RESEARCH ARTICLE

Combination suicide/cytokine gene therapy as adjuvants to a defective herpes simplex virus-based cancer vaccine

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We have used syngeneic, established bilateral subcutaneous tumor models to examine the antitumor activity of herpes simplex virus (HSV) vectors, including the induction of an immune response against non-inoculated distant tumors. In such a model with CT26 murine colon adenocarcinoma, unilateral intratumoral inoculation of replication-deficient HSV-1 tsK inhibited the growth of both the inoculated and noninoculated established tumors. To enhance this limited antitumor immune response, we generated a defective HSV vector, dvIL12-tk encoding both interleukin-12 (IL-12) and HSV thymidine kinase (TK), with tsK as the helper virus. In a 'suicide gene' strategy, ganciclovir (GCV) treatment after intratumoral inoculation of dvlacZ-tk/tsK, encoding *E. coli*

lacZ instead of IL-12, resulted in enhanced antitumor activity. Antitumor activity was also enhanced by local expression of IL-12 from dvIL12-tk/tsK. The combination of IL-12 cytokine therapy with GCV treatment was the most efficacious approach, with significantly greater inhibition of tumor growth than IL-12 or TK+ GCV alone. These results illustrate the power of combining different cancer therapy approaches; 'suicide gene' therapy, cytokine therapy, and HSV vector infection. HSV vectors are particularly well suited to this because they can accommodate the insertion of large and multiple gene sequences. Gene Therapy (2001) 8, 332–339.

Keywords: HSV; thymidine kinase; IL-12; cancer vaccine; CT26 adenocarcinoma

Introduction

A number of different strategies employing viruses in the immunotherapy of cancer are currently being developed, the goal being to stimulate the host immune response against cancer cells.¹ In murine tumor models, it is generally accepted that failure of an antitumor immune response is often due not to the absence of tumor-specific antigens, but rather to defects in immune regulation.² Systemic administration of cytokines can produce substantial antitumor activity, but the severe toxicity associated with this approach at effective doses is cause for concern.³ Transfer of cytokine genes into tumor cells using viruses or other vehicles has the advantage of producing concentrations of cytokines in the area of putative tumor antigens, thereby reducing the associated systemic toxicities.

IL-12 is a heterodimeric cytokine, composed of 35 kDa (p35) and 40 kDa (p40) subunits, that bind to receptors present on NK and T cells.⁴ IL-12 plays a multifunctional role in the immune system, augmenting the proliferation

and cytotoxic activity of T cells and NK cells, regulating IFN- γ production and promoting the development of CD4⁺ Th1 cells.^{5,6} The antitumor activity of IL-12 has been demonstrated in a number of different murine tumor models.^{7–9} In addition, IL-12 as an adjuvant has been shown to enhance the antimetastatic activity of recombinant vaccinia virus-based tumor vaccines,¹⁰ and vaccination with mutated p53 peptides has increased antitumor efficacy when IL-12 is given as an adjuvant, at doses that are ineffective when injected alone.¹¹

HSV TK has a relaxed substrate specificity compared with cellular TKs and can therefore utilize a number of modified nucleosides such as ganciclovir (GCV), acyclovir, and 1- β -D aravinofuranosylthymine (araT) which upon conversion to toxic metabolites are incorporated into nascent RNA and DNA chains and cause cell death.¹² HSV TK/GCV 'suicide' gene therapy not only kills cells transduced with TK but also neighboring non-TK expressing cells, the so called 'bystander effect'.¹³ The effect *in vivo* is likely mediated in some fashion by an immune response. Chen *et al*¹⁴ demonstrated that IL-2 acted synergistically with HSV TK/GCV gene therapy to induce a systemic antitumor immunity using adenoviral vectors in a murine model of hepatic metastases of colon cancer.

