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Two *Listeria monocytogenes* Vaccine Vectors That Express Different Molecular Forms of Human Papilloma Virus-16 (HPV-16) E7 Induce Qualitatively Different T Cell Immunity That Correlates with Their Ability to Induce Regression of Established Tumors Immortalized by HPV-16[1]

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Two recombinant *Listeria monocytogenes* (rLm) strains were produced that secrete the human papilloma virus-16 (HPV-16) E7 protein expressed in HPV-16-associated cervical cancer cells. One, Lm-E7, expresses and secretes E7 protein, whereas a second, Lm-LLO-E7, secretes E7 as a fusion protein joined to a nonhemolytic listeriolysin O (LLO). Lm-LLO-E7, but not Lm-E7, induces the regression of the E7-expressing tumor, TC-1, established in syngeneic C57BL/6 mice. Both recombinant E7-expressing rLm vaccines induce measurable anti-E7 CTL responses that stain positively for H-2D^b E7 tetramers. Depletion of the CD8$^+$ T cell subset before treatment abrogates the ability of Lm-LLO-E7 to impact on tumor growth. In addition, the rLm strains induce markedly different CD4$^+$ T cell subsets. Depletion of the CD4$^+$ T cell subset considerably reduces the ability of Lm-LLO-E7 to eliminate established TC-1 tumors. Surprisingly, the reverse is the case for Lm-E7, which becomes an effective anti-tumor immunotherapeutic in mice lacking this T cell subset. Ab-mediated depletion of TGF-β and CD25$^+$ cells improves the effectiveness of Lm-E7 treatment, suggesting that TGF-β and CD25$^+$ cells are in part responsible for this suppressive response. CD4$^+$ T cells from mice immunized with Lm-E7 are capable of suppressing the ability of Lm-LLO-E7 to induce the regression of TC-1 when transferred to tumor-bearing mice. These studies demonstrate the complexity of *L. monocytogenes*-mediated tumor immunotherapy targeting the human tumor Ag, HPV-16 E7. *The Journal of Immunology,* 2001, 167: 6471–6479.

Human papilloma viruses (HPV)[3] are known to infect the squamous epithelium of the mucocutaneous surface. Expression of the viral proteins E6 and E7 by a subset of HPV can immortalize infected cells and then may later ensure progression to malignant disease (1, 2). HPV strain 16 is associated with >50% of cervical cancer cases (3–5). E6 and E7 are expressed constitutively in HPV-16-induced cervical cancer (6) and are thus commonly investigated targets for cancer immunotherapy. The intracellular locations of E6 and E7 suggest that a cellular immune response is likely to be more efficacious than a humoral response.

Listeria monocytogenes infection is a classic model for the induction of a protective cellular immune response (7). As an intracellular pathogen, *L. monocytogenes* has direct access to the cytoplasm of APC. This ability to access the cytoplasm is largely due to the hemolytic activity of listeriolysin O (LLO) (8). LLO, a 529-aa protein with hemolytic activity, is secreted by *Listeria* and perforates the phagosomal membrane, allowing the bacterium to escape the vacuole and enter the cytoplasm. The hemolytic domain of LLO resides in the C-terminus of the protein. Proteins secreted by *L. monocytogenes* during this intracellular phase of its life cycle are effectively targeted by the cellular immune system (9). We have taken advantage of *L. monocytogenes* to target proteins to the cellular immune system by engineering the bacterium to secrete influenza nucleoprotein, NP, a model tumor Ag. Treatment of mice bearing tumors expressing NP with the NP-secreting *Listeria* recombinant (Lm-LLO-NP) resulted in the regression of Ag-bearing tumors (10–12). Although the influenza NP is a useful model Ag, it is not expressed by human tumors. Hence, we have turned our attention to relevant tumor-specific Ags, the HPV-16 proteins E6 and E7 that are constitutively expressed in HPV-16-associated tumors (6). E6 and E7 expression is sufficient to immortalize murine or human cells (13, 14). For example, the poorly immunogenic tumor, TC-1, is derived from murine lung cells immortalized with HPV-16 E6 and E7 (13, 15–17). TC-1 provides a model in which a human tumor-associated protein (E7), the expression of which is driven by the endogenous E7 promoter, serves as a target for immunotherapy.

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[3] Abbreviations used in this paper: HPV, human papilloma virus; CD62L, CD62 ligand; rLm, recombinant *L. monocytogenes;* LLO, listeriolysin O; Lm-E7, rLm strain that secretes HPV-16 E7; Lm-Gag, rLm strain that secretes HIV-1 Gag; Lm-LLO-E7, rLm strain that secretes a fusion protein consisting of HPV-16 E7 plus part of the hemolysin of *L. monocytogenes*; Lm-LLO-NP, rLm strain that secretes a fusion protein consisting of the nucleoprotein of influenza strain A/PR/8/34 plus part of the hemolysin of *L. monocytogenes*; NP, nucleoprotein.

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Here we describe two recombinant *L. monocytogenes* (rLm) strains, Lm-LLO-E7 and Lm-E7, that express and secrete E7. These recombinants differ enormously in their effectiveness as E7-specific tumor immunotherapeutics. Lm-LLO-E7 induces an immune response capable of causing the regression of established TC-1 tumors, whereas the other, Lm-E7, induces a response that suppresses anti-TC-1 immunity. In this study we have investigated the immune responses induced by the rLm strains and have established a system useful for comparing effective and ineffective induction of tumor immunity by recombinant *L. monocytogenes*.

Materials and Methods

Mice

Six- to 8-wk-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA).

