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ORIGINAL ARTICLE

Augmentation of anti-tumor responses of adoptively transferred CD8+T cells in the lymphopenic setting by HSV amplicon transduction

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Abstract Treatment of cancer with cytotoxic agents may induce lymphopenia. Adoptively transferred T cells have been reported to display enhanced anti-tumor efficacy in the lymphopenic setting. We reasoned that the anti-tumor effects of adoptively transferred cells in the lymphopenic host could be further augmented through local provision of an innate stimulus in the tumor bed. Utilizing a model in which mice were irradiated to induce lymphopenia, with limited shielding to allow tumor growth, we demonstrate that "triple" therapy consisting of radiation-induced lymphopenia, adoptive transfer of naïve CD8+ T cells, and intra-tumoral HSV amplicon injection resulted in reduced tumor growth compared to the combination of any two of the aforementioned interventions. To gain insight into the mechanism underlying this effect we studied the effects of HSV amplicon transduction into tumors on cytokine expression and on anti-tumor specific T cells. HSV amplicon transduction specifically induced several cytokine mRNAs including IFN-γ, and IP-10. Adoptively transferred transgenic OT-1 T cells directed against Ovalbumin were more effective against Ovalbumin-expressing tumors when combined with intra-tumoral HSV amplicon injections in the lymphopenic host. Following intra-tumoral HSV-amplicon injections, anti-tumor T cells secreted higher levels of interferon-γ in response to in-vitro re-stimulation with tumor cells, implying that HSV amplicon injection provided a strong signal for T cell activation. Combining adoptive transfer of naïve T cells in the lymphopenic setting with local T cell stimulation may facilitate expansion and activation of anti-tumor T cell populations in vivo, resulting in enhanced anti-tumor responses without the need to resort to prolonged in vitro T cell culture and/or manipulation.

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Introduction

Numerous anti-tumor immunologic strategies have been employed with limited clinical success including the adoptive transfer of cytolytic T cells, peptide vaccination, and dendritic cell vaccination, in order to induce a systemic anti-tumor immune response. Other investigators have used general activation of the immune system, for example, by inducing the innate immune system to stimulate an anti-tumor response [1, 2]. Vaccination strategies employed in the human setting to date have also been of limited effectiveness, particularly in the setting of established tumors. Several investigators have employed the adoptive transfer of immune anti-tumor effector cells with defined anti-tumor specificity [3–5]. While demonstrating improved results, this approach is contingent upon the availability of large fresh tumor samples, requires the manipulation of T cells in vitro for several weeks and is very cumbersome for general application.

Cytotoxic therapies remain the mainstay of treatment for metastatic disease, and are frequently accompanied by the induction of profound lymphopenia. Attempts to combine immunologic treatments with cytotoxic therapy therefore need to factor in the potential effects of chemotherapy-induced lymphopenia. Immunization of the lymphopenic host might initially seem counterproductive due to the potential depletion of anti-tumor lymphocytes. Paradoxically however, lymphoid depletion may in some settings actually facilitate an anti-tumor response through effects on the expansion of desirable tumor-specific T cell populations, the depletion of lymphocytes with an immunosuppressive role, such as regulatory T cells and B cells, and/or the depletion of tolerant T cell clones, all of which may assist in the emergence of an anti-tumor immune response [6–8]. Creation of "empty" lymphoid space may lead to the emergence of new populations of T cells in the periphery which may not otherwise expand in the absence of lymphopenia. Such "homeostatically expanded T cells" demonstrate a phenotype similar to partially activated T cells and may have a lower threshold for T cell activation [9–12]. This phenomenon has led to attempts to combine vaccination with lymphoid depletion to allow homeostatic expansion, in order to augment anti-tumor responses. Several investigators have studied application of immune therapy in the lymphopenic environment [13–17]. Studies by Dummer et al. [17], Fox and Hu [18, 19], suggest that adoptive T cell transfer may be more effective when performed in the lymphopenic setting. However, most of their studies were performed with T cells that were transferred before tumor implantation.

We wished to determine the effects of adoptive transfer in the setting of lymphopenia in the presence of pre-established tumor. In addition, we asked whether anti-tumor effects could be further amplified through the local provision of a strong innate stimulus within the tumor bed.

T cell transfer was performed into irradiated mice in which the tumor implant was selectively shielded from radiation in order to obtain lymphocyte depletion with minimal effects on the tumor itself. We analyzed the effects of the transferred T cells alone, or in combination with a strong innate immune stimulus, administered by local intra-tumoral injections of HSV-amplicons. Our studies demonstrate that the effectiveness of T cell transfer in the lymphopenic host is increased by local delivery of an innate stimulus, in the form of HSV amplicons. Utilizing tumor specific T cell adoptive transfer we demonstrate that local innate immune stimulation can result in increased activation of the anti-tumor T cells leading to enhanced effector function and anti-tumor response.

Material and methods

Mice and cell lines

C57BL/6, RAG-1, and B6 IFN-γ knockout mice were obtained from Jackson Laboratories. OT-I TCR transgenic mice [20], and GFP mice [21] (obtained from E. Podack, University of Miami), were bred to C57BL/6 mice to generate OT-I/GFP mice. OT-I mice express a transgenic TCR that is specific for OVA (257–264) (SIINFEKL) peptide bound to H-2K^b [20]. EL-4 cells were maintained in Iscove's Dulbecco modified Eagles Medium with 10% FCS. E.G7 (EL-4 expressing Ovalbumin) and LLC-OVA (Lewis lung carcinoma expressing Ovalbumin) were obtained from E Podack (University of Miami) and grown in IMDM with 10% FCS in the presence of G418.

HSV amplicons

The coding sequences for *Escherichia coli* β-galactosidase, (LacZ), were cloned into an HSV amplicon plasmid vector as described [22, 23]. HSV amplicon DNA was packaged into HSV-1 particles in RR1 cells as previously described [23]. Amplicon transduction titers for helper virus-packaged amplicon stocks were determined as follows: NIH 3T3 cells were plated in a 24-well plate at a density of 1×10^5cells/well and transduced with the HSV amplicon preparations; 24 h after transduction the monolayers were harvested for total DNA using lysis buffer (100 mM NaCl, 10 mM Tris, pH 8.0, 25 mM EDTA, 0.5% SDS), extracted with phenol–chloroform, and precipitated with ethanol. Real-time quantitative PCR was conducted on duplicate samples using primers corresponding to the gene encoding β-galactosidase present in the amplicon and amplicon titer calculated as previously published [23].

Cytokine mRNA induction by HSV amplicons

PCR primers for cytokines IFN-γ, IFN-β, IL-1β, IP-10, IL-6, and TNF-α were designed for real-time amplification of cDNA using the Web-based online version of Primer 3 (http://www-genome.wi.mit.edu/genome_software/other/primer3.html). Primer lengths were restricted to 20 bp and their corresponding amplified products average at 120 bp and crossed at least one intron junction. DNA oligonucleotide sequences were as follows:

IFN-γ- sense 5′-GCGTCATTGAATCACACCTG and anti-sense 5′-TGAGCTCATTGAATGCTTGG;
IFN-β- sense 5′- CCCTATGGAGATGACGGAGA and anti-sense 5′- GTCTCATTCCACCCAGTGCT;
IL-1β- sense 5′- AGTTGACGGACCCCAAAAG and antisense 5′- TACTGCCTGCCTGAAGCTCT;

IP10- sense 5′- AAGTGCTGCCGTCATTTTCT and anti-sense 5′- CCTATGGCCCTCATTCTCAC.

