Inhibition of Murine Prostate Tumor Growth and Activation of Immunoregulatory Cells With Recombinant Canarypox Viruses

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Background: Immunization with modified tumor cells carrying recombinant immunomodulatory genes is being explored as cancer immunotherapy. In this study, we examine whether canarypox ALVAC viruses carrying immunostimulatory cytokine genes (granulocyte–macrophage colony-stimulating factor, interleukin 2, interleukin 12, and tumor necrosis factor-α) can induce antitumor immunity (to rechallenge) in the RM-1 model of a highly aggressive, weakly immunogenic murine prostate cancer.

Methods: For antitumor activity studies, RM-1 murine prostate cancer cells were infected with the parental ALVAC virus or one or two recombinant ALVAC–cytokine viruses and then injected into male C57BL/6 mice. For rechallenge studies, other mice were first given an injection subcutaneously with irradiated (nonproliferating) recombinant ALVAC-infected RM-1 cells and then (10 days later) with untreated RM-1 cells. For the determination of which immune cells were required for antitumor activity, mice were immunodepleted of CD4, CD8, or natural killer (NK) NK1.1 cells with the corresponding monoclonal antibodies and were then given an injection of ALVAC–cytokine-infected RM-1 cells. For all experiments, tumor outgrowth and animal survival were monitored.

Results: After subcutaneous injection into mice, RM-1 cells infected with one (except ALVAC–interleukin 2) or two ALVAC–cytokine recombinants had statistically significantly greater antitumor activity than RM-1 cells infected with parental ALVAC (P<.001 for all; two-sided test). The antitumor activity of RM-1 cells infected with any two ALVAC–cytokine recombinants was greater than, but not statistically significantly different from, that of RM-1 cells infected with any one ALVAC–cytokine recombinant. NK1.1 cells were necessary for antitumor activity, but tumorspecific CD4+ regulatory T cells were also induced that inhibited CD8+ RM-1-specific cytotoxic T cells, resulting in the lack of immunity to a rechallenge by RM-1 cells. Discussion: Canarypox viruses can transfer immunostimulatory cytokine genes into RM-1 prostate cancer cells. When such cells were injected into mice, the cytokines induced an antitumor response against this highly aggressive, weakly immunogenic tumor. This response, however, did not protect the mouse against a rechallenge with RM-1 cells because suppressor CD4+ T cells were induced that inhibited tumor-specific CD8+ cytotoxic T cells. [J Natl Cancer Inst 2001;93:998–1007]
Several approaches have been used to develop cancer immunotherapy vaccines to activate a specific immune response against tumor-associated antigens that will eliminate the tumor, including genetically modified tumor cells (1), dendritic cells pulsed with tumor antigen (2), and heat-shock proteins derived from tumor cells (3). These vaccine strategies were designed to activate antigen-specific, antitumor immune responses, with preliminary observations indicating effectiveness, even in weakly immunogenic tumor model systems. The antigen-specific components in these antitumor responses are CD4+ and/or CD8+ T cells and, in some instances, natural killer (NK) cells (1–5).

Other immunotherapeutic approaches under development rely on viral vectors to transfer genes for tumor-associated antigens or immunostimulatory cytokines into target cells (4,6–9). Retroviral vectors, however, have been used with limited success (10,11) because these vectors do not infect all cells in a given population, target cells must be proliferating for viral integration, and there is an extended time between infection and expression of the transferred gene. Recombinant vaccinia virus has been used to deliver genes into tumor cells, but its use is limited because it replicates in tumor cells and thus toxic effects are associated with its systemic distribution (12). As an alternative, several poxvirus variants have been identified or developed that possess useful gene transfer qualities. The canarypox virus ALVAC is such a variant that does not replicate in mammalian cells and is an efficient gene-delivery vector (13). When carrying genes for rabies glycoprotein or measles hemagglutinin glycoproteins (14–16), ALVAC has proved to be a safe and effective vector in humans and in animals and can induce immune responses and protective immunity against challenge with the cognate pathogens (15,17). These characteristics suggest that ALVAC recombinants may be used for gene delivery in cancer therapy. Previous studies from this laboratory (18) have demonstrated that ALVAC vectors can efficiently insert immunostimulatory cytokine genes into prostate cancer cells and that the genes can produce high levels of the cytokine and thus can induce antitumor activity.

In this study, we used immunostimulatory cytokine genes for granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin 2 (IL-2), IL-12, tumor necrosis factor-α (TNF-α), and the highly aggressive and weakly immunogenic RM-1 prostate cancer model. We chose the ALVAC canarypox virus to deliver cytokine genes to RM-1 cells and explored the mechanism of the induced antitumor response.

**Materials and Methods**

**Tumor Cells and Animals**

The RM-1 mouse prostate tumor model, syngeneic to C57BL/6 mice, was obtained from Dr. Timothy Thompson (Baylor College of Medicine, Houston, TX). RM-1 cells, generated by transduction with the ras and myc oncogenes, produce a poorly differentiated carcinoma when they are implanted into C57BL/6 mice. The cells are cultured in Dulbecco’s modified Eagle medium (DMEM) (BioWhittaker, Inc., Walkersville, MD) containing 10% fetal calf serum (FCS) and 10 mM HEPES buffer (pH 7.0). Male C57BL/6 and severe combined immunodeficient (SCID) mice were purchased from Charles River Laboratories (Wilmington, MA) at 6–8 weeks of age. In all in vivo experiments, groups contained at least five randomly allocated animals, and experiments were repeated at least three times with similar results. All animal experiments were approved by the University of Iowa Animal Care Committees and met all animal care guidelines for the institution.