Cell lines

The C57BL/6 syngeneic TC-1 tumor was immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-*ras* oncogene (13). TC-1 expresses low levels of E6 and E7 and is highly tumorigenic. TC-1 was grown in RPMI 1640, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 50 μM 2-ME, 400 μg/ml G418, and 10% National Collection Type Culture-109 medium at 37° with 10% CO_2.

L. monocytogenes *strains and propagation*

The *Listeria* strains used in the E7 tumor Ag studies are Lm-LLO-E7 (*hly*-E7 fusion gene in an episomal expression system), Lm-E7 (single-copy E7 gene cassette integrated into *Listeria* genome), Lm-LLO-NP (*hly*-NP fusion gene in an episomal expression system), and Lm-Gag (single-copy HIV-1 Gag gene cassette integrated into the chromosome). Lm-LLO-NP, also known as DP-L2028 (18), and Lm-Gag, also known as ZY-18 (19), have been previously described. E7 was amplified by PCR using the primers 5′-GGCTCGAGCATGGAGATACACC-3′ (*Xho*I site is underlined) and 5′-GGGGACTAGTTTATGGTTTCTGAGAACA-3′ (*Spe*I site is underlined) and ligated into pCR2.1 (Invitrogen, San Diego, CA). E7 was excised from pCR2.1 by double digest with *Xho*I and *Spe*I and ligated into pGG-55. The expression system, pGG-55, is modeled on pDP-2028 (18). The *hly*-E7 fusion gene and *prfA* are cloned into pAM401, a multicopy shuttle plasmid, generating pGG-55. The *hly* promoter drives the expression of the first 441 aa of the *hly* gene product, LLO, which is joined by the *Xho*I site to the E7 gene. The result is a *hly*-E7 fusion gene that is transcribed and secreted as LLO-E7. By deleting the hemolytic C-terminus of LLO we have removed the hemolytic activity in the fusion protein. The pluripotential transcription factor, *prfA*, is also included on pGG-55. By transforming a *prfA* negative strain of *Listeria*, XFL-7 (a kind gift from Dr. Hao Shen, University of Pennsylvania), with pGG-55 we select for the retention of the plasmid in vivo (Fig. 2). The *hly* promoter and gene fragment were generated using primers 5′-GGGGGCTAGCC CTCCTTTGATTAGTATATTC-3′ (*Nhe*I site is underlined) and 5′-CTC CCTCGAGATCATAATTTACTTCATC-3′ (*Xho*I site is underlined). The *prfA* gene was PCR amplified using primers 5′-GACTACAAGGACGATGA CCGACAAGTGATAACCCGGGATCTAAATAAATCCGTTT-3′ (*Xba*I site is underlined) and 5′-CCCGTCGACCAGCTCTTCTTGGTGAAG-3′ (*Sal*I site is underlined). Lm-E7 was generated by introducing an expression cassette containing the *hly* promoter and signal sequence driving the expression and secretion of E7 into the *orfZ* domain of the *L. monocytogenes* genome. E7 was amplified by PCR using the primers 5′-GCGGATC CCATGGAGATACACCTAC-3′ (*Bam*HI site is underlined) and 5′-GCTCTAGATTATGGTTTCTGAG-3′ (*Xba*I site is underlined). E7 was then ligated into the pZY-21 shuttle vector. The resulting plasmid, pZY-21-E7, is an expression system that includes the previously described expression cassette inserted in the middle of a 1.6-kb sequence that corresponds to the *orfX, Y, Z* domain of the *L. monocytogenes* genome. *L. monocytogenes* strain 10403S was transformed with pZY-21-E7. The homology domain allows for insertion of the E7 gene cassette into the *orfZ* domain by homologous recombination. Clones were screened for integration of the E7 gene cassette into the *orfZ* domain. Bacteria were grown in brain heart infusion medium with (Lm-LLO-E7 and Lm-LLO-NP) or without (Lm-E7 and ZY-18) chloramphenicol (20 μg/ml). Bacteria were frozen in aliquots at −80°C.

Oligonucleotide primers

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

Synthetic peptides

Synthetic peptides were HPLC purified. Peptides were resuspended in DMSO or PBS (2 mg/ml) as solubility allowed.

Western blotting

Listeria strains were grown in Luria-Bertoni medium at 37°C and were harvested at the same OD measured at 600 nm. The supernatants were TCA precipitated and resuspended in 1× sample buffer supplemented with 0.1 N NaOH. Identical amounts of each cell pellet or each TCA-precipitated supernatant were loaded on 4–20% Tris-glycine SDS-PAGE gels (NOVEX, San Diego, CA). The gels were transferred to polyvinylidene difluoride and probed with an anti-E7 mAb (Zymed Laboratories, South San Francisco, CA). The secondary Ab was HRP-conjugated anti-mouse (Amersham Pharmacia Biotech, Little Chalfont, U.K.). Blots were developed with Amersham ECL detection reagents and exposed to Hyperfilm (Amersham Pharmacia Biotech).

Measurement of tumor growth

Tumors were measured every other day with calipers spanning the shortest and longest surface diameters. The mean of these two measurements was plotted as the mean tumor diameter in millimeters against various time points. Mice were sacrificed when the tumor diameter reached 20 mm. Tumor measurements for each time point are shown only for surviving mice.

Effects of Listeria *recombinants on established tumor growth*

Six- to 8-wk-old C57BL/6 mice (Charles River) received 2×10^5 TC-1 cells s.c. on the left flank. One week following tumor inoculation the tumors had reached a palpable size, 4–5 mm in diameter. Groups of eight mice were then treated with 0.1 LD_{50} i.p. Lm-LLO-E7 (10^7 CFU), Lm- E7 (10^6 CFU), Lm-LLO-NP (10^7 CFU), or Lm-Gag (5×10^5 CFU) on days 7 and 14 unless otherwise stated, or the mice were left untreated.