For in vitro measurements of cytokine mRNA expression, 4×10^6 LLC-OVA cells were treated with helper-containing HSV amplicons harboring recombinant LacZ (H+ HSV-LacZ) at a MOI concentration of 0.5×10^6 BFU or incubated in vitro with the innate ligands CpG, or PGN, or Poly (I:C). Following 36 h of treatment, LLC-OVA cells were lysed in the wells and processed for total RNA (Qiagen Austin Tx RNeasy Mini Kit) and analyzed for specific mRNA levels by real time RT-PCR using the cytokine primer pairs outlined above. For in vivo analysis real-time RT-PCR was used to measure specific mRNA levels extracted from tumors 24 h following $3–4 \times 10^6$ BFU of HSV-LacZ amplicons delivered by intra-tumoral injection.

CD8+ cell purification and adoptive transfer

CD8+ splenic cells from GFP or GFP/OT-I transgenic (Tg) mice were purified by positive column selection using MACS anti-CD8α MicroBeads (Miltenyi Biotech, Auburn, CA, USA) per manufacturer's instructions. Purified cells were >90% CD8+ by FACS analysis. 10^6 purified CD8+ cells were injected intraperitoneally at the indicated time point in each experiment.

Tumor treatment protocols

Radiation induced lymphopenia

10^6 EL-4, E.G7 or LLC-OVA tumor cells were injected subcutaneously (s.c.) on the shaved hind limbs of 6–10-week-old mice. When tumors reached approximately 5 mm in diameter, mice were grouped and 4×10^6 HSV-LacZ amplicons diluted in 100 µl PBS or PBS alone were inoculated intra-tumorally. Following the first injections, mice were anaesthetized by i.p. injection of Tribromoethanol/amylene hydrate. The mice were then irradiated to 550 cGy using a cobalt 60 source and tumors were shielded from irradiation using a specially constructed 5 half-value layer radiation protection device (Fig. 1a); 24 h post-irradiation 10^6 CD8+ cells or OT-I cells as indicated were transferred by intraperitoneal injection. HSV amplicons or PBS were then injected into the tumor bed, or where indicated injected extra-tumorally as a control, three times at 3-day intervals. Tumor area was assessed by bi-dimensional measurements using calipers.

Flow cytometry analysis and intracellular interferon-γ (IFN-γ) staining

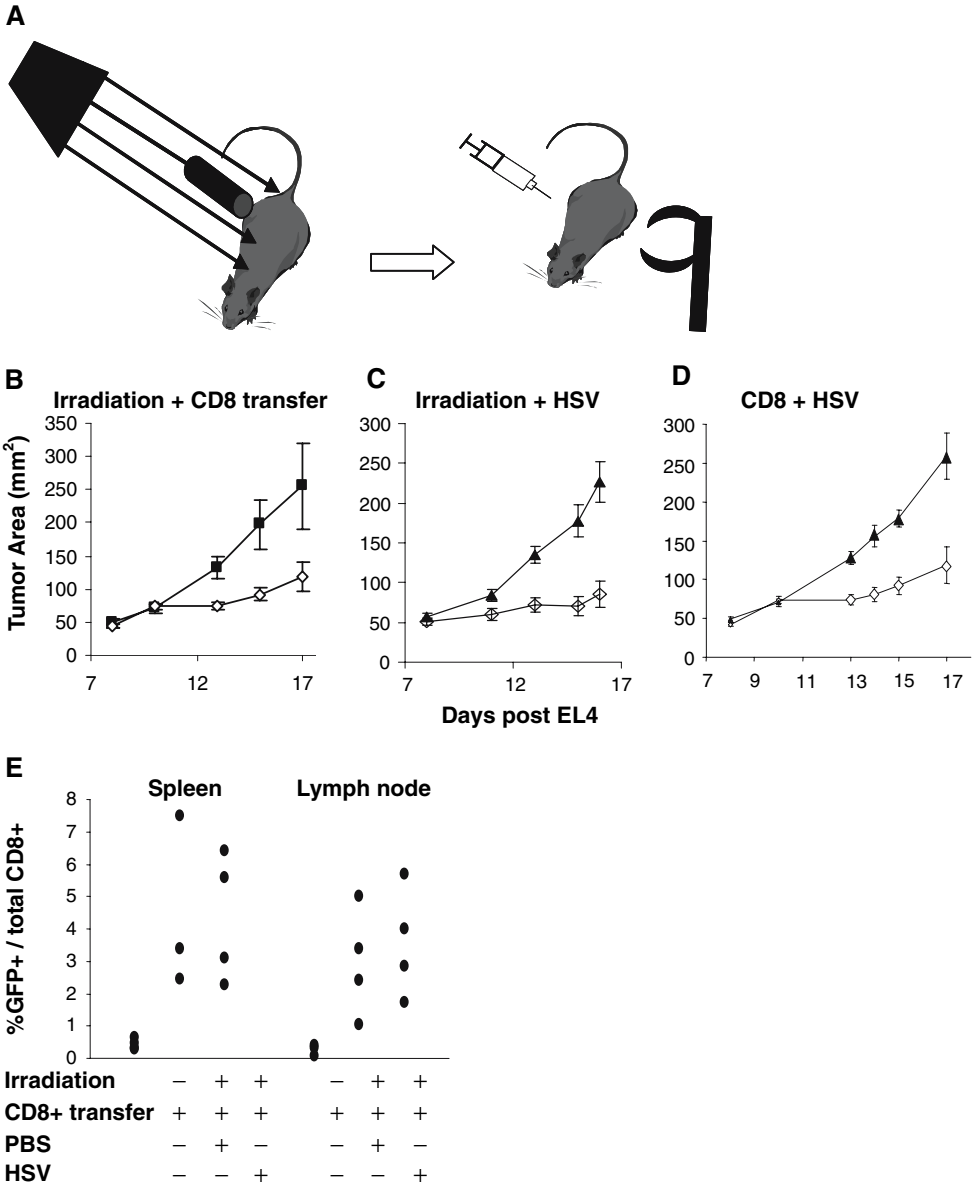
Splenocytes, lymph node cells and peripheral blood cells were prepared according to standard procedures, and then stained using directly conjugated monoclonal antibodies (BD Pharmingen). Mice bearing E.G7 were treated with varying combinations of irradiation with tumor shielding, T cell transfer and intra-tumoral HSV-amplicon or PBS injections as indicated. On day 15 following tumor implantation, single cell suspensions of splenocytes were co-incubated with irradiated (20,000 cGy) E.G7 or EL-4 cells in RPMI with 10% FCS for 2 days. Brefeldin A (10 µM) was then added for 4–5 h, cells washed and stained with the antibody for CD8α (PE-Cy-5, fixed in Cytofix/cytoperm buffer (BD Biosciences) for 15 min at 4°C, washed in 0.2% saponin, 1%FCS/PBS and re-stained with IFN-γ antibody (BD Biosciences) for 40 min at 4°C in 0.2% saponin, 1%FCS/PBS. Cells were then washed with PBS containing 2% FCS. Transferred CD8+/GFP + OT-I cells were identified based on positivity for CD8 and/or GFP expression and intracellular IFN-γ expression was detected by flow cytometry as above.

Results

Effects of radiation induced-lymphopenia, adoptive T cell transfer and HSV amplicon injection on EL-4 tumor growth

In order to study the effects of the adoptive transfer of T cells performed in the lymphopenic setting on the growth of *pre-established* tumors as well as the effects of local HSV amplicon injection on the behavior of adoptively transferred T cells, it was necessary to create a tumor model in which lymphopenia could be induced independently of effects on tumor growth. To assess effects of adoptive lymphocyte transfer on tumor growth, following the induction of lymphopenia, the direct effects of systemic irradiation on tumor growth had to be eliminated. To minimize direct effects of radiation on tumor growth we developed an apparatus in which the tumor implant was protected by a five half-value layer shield from radiation, while the rest of the animal was exposed to 550 cGy in order to induce transient lymphopenia (Fig. 1a). Tumors were unilaterally implanted in the flanks of animals, and mice were exposed to a cobalt source to induce systemic lymphopenia while the implanted tumors were selectively shielded from irradiation.