**Antibodies**

Monoclonal antibodies (MAbs) against the major histocompatibility complexes (MHCs) K*, D*, and I-A* and the costimulatory molecules CD80, CD86, intercellular adhesion molecules ICAM-1 and ICAM-2, Fas, and CD40 and cytokine-reactive antibodies against IL-2, IL-12, GM-CSF, and TNF-α for use in enzyme-linked immunosorbent assays (ELISAs) were purchased from PharMingen (San Diego, CA). The anti-CD4 MAb GK1.5 [rat IgG2b (19)], anti-CD8 MAb 2.43 [rat IgG2b (20)], and anti-NK1.1 MAb PK136 [mouse IgG2a (21)] were prepared in the laboratory from ascites fluids generated in SCID mice by precipitating immunoglobulins with 50% ammonium sulfate. After dialysis in phosphate-buffered saline (PBS), MAbs were diluted to 1 mg/mL in PBS.

**Flow Cytometry**

Flow cytometry was performed as described previously (22). Briefly, cultured tumor cells were harvested with 10 mM EDTA and washed with 10% horse serum in PBS. Cells were incubated with the various unlabeled primary MAbs (10 μg/mL) for 15 minutes on ice. After two washes with 1% FCS in PBS, primary antibody bound to the cells was detected with fluorescein isothiocyanate-conjugated secondary MAbs (Sigma Chemical Co., St. Louis, MO; 10 μg/mL for 15 minutes on ice). After two washes with 1% FCS in PBS, unfixed cells were analyzed immediately by flow cytometry on a FACSscan (Becton Dickinson, Mountain View, CA). To verify depletion of CD4+ and CD8+ T-cell subsets in vivo, we isolated splenocytes from antibody-treated or untreated C57BL/6 mice and stained them with either the anti-CD4 MAb GK1.5 or the anti-CD8 MAb 2.43, as described above. After two washes with 1% FCS in PBS, primary antibody bound to the cells was detected with fluorescein isothiocyanate-conjugated secondary MAb, as described above. After two washes with 1% FCS in PBS, cells were analyzed by flow cytometry on a FACScan. More than 95% of the CD4+ and CD8+ cells were depleted relative to the numbers present in untreated mice.

**Viral Vectors and Infection**

ALVAC is a canarypox virus-based vector that is productively replicated only in avian cells (13). Parental ALVAC (ALVAC-par) vector (ALVAC virus Cppp) and ALVAC vectors carrying a murine GM-CSF gene (vCP319), a murine IL-2 gene (vCP275), a murine IL-12 gene (vCP301), and a human TNF-α gene (vCP245) were developed at Aventis Pasteur (Toronto, ON, Canada), as described previously (13–16,23,24). After infection of RM-1 cells, the levels of proteins produced from the transgene of each vector were evaluated by ELISA.

RM-1 cells were harvested and cultured in DMEM containing 10% FCS and 10 mM HEPES buffer (pH 7.0) the day before infection. For infection with ALVAC, the medium was changed to DMEM with 2% FCS, and ALVAC–cytokine recombinants were added to the cells in various multiplicities of infection (MOIs). The cells were incubated for 24 hours before the measurement of cytokine production. For tumor therapy studies, cells were incubated with virus for 4 hours, and then the infected cells were removed from tissue culture plates, washed with PBS, resuspended to the desired cell density in PBS, and injected subcutaneously into mice on the flank.

**Tumor Inhibition Studies**

RM-1 cells infected with one or two of the ALVAC–cytokine recombinants described above were injected subcutaneously into C57BL/6 or SCID mice. For all experiments, uninfected RM-1 cells or RM-1 cells infected with the ALVAC-par virus served as controls. Tumor outgrowth and animal survival were monitored daily. Results are presented as the percentage of mice with palpable tumors. A palpable tumor had a diameter of at least 2–3 mm. Mice were killed when tumors reached a diameter of 20 mm or if the animals became moribund or cachectic.

**Immunization/Rechallenge Study**

RM-1 cells infected with one or two of the ALVAC–cytokine recombinants described above were injected subcutaneously into naive mice challenged with 5 × 10⁶ viable, uninfected RM-1 cells injected subcutaneously on the contralateral flank. Tumor outgrowth was monitored, as described above. Background values were determined by measurement of the response in naive mice challenged with 5 × 10⁶ uninfected RM-1 cells or ALVAC-parental-infected RM-1 cells.
Cytotoxic T Lymphocyte Assays