^{51}Cr release assay

C57BL/6 mice, 6–8 wk old, were immunized i.p. with 0.1LD_{50} Lm-LLO-E7, Lm-E7, Lm-LLO-NP, or Lm-Gag or were left untreated. Ten days postimmunization spleens were harvested. Splenocytes were established in culture with irradiated TC-1 cells (100:1, splenocytes:TC-1) as feeder cells. Following 5 days of in vitro stimulation, splenocytes were used in a standard ^{51}Cr release assay. Briefly, splenocytes were cultured with the following targets: EL-4, EL-4/E7, or EL-4 pulsed with E7 H-2^b peptide (RA HYNIVTF) (20). E:T cell ratios were 80:1, 40:1, 20:1, 10:1, 5:1, and 2.5:1. All dilutions were performed in triplicate. Following a 4-h incubation at 37°C, cells were pelleted, and 50 μl supernatant was removed from each well. The samples were assayed with a Wallac 1450 scintillation counter (Gaithersburg, MD). The percent specific lysis was determined as [(experimental counts per minute − spontaneous counts per minute)/(total counts per minute − spontaneous counts per minute)] × 100.

TC-1-specific proliferation

C57BL/6 mice were immunized with 0.1 LD_{50} and boosted by i.p. injection 20 days later with 1 LD_{50} Lm-LLO-E7, Lm-E7, Lm-LLO-NP, or Lm-Gag. Six days after the boost the spleens were harvested from immunized and naive mice. Splenocytes were established in culture at 5×10^5/well in flat-bottom 96-well plates with 2.5×10^4, 1.25×10^4, 6×10^3, or 3×10^3 irradiated TC-1 cells/well as a source of E7 Ag. Splenocytes were also established without TC-1 cells or with 10 μg/ml Con A. The cells were pulsed 45 h later with 0.5 μCi [^{3}H]thymidine/well. Plates were harvested 18 h later using a Tomtec harvester 96 (Orange, CT), and proliferation was assessed with a Wallac 1450 scintillation counter. The change in counts per minute was determined as experimental counts per minute − no Ag counts per minute.

Flow cytometric analysis

C57BL/6 mice were immunized i.v. with 0.1 LD_{50} Lm-LLO-E7 or Lm-E7 and boosted 30 days later. Three-color flow cytometry for CD8 (53-6.7, PE conjugated), CD62 ligand (CD62L; MEL-14, APC conjugated), and E7 H-$2D^b$ tetramer was performed using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, Mountain View, CA). Splenocytes harvested 5 days after the boost were stained at room temperature with

H-2D^b tetramers loaded with the E7 peptide (RAHYNIVTF) or a control (HIV-Gag) peptide. Tetramers were initially provided by Dr. Larry R. Pease (Mayo Clinic, Rochester, MN) and subsequently by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility and the National Institutes of Health AIDS Research and Reference Reagent Program. The tetramers were used at a 1/200 dilution. Cells were analyzed as described above comparing tetramer$^+$, CD8$^+$, CD62L^{low} cells generated by Lm-E7 or Lm-LLO-E7 immunization.

Depletions of specific immune components

CD8$^+$ cells, CD4$^+$ cells and IFN-γ were depleted in TC-1-bearing mice by injecting the mice with 0.5 mg 2.43 (11), GK1.5 (11), or xmg1.2 (21) mAb, respectively, on days 6, 7, 8, 10, 12, and 14 post-tumor challenge. CD4$^+$ and CD8$^+$ cell populations were reduced by 99% as measured by flow cytometric analysis. Also, CD25$^+$ cells and TGF-β were depleted from TC-1-bearing mice. The CD25$^+$ cells were depleted by i.p. injection of 0.5 mg/mouse anti-CD25 mAb (PC61, a gift of Andrew J. Caton (22)) on days 4 and 6 after tumor challenge. TGF-β was depleted by i.p. injection of the anti-TGF-β mAb (2G7, a gift from H. I. Levitsky), into TC-1-bearing mice on days 6, 7, 8, 10, 12, 14, 16, 18, and 20 following tumor challenge. Mice were treated with 10^7 Lm-LLO-E7 or Lm-E7 on day 7 following tumor challenge. Tumor growth was measured as described above.

Adoptive transfer

Donor C57BL/6 mice were immunized and boosted 7 days later with 0.1 LD_{50} Lm-E7 or Lm-Gag. The donor splenocytes were harvested and passed over nylon wool columns to enrich for T cells. CD8$^+$ T cells were depleted in vitro by incubating with 0.1 μg 2.43 anti-CD8 mAb for 30 min at room temperature. The labeled cells were then treated with rabbit complement. The donor splenocytes were >60% CD4$^+$ T cells, as determined by flow cytometric analysis. TC-1 tumor-bearing recipient mice were immunized with 0.1 LD_{50} 7 days post-tumor challenge. CD4$^+$-enriched donor splenocytes (10^7) were transferred 9 days after tumor challenge to each recipient mouse by i.v. injection. Tumor growth was measured as described previously.

Statistics

For comparisons of tumor diameters, the mean and SD of tumor size for each treatment group were determined, and statistical significance was determined by Student's *t* test (23). In all experiments, $p \leq 0.05$ was considered significant. The *p* values are reported in the figure legends.