Initial experiments were performed using the EL-4 tumor model. EL-4 tumors grew progressively when mice were treated with the combination of irradiation induced lymphopenia followed by transfer of naïve CD8+ T cells. However, when tumors were then also locally injected with HSV amplicons encoding β-galactosidase (HSV-LacZ), tumor growth was markedly impaired, compared to irradiated animals receiving CD8+ cells alone (Fig.1b). In the absence of CD8+ cell transfer, tumors also grew more



rapidly in irradiated mice despite injection with HSV amplicons, indicating a requirement for CD8+T cell transfer for maximal anti-tumor effects (Fig. 1c). Furthermore, radiation-induced lymphopenia was required in order to see a maximal reduction in tumor growth (Fig. 1d) even with CD8+T cell transfer and local HSV amplicon injection. These experiments demonstrated that local intra-tumoral injection of HSV amplicons, coupled with the adoptive delivery of CD8+ T cells in the setting of induced lymphopenia, resulted in markedly reduced tumor growth.

To further assess effects of lymphopenia and HSV amplicon injection upon the degree of expansion of adoptively transferred T cells, we transferred CD8+ cells derived from GFP+ transgenic mice in order to track transferred cells efficiently, and compared the percentage of transferred cells in different treatment groups. At day 16 following transfer, there were approximately ten-fold elevated percentages of GFP+/CD8+ cells relative to total CD8+ cells in the irradiated mice as compared to non-irradiated mice (Fig. 1e), which suggests that in the irradiated lymphopenic mice a significant percentage of CD8+ cells were derived from the transferred GFP+ cells.

Adoptive transfer of CD8+ T cells from tumor bearing mice

T cells from animals harboring growing tumors may differ from naïve T cells, in that they may have already been exposed to and/or tolerized to tumor. Therefore, we explored the efficacy of adoptive transfer of CD8+ cells obtained from tumor-bearing mice rather than naïve mice. GFP+/CD8+

◀ **Fig. 1** **a** Systemic irradiation and protection of tumor implants: EL-4, E.G7 or LLC-OVA tumor cells were cultured in vitro and 10^6 cells were injected subcutaneously on the dorsal side of the shaved hind limb of 6–10-week-old mice. Tumors were allowed to grow to approximately 5 mm (usually day 6–7) and injected with HSV amplicons as described in the Methods. The extended upper part of the hind limb, which included the tumor implant, was shielded by a specially constructed five half-value layer radiation protection device. The mice were irradiated using a cobalt 60 source to 550 cGy. At 24 h post-irradiation, 10^6 CD8+ cells were injected intraperitoneally as indicated. HSV amplicons or PBS was injected three additional times at 3-day intervals. Tumor area was assessed via bi-dimensional measurements using calipers. **b** Role of CD8+ transfer and HSV amplicon vector injection in tumor control: EL-4 tumors were implanted and allowed to grow to 5 mm, HSV amplicons were injected and CD8+ cells adoptively transferred and animals irradiated as described in the Methods. *Solid squares* combination of irradiation-induced lymphopenia followed by transfer of naïve CD8+ T cells; *open diamonds* tumors were locally injected with HSV amplicons encoding β-galactosidase (HSV-Lac) as indicated above, (tumor growth with HSV amplicon injection, was compared to irradiated animals receiving CD8+ cells alone, $P = 0.011$) (seven vs. six animals per group). Mean ± SEM for tumor area shown. **c** CD8+ transfer is required for tumor control. Mice with EL-4 implants were treated with irradiation and HSV amplicon injections with (*open diamond*) or without (*closed triangle*) CD8+ T cell transfer [15 animals in each group, results are combined from 3 different experiments; ($P = 0.011$)]. **d** Systemic irradiation is required for tumor control. Mice bearing EL-4 were treated with a combination of intratumoral HSV amplicon injection and CD8+ cell transfer without irradiation (*closed triangle*) or with irradiation and tumor shielding (*open diamond*) as described (eight mice per group). Results with or without systemic irradiation are compared ($P = 0.011$). **e** Effects of the lymphopenic state and HSV amplicon injection on expansion of adoptively transferred CD8+ T cells from naïve mice: To assess effects of lymphopenia and HSV amplicon injection upon expansion of adoptively transferred T cells, CD8+ cells derived from a GFP+ transgenic mouse were transferred and the percentage of GFP+ CD8+ cells detected in spleen and lymph nodes at day 16 following transfer were compared in different treatment groups as indicated. Detection of GFP+/CD8+ cells allowed discrimination between cells derived from the adoptively transferred population from resident CD8+ cells. The percentage of CD8+/GFP+/total CD8+ were assessed in spleen and lymph nodes in mice treated with irradiation with intratumoral HSV amplicon vectors, intratumoral PBS injections, or without irradiation and with HSV amplicon vectors as indicated. Values for individual mice are shown

T cells were harvested from the spleens of GFP+ transgenic mice bearing 3-week-old EL-4 tumors and transferred into tumor bearing irradiated mice. Similar percentages of transferred GFP+/CD8+ cells were noted at day 16 in mice treated with intra-tumoral injections of HSV amplicons, or controls (data not shown). In a representative experiment, T cell transfer alone from tumor bearing mice resulted in progressive tumor growth in five of seven mice, while tumors regressed in two of seven mice (Fig. 2a). This result is consistent with results by Dummer et al. [17] in which expanding T cells in a homeostatic environment demonstrated modest anti-tumor activity. In contrast, when tumors were injected locally with HSV-LacZ amplicons, five of six evaluable animals demonstrated complete tumor regression (Fig. 2b, $P = 0.036$). Therefore, adoptively transferred CD8+ cells derived from tumor-bearing mice could also mediate the anti-tumor effects seen following HSV amplicon injection.

The activation marker CD44 has been demonstrated by other investigators to be induced in T cells undergoing homeostatic expansion and may represent transition to a memory/activated T-cell-like phenotype [9]. CD8+ cells from tumor-bearing or non-tumor bearing mice demonstrated low levels of CD44 expression, while adoptively transferred GFP+/CD8+ cells recovered from tumor-bearing mice demonstrated substantially higher levels of CD44 staining (Fig. 2c), whether or not HSV amplicons were injected into the tumors. Adoptively transferred CD8+ cells derived from EL-4 tumor-bearing animals also demonstrated high levels of CD44 expression, when recovered from irradiated mice. This effect may primarily be attributable to expansion in the setting of lymphopenia, since both GFP- host and GFP+ transferred cells appeared to express CD44 at high levels (Fig. 2c).

Innate response to HSV amplicon transduction

We first wished to characterize the effects of transduction of tumor cells with HSV amplicons on induction of selected immune modulators. Transduction of LLC-OVA cells with HSV-LacZ amplicons induced a marked increase in induction of IFN-β, IL-1β, TNF-α, IL-6 and IFN-γ mRNA (Fig. 3a). The levels of cytokine mRNA induction were comparable to or exceeded levels induced by the TLR ligands PGN or LPS (Fig. 3a). We also analyzed mRNA levels from LLC-OVA tumors following direct intratumoral HSV amplicon injections in vivo. We noted HSV amplicon specific induction of several cytokine mRNAs including IFN-γ, and IP-10 (Fig. 3b).

