

# Regression of HPV-Positive Tumors Treated With a New *Listeria monocytogenes* Vaccine

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**Background:** Human papillomavirus (HPV) has been implicated in the pathogenesis of 15% to 23% of head and neck squamous cell carcinomas as well as most oropharyngeal carcinomas. The viral oncoproteins E6 and E7 are expressed in HPV-positive tumor cells and therefore provide ideal targets for tumor immunotherapy. Because of its unique ability to induce a cellular immune response, the intracellular bacteria *Listeria monocytogenes* has been studied as a potential HPV-positive tumor vaccine.

**Objective:** To present a new recombinant strain of *L monocytogenes* that is effective in treating HPV-positive tumors in a murine model.

**Design:** A new recombinant *L monocytogenes* vaccine, Lm-ActA-E7, was designed by transforming an attenuated *Listeria* strain with an E7 expression cassette. The cassette consists of the HPV-16 E7 sequence fused to the *Listeria* protein ActA. The resultant strain of bacteria secretes E7 antigen as a fusion protein with ActA.

**Methods:** Tumors were established in C57BL/6 mice with a syngeneic HPV-positive cell line prior to treatment with vaccine.

**Intervention:** The Lm-ActA-E7 vaccine was administered intraperitoneally to the mice 5 days after tumors were established. A booster dose was administered 7 days after the first dose. Tumor progression was measured in 2 dimensions periodically after the vaccination.

**Results:** In C57BL/6 mice, the administration of Lm-ActA-E7 caused the complete regression of HPV-positive tumors in 6 of 8 mice tested. A cytotoxic T-lymphocyte assay revealed that administration of the vaccine caused the generation of cytotoxic T cells specific for E7.

**Conclusion:** Our results demonstrate the ability of a new *Listeria*-based vaccine to generate a specific antitumor T-cell response and cause the regression of HPV-positive tumors in a murine model.

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**S**QUAMOUS CELL CARCINOMA OF the head and neck (HNSCC) remains a worldwide health problem. Recent estimates indicate more than 360 000 new cases of HNSCC worldwide annually, and more than 200 000 annual deaths.<sup>1</sup> In the United States, an estimated 37 200 new cases will be diagnosed in 2003, with 11 000 deaths.<sup>2</sup> Despite technological advances in surgery, radiation, and chemotherapy, 5-year survival rates remain less than 50% worldwide.<sup>1</sup>

New therapies to treat HNSCC are being developed and tested, including immunotherapeutic strategies that attempt to boost the immune response to eliminate malignant cancer cells.<sup>3-5</sup> An ideal cancer immunotherapeutic strategy would target a known tumor antigen expressed by the tumor cells but not by normal cells. The ideal therapeutic agent would signifi-

cantly enhance the host immune response to the antigen so that partial or complete tumor regression is achieved.

In this study we describe a novel immunotherapeutic strategy for the treatment of 1 subset of HNSCC. The targeted tumor antigen is the human papillomavirus (HPV) protein E7. Human papillomavirus DNA is present in roughly 15% to 23% of HNSCC,<sup>6-8</sup> and in over 50% of oropharyngeal SCCs.<sup>6-9</sup> The protein E7 is an ideal candidate for immunotherapeutic vaccine approaches because it is constitutively expressed in HPV-transformed tissues and is thought to be necessary to maintain the transformed state of these cells.<sup>10</sup> In addition, E7 has been shown to be immunogenic in humans.<sup>11,12</sup>

Central to the development of a therapeutic vaccine for HPV-associated tumors is the identification of a vector that provokes a strong cell-mediated immune

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response. Intracellular bacteria are among the potential vaccine vectors under investigation. These bacteria, such as *Listeria*, *Salmonella*, and bacillus Calmette-Guerin, have the ability to generate strong cell-mediated immunity in murine models.<sup>13</sup> They also produce toxins that could serve as an adjuvant in generating effective innate immunity and cytokine release.<sup>13,14</sup> Bacillus Calmette-Guerin is currently used as an immunotherapeutic agent for bladder cancer.<sup>15,16</sup>