Splenocytes (composed of red blood cells, T cells, B cells, NK cells, and interstitial cells) were harvested from control or experimental mice, as described above. Red blood cells in the preparation were lysed with ACK lysing buffer (0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM Na₂EDTA [pH 7.2]), and the remaining cells were cultured with IL-2 (10 U/mL) alone or with IL-2 and mitomycin C (40 μg/mL)-treated RM-1 cells as stimulator cells, at a splenocyte/stimulator ratio of 50:1. IL-2 stimulates the proliferation of primed precursor CD8⁺ cytotoxic T lymphocytes (CTLs) in the splenocyte preparation. After 48 hours, viable splenic effector cells were isolated by centrifugation (800g for 20 minutes at 4°C) in Ficoll. The effector cells were resuspended in complete medium and incubated with ⁵¹Cr-loaded experimental target cells (RM-1 or EL-4 cells) at various cell ratios for 4 hours. All assays were performed in round-bottom 96-well plates. Spontaneous or total release of ⁵¹Cr was determined in the presence of medium alone or 1% Nonidet P-40, respectively. The percent specific lysis was calculated as 100 × (experimental counts per minute [cpm] – spontaneous cpm)/(total cpm – spontaneous cpm). Experimental, spontaneous, and total cpm values used were the mean of triplicate wells, with the standard deviations being less than 10% of the calculated mean for each group, signifying accuracy within each culture condition. The spontaneous release of ⁵¹Cr from the target cells never exceeded 20% of maximal ⁵¹Cr release.

**In Vivo Depletion**

The following MAbs were used for the in vivo depletion studies: GK1.5, an anti-CD4 MAb; 2.43, an anti-CD8 MAb; and PK136, an anti-NK1.1 MAb. For the in vivo depletion of various cell populations, MAbs (100 μg/day) were injected into C57BL/6 mice daily for 5 days. Mice were allowed to rest for 2 days and then were given an injection of the same MAbs (100 μg/day) for an additional 5 days. Flow cytometry analysis (CD4⁺ and CD8⁺ T cells) and ⁵¹Cr-release assays (NK cells) were performed to verify which subset of cells was depleted. To determine antitumor activity in these mice, we shaved a region on the flank of each mouse, injected RM-1 cells subcutaneously at the shaved site, and monitored tumor growth and animal survival over time.

**Statistical Analyses**

An analysis of differences was performed by Dr. Justine Ritchie (University of Iowa Cancer Center, Iowa City), who used the log-rank test. Survival was estimated by the Kaplan–Meier method. The 95% confidence intervals (CIs) were based on the normal approximation but constrained to be between 0% and 100%. In experiments where no deaths were observed, an exact 95% one-sided binomial CI was used instead. All other statistical tests were two-sided.

**RESULTS**

**Characteristics of RM-1 Cells**

We assessed the ability of RM-1 murine prostate tumor cells to act as immunologic target cells by use of flow cytometry to analyze the expression levels of the following immunologically relevant cell surface proteins: MHC class I and class II antigens, the costimulatory molecules CD80 and CD86, the intercellular adhesion molecules ICAM-1 and ICAM-2, Fas receptor, and CD40. RM-1 cells constitutively expressed MHC class I antigens, and incubation with interferon γ (IFNγ) markedly increased the levels of these antigens (Fig. 1). In contrast, MHC class II antigens, ICAM-1, ICAM-2, CD80, and CD86 were not detected on RM-1 cells before or after stimulation with IFNγ. Fas receptor and CD40 were detected on unstimulated RM-1 cells, and their expression was slightly increased by incubation with IFNγ.

**Effect of ALVAC-Mediated Cytokine Gene Transfer on Growth of RM-1 Cells**

When implanted into immunocompetent mice, RM-1 cells are weakly immunogenic cells that form solid tumors; thus, RM-1 cells are suitable for immunologic gene therapy studies (18). We first determined whether RM-1 cells infected in vitro with the various ALVAC–cytokine recombinants secreted the corresponding cytokines. The day before infection, RM-1 cells were plated in six-well tissue culture plates at a density of 10⁶ cells per well. The cells were then infected with the ALVAC–cytokine recombinants ALVAC–TNF-α, ALVAC–GM-CSF, ALVAC–IL-2, or ALVAC–IL-12 at an MOI of 5, and the amount of corresponding cytokine was measured in the culture supernatants 24 hours later by ELISA. These cytokine vectors

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**Fig. 1.** Expression of cell surface markers (K<sup>b</sup>, D<sup>b</sup>, I-Ab<sup>+</sup>, CD80, CD86, intercellular adhesion molecules [ICAMs]-1 and -2, Fas, or CD40) on uninfected RM-1 prostate cancer cells was determined by flow cytometry. **Solid histograms** = cells labeled with monoclonal antibodies (MAbs) against K<sup>b</sup>, D<sup>b</sup>, I-Ab<sup>+</sup>, CD80, CD86, ICAMs-1 and -2, Fas, or CD40 after incubation with interferon γ for 48 hours; **open histograms** = unstimulated cells labeled with the same antibodies; **dashed-line histograms** = cells labeled with isotype control MAb.
were chosen because each transgene is known to stimulate antitumor responses. We found that $10^6$ ALVAC–IL-2-infected cells produced IL-2 at 28.6 ng/mL in 24 hours, $10^6$ ALVAC–IL-12-infected cells produced IL-12 at 7 ng/mL in 24 hours, $10^6$ ALVAC–GM-CSF-infected cells produced GM-CSF at 11.2 pg/mL in 24 hours, and $10^6$ ALVAC–TNF-α-infected cells produced TNF-α at 13 ng/mL in 24 hours. Infection at an MOI greater than 10 resulted in high cell death that was directly related to the high level of viral infection but not to actions of the cytokines, because infection with ALVAC-par at high MOIs was also toxic (data not shown). Levels of transgene-derived cytokines were also measured as a function of time. Five hours after infection, ALVAC–IL-2-infected RM-1 cells had produced, on average, about 10% the amount of IL-2 (2.9 ng/mL) that they had produced by 24 hours (28.6 ng/mL). Cytokine levels peaked 3 days after infection and were still detected 7 days after infection. Similar results were observed with the other ALVAC–cytokine vectors (data not shown). Thus, an MOI of 5 produced sufficient levels of cytokine with little associated cell death and was used for all subsequent experiments (18).

