analysis (Fig. 1B). HSV.LacZ transduction did not induce either B7.1 or 4-1BBL expression (Fig. 1B). HSV.B7.1 transduction did not induce 4-1BBL expression or vice versa (data not shown). To more accurately follow the effects of 4-1BBL on antigen-specific T cells, we used the adoptive transfer of CD8+ OT-1 T cells in our *in vivo* mouse experiments (32, 33). OT-1/GFP cells were derived from TCR-transgenic OT-1 mice that were back-crossed into GFP+ mice to facilitate monitoring once adoptively transferred. HSV.B7.1- or HSV.4-1BBL–transduced LLC/OVA tumor cells were used to stimulate CD8+ OT-1 T cells for 3 to 5 days (Fig. 1C). At day 3, markedly increased proliferation was seen for OT-1 cells stimulated with either HSV.B7.1- or HSV.4-1BBL–transduced tumors, compared with untransduced tumor (Fig. 1C). HSV.LacZ-transduced LLC/OVA did not augment proliferation of

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Phenotype of OT-1/GFP cells before adoptive transfer and experimental outline. A, CD8+ OT-1/GFP T cells were added to mitomycin C–treated LLC/OVA tumor cells. Three days later, OT-1/GFP T cells were stained for surface activation markers and analyzed by flow cytometry. Filled histogram, OT-1/GFP + HSV.4-1BBL–transduced LLC/OVA; bold solid line, OT-1/GFP + HSV.B7.1-transduced LLC/OVA; dashed line, OT-1/GFP + HSV.LacZ–transduced LLC/OVA; dotted line, naive OT-1/GFP. Data are representative of three separate experiments. B, LLC/OVA tumor cells were transduced with HSV.4-1BBL or HSV.B7.1 at an MOI of 1 and cultured for 1 d. Tumor cells were then washed and treated with mitomycin C. CD8+ OT-1/GFP cells were purified from spleen and cocultured with transduced tumor for 3 d. Stimulated OT-1/GFP cells were separated from tumor cells using anti-CD8 antibody-magnetic beads. OT-1/GFP cells were given i.v. into C57BL/6 mice bearing LLC/OVA tumor. LLC/OVA tumor was palpable following 2 to 4 d of s.c. injection. Peripheral blood, spleen, TDLN, and the tumor bed were examined for the presence of OT-1/GFP cells. Spleen was analyzed for cytolytic activity, and the phenotype of OT-1/GFP cells was characterized in the spleen, TDLN, and tumor bed.
OT-1 in vitro compared with untransduced LLC/OVA. OT-1 stimulated with HSV.4-1BBL–transduced LLC/OVA continued to proliferate vigorously at day 5 (data not shown). OT-1 cells which were cultured with parental LLC, with or without costimulatory ligands, did not proliferate, signifying an absolute requirement for signal one for T-cell activation (data not shown). OT-1 cells cultured with HSV.4-1BBL–transduced LLC/OVA did not show increased proliferation on days 3 (Supplementary Fig. S1) or 5 (data not shown), with the addition of soluble (2 μg) or plate-bound (0.2 μg) anti-CD28 antibody. Soluble anti-CD28 antibody instead
markedly inhibited proliferation elicited by HSV.B7.1-transduced LLC/OVA, but not that elicited by HSV.4-1BBL–transduced tumor, indicating that soluble anti-CD28 antibody blocked the interaction between CD28 on T cells and B7.1 on the tumor cells (Supplementary Fig. S1). Addition of soluble anti–CTLA-4 antibody (12 μg) to the cocultures of OT-1 cells with HSV.B7.1- or HSV.4-1BBL–transduced LLC/OVA did not further augment proliferation (Supplementary Fig. S1). Titration of OT-1 with HSV.4-1BBL–transduced LLC/OVA showed that tumor-specific OT-1 cells (10^5) could expand following serial dilution with normal CD8+ splenocytes (Fig. 1D). Dilutions of up to 1:32 (3,000 OT-1 cells per well) are shown for days 3 and 5 (Fig. 1D). On day 5, OT-1 cells continue to proliferate at a dilution of 1:32 even when the higher number of OT-1 have markedly decreased their proliferation due possibly to limited nutrients or lack of intact tumor cells. Therefore, tumor-specific cells can be stimulated to proliferate using this system even when present at lower numbers.