Among the intracellular bacteria, *Listeria monocytogenes* is the focus of our laboratory studies. It is a  $\beta$ -hemolytic, gram-positive, facultative bacterium that infects many human cells, including antigen-presenting cells such as dendritic cells and macrophages. This strain of bacteria has an unusual life cycle that leads to the activation of the 2 main T-cell populations: cytotoxic (CD8<sup>+</sup>) and helper (CD4<sup>+</sup>) T cells (Figure 1).<sup>17</sup> Because of its unique immunologic properties, *Listeria* has been studied as a vaccine vector by several different laboratories for the treatment of different infectious and neoplastic diseases.<sup>18-23</sup>

Herein we describe a recombinant strain of *L monocytogenes* engineered to express and secrete HPV-16 E7. In this strain, Lm-ActA-E7, E7 is secreted as a fusion protein with ActA, an *L monocytogenes* protein necessary for the assembly of the actin filaments that propel *Listeria* to the periphery of an infected cell. We investigate the ability of this vaccine vector to cause tumor regression and to elicit an E7-specific immune response in a murine model.

## METHODS

### MICE

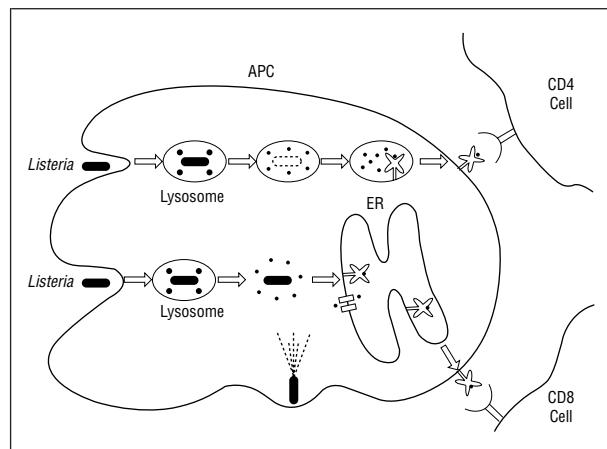
Our experimental mice were 6- to 8-week-old C57BL/6 mice purchased from Charles River Laboratories (Wilmington, Mass.). All animal experiments were performed under approved protocols from the institutional animal care and use committee of the University of Pennsylvania.

### CELL LINES

The TC-1 cell line (provided by T. C. Wu, MD, PhD, Johns Hopkins, Baltimore, Md)<sup>24</sup> was derived from C57BL/6 mice lung epithelial cells. The cells were immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene. They express both E6 and E7 and are highly tumorigenic.<sup>24</sup> The EL4 cell line is established from a lymphoma induced in a C57BL mouse. The EL4/E7 cell line was derived from the EL4 cell line that expresses the E7 protein (provided by Robert Tindle, PhD, Royal Children's Hospital, Brisbane, Queensland, Australia).<sup>25</sup> All of these cell lines were grown in culture with RPMI 1640, 10% fetal calf serum, 2mM L-glutamine, 100 U/mL of penicillin, 100  $\mu$ g/mL of streptomycin, 100  $\mu$ M nonessential amino acids, and 1mM sodium pyruvate at 37°C and 10% carbon dioxide.

### LISTERIA MONOCYTOGENES STRAINS AND PROPAGATION

The 2 *Listeria* strains used in this study were Lm-ActA-E7 and Lm-LLO-NP. The latter construct, also known as DP-L2028, has been previously described.<sup>26</sup> Lm-ActA-E7 refers to a recombinant strain of *L monocytogenes* that carries a plasmid that expresses the E7 protein fused to the ActA protein.



**Figure 1.** Antigens secreted by *Listeria monocytogenes* are presented to both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. After phagocytosis into an antigen-presenting cell (APC), *Listeria* are either destroyed within the phagolysosome (top pathway within the cell), where peptides are loaded onto major histocompatibility complex class II molecules and presented to CD4<sup>+</sup> T cells, or they escape into the cytoplasm of the cell (bottom pathway). Here, *L monocytogenes* secretes antigens (black dots) that will be transported to the endoplasmic reticulum (ER) for loading onto class I molecules and presentation to CD8<sup>+</sup> T cells.