For the determination of whether this method of gene transfer affected RM-1 cell growth in vivo, RM-1 cells were infected with the various ALVAC–cytokine recombinants in vitro. Four hours later, the infected cells were washed and then injected subcutaneously into the flank of male C57BL/6 mice. As shown in Fig. 2, A, mice given an injection of RM-1 tumor cells infected with ALVAC–IL-12, ALVAC–GM-CSF, or ALVAC–TNF-α had a statistically significant survival advantage (P < .001 for each) compared with mice given an injection of RM-1 cells infected with ALVAC-par. In contrast, there was no statistically significant survival advantage among mice given an injection of ALVAC–IL-2-infected RM-1 cells (P = .102). Mice given an injection of RM-1 cells infected with each ALVAC–cytokine vector were monitored for 100 days, with no further change in tumor outgrowth. In addition, all mice behaved normally; no mouse demonstrated a behavioral change indicative of cytokine-induced toxicity after the injection of ALVAC–cytokine-infected RM-1 cells (data not shown).

In previous studies, single cytokine therapy with ALVAC–TNF-α inhibited RM-1 tumor growth better than that with ALVAC–IL-2, ALVAC–IFN γ, and ALVAC–B7-1 (18). Consequently, for double cytokine therapy, we investigated combinations of ALVAC–TNF-α with ALVAC–IL-2, ALVAC–IL-12, or ALVAC–GM-CSF (maintaining a constant final MOI of 5). Mice receiving RM-1 cells infected with the combinations ALVAC–TNF-α/ALVAC–IL-2, ALVAC–TNF-α/ALVAC–IL-12, or ALVAC–TNF-α/ALVAC–GM-CSF showed a statistically significant survival advantage compared with mice receiving uninfected RM-1 cells or RM-1 infected with ALVAC-par virus (Fig. 2, B; P < .001 for all three combinations). The survival of mice receiving RM-1 cells infected with ALVAC–TNF-α/ALVAC–GM-CSF was minimally, but reproducibly, decreased but not statistically significantly different from the survival of mice receiving RM-1 cells infected with ALVAC–TNF-α/ALVAC–IL-2 (P = .589) or ALVAC–TNF-α/ALVAC–IL-12 (P = .072). In contrast, in all experiments, control mice that received uninfected RM-1 cells or RM-1 cells infected with ALVAC-par were dead within 39 or 67 days, respectively. Furthermore, we found that use of a single type of recombinant ALVAC viruses carrying genes for a single cytokine (TNF-α, IL-2, IL-12, or GM-CSF) was reproducibly, but not statistically significantly greater than 10 resulted in high cell death that was directly related to the high level of viral infection but not to actions of the cytokines, because infection with ALVAC-par at high MOIs was also toxic (data not shown). Levels of transgene-derived cytokines were also measured as a function of time. Five hours after infection, ALVAC–IL-2-infected RM-1 cells had produced, on average, about 10% the amount of IL-2 (2.9 ng/mL) that they had produced by 24 hours (28.6 ng/mL). Cytokine levels peaked 3 days after infection and were still detected 7 days after infection. Similar results were observed with the other ALVAC–cytokine vectors (data not shown). Thus, an MOI of 5 produced sufficient levels of cytokine with little associated cell death and was used for all subsequent experiments (18).

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Journal of the National Cancer Institute, Vol. 93, No. 13, July 4, 2001 ARTICLES 1001
significantly, less effective than use of a combination of two types of ALVAC–cytokine viruses (TNF-α/IL-2, TNF-α/GM-CSF, or TNF-α/IL-12). It should be noted that all mice bearing tumors 24 days after RM-1 cell injection ultimately died and that no late tumor regression was observed. Thus, when compared with mice receiving uninfected RM-1 cells or ALVAC-par-infected RM-1 cells, mice receiving RM-1 cells infected with one or two types of ALVAC viruses carrying immunostimulatory cytokine genes had statistically significantly longer survival and smaller RM-1 tumors.

**Mechanism of Tumor Regression**

To determine whether mice receiving RM-1 cells infected with ALVAC–cytokine recombinants also developed immunity to RM-1 cells on rechallenge, we first immunized mice with γ-irradiated (70 Gy for 30 minutes) ALVAC–cytokine (TNF-α/IL-2, TNF-α/IL-12, or TNF-α/GM-CSF)–infected RM-1 cells. The γ-irradiated, ALVAC–cytokine-infected RM-1 cells produced the corresponding cytokines (TNF-α/IL-2, TNF-α/IL-12, or TNF-α/GM-CSF) at levels equivalent to those produced by nonirradiated-infected cells (data not shown). Control mice were immunized with irradiated uninfected RM-1 cells or irradiated ALVAC-par-infected RM-1 cells. Ten days after receiving the irradiated cells, all groups were challenged with normal (i.e., nonirradiated and uninfected) RM-1 cells, and tumor outgrowth was followed. We did not observe increased survival in any group (Fig. 3). A similar lack of protection against rechallenge was observed in mice that had shown antitumor activity against viable, ALVAC-infected RM-1 tumor cells (data not shown). These results suggest that antigen-specific, T-cell-mediated immunity was not induced against RM-1 tumor cells in these mice.