Antigen-specific OT-I CD8+ T cell expansion in the lymphopenic host

In order to further characterize the fate of naïve T cells with a defined anti-tumor specificity in the presence of a cognate tumor antigen, we studied the behavior of adoptively transferred CD8+ OT-I T cells. OT-I T cells carry a transgenic T cell receptor for the Ovalbumin-specific peptide (SIINFEKL) presented on the H-2K^{b}histocompatibility locus [20]. The OT-I transgenic T cell model has been used in numerous studies regarding different aspects of T cell functions and several initial studies on T cell "homeostatic" proliferation used this model [24, 25]. Podack and colleagues used OVA secreting tumor cells (including E.G7 and LLC-OVA) and transgenic OT-I cells to demonstrate that perforin and NK cells are required for innate and adaptive immunity induced by a soluble version of the heat shock protein gp96 [26]. Others have used this system to

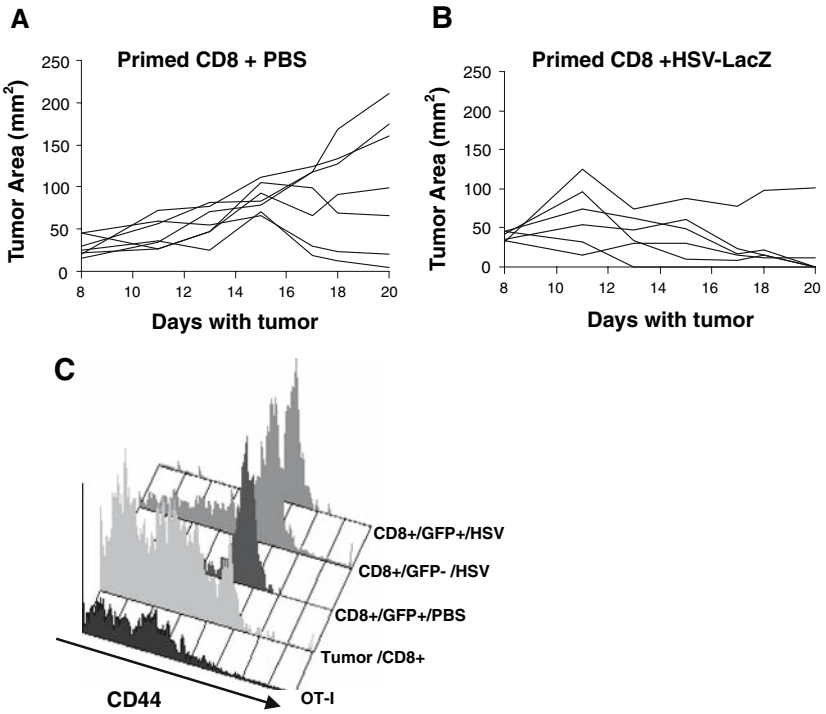


Fig. 2 Induction of anti-tumor responses by CD8+ cell transfer from tumor bearing mice into irradiated mice bearing tumors. **a**, **b** Mice harboring EL-4 tumors, were irradiated (with tumor shielding), and injected with 3×10^6 CD8+ cells from EL-4 bearing mice as described in the Methods. **a** Tumor growth curves in mice injected intratumorally with PBS ($n = 7$); **b** tumor growth curves in mice injected intratumorally with HSV amplicons ($n = 6$), (results of **a**, **b** are compared, $P = 0.036$); **c** CD44 expression following adoptive transfer of CD8+ cells derived from EL-4 tumor bearing animals into irradiated mice. CD44 expression on CD8 + cells in the peripheral blood following transfer was measured on day 20 following tumor implant. From front to back: Naïve CD8+/GFP+ OT-I cells, CD8+ cells of EL-4 tumor bearing mice, CD8+/GFP+ cells after adoptive transfer to a mouse injected intratumorally with PBS, CD8+/GFP- cells from mouse injected intratumorally with HSV amplicons, CD8+/GFP+ cells following transfer from a mouse injected intratumorally with HSV amplicons

determine which T cell effector molecules are important for tumor eradication [24]. Thus the OT-I model has proved very useful for characterizing T cell anti-tumor responses. We compared the effects of OT-I transfer in the presence or absence of HSV amplicon injection, in irradiated mice with induced lymphopenia, or following transfer into constitutively lymphopenic $RAG^{-/-}$ mice which lack normal T or B cells. We utilized two tumor models which were engineered to express Ovalbumin, LLC-OVA and the EL-4 derived E.G7 model. Using the LLC-OVA model, OT-I T cell transfer into irradiated hosts (Fig. 4a), or into $RAG^{-/-}$ mice (Fig. 4b), coupled with the local intra-tumoral injection of HSV amplicons (i.e. "triple therapy"), markedly reduced the growth of tumor as compared to either individual treatments, or to the combination of any two approaches ($P < 0.01$ for difference in size on day 19). Similar results were seen using the E.G7 model (data not shown).

Local innate response was required for inhibition of tumor growth

We next sought to determine whether local innate response was instrumental in facilitating tumor rejection. We asked whether direct *intra-tumoral* injection of HSV amplicons was required for the anti-tumor effects seen with the adoptive T cell transfer or whether similar effects would be observed with extra-tumoral HSV amplicon injections. We implanted E.G7 tumors, followed by OT-I transfer. HSV amplicon injections were then performed intra-tumorally, or injected subcutaneously into uninvolved skin on the mouse abdomen. OT-I transfer and intra-tumoral injection of HSV amplicons resulted in improved tumor control and in the survival of approximately of 60% of the animals. In contrast, HSV amplicon injection intra-tumorally in the absence of OT-I T cell transfer, or the combination of OT-I T cell transfer with extra-tumoral injection of HSV amplicons, did not control tumor growth, resulting in markedly decreased survival (Fig. 5a, $P = 0.03$). This suggested that a local innate stimulus provided by HSV amplicon injection was required for induction of optimal anti-tumor effects by the adoptively transferred CD8+ cells.

We compared the percentage of OT-I/GFP^+ T cells and CD44 expression levels at day 30 in the peripheral blood of irradiated animals receiving OT-I cells by adoptive transfer to those in irradiated animals cured of implanted tumors by HSV amplicon injection and adoptive transfer of OT-I