The *actA-E7* DNA insert was generated by gene splicing by overlap extension in several steps. First, the promoter (*pHly*) and signal sequence from the *L monocytogenes* gene *hly* was amplified via polymerase chain reaction (PCR) from pGG55 (pLLO-E7, a construct from our laboratory previously described<sup>20</sup>) using primer 5'-GGGGTCTAGACCTCCTTGATTAGTATA TTC-3' (*Xba*I site is underlined) and primer 5'-ATC **TTCGCTATCTGCGCCGGCGC**GTGCTTCAGT TTGTTGCGC-3' (*Not*I site is underlined; primer sequence shown in bold is the *actA* gene overlap).

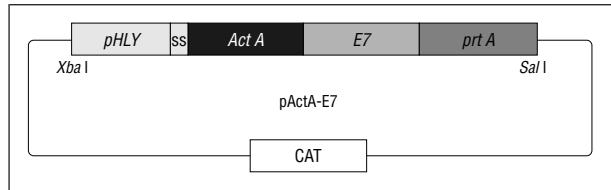
The *actA* gene was PCR amplified from the wild-type *L monocytogenes* 10403s genome using primer 5'-GCG CAACAAACTGAAGCAGCGGCCGCGACAGATA GCGAAGAT-3' (*Not*I site is underlined; primer sequence shown in bold is the *pHly* overlap) and primer 5'-**TGT AGGTG**TATCTCCATGCTCGAGAGCTAGGCGAT CAATTTC-3' (*Xho*I site is underlined; primer sequence shown in bold is the *E7* gene overlap). These primers amplify the first 420 amino acids of the ActA protein.

The *E7* gene was PCR amplified from pGG55 (pLLO-E7) using primer 5'-**GGAATTGATCGCCTAGCTCT** CGAGCATGGAGATACACCTACA-3' (*Xho*I site is underlined; primer sequence shown in bold is the *actA* gene overlap) and primer 5'-**AAACGGATT**TATTAGATCCGGGTTATGGTTCTGAGAACA-3' (*Xma*I site is underlined; primer sequence shown in bold is the *prfA* gene overlap).

The *prfA* gene was PCR amplified from the wild-type *L monocytogenes* 10403s using primer 5'-**TGTTCTCAGAAA** CCATAACCCGGGATCTAAATAATCCTTT-3' (*Xma*I site is underlined; primer sequence shown in bold is the *E7* gene overlap) and primer 5'-**GGGGT**CGACCAGCTTTCTTG-GTGAAG-3' (*Sall*I site is underlined).

The *pHly* fused to the *actA* gene (*pHly-actA*) was PCR generated and amplified from purified *pHly* DNA and purified *actA* DNA using the *pHly* primer (upstream) 5'-GGGGTCTCTTGATTAGTATATTC-3' (*Xba*I site is underlined) and *actA* primer (downstream) **TGTAGGT** GTATCTCCATGCTCGAGAGCTAGGCGATCAATTTC-3' (*Xho*I site is underlined).

The *E7* gene fused to the *prfA* gene (*E7-prfA*) was PCR generated and amplified from purified *E7* DNA and purified *prfA* DNA using the *E7* primer (upstream) GGAATTGAT



**Figure 2.** A schematic representation of the plasmid pActA-E7. The plasmid was used to transform the *Listeria monocytogenes* strain XFL-7 to create Lm-ActA-E7. It includes the promoter (*pHly*) and signal sequence (ss) from the *hly* gene, the *actA* gene, the human papillomavirus 16 *E7* gene, and the transcription factor *prfA*. XFL-7 is a *prfA*-negative strain of *L monocytogenes*. Thus, only bacteria that retain the plasmid will replicate *in vivo*.

CGCCTAGCTCTGAGCATGGAGATAACACCTACA-3' (XbaI site is underlined) and *prfA* gene primer (downstream) GGGGGTCGACCAGCTCTTGGTGAAG-3' (SalI site is underlined).