HSV vectors have been used for direct oncolytic anticancer therapy and as gene delivery vehicles for immunotherapy of tumors.^{15–18} We recently found that intratumoral infection with G207, an attenuated replication-competent HSV vector, in immune-competent

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mice induces a potent tumor-specific immune response that involves CD8⁺ CTL activity and provides protective immunity to tumor rechallenge.^{19,20} We have referred to this as *in situ* cancer vaccination. Furthermore, intratumoral expression of IL-12 can significantly enhance the anti-tumor activity of G207.²¹ In these studies, cytokine genes were expressed from defective HSV vectors.^{21,22} Defective HSV vectors are generated from an amplicon plasmid containing HSV *cis*-acting elements (origin of DNA replication and cleavage/packaging signal) for amplification and packaging into viral particles in the presence of helper HSV.²³ Virus stocks of defective HSV contain both 'defective particles' and helper virus. Here, we have generated a defective HSV vector encoding both IL-12 polypeptides and HSV TK using a replication-deficient helper virus, HSV tsK.²⁴ The combination of IL-12 expression, HSV TK/GCV treatment and HSV infection provides a substantial improvement in efficacy. The presence of both IL-12 and TK in the same vector also provides a safety feature, whereby cells expressing IL-12 could be eliminated by GCV treatment under conditions of IL-12 toxicity, as has been done for donor T cell depletion after bone marrow transplantation.^{25,26}

Results

Generation of defective HSV vectors

Defective HSV vectors, dvlacZ-tk/tsK and dvIL12-tk/tsK, were generated from amplicon plasmids, pHCL-tk and pHCIL12-tk, respectively, using HSV tsK as helper virus. The ratio of dvlacZ-tk to tsK was 0.3:1. The expression and secretion of IL-12 was determined by ELISA assay after infection of tumor cells in culture. Infection of CT26 (murine colon adenocarcinoma), MCA38 (murine colon adenocarcinoma), MCA207 (murine fibrosarcoma), and Vero cells with dvIL12-tk/tsK resulted in secretion of up to 81 pg murine IL-12/10⁵ tumor cells/24 h (Figure 1). In a separate experi-

ment, dvIL12-tk/tsK infection of Vero cells resulted in 86 and 353 pg IL-12/10⁵ cells at 24 and 48 h after infection, respectively. No IL-12 expression was detected in supernatants from cell cultures infected with dvlacZ-tk/tsK, nor in the supernatants of uninfected tumor cells.

Treatment of established bilateral subcutaneous tumors

We first evaluated the antitumor activity of the helper virus, tsK alone (in the absence of defective vector) in the CT26 mouse colon adenocarcinoma established bilateral subcutaneous tumor model. BALB/c mice were injected subcutaneously with 1 × 10⁵ CT26 cells in their bilateral flanks and when tumors were palpable, they underwent unilateral intratumoral inoculation with tsK (1 × 10⁵ p.f.u.), followed by a second inoculation 7 days later in the same tumor. Both the inoculated tumors and their noninoculated contralateral counterparts demonstrated a limited, but significant reduction in tumor growth with tsK compared with mock (Figure 2).

We next evaluated the antitumor efficacy of local IL-12 expression, from dvIL12-tk with tsK as helper virus, in the same established bilaterally tumor model. BALB/c mice with palpable bilateral tumors were inoculated with dvlacZ-tk/tsK (*lacZ* expression as a control) or dvIL12-tk/tsK (2 × 10⁵ p.f.u. of tsK helper virus) into the right flank tumors, followed by a second inoculation 7 days later. The growth of the inoculated tumors (Rt, Figure 3a), as well as the noninoculated tumors (Lt, Figure 3a) was significantly inhibited by infection with dvlacZ-tk/tsK as compared with mock, similar to what occurred with tsK helper virus alone. Inoculation of dvIL12-tk/tsK was much more effective in inhibiting bilateral tumor growth than dvlacZ-tk/tsK (Figure 3a). Two of six of the dvIL12-tk/tsK-inoculated tumors regressed to an undetectable size. Mice treated with dvIL12-tk/tsK survived significantly longer than either dvlacZ-tk/tsK or mock treated mice, while dvlacZ-tk/tsK treated mice survived longer than mock treated mice (Figure 3b).