Results

Construction of L. monocytogenes *strains that secretes HPV-16 E7*

We have designed and constructed two rLm strains that express and secrete the HPV-16 E7 gene product. The constructs differ in their expression system as well as in the form of the secreted E7 tumor Ag. Lm-E7 has a single copy of the E7 gene integrated into the genome, which expresses the E7 protein preceded only by the LLO signal sequence to ensure secretion of E7 (Fig. 1*A*). Lm-LLO-E7 uses a multicopy episomal expression system to secrete a fusion protein consisting of a nonfunctional LLO joined at the C-terminus to E7 (Fig. 1*B*). The rLm construct, Lm-E7, is modeled after the Lm-Gag recombinant that has previously been demonstrated to induce effective anti-viral immunity (19, 24, 25). Lm-LLO-E7 is modeled after the Lm-LLO-NP rLm strain that has shown remarkable effectiveness as an immunotherapeutic targeting the artificial tumor Ag, NP (10–12). Lm-LLO-E7 expresses and secretes a 67-kDa LLO-E7 fusion protein, and Lm-E7 secretes E7 that migrates at approximately 14 kDa, as verified by anti-E7 Western blot (Fig. 2). The virulence of Lm-LLO-E7 and Lm-E7 is significantly decreased compared with that of the wild-type strain 10403S, but is similar to that of the respective control strains, Lm-LLO-NP and Lm-Gag. We hypothesized that the expression system may influence the effectiveness of the rLm as a tumor therapeutic.

Lm-LLO-E7 induces complete regression of established TC-1 tumors

Lm-E7 and Lm-LLO-E7 were compared for their abilities to impact on TC-1 growth. Subcutaneous tumors were established on the left flank of C57BL/6 mice. Seven days later tumors had reached a palpable size of 4–5 mm in diameter. The mice were treated on days 7 and 14 with 0.1 LD_{50} Lm-E7, Lm-LLO-E7, or, as controls, Lm-Gag and Lm-LLO-NP. While Lm-E7 had no effect on tumor growth compared with the Lm-Gag control, Lm-LLO-E7 induced complete regression of 75% of established TC-1 tumors (Fig. 3). The slowing of TC-1 growth in Lm-E7-treated mice compared with naive controls is clearly due to innate immune mechanisms, since the isogenic control, Lm-Gag, slows tumor growth to the same extent.

Lm-LLO-E7 and Lm-E7 induce similar levels of CTL activity

To determine whether Lm-LLO-E7 was more effective than Lm-E7 at inducing an E7-specific CTL response, we compared CTL levels induced by the two recombinants with a ^{51}Cr release assay using syngeneic EL-4 target cells. The results, shown in Fig. 4, demonstrate that both strains induce similar levels of E7-specific lytic activity. EL-4/E7 and EL-4 pulsed with the peptide, RA HYNIVTF, were effectively lysed by splenocytes from Lm-E7- or Lm-LLO-E7-immunized mice, while splenocytes from control immunized mice produced little or no lysis. Similarly, EL-4 without

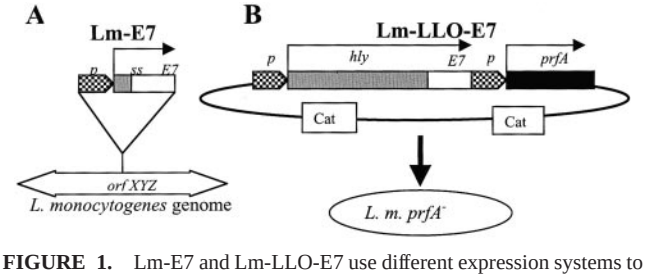

FIGURE 1. Lm-E7 and Lm-LLO-E7 use different expression systems to express and secrete E7. Lm-E7 was generated by introducing a gene cassette into the *orfZ* domain of the *L. monocytogenes* genome (*A*). The *hly* promoter drives expression of the *hly* signal sequence and the first five amino acids of LLO followed by HPV-16 E7. *B*, Lm-LLO-E7 was generated by transforming the *prfA*$^-$ strain XFL-7 with the plasmid pGG-55. pGG-55 has the *hly* promoter driving expression of a nonhemolytic fusion of LLO-E7. pGG-55 also contains the *prfA* gene to select for retention of the plasmid by XFL-7 in vivo.

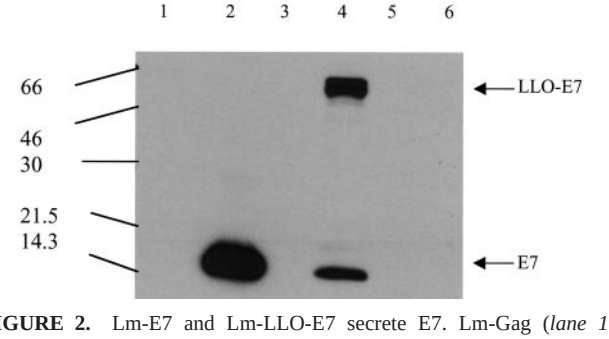

FIGURE 2. Lm-E7 and Lm-LLO-E7 secrete E7. Lm-Gag (*lane 1*), Lm-E7 (*lane 2*), Lm-LLO-NP (*lane 3*), Lm-LLO-E7 (*lane 4*), XFL-7 (*lane 5*), and 10403S (*lane 6*) were grown overnight at 37°C in Luria-Bertoni broth. Equivalent numbers of bacteria, as determined by OD at 600 nm absorbance, were pelleted and 18 ml of each supernatant was TCA precipitated. E7 expression was analyzed by Western blot. The blot was probed with an anti-E7 mAb, followed by HRP-conjugated anti-mouse (Amersham). The blot was developed using ECL detection reagents (Amersham).