The *pHly-actA* fusion DNA product fused to the *E7-prfA* fusion DNA product is PCR generated and amplified using the *pHly* primer (upstream) 5'-GGGGTCTAGACCTCCTT-GATTAGTATATTTC-3' (XbaI site is underlined) and *prfA* gene primer (downstream) 5'-GGGGGTCGACCAGCTCTTGGTGAAG-3' (SalI site is underlined) and ligated into pCRII (Invitrogen, San Diego, Calif). After TOP10/F (a competent *Escherichia coli* strain from Invitrogen) was transformed with the pCRII-ActAE7, the isolated plasmid was screened by restriction analyses using *Bam*H1 (expected fragment sizes, 770 base pairs [bp] and 6400 bp [or when the insert was reversed into the vector, 2500 bp and 4100 bp]) and *Bst*X1 (expected fragment sizes, 2800 bp and 3900 bp) and also screened with PCR analyses using the *pHly* primer (upstream) 5'-GGGGTCTAGACCTCCTT-GATTAGTATATTTC-3' and *prfA* gene primer (downstream) 5'-GGGGGTCGACCAGCTCTTGGTGAAG-3'.

The *pHly-ActA-E7-prfA* DNA insert was excised from pCRII by double digestion with *Xba*I and *Sal*I and ligated into the vector pDP-2028. The resulting plasmid was named pActAE7 (Figure 2). After TOP10/F was transformed with plasmid pActAE7, chloramphenicol-resistant clones were screened by PCR analysis using the *pHly* primer (upstream) 5'-GGGGTCTAGACCTCCTT-GATTAGTATATTTC-3' and the *prfA* gene primer (downstream) 5'-GGGGGTCGACCAGCTCTTGGTGAAG-3'. A clone containing pActAE7 (Figure 2) was grown in brain-heart infusion medium with chloramphenicol (20 µg/mL), and pActAE7 was isolated from the bacteria cell using a midiprep DNA purification system kit (Promega, Madison, Wis).

After a *prfA*-negative strain of penicillin-treated bacteria *Listeria*, XFL-7, was transformed with expression system pActAE7, clones were selected for the retention of the plasmid *in vitro*, and secretion of the ActA-E7 fusion protein was verified by Western blot analysis. Lm-ActA-E7 was grown in brain-heart infusion medium with 20 µg/mL of chloramphenicol at 37°C. Bacteria were frozen in aliquots at -80°C.

Primers were synthesized by Operon Technologies, Alameda, Calif, and were resuspended in Tris-EDTA and stored at -20°C.

#### WESTERN BLOTTING ANALYSIS

*Listeria* strains were grown in Luria-Bertoni medium at 37°C. After centrifugation, protein was precipitated from the supernatant with trichloroacetic acid (TCA) and resuspended in 1× sample buffer with 0.1N sodium hydroxide. Identical amounts of each TCA precipitated supernatant were loaded on 4% to 20% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (NOVEX, San Diego, Calif). The gel was transferred to polyvinylidene difluoride mem-

branes and probed with anti-E7 monoclonal antibody (Zymed Laboratories, South San Francisco, Calif), diluted to 1:2500. The secondary antibody was horseradish peroxidase-conjugated antimouse IgG (Amersham Pharmacia Biotech, Little Chalfont, England), diluted to 1:5000. Blots were developed with Amersham enhanced chemiluminescence detection reagents and exposed to autoradiography film (Amersham).

#### EFFECTS OF Lm-ActA-E7 ON ESTABLISHED TUMOR GROWTH

Six- to 8-week-old C57BL/6 mice received  $2 \times 10^5$  TC-1 cells subcutaneously on the left flank. Seven days after tumor challenge most tumors were between 4 and 5 mm in size. Groups of 8 mice were then treated intraperitoneally with 0.1 median lethal dose ( $LD_{50}$ ) of Lm-ActA-E7 ( $2 \times 10^8$  colony-forming units [cfu]) or Lm-LLO-NP ( $2 \times 10^7$  cfu) on days 7 and 14 or left untreated.