Combination 'suicide' and cytokine gene therapy

The antitumor efficacy of 'suicide' and cytokine gene therapies in combination with HSV infection was evaluated in the established bilateral CT26 subcutaneous tumor model. Animals with palpable bilateral tumors received a single intratumoral inoculation of defective HSV vector (2 × 10⁵ p.f.u. of tsK helper virus) in the right flank tumor (day 0). GCV (50 mg/kg daily) or saline was injected intraperitoneally daily from day 2 until day 9 after infection, and tumor volumes were compared on day 21 after infection (Figure 4a). HSV TK is present on both of the defective vectors, as well as the helper virus tsK. However, because of the temperature-sensitive mutation in tsK, TK should not be expressed in tsK infected cells. GCV treatment had no impact on the growth of mock-inoculated tumors. A single inoculation of dvlacZ-tk/tsK had only a limited effect on growth compared with mock inoculation (Figure 4a, $P = 0.1$ (Rt); $P = 0.07$ (Lt), unpaired *t* test). This can be contrasted with the inhibition seen with two inoculations dvlacZ-tk/tsK (Figure 3). Even though twice as much total vector was inoculated with two injections, we believe that the difference is more likely due to multiple treatments.²⁷ With only a single inoculation of dvlacZ-tk/tsK, GCV treatment significantly reduced the growth of both inoculated and noninoculated tumors compared with saline treat-

Figure 1 Synthesis of murine IL-12 by various mouse tumor cell lines (CT26, MCA38, and MCA207) and monkey kidney cells (Vero) infected with dvIL12-tk/tsK at a multiplicity of infection (MOI) of 1 of tsK. Culture supernatants were collected after 24 h and assayed for murine IL-12 using a sandwich ELISA.

Figure 2 Bilateral established subcutaneous CT26 tumor therapy in syngeneic BALB/c mice. When bilateral subcutaneous tumors reached approximately 5 mm in diameter, mice underwent unilateral inoculation into the right side tumor with tsK virus (1×10^5 p.f.u.) or mock-infected extract (Mock), on day 0, followed by a second inoculation on day 7. Both the inoculated tumors (Rt) and their non-inoculated contralateral counterparts (Lt) demonstrated significant tumor growth reduction after infection with tsK compared with mock ($P < 0.05$ on day 21 after infection; unpaired *t* test).

ment (Figure 4a) and increased survival (Figure 4b). GCV treatment in combination with dvIL12-tk/tsK further enhanced the efficacy (Figure 4a), with one of six animals having both tumors regress (Figure 4b). In this and prior studies with dvIL12 and CT26 we have never seen both tumors regress.²¹ Six months later, this 'cured' mouse resisted a tumor rechallenge with 5×10^5 tumor cells, whereas all three control naive animals developed tumors within 5 to 6 days after implantation.

To test whether inhibition of subcutaneous tumor growth was associated with increased CTL activity, we evaluated CT26-specific CTL activity *in vitro* using a ^{51}Cr release assay. Splenocytes were obtained from dvIL12-tk/tsK inoculated mice that had been treated with GCV or saline and cultured *in vitro* with mitomycin C-treated CT26. These effector cells exhibited specific lysis of CT26 tumor cells or A20 lymphoma cells pulsed with AH1, the immunodominant peptide for CT26, but not A20 cells pulsed with P815AB, the immunodominant peptide derived from murine mastocytoma P815 cells (Figure 5). There was no significant difference between animals treated with GCV or saline (Figure 5), even though there was enhanced antitumor efficacy after GCV treatment (Figure 4).