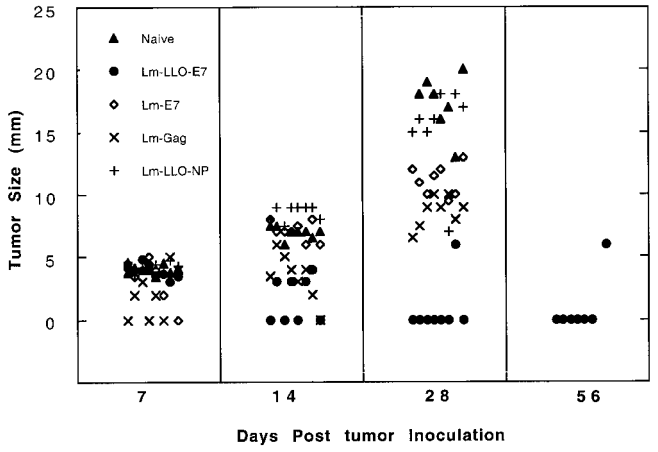

FIGURE 3. Lm-LLO-E7 induces complete regression of established TC-1 tumors. C57BL/6 mice (eight per group) received 2×10^5 TC-1 cells by s.c. injection on the left flank. Mice were treated on days 7 and 14 following tumor challenge with 0.1 LD_{50} Lm-LLO-E7, Lm-E7, Lm-LLO-NP, or Lm-Gag or were left untreated. The average tumor diameter was measured with calipers and is shown for each mouse. Mice were sacrificed when tumor diameter reached approximately 2.0 cm. Tumor measurements for each time point are shown only for surviving mice. Depicted is one of five experiments.

the E7 peptide failed to be lysed during the duration of the ^{51}Cr release assay.

Lm-E7 and Lm-LLO-E7 stimulate similar levels of E7-specific CD8$^+$ T cells

To further analyze the abilities of the two recombinants to induce E7-specific $CD8^+$ T cells, mice were immunized and boosted with Lm-E7 or Lm-LLOE7, and their splenocytes were stained with H-$2D^b$ tetramers loaded with the E7 peptide. When activated $CD8^+$ T cells were analyzed for tetramer staining, Lm-E7 and Lm-LLO-E7 induced similar levels of tetramer-positive T cells (Fig. 5).

CD8$^+$ T cells and IFN-γ are necessary for TC-1 regression

In the NP model system both $CD8^+$ T cells and IFN-γ were necessary for the regression of established NP-bearing tumors (11, 21). When $CD8^+$ T cells were depleted by the in vivo administration of Ab 2.43 following TC-1 challenge, Lm-LLO-E7 had little or no impact on tumor growth (Fig. 6*A*). Likewise, when IFN-γ was depleted using the Ab xmg1.2 following TC-1 challenge, Lm-LLO-E7 had little or no impact on tumor growth (Fig. 6*B*). Depletion of $CD8^+$ T cells or IFN-γ had no effect on the incapacity of Lm-E7 to influence the growth of TC-1 in C57BL/6 mice (data not shown).

Depletion of CD4$^+$ cells improves the effectiveness of Lm-E7 treatment

Depletion of $CD4^+$ cells significantly decreases the effectiveness of Lm-LLO-E7 treatment on TC-1-bearing mice. None of eight mice depleted of $CD4^+$ cells exhibited complete tumor regression, while five of eight nondepleted, Lm-LLO-E7-treated mice had complete tumor regression (Fig. 7*A*). Surprisingly, depletion of $CD4^+$ cells in Lm-E7 mice improved the anti-TC-1 response. Following depletion, three of eight Lm-E7-treated mice had complete regression of established TC-1 tumors (Fig. 7*B*). Also, mice with growing tumors in the Lm-E7-treated, $CD4^+$-depleted group showed slower tumor growth compared with the nondepleted, Lm-E7 mice. The difference in tumor size in the $CD4^+$-depleted compared with the undepleted group was statistically significant ($p < 0.001$) on day 27 (Fig. 7B). The effects demonstrated by the depletion experiments are not simply due to an inability of the depleted animals to clear the Lm-LLO-E7 infection, as the rLm are largely cleared by innate immunity. SCID mice infected with 10^7 Lm-LLO-E7 rapidly reduced numbers of Lm-LLO-E7 in the spleen to below detectable levels within 2 days following infection (G. R. Gunn, et al., unpublished observations).