#### MEASUREMENT OF TUMOR GROWTH

Tumors were measured twice per week with calipers spanning the shortest and longest surface diameters. The mean of these 2 measurements was plotted as the mean tumor diameter in millimeters against various time points. Mice were killed if the tumor diameter reached 20 mm. Tumor measurements for each time point are shown for surviving mice only. For comparisons of tumor diameters between groups, the mean and SD of tumor size for each treatment group were determined, and statistical significance was determined by *t* test. In all experiments,  $P < .05$  was considered significant. The *P* values are reported in the figure legends.

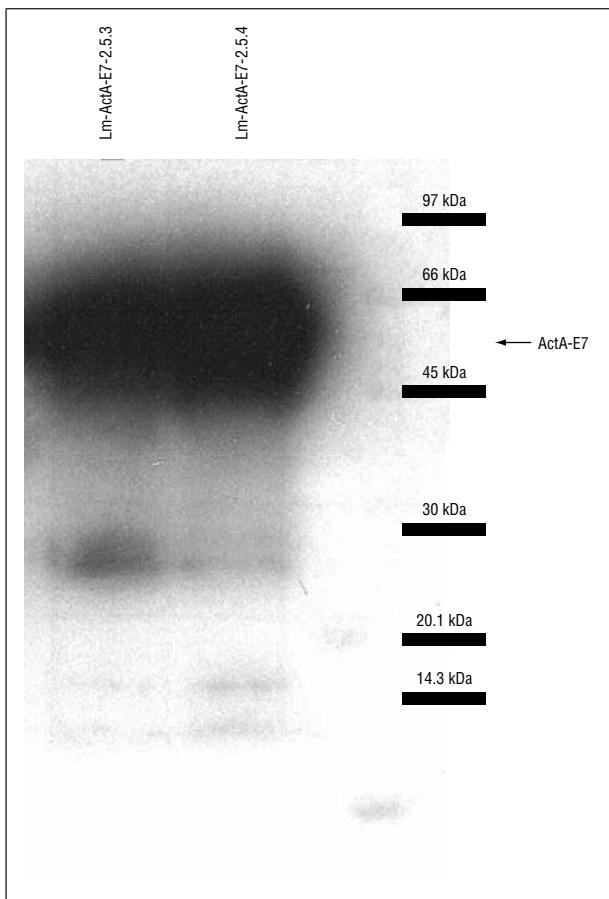
#### CHROMIUM 51 RELEASE ASSAY

The C57BL/6 mice, 6 to 8 weeks old, were immunized intraperitoneally with 0.1  $LD_{50}$  Lm-ActA-E7 or Lm-LLO-NP or left untreated. One week later, a booster dose was administered. Seven days after immunization, spleens were harvested. Splenocytes were established in culture with irradiated TC-1 cells as feeder cells (100 splenocytes/1 TC-1 cell). After 6 days of *in vitro* stimulation, the splenocytes were used in a standard chromium 51 ( $^{51}\text{Cr}$ ) release assay. Briefly, target cell lines EL-4 or EL-4/E7 were incubated for 1 hour with  $^{51}\text{Cr}$ . The stimulated splenocytes were then cultured with the radioactive targets for 4 hours at various effector-target ratios. Following incubation, the cells were pelleted, and 100 µL of supernatant removed. The samples were assayed with a Wallac 1450 scintillation counter (Gaithersburg, Md). The percentage of specific lysis was determined as (experimental counts per minute - spontaneous counts per minute)/(total counts per minute - spontaneous counts per minute) × 100.

#### RESULTS

#### CONSTRUCTION OF Lm-ActA-E7

Lm-ActA-E7 is a new recombinant strain of *L monocytogenes* that uses a multicopy episomal expression system to secrete the HPV protein E7 fused to the *Listeria* protein ActA. Lm-ActA-E7 was generated by introducing a recombinant plasmid vector, pActAE7, into the *L monocytogenes* strain XFL-7. pActAE7 carries a DNA insert that contains the following genes: (1) the 310-bp promoter from the *hly* gene and the *hly* signal sequence; (2) the 1171-bp *actA* gene; (3) the 300-bp *E7* gene; (4) the 1019-bp *prfA* gene; and (5) a chloramphenicol resis-