**OT-1 cells costimulated ex vivo with 4-1BBL display an effector phenotype.** Expanded OT-1/GFP cells were characterized following 3 days of coinoculation with transduced LLC/OVA (Fig. 2A). CD44 is expressed on activated T cells and functions in lymphocyte homing and adhesion. CD25 (IL-2R) is a component of the high-affinity IL-2 receptor up-regulated on effector T cells. Granzyme B is a serine protease stored in the granules of CTLs along with perforin (34). CD107a (LAMP-1) is a widely expressed intracellular antigen that appears on CD8+ CTLs following activation-induced degranulation (35). OT-1/GFP stimulated ex vivo with either HSV.4-1BBL- or HSV.B7.1-transduced LLC/OVA expressed high levels of CD44, intracellular granzyme B, and CD107a and modestly increased levels of CD25, indicating that they were activated and capable of cytotoxic activity (Fig. 2A). HSV.LacZ-stimulated OT-1/GFP expressed CD44 and CD107a at lower levels than those stimulated with HSV.4-1BBL or HSV.B7.1. 4-1BB was expressed on OT-1 cells stimulated for 3 days with HSV.4-1BBL-transduced LLC/OVA, but was not detected on naive OT-1 cells (Fig. 2A). Naive OT-1/GFP did not express any of the aforementioned activation markers.

Ly-6C is a marker for previously activated T cells and memory CD8+ T cells (36). Expression of Ly-6C was highest in HSV.4-1BBL–stimulated OT-1/GFP, compared with HSV.B7.1- and HSV.LacZ-stimulated OT-1/GFP and naive OT-1/GFP (Fig. 2A). Because Ly-6C can be up-regulated on T cells by type I IFN secretion by HSV amplicon-transduced tumors (37), supernatant from cocultures of OT-1 and LLC/OVA transduced with HSV.4-1BBL or HSV.LacZ was collected on days 1 to 3. IFN-α was not detected by ELISA at a detection threshold of 12.5 pg/mL (data not shown). More likely, IFN-γ (38) produced by 4-1BBL–stimulated T cells induced Ly-6C expression.

**T cells costimulated with 4-1BBL ex vivo expand in vivo in response to tumor.** Ex vivo stimulated OT-1/GFP cells were adoptively transferred into LLC/OVA tumor-bearing mice, and the extent of expansion and antitumor response were measured (Fig. 2B). Mice were bled at several time points following transfer of OT-1/GFP cells to detect expansion (Fig. 3A). Six days following transfer, the number of OT-1/GFP in the peripheral blood was significantly greater in the LLC/OVA tumor-bearing group receiving HSV.4-1BBL–stimulated OT-1/GFP cells compared with tumor-bearing groups receiving naive (P = 0.007) or HSV.B7.1-stimulated OT-1/GFP (Fig. 3A). Greater numbers of OT-1/GFP cells were observed following transfer of 4-1BBL–stimulated OT-1/GFP cells into non–tumor-bearing mice than naive or B7.1-stimulated OT-1/GFP cells, indicating continued proliferation of 4-1BBL–stimulated OT-1/GFP cells in vivo in the absence of tumor. The number of 4-1BBL–stimulated OT-1/GFP cells was significantly greater in the tumor-bearing mice compared with non–tumor-bearing mice (P = 0.017), suggesting that 4-1BBL-stimulated OT-1/GFP cells can respond in vivo to tumor-specific antigen (Fig. 3A).

The total numbers and percentages of tumor-specific OT-1/GFP cells in relation to the CD8+ population were determined in the spleen of tumor-bearing mice 17 days post-transfer. Representative mice are shown in Fig. 3B. The percentage of GFP+ cells in the spleen was the greatest in the tumor-bearing group given OT-1/GFP cells stimulated with HSV.4-1BBL–transduced tumor (19.8%) compared with HSV.B7.1-stimulated OT-1/GFP, HSV.LacZ-stimulated OT-1/GFP (Fig. 2B). Numbers of OT-1/GFP cells were also detected in the spleen of non–tumor-bearing mice 17 days after transfer of 4-1BBL-stimulated OT-1/GFP cells (7% of CD8+ cells), suggesting that tumor-specific 4-1BBL–stimulated T cells can also persist in vivo in the absence of antigen.
In addition, there were greater absolute numbers of OT-1/GFP cells present in the spleen for the 4-1BBL-stimulated OT-1/GFP group (0.9 ± 0.4 × 10^6 cells per spleen) versus B7.1-stimulated (0.9 ± 0.7 × 10^6) or naive OT-1/GFP groups (1 ± 0.3 × 10^6).