To determine whether RM-1-specific CD8+ T-cell immunity was induced by this protocol, we used in vitro cytolytic assays. In these experiments, splenocytes were isolated from mice 7 days after the mice were given an injection of ALVAC–TNF-α/ALVAC–IL-2-infected RM-1 cells. To expand the number of primed precursor CD8+ CTLs, we cultured the splenocyte preparations for 5 days with mitomycin C–treated RM-1 cells at a splenocyte/stimulator RM-1 cell ratio of 50:1. Consistent with the lack of protective immunity, no RM-1-specific CTLs were detected in mice immunized with RM-1 cells infected with ALVAC–TNF-α combined with ALVAC–IL-2, ALVAC–IL-12, or ALVAC–GM-CSF or in mice that had rejected RM-1 cells infected with any of the above combinations of ALVAC–cytokine recombinants (data not shown). Because the MOI of ALVAC viruses used did not affect the in vitro proliferation or alter the viability of the RM-1 cells (data not shown), these results suggest that immunologic mechanisms are involved in the observed antitumor activity, even though the induction of immunity or activation of antigen-specific CTLs was not detected.

**Effector Cells Mediating the ALVAC-Induced Anti-RM-1 Response**

With the observation that ALVAC-infected RM-1 cells failed to establish tumors without the induction of immunity or activation of antigen-specific CTLs, it was then expected that the absence of the cells responsible for adaptive immunity would not alter the outcome of the cytokine gene therapy procedure. We first explored the immunologic mechanism of the antitumor activity by determining whether T or B cells participated in the antitumor response against the ALVAC-infected RM-1 cells. RM-1 cells infected with various combinations of ALVAC recombinants were injected into SCID (T- and B-cell deficient) mice and C57BL/6 mice, and tumor growth was monitored. We observed that all cytokine combinations inhibited tumor outgrowth equally in C57BL/6 and SCID mice (Fig. 4), suggesting that neither T nor B cells were required for the antitumor effect.

Additional studies were performed in C57BL/6 mice that were depleted of specific cell populations (CD4+, CD8+, and NK1.1+ cells) by infusing subset-specific antibodies. Depletion of CD4+ and CD8+ cells was verified by flow cytometry, and depletion of NK1.1+ cells was verified by a cytolytic assay before uninfected RM-1 cells or RM-1 cells infected with the various ALVAC recombinants were implanted (data not shown). Tumor outgrowth of ALVAC–IL-2-infected RM-1 cells in normal C57BL/6 mice and mice treated with anti-CD8, anti-CD4, or anti-NK1.1 was compared. The results indicate that NK1.1+ cells are the primary effector cells for ALVAC recombinant-induced antitumor activity, since depletion of this population abrogated the antitumor effects of ALVAC infection (anti-NK1.1 versus ALVAC–TNF-α/ALVAC–IL-2; P<.001; Fig. 5), whereas depletion of the other populations did not. Although NK cells were the predominant effector cell type, depletion of either CD4+ or CD8+ T cells enhanced tumor outgrowth but not statistically significantly (ALVAC–TNF-α/ALVAC–IL-2 versus anti-CD4 or anti-CD8; P = .075; Fig. 5). Thus, ALVAC-mediated cytokine gene transfer into RM-1 cells before implantation into mice appears to lead to the induction of innate anti-tumor mechanisms (i.e., activation of NK 1.1+ cells) that are necessary for the prevention of tumor outgrowth, but T cells also

Fig. 3. Growth of uninfected parental RM-1 cells in C57BL/6 mice immunized with cytokine-expressing canarypox virus ALVAC–cytokine-infected RM-1 cells. Mice (15 mice/group) were immunized with 5 × 10⁸ RM-1 cells that had been infected with parental ALVAC (ALVAC-par) or a combination of ALVAC–tumor necrosis factor-α (TNF-α) and ALVAC–interleukin 2 (IL-2), ALVAC–interleukin 12 (IL-12), or ALVAC–granulocyte–macrophage colony-stimulating factor (GM-CSF) and then γ irradiated (70 Gy for 30 minutes) before injection. Ten days later, the mice were given a subcutaneous injection of 5 × 10⁵ untreated RM-1 cells on the contralateral flank. Naive (unimmunized) mice (15 mice/group) were given an injection of 5 × 10⁵ uninfected RM-1 cells, as another control. Tumor growth and animal survival were monitored over time. The ALVAC–cytokine-infected RM-1 cells were compared with irradiated RM-1 cells alone or irradiated RM-1 cells infected with ALVAC-par. Because of the similarity of effects, single curves may represent several conditions. The growth of RM-1 tumors in naive mice was not appreciably different from that in mice immunized with irradiated RM-1 cells.
Reduced helper T-cell activity, as reported for other systems (6,24). Thus, we attempted to provide the necessary cytokine support for the expression of immunity to a secondary tumor challenge in immunized mice. Mice that had rejected an initial challenge with ALVAC-TNF-α/ALVAC-IL-2-infected RM-1 cells or mice immunized with irradiated RM-1 cells were rechallenged with ALVAC-TNF-α/ALVAC-IL-2-infected RM-1 cells 50 days after the initial challenge. Naïve mice challenged with ALVAC-TNF-α/ALVAC-IL-2-infected RM-1 cells or ALVAC-par-infected RM-1 cells served as control subjects. Surprisingly, the growth of ALVAC-TNF-α/ALVAC-IL-2-infected RM-1 tumors was not inhibited in mice implanted previously with ALVAC-TNF-α/ALVAC-IL-2-infected RM-1 cells (naïve mice challenged with ALVAC-par-infected RM-1 cells versus mice given an injection of ALVAC-TNF-α/IL-2-infected RM-1 cells challenged with ALVAC-TNF-α/IL-2-infected RM-1 cells; P = 0.87; Fig. 6), and rapid tumor outgrowth was observed. In contrast, the expected antitumor effect of ALVAC-TNF-α/ALVAC-IL-2 treatment was observed only in the naïve mice, suggesting that injection of ALVAC recombinant-infected RM-1 cells actively inhibited the induction of tumor immunity.