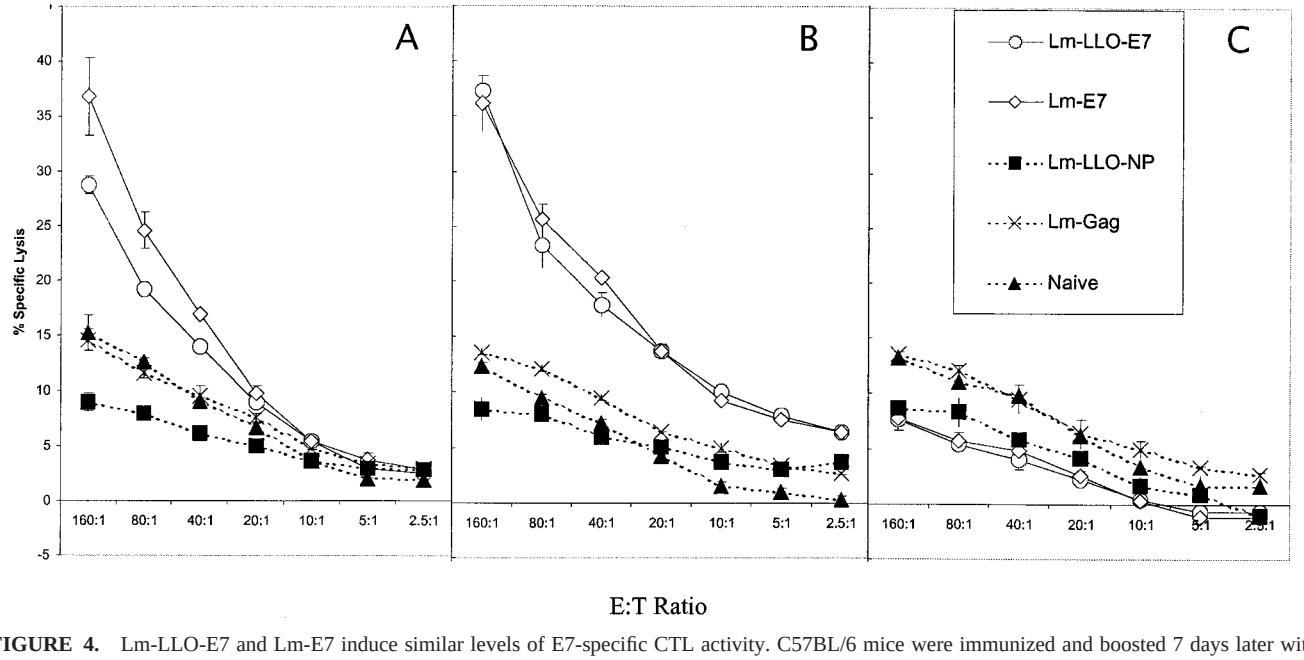

FIGURE 4. Lm-LLO-E7 and Lm-E7 induce similar levels of E7-specific CTL activity. C57BL/6 mice were immunized and boosted 7 days later with 0.1 LD_{50} Lm-LLO-E7, Lm-E7, Lm-LLO-NP, or Lm-Gag. Splenocytes were harvested 10 days after the boost and established in primary culture with irradiated TC-1 cells for 5 days. Following the primary culture, CTL activity was assayed by 4-h ^{51}Cr release from EL-4/E7 (*A*), EL-4 and E7 peptide (RAHYNIVTF; *B*), or EL-4 (*C*) targets. Results are expressed as the mean of triplicate cultures. These results are representative of three experiments.

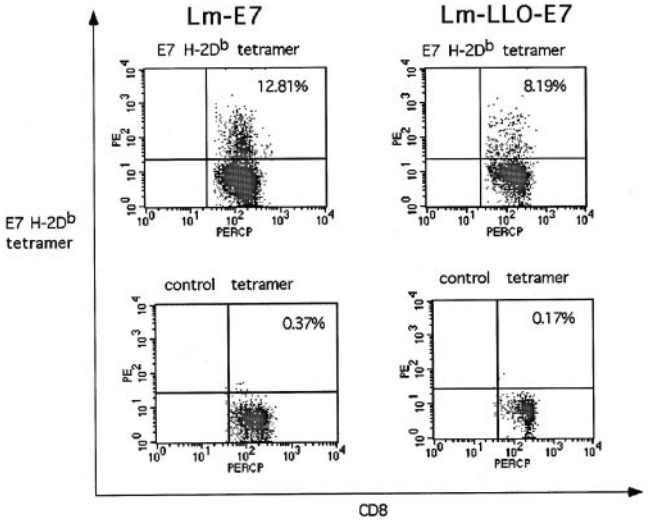

FIGURE 5. Lm-E7 and Lm-LLO-E7 induce similar levels of tetramer$^+$, CD8$^+$ T cells. C57BL/6 mice were immunized and boosted 21 days later with 0.1 LD_{50} Lm-E7 or Lm-LLO-E7. Ex vivo splenocytes were stained with an H-2D^b E7 tetramer, anti-CD8, and anti-CD62L. The population analyzed in the figure is CD8$^+$CD62L^{low}.

Lm-LLO-E7 treatment elicits TC-1 specific splenocyte proliferation

The dramatic difference in the effects of CD4$^+$ T cells induced by Lm-E7 vs Lm-LLO-E7 on tumor growth prompted us to explore the induction of T cells by the rLm strains. We assessed the TC-1-specific proliferative response of splenocytes from rLm-immunized mice. Proliferation of primed splenocytes in response to stimulation with exogenous Ag is a measure of Ag-specific immunocompetance. Ag-specific proliferation is largely mediated by the release of IL-2 from T cells responding to the Ag presented by APCs (26). The results indicate that splenocytes from Lm-LLO-E7-immunized mice proliferate when exposed to irradiated TC-1 cells as a source of E7 (Fig. 8). Conversely, splenocytes from Lm-E7 and rLm control immunized mice exhibited little or no proliferative response to TC-1 cells. Lm-LLO-E7-induced proliferation was evident at splenocyte to TC-1 ratios of 20:1, 40:1, 80:1, and 160:1. These data suggest that Lm-E7 does not induce a Th cell response to the E7 Ag expressed by TC-1.