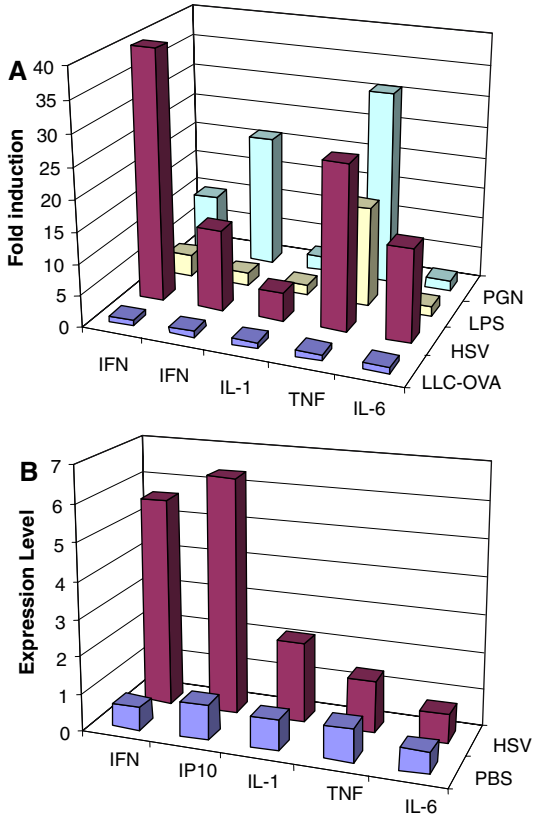


Fig. 3 Innate cytokine response to HSV amplicon transduction. **a** HSV amplicons induce cytokine production by tumor cells in vitro. Following 36 h of in vitro exposure to recombinant HSV amplicon virions at a MOI of 0.5, LLC-OVA cells were processed for total RNA and analyzed for cytokine cDNA by real-time RT-PCR. Parallel control cultures were assessed after no treatment or after stimulation with TLR2 or TLR4 ligands, PGN and LPS, respectively. Expression levels are graphed as fold-induction above untreated cells. Values ± SD for cytokine mRNA induction were: IFN-γ (HSV 105.2 ± 5.2; LPS 3.5 ± 1.5; PGN 9.9 ± 2.1), IFN-β (HSV 13.0 ± 1.8; LPS 2.0 ± 0.2; PGN 21.1 ± 3.3), IL-1β (HSV 4.6 ± 1.0; LPS 1.6 ± 0.8; PGN 2.2 ± 1.1), TNF (HSV 26.3 ± 3.3; LPS 15.8 ± 1.9; PGN 30.9 ± 2.5), IL-6 (HSV 14.9 ± 2.5; LPS 1.5 ± 0.2; PGN 1.6 ± 0.2). This is one representative experiment out of 3. **b** Intratumoral HSV amplicon injections stimulate cytokine expression in vivo. Mice bearing implanted tumors of 1-week's duration were treated twice with HSV-LacZ or PBS on day 1 and day 11 as described in the Methods. Twenty-four hours after the second injection, they were euthanized, and the tumors were processed for total RNA, which was analyzed as cDNA by real-time RT-PCR for cytokine expression. mRNA expression levels were normalized to PGK1. Values ± SD for cytokine mRNA induction were: IFN-γ (HSV 5.6 ± 0.7; PBS 0.6 ± 0.1), IP10 (HSV 6.4 ± 0.8; PBS 0.9 ± 0.2), IL-1β (HSV 2.2 ± 0.3; PBS 0.8 ± 0.1), TNF-α (HSV 1.4 ± 0.3; PBS 0.9 ± 0.3), IL-6 (HSV 0.8 ± 0.2; PBS 0.6 ± 0.2)

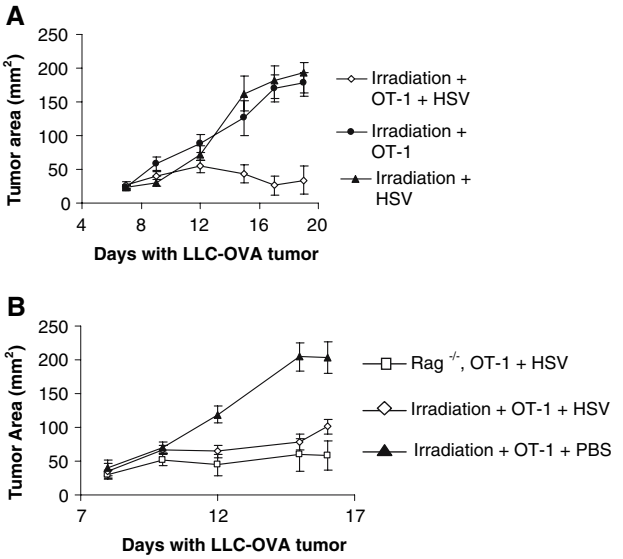


Fig. 4 Effect of intra-tumoral injection of HSV amplicons on anti-tumor activity of adoptively transferred anti-tumor specific OT-I CD8+ cells against LLC-OVA. **a** Irradiated LLC-OVA bearing mice were treated with adoptively transferred CD8+ cells derived from OT-I mice injected with PBS (eight mice), or with intra-tumoral HSV amplicon injection without adoptive OT-I transfer (five mice), or with a combination of intra-tumoral HSV amplicons and OT-I CD8+ cell transfer (11 mice). The figure represents a compilation of two separate experiments. Details are in text. **b** Rejection of tumor in irradiated or RAG$^{-/-}$ lymphopenic mice: LLC-OVA bearing mice were treated with irradiation/tumor shielding, and a combination of OT-I transfer and PBS injections (ten mice) or intratumoral HSV amplicon injection (ten mice). In a third group RAG-1$^{-/-}$ mice bearing implanted LLC-OVA were treated with HSV amplicon injections and OT-I transfer (five mice) as indicated. Details are in text

T cells. Higher percentages of OT-I/GFP$^+$CD8$^+$ cells were noted in the peripheral blood of cured mice (Fig. 5b right panel; 5 ± 3.5%), compared to irradiated hosts that had not received tumor (Fig. 5b, center panel; 1.25 ± 0.4%); one representative experiment of three is shown in Fig. 5b). Higher levels of CD44 expression in CD8+ T cells were found in animals that had received OT-I cells followed by irradiation and adoptive transfer, or in animals which had been irradiated, bearing tumor which had been injected with HSV amplicons, as compared to naive OT-I donors (Fig. 5c).

Mice that have been irradiated and were devoid of tumors, following treatment with a combination of OT-I transfer and intra-tumoral HSV amplicon injections, and mice which had been irradiated and treated with OT-I transfer alone were re-analyzed for CD44 levels 6 months following T cell transfer. Higher levels of CD44 expression were detected on CD8$^+$GFP$^+$ T cells in the peripheral blood of cured animals compared to CD44 levels on CD8$^+$GFP$^-$ T cells (Fig. 5d). This suggested that the OT-I CD8+ T cell population had undergone selective expansion, and had acquired persistently high expression levels of CD44. These results are consistent with previously published studies of homeostatic T cell expansion [9–12], and suggest that anti-tumor T cells had acquired a CD44+ memory phenotype.