Because of the minor role played by T cells in the inhibition of tumor outgrowth (Fig. 5) and because of the apparently active inhibition of the antitumor response (Fig. 6), we hypothesized that addition of the antigen (RM-1 cells) to an in vitro culture system intended to stimulate the proliferation of primed cytolytic precursor cells would have the opposite effect (i.e., inhibit the proliferation or activation of antigen-specific CD8⁺ CTLs).

**Inhibition of Tumor-Specific CTL Activity by CD4⁺ T Cells**

One possible explanation for the absence of protection against a secondary challenge (shown in Fig. 3) might involve reduced helper T-cell activity, as reported for other systems (6,24). Thus, we attempted to provide the necessary cytokine support for the expression of immunity to a secondary tumor challenge in immunized mice. Mice that had rejected an initial challenge with ALVAC-TNF-α/ALVAC-IL-2-infected RM-1 cells or mice immunized with irradiated RM-1 cells were rechallenged with ALVAC-TNF-α/ALVAC-IL-2-infected RM-1 cells 50 days after the initial challenge. Naïve mice challenged with ALVAC-TNF-α/ALVAC-IL-2-infected RM-1 cells or ALVAC-par-infected RM-1 cells served as control subjects. Surprisingly, the growth of ALVAC-TNF-α/ALVAC-IL-2-infected RM-1 tumors was not inhibited in mice implanted previously with ALVAC-TNF-α/ALVAC-IL-2-infected RM-1 cells (naïve mice challenged with ALVAC-par-infected RM-1 cells versus mice given an injection of ALVAC-TNF-α/IL-2-infected RM-1 cells challenged with ALVAC-TNF-α/IL-2-infected RM-1 cells; P = 0.87; Fig. 6), and rapid tumor outgrowth was observed. In contrast, the expected antitumor effect of ALVAC-TNF-α/ALVAC-IL-2 treatment was observed only in the naïve mice, suggesting that injection of ALVAC recombinant-infected RM-1 cells actively inhibited the induction of tumor immunity.

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**Inhibition of Tumor-Specific CTL Activity by CD4⁺ T Cells**

One possible explanation for the absence of protection against a secondary challenge (shown in Fig. 3) might involve
We took advantage of the fact that CD8+ CTL activity can be detected if primed cells, which express the high-affinity IL-2 receptor and thus proliferate in response to IL-2, are cultured for 48 hours in medium containing IL-2 but in the absence of the priming antigen (25). To determine whether RM-1 cells infected with ALVAC–cytokine recombinants primed CTL precursors, we first immunized mice with ALVAC–TNF-α/ALVAC–IL-2-infected RM-1 cells. Seven days later splenocytes were isolated, and the cells were cultured with IL-2 for 5 days and then tested for their lytic activity. When primed spleen cells were cultured with IL-2 alone, effector (CTL) cells lysed RM-1 target cells but not EL-4 cells, an MHC class I-matched, antigenically distinct cell line (Fig. 7, A). If splenocytes from unimmunized (naive) mice were used in the same experiment, we detected no lytic activity (data not shown). Likewise, when cultured with IL-2 alone, spleen cells isolated from mice immunized with ALVAC–TNF-α/ALVAC–IL-2-infected RM-1 cells or ALVAC–TNF-α/ALVAC–GM-CSF-infected RM-1 cells specifically lysed RM-1 target cells (data not shown). No cytolytic activity against RM-1 target cells was measured when spleen cells cultured with IL-2 and depleted of CD8+ cells were used, indicating that the lysis of RM-1 target cells was mediated by classic CD8+ CTLs (Fig. 7, B). These results also suggest that the RM-1 cells inhibit the proliferation of CD8+ CTLs. To test this hypothesis, we added RM-1 cells to the splenocyte preparation cultured with IL-2 and then tested the ability of the effector cells in this culture to lyse RM-1 target cells. Under these conditions, the ability of the effector cells to lyse RM-1 target cells was completely abrogated, suggesting that the addition of RM-1 cells inhibited the generation of RM-1-specific CTLs from primed cytolytic precursors (Fig. 7, C).