Discussion

The expression of cytokines within the microenvironment of a tumor is thought to facilitate, through paracrine action, the priming of naive or immunoreactive, but dysfunctional, antitumor effector cells.^{2,28} Genetic modification of tumor cells can be accomplished by *ex vivo* or *in vivo* transduction of cytokine genes using viral vectors.^{7,8,21,29} The induction of systemic antitumor immunity that can resist tumor cell challenge after cancer vaccination has been well documented.³⁰ However, the capacity of cancer vaccines to limit the progression of established tumors at a distant site has been less successful. Using a bilateral established subcutaneous tumor model, with CT26 colon adenocarcinoma, M3 melanoma, or N18 neuroblastoma, we have demonstrated that intra-

tumoral inoculation of the attenuated, replication-competent HSV-1 vector G207 can induce a systemic antitumor immune response that inhibits the growth of noninoculated distant tumors.^{19,20}

As yet, there is no clearly defined mechanism of how virally modified tumor cells *in situ* induce specific antitumor immunity. It has been demonstrated that the priming of an immune response against a MHC class I-restricted tumor antigen involves the transfer of that antigen to antigen-presenting cells (APCs) before its presentation to CD8⁺ T cells.³¹ A subset of macrophages are able to present exogenous antigens on MHC class I molecules to CD8⁺ T cell clones.³² Local HSV infection of the tumor might induce circulating precursors to differentiate into APCs which would pick up tumor antigens and traffic to the draining lymph nodes for presentation to CD8⁺ T cells. Associative recognition of HSV-specific and tumor-specific antigens might also play a role in the strength of the response.

In these studies, we examined the ability of tsK, a temperature-sensitive HSV-1 mutant that is unable to replicate at 39.5°C,³³ to induce an antitumor immune response, in combination with IL-12 and/or HSV TK expression or alone. Even though tsK does not replicate *in vivo*, it does induce an inflammatory response.³⁴ The mutant ICP4 gene product of tsK can induce a stress response in infected cells³⁵, and immediate-early gene products, which are overexpressed after tsK infection, induce cytopathic changes resulting in cell death and tissue necrosis.³⁶ Finally, immediate-early gene products, including ICP4, are targets for cell-mediated immunity.^{37,38}

Some dose effect in inducing systemic immunity was noted, as two inoculations with dvlacZ-tk/tsK caused significant growth inhibition of distant tumors, whereas one inoculation did not. Similarly, the enhanced antitumor activity of IL-12 expression compared with dvlacZ-tk/tsK inoculation was only apparent after two inoculations. With HSV-1 G207, multiple inoculations are significantly more efficacious than a single inoculation of the same total dose,²⁷ and efficacy roughly correlates with

Figure 3 Defective HSV vector treatment of bilateral established subcutaneous CT26 tumors. When bilateral subcutaneous tumors reached approximately 5 mm in diameter, mice underwent unilateral intratumoral inoculation with defective HSV vectors (2×10^5 p.f.u. of helper virus tsK) or virus buffer (Mock) into the right side tumor on day 0, followed by a second inoculation on day 7. (a) Both the inoculated tumors (Rt), as well as their non-inoculated contralateral counterparts (Lt), demonstrated significant tumor growth reduction after infection with *dvlacZ-tk/tsK* (*dvlacZ-tk*) compared with mock ($P < 0.005$ (Rt), $P < 0.01$ (Lt) on day 22 after infection; unpaired *t* test). Inoculation with *dvIL12-tk/tsK* (*dvIL12-tk*) was more effective in inhibiting bilateral tumor growth than inoculation with *dvlacZ-tk/tsK* ($P < 0.001$ on day 22 after infection; unpaired *t* test). Bars represent means $\pm$ s.e.m. ($n = 6$ per group). (b) Survival of mice treated with defective HSV vectors. When mice became moribund or tumors reached >18 mm in diameter animals were killed. Survival of mice treated with *dvIL12-tk/tsK* (*dvIL12-tk*) was significantly greater than mice treated with *dvlacZ-tk/tsK* (*dvlacZ-tk*) or Mock ($P < 0.01$, Cox Mantel test). *DvlacZ-tk/tsK* treated mice survived longer than mock-treated mice ($P < 0.01$, Cox Mantel test).

dose.²⁰ The antitumor activity of IL-12 (recombinant protein, transduced cells, or viral vector delivered) has been shown to be dose-dependent in a number of animal tumor models.^{9,39–41}