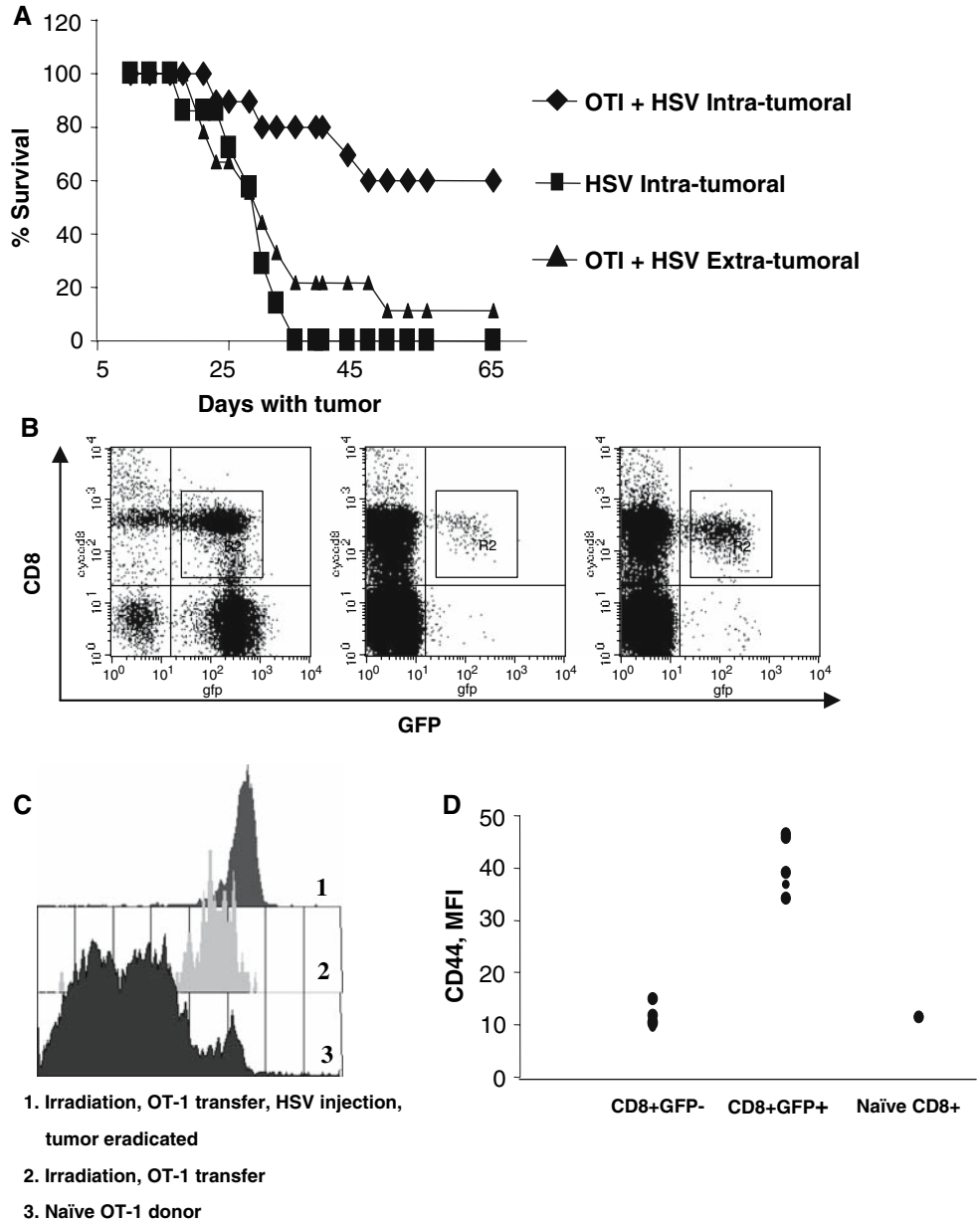


Fig. 5 Effects of intra-tumoral compared to extra-tumoral HSV amplicon injection on E.G7 tumor growth. **a** E.G7 bearing mice were irradiated and treated with intratumoral HSV-amplicons alone (seven mice), intra-tumoral HSV amplicons and OT-I cell transfer (ten mice), or with extra-tumoral HSV amplicon injection and OT-I transfer (nine mice). Mice were sacrificed when the tumors exceeded 15 mm in diameter. The percentage of surviving mice at the indicated dates is plotted. Comparison is made between intra- and extra-tumoral injection of HSV amplicons ($P = 0.035$). **b** Peripheral blood flow cytometry of mice at day 30 post OT-I CD8+ T cell transfer. Results are shown for a naïve donor OT-I/GFP mouse (*left panel*), a mouse irradiated and followed by OT-I/GFP transfer in the absence of implanted EG.7 (*center panel*), or a mouse cured of E.G7 by the combination of OT-I, intra-tumoral injections of HSV-amplicons, and irradiation (*right panel*). Details are in text. **c** Histogram depicting the levels of CD44 staining on GFP+ OT-I transferred cells in **b**. Analysis of CD44 expression on peripheral blood CD8+ cells from individual mice cured of E.G7 six months prior to the analysis. Results (MFI for CD44) for the host (GFP^-) and OT-I derived cells (GFP^+), and for CD8+ T cells in naïve mice are shown. Each point represents MFI for CD44 for an individual mouse

Re-challenge of mice with E.G7 tumor following initial tumor eradication

To determine whether mice that were "cured" in the previously mentioned experiments using a combination of OT-I transfer, irradiation and intra-tumoral injection of HSV amplicons had developed durable immunity, we re-challenged mice that had previously rejected E.G7 following adoptive transfer of OT-I and HSV amplicons. E.G7 tumor grew in all naive animals, and in 9 of 11 animals previously

implanted with OT-I, albeit with a modest delay in kinetics, but in only 1 of 11 animals which had previously rejected tumors following the triple therapy of irradiation, OT-I transfer and HSV amplicon injection therapy ($P < 0.01$ for comparison with control group and $P = 0.035$ for comparison with "irradiation + OT-I transfer" controls) (Fig. 6a).

GFP^+CD8^+ OT-I cells in the peripheral blood were compared in control mice, previously injected with OT-I cells in the absence of prior tumor, and in previously "cured" mice, at 7 days following E.G7 re-challenge. Markedly increased levels of GFP^+OT-I T cells were detected in the peripheral blood of previously cured mice (Fig. 6b). This finding suggests that transferred OT-I T cells from animals that had rejected tumor could undergo recall and re-expansion in response to tumor re-challenge.

"Epitope spreading" following OT-I transfer and tumor eradication

E.G7 is derived from the immunogenic EL-4 tumor, and in the course of challenge with E.G7, animals may be sensitized to antigens in addition to those recognized by the transferred OT-I T cells. To determine whether "epitope spreading" had occurred, we rechallenged a subset of animals that had been "cured" of E.G7 with parental EL-4 tumors lacking Ovalbumin. In control groups (a group of animals harboring contralaterally implanted E.G7 tumor, or naive mice group) EL-4 grew progressively in all mice. In contrast, in mice previously cured of E.G7 by "triple therapy", 1 month, or >6 months prior to re-challenge, the majority of EL-4 tumors stabilized in size and rejection was ultimately observed in over 50% of tumors (Fig. 6c, $P < 0.01$). These results are similar to those reported by