Spleens were sectioned and examined for GFP+ cells. Spleens from mice given adoptively transferred OT-1/GFP showed increased infiltration with GFP+ cells (Fig. 3C). Analysis by flow cytometry indicated that 9.4% of the CD8e spleenocytes in the HSV-4.1BBL-stimulated OT-1/GFP group were GFP+, compared with 0.1% and 0.2% GFP+ in the naive OT-1/GFP and HSV.B7.1-stimulated OT-1/GFP groups, respectively. These results indicate that tumor-specific T cells stimulated ex vivo with HSV-4.1BBL–transduced tumor could expand and persist in vivo.

The tumor bed was also analyzed for the presence of OT-1/GFP cells. Six days post-transfer, flow cytometry analysis of dispersed tumor showed that the percentage of GFP+ cells was 7- to 8-fold greater in mice that received the 4-1BBL–stimulated OT-1/GFP cells compared with those receiving naive cells (Fig. 3D). Later time points could not be examined due to tumor regression in the HSV-4.1BBL–stimulated OT-1/GFP group.

BrdUrd is incorporated by adoptively transferred cells. To determine whether adoptively transferred OT-1 divided in vivo, we characterized the BrdUrd uptake of transferred cells in separate experiments. Thy1.2+ OT-1 cells were transferred into Thy1.1+ mice, which were pulsed with BrdUrd on days 7 and 10. Mice were sacrificed on day 12 to determine in vivo proliferation of OT-1 CD8e cells and their distribution in the spleen, TDLNs, and non-TDLNs (Fig. 4A). Significantly higher percentages of OT-1 cells that had incorporated BrdUrd were present in the TDLNs in the 4-1BBL group compared with the naive OT-1 group (Fig. 4A).

The presence of proliferating OT-1 cells in the tumor bed was assessed by pulsed mice on days 3 and 6 with 1 mg of BrdUrd and harvesting the tumor on day 8. Overall, more Thy1.2+ OT-1 cells were present in the 4-1BBL–stimulated group as compared with the naive OT-1 or B7.1-stimulated group, and these cells had incorporated BrdUrd (Fig. 4B).

CD8e T cells expanded in vitro with 4-1BBL possess cytolytic activity and markedly decrease tumor growth. We measured CTL activity from spleenocytes of mice that received 2 × 10^6 OT-1/GFP cells by adoptive transfer. LLC/OVA tumor-bearing mice treated with HSV-4.1BBL–activated OT-1/GFP cells harbored lower tumor burden at day 14 than untreated mice (P < 0.0001) or mice treated with an identical number of HSV.B7.1-stimulated OT-1/GFP (P < 0.0001) or naive OT-1/GFP cells (P = 0.0003; Fig. 5A). Naive OT-1/GFP treatment did not reduce tumor burden significantly when compared with the untreated group (P > 0.05). Administration of HSV.B7.1-activated OT-1/GFP cells did not have major inhibitory effects on tumor size when compared with no treatment or to naive OT-1/GFP transfer (P > 0.05; Fig. 5A).

Splenocytes from the HSV-4.1BBL–activated OT-1/GFP group showed substantially higher CTL activity against LLC/OVA compared with spleenocytes from the HSV.B7.1-stimulated OT-1/GFP, naive OT-1/GFP, and untreated groups (Fig. 5B). These results suggest that T cells expanded with HSV-4.1BBL–transduced tumor cells may have more favorable effector characteristics than those obtained through HSV.B7.1-mediated stimulation.