The use of HSV as a vaccine, as well as a gene expression vector is a novel and attractive approach for cancer immunotherapy. The defective HSV vector stock contains both defective HSV particles and helper HSV. There are a number of advantages to this strategy for the immunotherapy of cancer; the helper virus has the potential to induce antitumor immunity, acting as an *in situ* cancer vaccine,^{19,20} and the defective HSV vector has capacity to transduce large or multiple DNA sequences, delivering multiple copies of the transducing gene per virion.^{21,22,42} Combination suicide and cytokine gene therapy, using multiple adenovirus vectors, has been shown to be more effective than either treatment alone.^{43,44} In our studies, we were able to deliver both therapeutic genes in the same vector so that every infected cell would express the combination of gene products. The triple

combination of IL-12, HSV TK + GCV and tsK was more effective than either of the treatments alone or as pairs.

The addition of GCV treatment to either *dvlacZ-tk/tsk* or *dvIL12-tk/tsK* tumor-inoculated animals increased the therapeutic efficacy, even though the vector dose was very low. The mechanism behind the 'bystander' effect of HSV TK + GCV therapy⁴⁵ involves multiple pathways, including: a cellular immune response that is lacking in athymic mice,^{46,47} increased expression of MHC class I antigens,⁴⁸ IL-6 and TNF α ,⁴⁹ infiltration of macrophages and T cells,^{43,49} and induction of apoptosis.^{50,51} In a study treating hepatic metastases with adenovirus vectors expressing HSV TK or TK + GM-CSF, no significant induction of CTL activity was found, even though there was significant inhibition of tumor growth.⁴³ We similarly found no enhancement in CTL activity in *dvIL12-tk/tsK* inoculated animals after GCV treatment, even though there was increased inhibition of tumor growth.

As for the safety of this strategy, HSV DNA does not integrate into the infected cell genome and expression of

Figure 4 Combination suicide and IL-12 gene therapy. Bilateral tumors were established in the flanks of BALB/c mice by the implantation of 10^5 CT26 cells per side. Treatment was initiated when subcutaneous tumors reached approximately 5 mm in diameter. Each animal then underwent one unilateral intratumoral inoculation of defective vector stock (2×10^5 p.f.u. of helper virus tsK) in the right flank (day 0). GCV or saline was injected intraperitoneally daily from day 2 until day 9 after infection. (a) Tumor volumes were compared on day 21 after infection. GCV treatment had no impact on the growth of mock-inoculated tumors. GCV treatment with *dvlacZ*-tk/tsK (*dvlacZ*-tk + GCV) inhibited the growth of both the inoculated (Rt) and contralateral noninoculated (Lt) tumors compared with *dvlacZ*-tk/tsK with saline (*dvlacZ*-tk + saline). The antitumor effect of GCV treatment with *dvIL12*-tk/tsK (*dvIL12*-tk + GCV) was significantly greater than that of *dvlacZ*-tk + GCV. *, $P < 0.05$; **, $P < 0.005$; ns, not significant; unpaired *t* test. Bars represent means $\pm$ s.e.m. ($n = 6$ per group). (b) Survival of mice treated with combination therapy. When mice became moribund or tumors reached >18 mm in diameter they were killed. Mice treated with *dvIL12*-tk + GCV survived significantly longer than *dvIL12*-tk + saline or *dvlacZ*-tk + GCV treated mice (*dvIL12*-tk + saline: $P < 0.05$; *dvlacZ*-tk + GCV: $P < 0.01$; Wilcoxon test). *DvlacZ*-tk + GCV-treated mice survived significantly longer than *dvlacZ*-tk + saline treated mice ($P < 0.05$; Wilcoxon test).