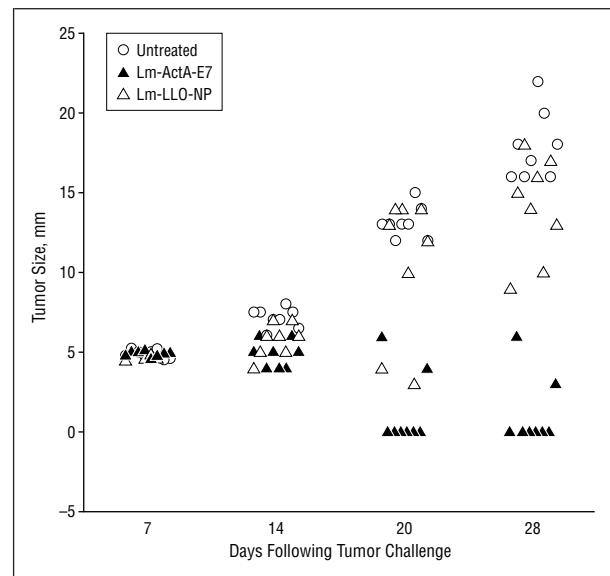


**Figure 3.** Lm-ActA-E7 secretes E7 as a fusion protein. Two bacterial clones that retained the plasmid pActAE7, Lm-ActA-E7-2.5.3 and Lm-ActA-E7-2.5.4, were grown overnight at 37°C in Luria-Bertoni medium. Bacteria were pelleted by centrifugation, and each supernatant was precipitated with trichloroacetic acid. E7 expression was analyzed by Western blot. The blot was probed with anti-E7 monoclonal antibody followed by horseradish peroxidase-conjugated antimouse antibody (Amersham Pharmacia Biotech, Little Chalfont, England). The blot was developed using enhanced chemiluminescence detection reagents (Amersham). Lm-ActA-E7-2.5.4 was used in all subsequent experiments.

tance gene (CAT). The promoter that drives the expression and secretion of this fusion protein is derived from the *hly* gene, an *L moncytogenes* gene that encodes the protein listeriolysin O (LLO). Using gene splicing by overlap extension PCR methods, this promoter was spliced to *actA*, a *Listeria* gene that encodes the protein ActA, which is a surface protein necessary for actin assembly. The *E7* gene encodes the HPV protein that is the target of our immunotherapeutic strategies. *prfA* is a transcription factor without which the *L moncytogenes* strain XFL-7 will not replicate. Thus, during *in vivo* propagation only those bacteria that contain the DNA insert will reproduce. This recombinant strain is highly attenuated, with an LD<sub>50</sub> of  $2.5 \times 10^9$  cfu, compared with the wild-type *L moncytogenes* strain 10403s, which has an LD<sub>50</sub> of  $5 \times 10^4$  cfu (data not shown).

#### Lm-ActA-E7 SECRETES A FUSION ActA-E7 PROTEIN

We predicted that, driven by the *pHly*, our recombinant *Listeria* vaccine vector would produce a fusion protein 58 kDa in size (ActA, 46 kDa; E7, 12 kDa). Owing to the



**Figure 4.** Lm-ActA-E7 causes regression of established TC-1 tumors. C57BL/6 mice received  $2 \times 10^5$  TC-1 cells subcutaneously on the left flank. Tumors grew to 5 mm after 7 days. The mice were then treated with 0.1 median lethal dose of Lm-ActA-E7 or Lm-LLO-NP as a negative control on day 7, and a booster dose was given on day 14. The third and final group was left untreated. The average tumor diameter was measured with calipers and is shown for each mouse. The difference in tumor sizes between the Lm-ActA-E7 group and either control group at days 20 and 28 is statistically significant ( $P < .005$  and  $P < .001$ , respectively). Depicted is 1 experiment representative of 4.

presence of the signal sequence, our vector should also secrete this protein. Western blot analysis of the supernatant of pelleted bacteria using an anti-E7 monoclonal antibody confirmed that Lm-ActA-E7 expresses and secretes a 58-kDa fusion protein (**Figure 3**).