In a second experiment, 4 × 10^6 cells OT-1/GFP cells, either naive or stimulated with HSV-4.1BBL–transduced tumor, were transferred into LLC/OVA tumor-bearing mice to determine the effects on tumor volume (Fig. 5C). Transfer of HSV.4-1BBL–stimulated OT-1/GFP resulted in a statistically significant decrease in tumor growth compared with no treatment (P < 0.001) or to naive OT-1/GFP transfer (P < 0.001; Fig. 5C). The estimated rate of growth for the HSV-4.1BBL OT-1/GFP group from the regression model was significantly lower than that for the naive OT-1/GFP group (0.013 versus 0.130; P < 0.001). By day 30, 88.9% (8/9) mice in the HSV-4.1BBL OT-1/GFP group were tumor-free, compared with 0% (0/7) tumor-free in the no-treatment group and 12.5% (1/8) tumor-free in the naive OT-1/GFP group. Analysis using Fisher’s exact test show that these differences between the HSV-4.1BBL OT-1/GFP and the no-treatment group (P = 0.001) or the naive OT-1/GFP group (P = 0.003) were statistically significant (Fig. 5D).

4-1BBL–stimulated CD8e OT-1 cells display memory phenotypic characteristics. The phenotype of OT-1/GFP cells in each group was characterized post-adoptive transfer (Fig. 6). On day 6, GFP+ cells in the spleen and tumor bed were analyzed for Ly-6C (Fig. 6A). The HSV-4.1BBL–stimulated OT-1/GFP group had a greater percentage of Ly-6C+GFP+ cells present in the spleen and tumor bed than the naive OT-1/GFP group. On day 17, OT-1/GFP+ cells in tumor-bearing and non–tumor-bearing mice were studied for levels of CD44, Ly-6C, and CD62L (1-selectin; Fig. 6B and Supplementary Fig. S2). Significantly higher levels of CD44+ OT-1/GFP were observed in the splenocytes of mice that had received HSV-4.1BBL–stimulated OT-1/GFP (12.7% in tumor-bearing mice, 3.8% in non–tumor-bearing mice) than were seen for either HSV.B7.1-stimulated (0.1% in tumor-bearing and non–tumor-bearing mice) or naive OT-1/GFP transfer (0.4% in tumor-bearing mice, 0.1% in non–tumor-bearing mice; Fig. 6B and Supplementary Fig. S2). HSV-4.1BBL–stimulated OT-1/GFP cells continued to show higher levels of Ly-6C expression on day 17 in comparison to naive or HSV.B7.1-stimulated OT-1/GFP transferred cells (Fig. 6B and Supplementary Fig. S2). Further analysis showed that OT-1/GFP cells persisting in the spleen were CD62L negative (Fig. 6B and Supplementary Fig. S2), as well as CD25 negative and CD127 (IL-7Rα) negative (data not shown). Similar characteristics, indicating the generation of effector memory cells, were observed in OT-1/GFP cells present in the spleen at day 32 (data not shown). This indicates that upon adoptive transfer of 4-1BBL–stimulated T cells, the T cells persist at high levels and exhibit phenotypic attributes of effector memory cells.

To determine whether this strategy could be used in a nontransgenic setting, we inoculated GFP+ mice with LLC/OVA s.c. for 8 days and then harvested spleens and TDLNs. CD8e T cells in the spleen or lymphocytes from TDLNs were studied ex vivo with untransduced LLC/OVA or LLC/OVA transduced with HSV-4-1BBL. Increased Ly-6C expression was observed in vitro on the CD8e spleenocytes and, to a lesser degree, on the CD8e TDLN cells following 5 days of cultivation with HSV-4.1BBL–transduced LLC/OVA compared with coculture with untransduced tumor (data not shown). Although 4-1BBL–transduced LLC/OVA induced proliferation and modest expansion of GFP+ lymphocytes in vitro, levels were ~20-fold lower than with OT-1, preventing adequate harvest for adoptive transfer. Adoptive transfer of 0.5 × 10^6 cocultured T cells showed modest effects on tumor growth. This may be due to low precursor frequency and reduced expansion. Therefore, further optimization of the ability to generate tumor-specific T cells and/or modification of transferred dose and dose schedules will be necessary to improve tumor control.