This inhibitory effect could be mediated by the production of soluble inhibitory products, such as transforming growth factor-β (TGF-β), or the activation of regulatory cells, such as CD4+ T cells. To test whether TGF-β was involved in this effect, we isolated splenocytes from mice that had rejected an ALVAC–TNF-α/ALVAC–IL-2-infected RM-1 tumor, cultured these primed cells in medium that contained up to 50% conditioned medium from RM-1 cells (which contained TGF-β at 46.5 pg/mL), and then determined whether RM-1-specific CTLs had been generated. We found that RM-1-specific CTLs had been generated, which would rule out the first mechanism of inhibition by soluble products (data not shown).

Numerous reports (26–35) have described CD4+ T-cell populations that regulate the activation and functions of CD8+ T cells in various settings. Thus, we investigated whether CD4+ T cells contributed to the RM-1 cell-mediated inhibition of CTL activity by injecting mice with ALVAC–TNF-α/ALVAC–IL-2-infected RM-1 cells and then isolating splenocytes 7 days later. One part of the splenocyte preparation was cultured with RM-1 cells, and the other part was depleted of CD4+ T cells and then cultured with RM-1 cells. Flow cytometry showed that, in the depleted preparations, CD4+ T cells were less than 3% of the population (data not shown). Effector cells from both cultures were harvested after 3 days and tested for lytic activity. Primed, unfractionated splenocytes cultured with IL-2 and RM-1 cells had no lytic activity (see Fig. 7, C). When CD4+ T cells were depleted before in vitro stimulation, however, cells capable of killing RM-1 target cells but not EL-4 cells (Fig. 7, D) were observed, suggesting that a CD4+ regulatory T cell was inhibiting RM-1-specific CD8+ CTLs in animals immunized with ALVAC recombinant-infected RM-1 cells.

**DISCUSSION**

Development of immunologically and clinically relevant mouse prostate tumor models has made it possible to investigate the roles of immune system activation and effector cell function in prostate cancer. Although such model systems may appear to be cumbersome and highly intricate, they provide a critical step in the evaluation of new treatments of prostate cancer, one of the most prevalent cancers among men in the United States with annual death rates currently estimated at more than 40,000 (36). Current treatment of localized prostate cancer is limited to surgery or radiation therapy, and androgen ablation is generally accepted as the best method for treating metastatic prostate cancer. Unfortunately, many patients with advanced prostate cancer fail to respond to androgen ablation therapy because prostatic cancer cells often become androgen independent. Consequently, an alternative or adjuvant therapy for prostate cancer is needed.

In this article, we demonstrate that genes for several immunostimulatory cytokines transferred into the prostate tumor cells with a canarypox vector before the cells are injected into mice can mediate an antitumor response against a highly aggressive and weakly immunogenic prostate cancer tumor. We found that use of a single type of recombinant ALVAC
Fig. 7. Measurement of interleukin 2 (IL-2)-stimulated antigen-specific cytotoxic T-lymphocyte (CTL) activity after combination canarypox virus ALVAC treatment. Culture conditions were as follows. Panel A: Splenocytes from C57BL/6 mice immunized with ALVAC–tumor necrosis factor-α (TNF-α)/ALVAC–IL-2-infected RM-1 cells were cultured for 48 hours with IL-2 (10 U/mL). Panel B: Depletion of CD8+ cells abrogates CTL activity. Untreated splenocytes or splenocytes depleted of CD8+ cells before culture from mice given an injection of ALVAC–TNF-α/ALVAC–IL-2-infected RM-1 cells were cultured for 48 hours with IL-2 (10 U/mL). Panel C: Antigen-specific CTL activity of splenocytes cultured with RM-1 cells and IL-2 is inhibited by CD4+ T cells. Splenocytes from mice given an injection of ALVAC–TNF-α/ALVAC–IL-2-infected RM-1 cells were cultured for 48 hours with mitomycin C-treated RM-1 cells (at a splenocyte to RM-1 cell ratio of 50:1) and IL-2 (10 U/mL). Panel D: Depletion of CD4+ cells restores CTL activity. CD4-depleted splenocytes from mice given an injection of ALVAC–TNF-α/ALVAC–IL-2-infected RM-1 cells were cultured for 48 hours with mitomycin C-treated RM-1 cells (at a splenocyte to RM-1 cell ratio of 50:1) and IL-2 (10 U/mL). After these culture periods and for all four experiments, splenocytes were then tested for lytic activity by incubation with 51Cr-labeled RM-1 or EL-4 cells for 4 hours at various effectors (splenocytes) to target (RM-1 cell) ratios. Spontaneous or total release of 51Cr was determined in the presence of medium alone or 1% Nonidet P-40, respectively. The percent specific lysis was calculated as 100 × (experimental counts per minute [cpm] – spontaneous cpm)/total cpm – spontaneous cpm). Experimental, spontaneous, and total cpm values used were the mean of tripli-


virus carrying a single cytokine gene (TNF-α, IL-2, IL-12, or GM-CSF) was reproducibly, but not statistically significantly, less effective than use of a combination of two types of ALVAC–cytokine viruses (TNF-α/IL-2, TNF-α/GM-CSF, or TNF-α/IL-12), with the combinations of ALVAC–TNF-α/ALVAC–IL-2 or ALVAC–TNF-α/ALVAC–IL-2 giving the best results. Inhibition of tumor outgrowth was mediated predominantly by NK cells, with a minor contribution from T cells. Results of in vivo experiments designed to boost the minimal T-cell response suggested that T-cell immunity was being actively suppressed, because a secondary tumor challenge was not inhibited in animals immunized with γ-irradiated- or ALVAC–TNF-α/ALVAC–IL-12-infected RM-1 cells. Additional experiments identified a CD4+ regulatory cell in splenocyte preparations that inhibited RM-1-specific CTL activity. When splenocyte preparations were depleted of CD4+ cells before coculturing with RM-1 cells and IL-2, antigen-specific CTL activity reappeared.