Figure 5 Induction of specific CTL response after intratumoral inoculation of *dvIL12*-tk/tsK. BALB/c mice with established subcutaneous CT26 tumors were inoculated on day 0 and then treated intraperitoneally with GCV or saline from day 2 until day 9. Splenocytes were harvested on day 12 and stimulated *in vitro* with mitomycin C-treated CT26 cells. After 6 days in culture, a ^{51}Cr release assay was performed using as targets CT26 cells or A20 cells pulsed with AH1 or P815Ab peptides. Intratumoral inoculation of *dvIL12*-tk/tsK induced a significant CTL activity against CT26 cells or A20 cells pulsed with AH1, but not the nonspecific peptide P815AB. There was no significant difference between animals receiving GCV or saline.

the gene product is transient and local. When used to deliver cytokines in immunotherapy, the vector would therefore not result in chronic stimulation of the immune system or high levels of systemic cytokine. Moreover, GCV treatment would eliminate HSV and any associated viral toxicity, as well as HSV TK expressing cells, generate a bystander effect and function as an adjuvant to the HSV-based cancer vaccine. We conclude that the combination of suicide and cytokine gene therapies using the defective HSV vector system is an effective adjuvant to enhance the antitumor activity mediated by helper HSV.

Materials and methods

Cell culture

African green monkey kidney (Vero) cells (kindly provided by Dr D Knipe, Harvard Medical School, Boston, MA, USA) were cultured in Dulbecco's minimum essential medium (DMEM) containing 10% calf serum (CS) (Hyclone, Logan, UT, USA). CT26 is a colon epithelial tumor cell line derived by intrarectal injections of *N*-nitroso-*N*-methylurethane in BALB/c mice (H-2^d)⁵² (kindly provided by Dr NP Restifo, National Institute of Health, Bethesda, MD, USA), MCA38 murine colon adenocarcinoma (obtained from NP Restifo) and MCA207 murine fibrosarcoma⁵³ (obtained from NP Restifo) were grown in DMEM containing 10% heat-inactivated fetal calf serum (FCS) (Hyclone) and penicillin-streptomycin (Sigma Chemical Co, St Louis, MO, USA).

Defective HSV vectors

The double-cassette amplicon plasmids pHCL-tk, encoding *E. coli lacZ* and HSV-1 TK, and pHCIL12-tk, encoding IL-12 and HSV-1 TK, were described previously.²¹ HSV tsK, a strain 17-derived temperature-sensitive ICP4 mutant (obtained from J Subak-Sharpe, Institute of Virology, Glasgow, UK),³³ was propagated in Vero cells in DMEM containing 1% inactivated fetal calf serum at 31.5°C. To isolate tsK DNA, the virus was treated with 20 µg/ml DNase I (Sigma) and 20 µg/ml RNase A (Sigma) to digest cellular DNA and RNA, followed by 200 µg/ml proteinase K at 37°C for 12–16 h, and the DNA was gently extracted with phenol:chloroform and then ethanol precipitated.

Defective HSV was generated by co-transfecting Vero cells with 1.5 µg each of amplicon plasmid and tsK DNA using lipofectamine (GIBCO-BRL, Rockville, MD, USA). The transfected cells were then incubated at 31.5°C, permissive temperature for tsK, until complete cytopathic effect. The virus was passaged at a 1:4 dilution in Vero cells until inhibition of helper virus tsK replication was observed. After a freeze-thaw/sonication regime, cell debris was removed by low-speed centrifugation (2000 g for 10 min at 4°C). Mock-infected extracts were prepared identically, except buffer was used in place of virus during the infection step. The defective vectors (dv) generated with pHCL-tk and pHCIL12-tk are referred to as dvlacZ-tk and dvIL12-tk, respectively. TsK was titrated on Vero cells by plaque assay at the permissive temperature (31.5°C). The helper virus titers of the defective HSV stocks used were: dvlacZ-tk/tsK, 9×10^6 plaque forming units (p.f.u.)/ml and dvIL12-tk/tsK, 2×10^7 p.f.u./ml. The defective titer of dvlacZ-tk, 3×10^6 defective particle units (d.p.u.)/ml, was determined by counting X-gal his-

tochemistry positive cells after infection at 39°C, the non-permissive temperature for tsK.