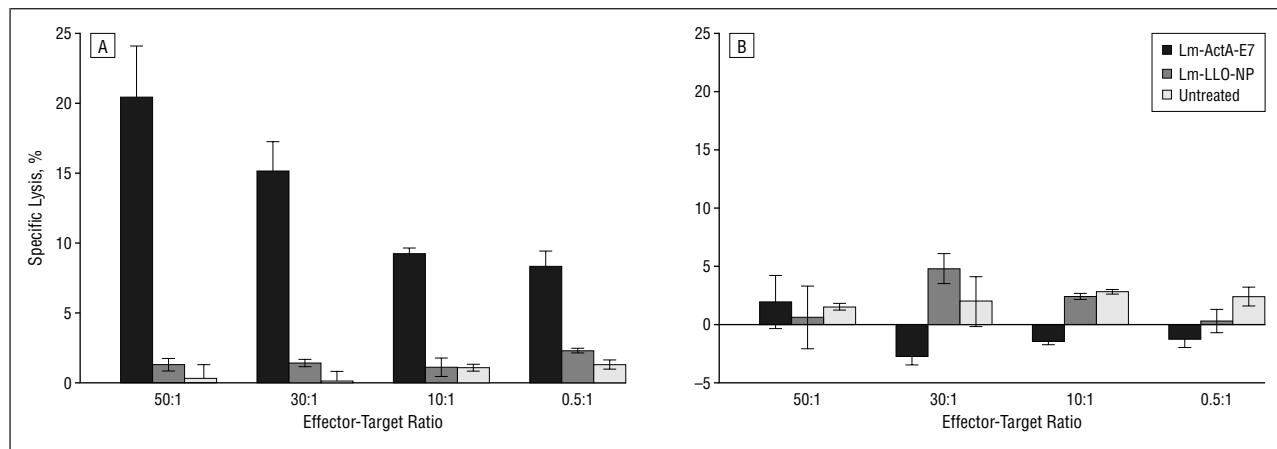
#### Lm-ActA-E7 INDUCES COMPLETE REGRESSION OF ESTABLISHED TC-1 TUMORS

The HPV-positive syngeneic cell line TC-1 was used to establish tumors in the left flank of C57BL/6 mice. Seven days later, most tumors had reached a palpable size of 4 to 5 mm in diameter. The mice were treated with 2 doses, 1 week apart, of either Lm-ActA-E7 or a negative control bacterial vector, Lm-LLO-NP, at 0.1 LD<sub>50</sub>. Lm-LLO-NP is a recombinant *Listeria* strain that expresses the irrelevant antigen influenza nucleoprotein. For each experiment, 1 group was left untreated.

While Lm-LLO-NP had little effect on tumor growth compared with the untreated group, those mice treated with Lm-ActA-E7 had significantly smaller tumors after 28 days ( $P < .001$ ). In 6 of 8 mice, the tumors had completely disappeared; in the control groups, none of the tumors regressed (**Figure 4**).

#### Lm-ActA-E7 INDUCES E7-SPECIFIC CYTOTOXIC T-LYMPHOCYTE ACTIVITY

To determine whether the Lm-ActA-E7 was able to induce an E7-specific cytotoxic T-lymphocyte (CTL) response, a CTL assay was performed. C57BL/6 mice were vaccinated with 0.1 LD<sub>50</sub> of Lm-ActA-E7 or the negative control vector, Lm-LLO-NP, or left untreated. One week



**Figure 5.** A, Lm-ActA-E7 induces E7-specific cytotoxic T-lymphocyte (CTL) activity. C57BL/6 mice were immunized with 0.1 median lethal dose of Lm ActA-E7 or Lm-LLO-NP. A separate group of mice was left untreated. A booster immunization was administered 7 days later. Splenocytes were harvested 7 days after the booster and established in primary culture with irradiated TC-1 cells for 7 days. Following the primary culture, CTL activity was assayed via chromium  $^{51}\text{Cr}$  release from EL4-E7 cells. The CTL activity was significantly higher in those mice that were vaccinated with Lm-ActA-E7 than in controls ( $P < .05$ ). Results are expressed as the mean of triplicate cultures. These results are representative of 3 experiments. B, In a control experiment, splenocytes harvested from mice immunized with Lm-ActA-E7 or Lm-LLO-NP and untreated mice were cultured with EL4 target cells that do not express E7. A standard  $^{51}\text{Cr}$  assay was performed after culture with irradiated TC-1 cells for 7 days. Negligible CTL activity against EL4 cells was detected. Results are expressed as the mean of triplicate cultures. These results are representative of 2 separate experiments.