Discussion

The overall purpose of these studies was to understand the potential utility of 4-1BB as a means of expanding and activating 4-1BBL in Adoptive Immunotherapy
tumor-specific CD8+ T cells for adoptive immunotherapy. 4-1BB stimulation has been used to sustain nascent responses and prevent activation-induced cell death in T cells (39). 4-1BB stimulation triggers TRAF2 signaling and nuclear factor-κB activation (40,41), which may, in turn, induce Bcl-xL and Bfl-1, two prosurvival members of the Bcl-2 family. We reasoned that cells expanded using 4-1BB costimulation might have favorable effector characteristics and persist in vivo, potentially leading to improved tumor control.

Figure 5. Cytolytic activity and tumor volumes in mice following adoptive transfer of OT-1/GFP T cells. CD8+ OT-1/GFP T cells were activated in vitro with transduced LLC/OVA at an E:T ratio of 3:2 for 3 days. A and B, activated OT-1/GFP or naive OT-1/GFP (2 × 10^6) cells were given into the tail vein of mice injected with tumor s.c. 4 days previously. A, tumors were measured at the time points indicated. bars, SE. P values were determined from regression analysis. HSV-4-1BBL OT-1/GFP, 10 mice; HSV.B7.1 OT-1/GFP, 5 mice; naive OT-1/GFP, 5 mice; No OT-1/GFP, 3 mice. B, at 22 d following tumor injection, splenocytes were harvested and cultured with recombinant human IL-2 (10 units/mL) and mitomycin C–treated LLC/OVA cells at an E:T ratio of 10:1 for 6 d. LLC/OVA cells were used as targets in an 8-h 51Cr release assay. Percent lysis with SD is shown. Data represent two separate experiments. C and D, activated OT-1/GFP or naive OT-1/GFP (4 × 10^6) were given into the tail vein of mice injected with LLC/OVA tumor (1 × 10^6) s.c. 2 d previously. Tumors were measured every 2 to 4 d, and mice were sacrificed when tumor diameter reached 15 mm. Data are representative of three separate experiments. Dotted lines, individual mice; solid line, fitted growth curve.
HSV.4-1BBL amplicons were used to transduce LLC/OVA tumors in vitro for purposes of ex vivo expansion of tumor-specific OT-1 T cells. OT-1 cells responded to coculture with HSV.4-1BBL–transduced LLC/OVA by proliferating and expressing activation markers, namely, CD44, CD25, Ly-6C, CD107a, 4-1BB, and intracellular granzyme B, indicating priming and differentiation into cytolytic effectors.

Adoptive transfer of HSV.4-1BBL–stimulated OT-1/GFP T cells significantly conferred greater protection against LLC/OVA growth compared with naive or HSV.B7.1-stimulated OT-1/GFP cells. Mice treated with HSV.4-1BBL–expanded OT-1/GFP cells exhibited greater CTL activity and showed higher percentages of tumor-specific OT-1/GFP+ cells in the spleen and tumor bed. Following HSV.4-1BBL stimulation, CD8+ OT-1 T cells expanded in vivo, incorporated BrdUrd, and expressed high levels of CD44 and the CD8+ memory marker Ly-6C. Ly-6C has been shown to regulate homing of CD8+ T cells to lymph nodes (42) and perhaps augments the homing of transferred HSV.4-1BBL–stimulated OT-1/GFP cells to secondary lymphoid organs, where they can be found on days 6, 17, and 32 post-transfer. Most transferred OT-1/GFP cells were CD62L negative even after 20 days post-tumor eradication in the host, indicating, in combination with Ly-6C expression, an effector memory phenotype. Perhaps, longer observations can show the conversion of tumor-specific cells to CD44hiLy-6ChiCD62Lhi central memory cells. In these studies, we show that T-cell costimulation with 4-1BBL expressed on tumor cells may be useful in facilitating the expansion of tumor-specific T cells in vivo as well as in vitro.