IL-12 expression

Cells were infected with dvIL12-tk/tsK at a multiplicity of infection (MOI) of 1 p.f.u. tsK per cell, incubated at 39.5°C, and at 24 h after infection aliquots of infected-cell supernatant were removed, quick frozen in a dry-ice/ethanol bath, and stored at -80°C for detection of IL-12. Immunoreactive IL-12 levels were determined by sandwich ELISA, using antibody pairs kindly provided by DH Presky (Hoffmann-La Roche, Nutley, NJ, USA).⁵⁴ Briefly, 96-well plates coated with an antimouse IL-12 monoclonal antibody (9A5) were incubated overnight at room temperature with the test samples. After washes, the plates were incubated with peroxidase-labelled antimouse IL-12 p40-antibody (5C3) for 2 h and developed. Absorbance was measured at 450 nm.

Subcutaneous tumor model

BALB/c mice were obtained from the National Cancer Institute or Charles River (Wilmington, MA, USA). All animal procedures were approved by the Georgetown University Animal Care and Use Committee. For surgical procedures, each mouse was anesthetized with an intraperitoneal injection of a 0.25–0.30-ml solution consisting of 84% bacteriostatic saline, 10% sodium pentobarbital (1 mg/ml; Abbott Laboratories, Chicago, IL, USA), and 6% ethyl alcohol. CT26 tumor cells (1×10^5) were injected subcutaneously in the bilateral flanks of BALB/c mice. When subcutaneous tumors were palpably growing (approximately 5 mm in diameter), mice were unilaterally inoculated into the right side tumor with either 50 µl of defective HSV vector in virus buffer (150 mM NaCl, 20 mM Tris, pH 7.5) or with 50 µl of virus buffer, followed by a second injection 7 days later. For some studies, where indicated, mock extract⁵⁵ was used in place of virus buffer. For 'suicide' gene therapy, intraperitoneal ganciclovir (GCV; 50 mg/kg daily) treatment was initiated 48 h after the first defective HSV vector inoculation and continued for 7 consecutive days. Tumor size was measured by external caliper and tumor volume was calculated ($V = h \times w \times d$). If animals appeared moribund or the diameter of their subcutaneous tumors reached 18 mm, they were killed and this was recorded as the date of death for survival studies. Statistical differences were calculated using StatView 4.5 (Abacus Concepts, Berkeley, CA, USA) where mean tumor volume was assessed by unpaired *t* test and differences in survival by logrank (Mantel-Cox) or Wilcoxon test.

CTL assays

To generate lymphocytes for CTL assays, mice were killed 12 days after vector inoculation and their spleens isolated. Single-cell suspensions of splenocytes from individual inoculated mice were prepared in ACK lysing buffer (BioWhittaker, Walkersville, MD, USA) followed by washing in RPMI 1640 medium containing 10% heat-inactivated FCS, and cultured in RPMI 1640 medium, 10% heat-inactivated FCS, 20 µM 2-ME, 2 mM glutamine, 20 mM HEPES, penicillin-streptomycin in 24-well plates at a concentration of 3×10^6 cell per ml, with 10^6 mitomycin C-treated CT26 cells for stimulation. CT26 cells were inactivated by incubation for 1 h in culture medium containing 100 µg/ml of mitomycin C and then washed

twice. Effector cells were harvested after 6 days of *in vitro* culture. Four-h ^{51}Cr release assays were performed as previously described.²¹ A20 target cells were pulsed with 1 $\mu\text{g}/\text{ml}$ of L^d-restricted peptides AH1 or P815AB for 1 h before labelling. Peptide AH1 (SPSYVYHQF), an immunodominant antigen identified in CT26 cells,⁵⁶ was synthesized by Peptide Technologies (Washington, DC, USA) to >99% purity, and peptide P815AB (LPYLGWLVF), an immunodominant antigen derived from murine mastocytoma P815 cells,⁵⁷ was kindly provided by Dr NP Restifo. The amount of ^{51}Cr release was determined by γ counting and the percent specific lysis was calculated from triplicate samples as follows: [(experimental c.p.m. – spontaneous c.p.m.)/(maximum c.p.m. – spontaneous c.p.m.)] $\times$ 100.

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