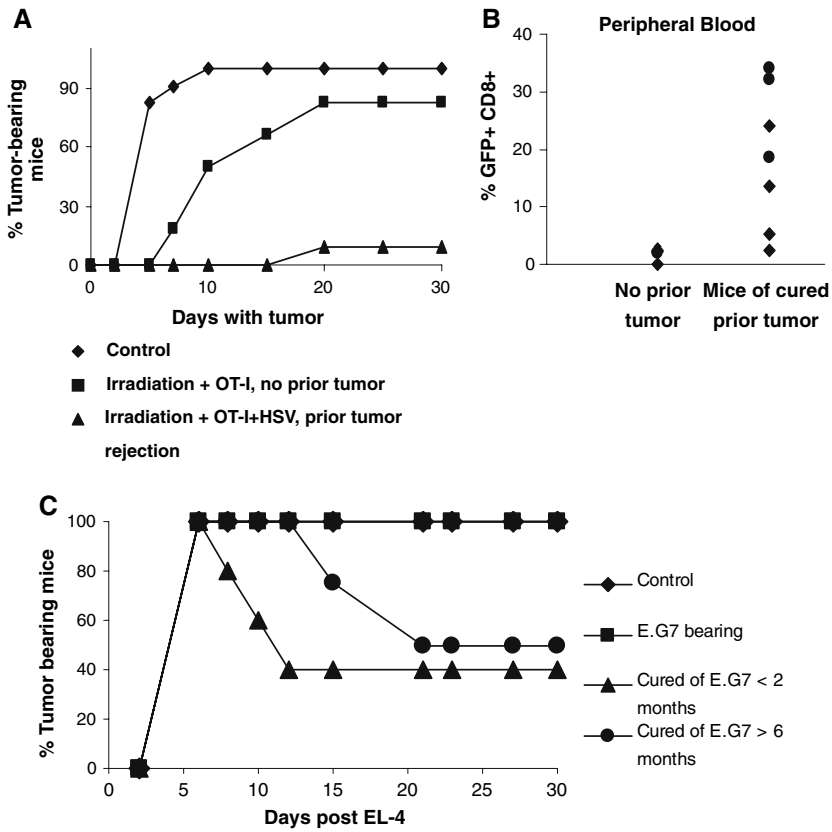


Fig. 6 Re-challenge experiments following tumor eradication. **a** Re-challenge with E.G7 tumors. Control mice ($n = 11$), mice previously irradiated and injected with OT-I cells in the absence of tumors ($n = 11$), and mice which had previously rejected implanted E.G7 tumors using triple therapy, e.g. a combination of irradiation, OT-I cell transfer and HSV amplicon injection ($n = 11$ animals), were injected with E.G7, and tumor growth was measured as indicated (results are combined from two separate experiments). Details are in text. **b** OT-I percentage of total CD8 cells (as determined by GFP+/CD8+ cells) in the peripheral blood of mice treated with irradiation and OT-I/GFP transfer (*left lane*), or "cured" of tumor by the combination of irradiation, OT-I/GFP transfer and intratumoral HSV amplicon injections (*right lane*). Details are in text. **c** Rechallenge with EL-4 tumors. Four groups of mice as indicated were rechallenged with EL-4 tumors: naïve controls ($n = 5$); mice bearing E.G7 tumors ($n = 8$), were challenged with EL-4 on the contralateral flank; mice which had previously rejected E.G7 tumors following'triple'therapy—five animals were cured less than 2 months and four mice cured more than 6 months before rechallenge with EL-4. The percentage of tumor-bearing mice is plotted. Details are in text

Eisenbach and colleagues, who demonstrated that during CTL-mediated rejection of E.G7 tumor cells, mice acquired a diversified CTL response to additional epitopes [27]. Therefore a highly plausible explanation for the increased rejection of EL4 tumors in mice cured from EG.7 by the "triple therapy" is that following tumor eradication by OVA specific T cells, new populations of anti-tumor T cells appear which recognize tumor derived epitopes unrelated to OVA.

T-cell activation following transfer: the role of IFN-γ

The overall number of OT-I T cells detected in the peripheral blood appeared to correlate primarily with induction of a lymphopenic state, rather than with HSV amplicon injection, and also correlated to a lesser degree with the presence of Ovalbumin-expressing tumor. Therefore, we postulated that differences seen with respect to tumor rejection could primarily be due to changes in activation and differentiation into effectors. We tested whether the transferred T cells differed phenotypically with regard to T cell "activation" and/or cytokine secretion. We assessed "activation" by restimulating splenocytes obtained from tumor-bearing mice with irradiated E.G7 cells or control EL-4 cells, and measured intracellular IFN-γ staining. Markedly higher percentages of GFP+ OT-I derived T cells secreting IFN-γ were detected following tumor restimulation in vitro using splenocytes derived from mice treated with intra-tumoral HSV amplicon injection, than from control PBS injected mice (56.0 ± 12.8 vs. $23.5 \pm 5.8\%$ for the average percentage of IFN-γ secreting cells following restimulation with E.G7 cells versus EL-4 cells (Fig. 7a, b, $P < 0.01$).

We wished to differentiate between the effects of IFN γ secretion by adoptively transferred T cells and the secretion of IFN γ by the tumor following local injections of HSV amplicons (see Fig. 3). We have consistently demonstrated that intra-tumoral HSV amplicon injection alone was insufficient to eradicate tumors, and that the addition of adoptively transferred T cells was needed for anti-tumor responses (see Figs. 1, 2, 4, 5). In order to determine if T cell derived IFN-γ was important for the anti-tumor responses, we tested for the effects of triple therapy (e.g. irradiation, T cell transfer and HSV amplicon injection) in the EL-4 model, using adoptive transfer of T cells derived from either IFN-$\gamma^{-/-}$ knockout mice or control mice. Tumor growth was markedly impaired following transfer of normal CD8+ T cells compared to that observed with adoptive transfer of IFN-$\gamma^{-/-}$ CD8+ T cells ($P < 0.01$) (Fig. 7c).

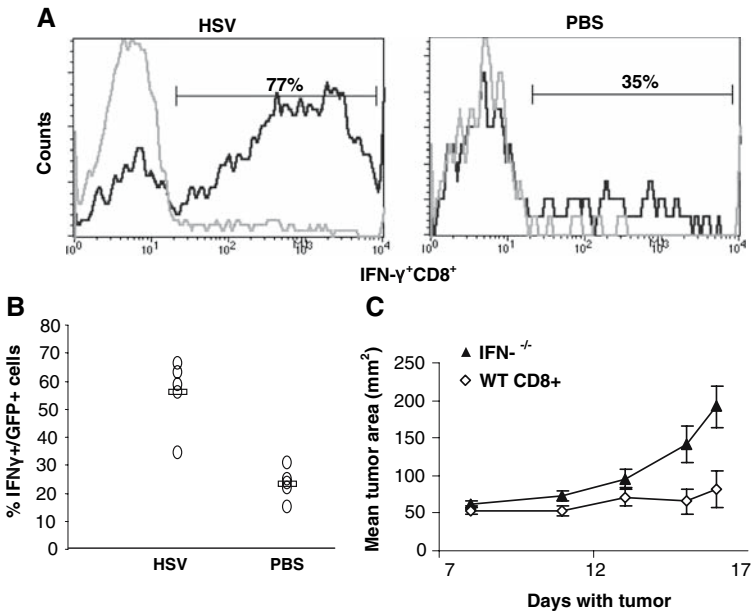


Fig. 7 Adoptive transfer of IFN-γ secreting T cells are required for tumor rejection: **a** Cytokine secretion following adoptive transfer: E.G7 tumor-bearing mice were irradiated with tumor shielding, followed by OT-I cell transfer and intra-tumoral injection of HSV-amplicons (*left panel*) or PBS (*right panel*). On day 15 post tumor implantation, splenocytes were isolated from the mice and incubated for 2 days in the presence of irradiated EL-4 (*gray line*) or E.G7 cells (*black line*). IFN-γ intracellular staining of the transferred OT-I cells was then assessed by flow cytometry as described in the methods. Representative mice are shown. **b** A dot plot of the percentage of (OT-I/IFN-γ+ cells)/total OT-I following in vitro incubation with E.G7 (expressing target antigen) or EL-4. The value shown is of the percentage of GFP+/IFN γ+ cells incubated with E.G7 minus the % of GFP+/IFN γ+ cells seen following incubation with EL-4 (nonspecific background). Each dot represents results obtained from splenocytes of a single animal ($P < 0.01$). **c** Effects of CD8+ cell transfer from IFN-$\gamma^{-/-}$ mice. EL-4 bearing mice were treated with a combination of irradiation (with tumor shielding), HSV amplicon injection and transfer of either IFN-$\gamma^{-/-}$ CD8+ or normal CD8+ cells (combined results from two experiments with 13 animals for each group). ($P < 0.01$ for comparison between groups)

This suggested that IFN-γ secreting T cells play an important role in the tumor rejection seen in this model. Dutton and colleagues have performed an extensive study on factors influencing tumor rejection by activated OT-I T cells. In their studies adoptively transferred OT-I T cells derived from perforin-, Fas ligand-, or TNF-α -deficient transgenic TCR mice behaved similarly with respect to tumor eradication. Diminished tumor eradication was noted only when using OT-I T cells lacking IFN-γ [30]. Thus our results support the notion that IFN-γ which is produced in higher levels by the transferred T cells is important for the observed antitumor responses.

Discussion

Selection and expansion ex-vivo of specific anti-tumor T cells is relatively impractical for treatment of large numbers of cancer patients. In the current study, we explored adoptive T cell transfer, which does not demand the selection and in vitro enrichment of tumor specific T cells. We demonstrate here that the adoptive transfer of T cells into lymphopenic mice in combination with the delivery of a strong local innate immune stimulus within the tumor bed could be used to elicit a potent anti-tumor response.