Depletion of CD25$^+$ cells improves effectiveness of Lm-E7 treatment

A population of CD4$^+$ T cells expressing the marker CD25 (IL-2Rα) has been implicated in the maintenance of immunological self-tolerance (27–29). Furthermore, CD4$^+$CD25$^+$ T cells have been shown to contribute to tumor growth by suppressing anti-tumor immune responses (30). We hypothesized that this suppressive population is associated with the lack of T cell help in mice treated with Lm-E7. To address this question we depleted mice of CD25$^+$ cells with an anti-CD25 mAb. One difficulty associated with this depletion is that the IL-2Rα is up-regulated on activated T cells. Therefore, it was necessary to deplete the CD25$^+$ cells from naive, tumor-bearing mice before priming this subset with Lm-E7. TC-1-bearing mice were treated with anti-CD25 or the control Ab, anti-β-galactosidase. While the depletion of CD25$^+$ cells had no effect on the growth of the TC-1 tumors in naive mice, anti-CD25-treated mice receiving Lm-E7 exhibited significantly slower tumor growth than the anti-β-galactosidase-treated controls (which were also treated with Lm-E7; Fig. 9). These data suggest that the CD4$^+$CD25$^+$ population may be playing a role in the poor

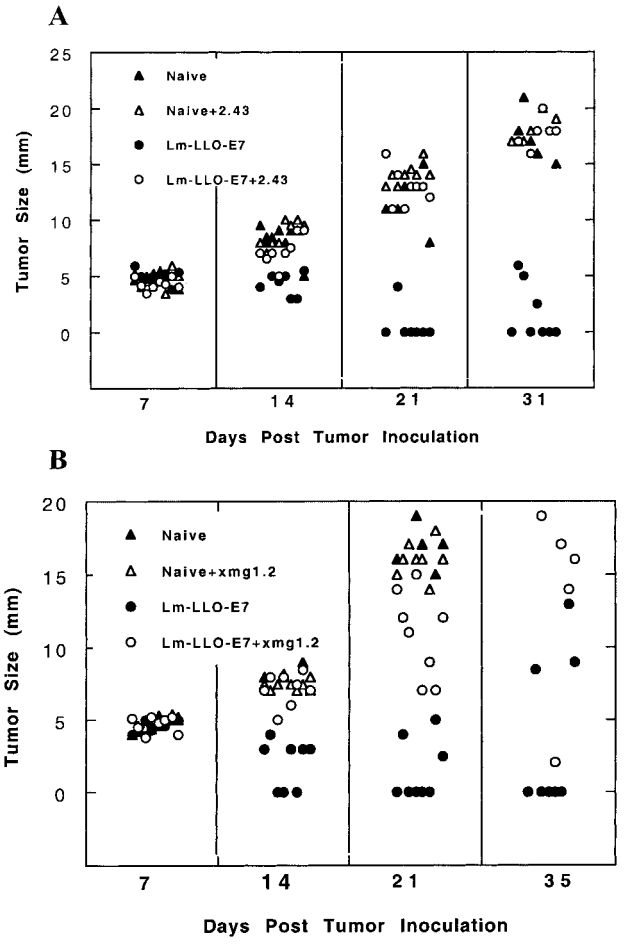

FIGURE 6. CD8$^+$ T cells and IFN-γ are necessary for Lm-LLO-E7-induced tumor regression. C57BL/6 mice (eight mice per group) received 2×10^5 TC-1 cells by s.c. injection in the left flank. Mice were either left untreated or treated on day 7 with Lm-LLO-E7. Mice were treated with 0.5 mg 2.43 (anti-CD8; *A*) or xmg1.2 (anti-IFN-γ; *B*) on days 6, 7, 8, 10, and 12 following tumor challenge. Mice were sacrificed when tumor diameter reached approximately 2.0 cm. Tumor measurements for each time point are shown only for surviving mice. Depicted is one of two similar experiments.

anti-tumor immune response generated by Lm-E7 treatment. However, in contrast to the depletion of CD4$^+$ T cells, the depletion of CD25$^+$ cells in Lm-E7-treated mice did not result in the mice becoming tumor free. This may be due to the efficiency and/or timing of the depletions or to the fact that there are other CD4$^+$ CD25$^-$ T cells that have suppressive effects.

Depletion of TGF-β in vivo greatly improves the efficacy of Lm-E7 treatment

TGF-β has been implicated in the escape of tumors in immunocompetent hosts (31, 32). Furthermore, TGF-β was reported to be secreted by CD4$^+$, CD25$^+$, suppressive T cells and was necessary for the immune suppressive function of these cells (33). We depleted TGF-β to ascertain its role in the tumor escape exhibited in TC-1-bearing mice treated with Lm-E7. TC-1-bearing mice were treated with an anti-TGF-β mAb (2G7) before and after administration of Lm-E7 or Lm-LLO-E7. While treatment with 2G7 had no apparent effect on mice that received Lm-LLO-E7 (six of eight mice were tumor free without 2G7 and seven of eight were tumor free with 2G7), anti-TGF-β treatment had a profound effect on mice receiving Lm-E7 (Fig. 10). On day 21, the day after the last

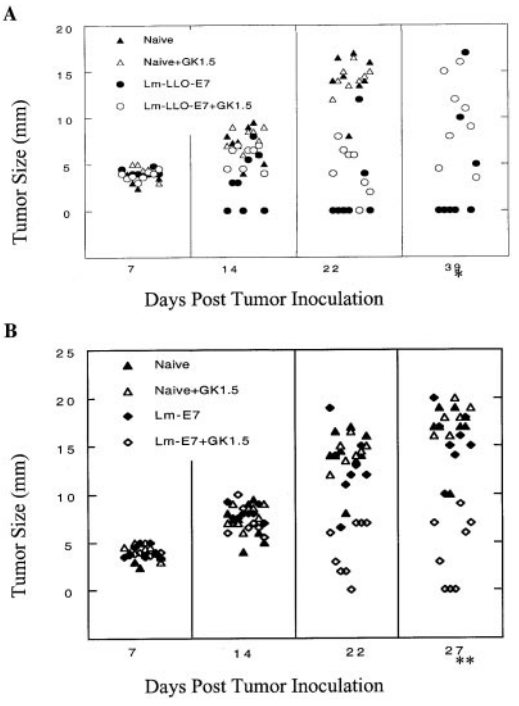

FIGURE 7. TC-1-bearing, Lm-LLO-E7- and Lm-E7-treated mice respond differently to depletion of $CD4^+$ cells. C57BL/6 mice received 2×10^5 TC-1 cells s.c. on the left flank. Mice were left untreated or were treated with Lm-LLO-E7 (*A*) or Lm-E7 (*B*) on day 7 post-tumor challenge. $CD4^+$ cells were depleted by administering 0.5 mg i.v. of the anti-CD4 mAb, GK1.5, on days 6, 7, 8, 10, and 12 following tumor challenge. *, $p < 0.05$; **, $p < 0.001$. Depicted is one of two similar experiments. Mice were sacrificed when tumor diameter reached approximately 2.0 cm. Tumor measurements for each time point are shown only for surviving mice.