Other groups have examined the possibility of using 4-1BB costimulation for the generation of tumor-reactive T cells for adoptive immunotherapy. Addition of an agonistic anti–4-1BB antibody to in vitro cultures of TDLN cells and anti-CD3/anti-CD28 antibodies enhanced expansion, production of type 1 cytokines,
and survival of T cells (43). When anti-CD3/anti-CD28/anti–4-1BB–expanded TDLN cells were adoptively transferred into MCA 205 tumor-bearing mice, significantly fewer metastatic lesions and prolonged survival of mice were observed compared with TDLN cells stimulated without anti–4-1BB (43). Strome et al. (16) also observed that the combined use of anti-CD3/anti-CD28/anti–4-1BB in activating T cells for adoptive immunotherapy resulted in the generation of T cells that were more effective than those activated by anti-CD3 alone or anti-CD3/anti-CD28 in mediating antitumor reactivity.

However, when groups used anti-CD3/anti-4-1BB antibodies, without anti-CD28 antibody, to expand a polyclonal T-cell population in culture, they were not successful in generating tumor-reactive T cells due to nonspecific expansion (16, 17). Our studies indicate that it may be possible to specifically generate tumor-reactive clones, which can persist in vivo, using HSV-4-1BBL–transduced tumor cells in the absence of additional CD28 costimulation. Whether the low levels of B7.1 present on LLC/OVA tumor are adequate for CD28 stimulation to work in combination with 4-1BBL costimulation is not known (44).

Maus et al. (11) have shown ex vivo expansion of human polyclonal and MHC tetramer–sorted antigen–specific CTL using artificial antigen presenting cells, specifically K562 erythroleukemia cell lines stably transfected to express 4-1BBL and the Fcy receptor CD32 to bind anti-CD3 and anti-CD28 antibodies on the surface. In contrast to the work by Maus et al., the highly efficient HSV amplicon system provides a theoretical means by which to selectively expand tumor–specific effector populations using autologous tumor from patients without the need for presorting for tumor–reactive T cells.

In addition to 4-1BB costimulation, HSV-4-1BBL amplicon transduction of tumor may provide other stimuli that facilitate the generation of effector cells. HSV amplicons can impart a strong innate response to transduced cells, including macrophage cell lines and human CLL cells, resulting in cytokine secretion and NK2D-L expression by the transduced cells (23). HSV possesses at least three molecular components capable of activating the innate immune system: (1) dsRNA generated through self–hybridization of viral genes transcribed from complementary DNA strands (45); (2) envelope glycoproteins recognized by TLR2 (46); and (3) unmethylated CpG motifs encoded in the viral genome that activate TLR9 (47). Due to the fact that HSV amplicon DNA is concatamerized, CpG effects on TLR9 may be quite potent. The enhanced capacity of transduced tumors to stimulate an innate immune response may lead to an improved adaptive response.

We did not test whether HSV.B7.1 will further augment effects seen with HSV-4-1BBL stimulation. HSV.B7.1–stimulated OT-1 failed to expand in vivo and inhibit tumor growth. Because B7.1 serves as a ligand for both CD28 and CTLA-4, B7.1 may have also bound to CTLA-4 expressed on activated OT-1 cells, inhibiting expansion and survival. We, however, did not observe greater proliferation in the presence of soluble anti–CTLA-4 antibody (Supplementary Fig. S1).

In summary, our studies suggest that costimulation with 4-1BBL may be employed to enhance expansion and cytolytic activity of tumor–specific CD8+ T cells for the generation of tumor–specific immunity. We used the adoptive transfer of TCR-transgenic OT-1/GFP T cells to more accurately follow the effects of 4-1BBL on T cells that were specific for defined tumor–related antigens in our in vivo mouse experiments, because antigen–specific CD8+ T cells are normally present in very low numbers (32, 33) Although the OT-1 model was useful in demonstrating expansion of a tumor–specific response, results obtained with OT-1 may differ as compared with what would be observed using a polyclonal T cell population as a source of expanded T cells. Such a population might contain relatively fewer precursor T cells with antitumor activity, and expansion of relevant effectors might prove more difficult. Nevertheless, the vigorous cytolytic effector function as well as the increased expansion and persistence seen using HSV amplicon–transduced tumor suggest that this method should be explored further and may be potentially applicable in the human setting. Because HSV vectors are theoretically safe and a highly efficient means of gene transfer, the laboratory is pursuing preclinical development of these vectors for potential human use in CLL. Further optimization may allow for efficient expansion of relatively rare precursor antitumor T cells.

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