In our experiments, the adoptive transfer of CD8+ cells or of tumor-specific OT-I into tumor-bearing lymphopenic mice alone had modest effects on tumor growth (Figs. 1, 2). We therefore provided an innate immune stimulus, in the form of intra-tumoral HSV amplicon injections to boost response.

We previously demonstrated the ability of intra-tumorally injected HSV amplicons, expressing known immune stimulatory genes such as chemokines or co-stimulatory molecules (e.g., SLC, Rantes, B7.1, CD40L), to induce eradication of established tumors [28, 29]. In previous studies, only a minority of animals receiving HSV amplicon vectors encoding β-galactosidase experienced anti-tumor responses. These responses were less effective than responses induced by the HSV amplicons expressing immune stimulatory molecules such as CD40L or the chemokine SLC. However, in our studies, we demonstrate that coupling of adoptive transfer with HSV-LacZ amplicon injection can produce reliable tumor responses, and that HSV amplicons can induce strong local innate responses (Fig. 3). Adoptive T cell transfer alone did not induce effective tumor control (Figs. 1, 2, 4). However, when we combined intra-tumoral HSV amplicon injection with T cell transfer into the lymphopenic host, we observed enhanced inhibition of tumor growth (Figs. 1, 2, 4). Innate immune responses induced by HSV-LacZ amplicons may in part be due to the effects of activating CpG motifs contained within β-galactosidase and within HSV amplicon DNA sequences. This suggests that exposure of transferred T cells to the innate response evoked within HSV amplicon-transduced tumors can lead to changes in T cell number and/or activation, which in turn promote tumor rejection.

One consequence of the innate response evoked by HSV amplicons may be the induction of IFN-γ secreting CD8+ anti-tumor effector cells. Dutton et al. [30] have shown that anti-tumor responses in subcutaneously implanted E.G7 tumors treated with activated OT-I T cells are dependent upon IFN-γ production. In our experiments, the transfer of IFN-$\gamma^{-/-}$ CD8+cells was relatively ineffective in eradicating EL-4 tumors compared to wild type CD8+ transfer. Therefore, IFN-γ appears to be needed for the anti-tumor effects we observed. We also noted diminished responses in mice treated with CD8+ transfer and HSV amplicon injection if the mice were not irradiated prior to the adoptive CD8+ transfer (Fig. 1). Since tumor implants were protected from irradiation, the increased anti-tumor effect induced in those experiments was likely a result of the induced leukopenia, and not due to direct radiation effects on the tumor.

Transferred cells from tumor-bearing animals treated with HSV amplicon injections (Figs. 2c, 5c, d) expressed high levels of CD44, as has been described previously for homeostatically expanded T cells [9, 10]. It is interesting to note that a subset of the CD44+ high population in naïve mice resemble conventional memory T cells with regard to the ability to rapidly produce IFN-γ upon TCR stimulation [31]. CD44+ high T cells, express low or undetectable levels of NK receptors prior to activation, but upon activation with IL-2, they express significant levels of activating NK receptors including 2B4 and NKG2D [24]. Transferred OT-I CD44 expression was higher in irradiated E.G7 tumor-bearing mice regardless of whether they were treated with HSV amplicons when compared to non-tumor bearing mice (Fig. 5). It is possible that proliferation of OT-I cells in irradiated animals alone does not lead to the same degree of CD44 induction as that in mice in which high levels of the T cell specific antigen are produced. CD8+CD44^{hi} cells derived from the tumor-bearing animals (Fig. 2) or from OT-I mice (Fig. 5) may have a lower threshold for TCR-mediated activation (as demonstrated by Dummer et al. [17]) or possess nonspecific killing capacity distinct from that mediated through the TCR [24, 31]. We also note that following eradication of tumor by transferred OT-I cells, cured animals remained resistant to tumor re-challenge (Fig. 6), implying the emergence of active immune memory.

We used OT-I transfer as a model to allow further analysis of underlying mechanisms of increased effectivity of the combined treatment. In both the E.G7 or LLC-OVA tumor models, OT-I T cell anti-tumor effects were further amplified by direct intra-tumoral injections with HSV-amplicons. We analyzed IFN-γ production by the transferred OT-I

cells. The fraction of transferred cells secreting IFN-γ in response to E.G7 was substantially higher in the mice treated with the HSV-amplicons (Fig. 7). An explanation for this higher level of activation of the anti-tumor T cells can be delivery of a so-called "third signal" induced by the HSV-amplicon injection. Mescher and colleagues demonstrated that for full activation of T cells, a third signal besides the TCR and co-stimulator activation was needed [32–34]. Cytokine production, induced in response to local HSV amplicon injection could provide this third signal. The presence of large amounts of antigen (such as Ovalbumin from over-expressing tumors) may allow T cell proliferation to take place independently of a "third signal", without causing full T cell activation (as measured by the production of IFN-γ and CTL activity). This may explain why we did not detect higher OT-I percentages in mice treated with HSV amplicons, compared to controls when Ovalbumin secreting tumors were implanted. Our results suggest that local injections of HSV vectors might provide a crucial "third" signal needed for full T cell activation.

Mice cured by the combination of OT-I cells, radiation and intratumoral HSV injections, also displayed anti-tumor activity against the parental lymphoma, EL-4. These results suggest the occurrence of "epitope spreading" which may be relevant to tumor eradication. Similar results were published by Eisenbach and colleagues, who demonstrated that following vaccination of mice with irradiated cells loaded with the SIINFEKL peptide (the Ovalbumin-derived dominant T cell antigen) and EG7 tumor rejection there was also rejection of EL-4 cells due to reactive T-cells directed at previously unrecognized epitopes [27]. Thus a possible advantage to the specific T cell mediated tumor rejection would be the generation of additional potent anti-tumor clones with specificities independent from those of the original transferred T cells.

Treatments that induce lymphopenia to a variable degree are frequently employed in cancer therapy. In humans intratumoral injection of viral vectors has been performed in numerous clinical trials. The effects of the transferred transgene versus the effects of innate immune stimulus delivered as a result of local viral vector injection have not been extensively evaluated in humans, since most trials have not been performed with control "empty" viral vectors. An interesting phase II study has been recently reported by Hortobagyi and colleagues who combined systemic therapy with intratumoral injections of AdCMV-p53 into breast cancer before surgery [25]. All 12 evaluable patients achieved an objective clinical response. The surgical specimens revealed scattered tumor cells with extensive tumor-infiltrating leukocytes (predominantly T lymphocytes) [25]. Our study suggests that adoptive transfer of T cells during a lymphopenic period might increase the efficacy of intratumoral viral vector injection.

Dedicated agents such as the TLR9 stimulator CpG 7909 designed to act as innate immune stimulators are currently being tested in clinical trials [35]. Following the results obtained in a randomized phase IIB trial of CpG 7909 in combination with chemotherapy in 112 patients with lung cancer, a large scale clinical trial has been initiated [35]. Results from our study suggest that one way to increase efficacy would be to combine intratumoral injections of an innate immune stimulator with adoptive T cell transfer.

Our studies suggest that intra-tumoral innate immune stimulation might also have a beneficial effect on the induction of tumor specific T cells. This approach could be applied as an alternative to high dose IL-2 therapy used by Rosenberg and colleagues to activate adoptively transferred tumor specific T cells (in the lymphopenic setting) as the systemic toxicities of IL-2 may pose a problem [3–5]. Our results suggest that a combination of intra-tumoral innate immune stimulation with adoptive T cell transfer in the lymphopenic setting could be incorporated into a variety of clinical strategies.

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