later, booster immunizations were administered. Splenocytes were then harvested 7 days after the second vaccination and incubated with irradiated TC-1 feeder cells. After 7 days of in vitro stimulation, a standard  $^{51}\text{Cr}$  release assay was performed using EL4 or EL4/E7 as the target cells and the harvested splenocytes as the effector cells. The results showed that the target cells that expressed E7 were effectively lysed by splenocytes from Lm-ActA-E7-vaccinated mice but not by splenocytes from the control and untreated mice (Figure 5A). The target cells that do not express E7 were not lysed by splenocytes from any of the treatment groups (Figure 5B).

#### COMMENT

In this study we describe the creation of a new recombinant *L monocytogenes* tumor vaccine, Lm-ActA-E7. In a murine model, the administration of Lm-ActA-E7 results in the induction of E7-specific CTLs and the regression of HPV-positive tumors. The induction of tumor-specific CTLs is crucial to any therapeutic vaccine strategy because these lymphocytes are capable of eliminating cells that express foreign peptide in the context of major histocompatibility complex class I molecules.

Lm-ActA-E7 secretes E7 in the context of a fusion protein, including both ActA and E7. One reason a fusion protein strategy was developed is because previous studies showed that different fusion protein constructs were more effective than vaccines that secreted tumor antigen alone.<sup>20</sup> The reasons that the fusion proteins generate a more potent immune response are unclear and are under investigation.

*Listeria monocytogenes* is a ubiquitous gram-positive bacterium that can be readily isolated from soil, water, and vegetation.<sup>27</sup> It has been shown that humans incidentally ingest *Listeria* via contaminated food or water frequently.<sup>28,29</sup> Pathologic human *Listeria* infection, or listeriosis, is rare, estimated to occur at a rate of less than 1 per 100 000 population per year in North America.<sup>30</sup>

The clinical manifestations most often include fever and gastroenteritis.<sup>31</sup> Immunocompromised persons and pregnant women and their fetuses are more susceptible than the population at large. In these susceptible patients listeriosis can have more severe consequences, such as meningitis or sepsis.<sup>29</sup>

Despite the potential for listeriosis, we believe that use of an attenuated *L monocytogenes* vector is feasible for human trials. Oral inoculation of the bacteria in adult volunteers has been investigated and found to be safe.<sup>32</sup> Though work presented here is based on parenteral administration, the mice tolerated the vaccine well with no signs of toxic effects or illness throughout the course of the experiments (data not shown). Certainly, a clinical trial would exclude immunocompromised individuals and women of childbearing age. Our recombinant strain of *Listeria* is highly attenuated compared with wild type, and *Listeria* remains exquisitely sensitive to standard antibiotic therapy in the event of clinical listeriosis.<sup>33</sup>

Because HPV DNA is not present in all head and neck cancers, this particular vaccine strategy is not feasible for all patients with HNSCC. However, most patients with oropharyngeal HNSCC will have HPV-associated tumors. Because surgical treatment of tumors in this region often results in severe functional morbidity, non-surgical treatments are often used. Our vaccine strategy would complement existing modalities, providing specific eradication of malignant cells without damage to normal tissues. Local intratumoral injections or oral inoculations of Lm-ActA-E7 are also potential methods of administering the vaccine in oropharyngeal tumors. Animal studies investigating the efficacy of these routes of administration are currently under investigation in our laboratory.

In summary, Lm-ActA-E7 is a new cancer immunotherapeutic agent capable of treating HPV-positive tumors. Given that many head and neck tumors are associated with HPV infection, this cancer vaccine has a potential role to play in the treatment of HNSCC.

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