ALVAC is a canarypox virus that can infect mammalian cells but cannot replicate in them (13,37), and thus no progeny virus is produced by infected mammalian cells. Indeed, several studies (38–40) that examined the consequences of ALVAC infection reported no viral vaccine-associated or virus-induced local or systemic reactions. In contrast, replication-competent recombinant vaccinia virus has also been used as a “live” vaccine, but severe adverse reactions (progressive vaccinia, eczema vaccinatum, and encephalitis) have been reported with its use (12,41). The induction of immune responses and protective immunity against challenge with the cognate pathogen, with essentially no local or systemic reaction against the ALVAC vector, have been reported in studies using ALVAC vectors to carry genes for the rabies virus glycoprotein (14,15), the measles virus fusion and hemagglutinin glycoproteins (16), the feline leukemia virus env and gag proteins (23), and the human immunodeficiency virus-1 envelope glycoprotein (17,24). Thus, ALVAC is a useful tool for gene delivery.

NK1.1+ cells were required for the antitumor response. Depletion of NK1.1+ cells in vivo abrogated the antitumor effects of ALVAC infection. Experiments with SCID mice supported this observation, because the ALVAC combination therapy was equally effective in preventing tumor outgrowth in SCID and in normal mice. Of interest, depletion of either CD4+ or CD8+ T cells consistently reduced the effectiveness of the vaccine in normal mice but not in SCID mice. Enhanced NK cell activity has been reported in SCID mice in other model systems (1,3,5,23), but the reason(s) for the increase in activity are not known. NK cells are the effector cells in the elimination of primary tumor implants [(3,5); this study] and also in CD8-mediated antigen-specific control of metastatic tumor foci and tumor challenge after immunization (3,42). When examined (5,23), tumor cell susceptibility to NK cells was linked to a decrease in MHC class I expression or to an increase in B7 expression. In an investigation of MHC class I modulation of NK cell activity (23), the loss of only a single allele (H-2Kb) was sufficient to induce NK-cell cytolytic activity, and only tumor cells with diminished H-2Kb expression were susceptible to NK cell-mediated lysis. RM-1 cells express moderate levels of H-2Kb, which can be increased by incubation with IFN-γ (Fig. 1). Because inflammation is induced at the injection site of the ALVAC vaccine (24), perhaps levels of H-2Kb also increase in vivo. Indeed, when ALVAC–IL-2-infected RM-1 cells were examined after they were injected into mice, the expression of H-2Kb was enhanced (data not shown). Thus, it can be suggested that the ALVAC–cytokine response is biphasic, where the NK
cells respond first to the tumor and then to tumor antigen-specific T cells. The increased NK cell activity in the SCID mice was sufficient to eliminate all of the tumor cells, but "normal" NK cell activity in C57BL/6 mice required a second wave of help from the tumor-antigen-specific T cells to eradicate the tumor.

Animals that received ALVAC-infected RM-1 cells, which lead to the inhibition of tumor outgrowth, had no immunity to a secondary challenge with RM-1 cells. In additional in vitro experiments, tumor-specific CTL activity was not detected when spleen cells from ALVAC vaccine-treated mice were cultured with the antigen (mitomycin C-treated RM-1 cells); CTL activity was observed only when CD4+ T cells were depleted before incubation with the antigen. These results suggest that activation of an immunoregulatory, or suppressor, T-cell population inhibits the activation or proliferation of RM-1-specific CTLs. Although once controversial, the concept of T-cell-mediated suppression of the activation and/or expansion of antigen-specific T cells is now being accepted (26,27). Much of the disbelief in suppressor T cells was largely due to the failure to accurately define a suppressor T-cell lineage, their antigen specificity, and mechanism of action. Recent advances have identified molecular mechanisms by which one population of T cells can regulate another, such as the production of soluble antigen-specific immunosuppressive factors encoded by T-cell receptor α and/or β genes (28–31). Alternatively, immunosuppression may be induced through cell–cell contact in which a noncytolytic negative signal is sent from the regulatory T cell to the target cell. CD4+ CD25+ regulatory T cells have been proposed to function in this manner (32–34), by competing for or altering the function or expression of costimulatory molecules on antigen-presenting cells (35). Either of these mechanisms would interfere with the activation or proliferation potential of other T cells responding to antigens expressed on the antigen-presenting cells. The mechanism by which the CD4+ T-cell population regulates the lytic function of CD8+ T cells in our prostate cancer model is under investigation.

ALVAC is a useful means of delivering gene sequences in immunotherapeutic protocols because of its inability to replicate productively in mammalian cells, while retaining high infection efficiency and transgene product expression. The ability of recombinant ALVAC vectors to activate an antitumor response after immunization was demonstrated herein by use of a prostate tumor cell vaccine model. While our data provide further evidence for the feasibility of using recombinant ALVAC vectors in a tumor cell vaccine protocol, studies characterizing the immunoregulatory CD4+ T cells induced in our model system are under way to determine the potential ramifications of activating such a regulatory cell population in clinical patients with the use of this type of tumor cell vaccination protocol.

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NOTES

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