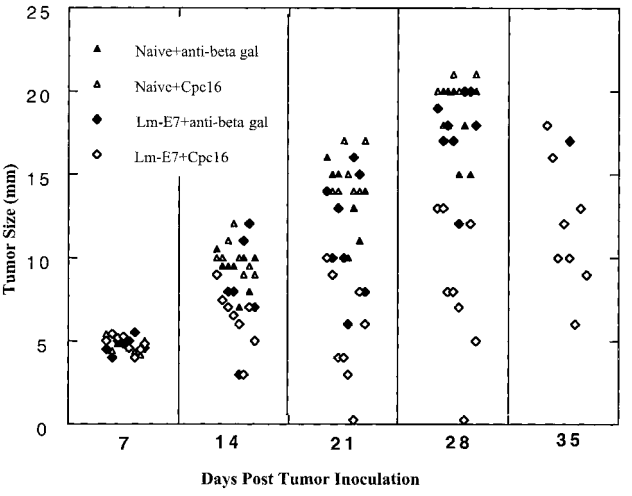

FIGURE 9. CD25 depletion improves Lm-E7 therapeutic efficacy. Following the s.c. injection of 2×10^5 TC-1 cells each mouse was given 0.5 mg anti-CD25 mAb (Cpc16; filled symbols) or 0.5 mg control anti-β-galactosidase mAb (open symbols) on days 4 and 6. The C57BL/6 mice (eight per group) were treated with Lm-E7 on day 7 (diamonds) or were left untreated (triangles). The slowing of tumor growth in mice depleted of $CD25^+$ cells and treated with Lm-E7 is significant compared with that in Lm-E7-treated controls ($p < 0.01$). Mice were sacrificed when tumor diameter reached approximately 2.0 cm. Tumor measurements for each time point are shown only for surviving mice. Depicted is one of two similar experiments.

administration of 2G7, five of eight Lm-E7 mice were tumor free, while none of eight of the Lm-E7 group that did not receive 2G7 were tumor free. However, soon after Ab administration was stopped, three of the tumors that had regressed in response to Lm-E7/2G7 treatment grew out (Fig. 10). These data suggest that TGF-β plays a major role in the suppression of the Lm-E7-mediated, anti-TC-1 response.

Transfer of $CD4^+$ cells from Lm-E7-immunized mice to Lm-LLO-E7-treated mice abrogates the anti-TC-1 response

We hypothesized that the suppressive anti-TC-1 response induced by Lm-E7 treatment could be recapitulated in Lm-LLO-E7-treated mice by cell transfer. To address this hypothesis we immunized

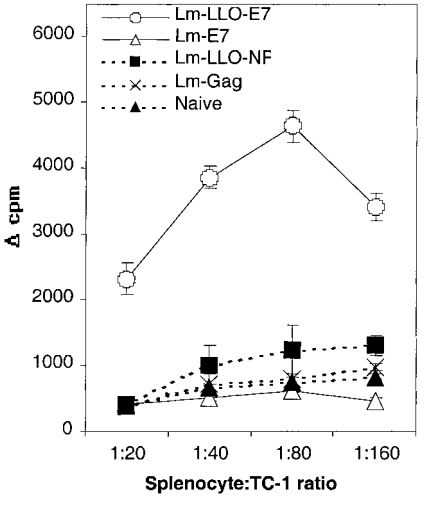

FIGURE 8. Splenocytes from Lm-LLO-E7-immunized mice proliferate when exposed to TC-1 cells. C57BL/6 mice were immunized and boosted with Lm-LLO-E7, Lm-E7, or control rLm strains. Splenocytes were harvested 6 days after the boost and plated with irradiated TC-1 cells at the ratios shown. The cells were pulsed with $[^3H]$thymidine and harvested. Δ cpm is defined as (experimental cpm) $-$ (no-TC-1 control). Depicted is one of two identical experiments.

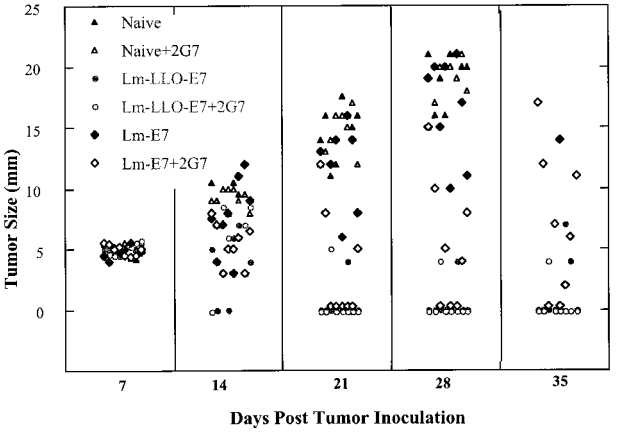

FIGURE 10. TGF-β depletion improves Lm-E7 therapeutic efficacy. Following the s.c. injection of 2×10^5 TC-1 cells, each mouse was given 0.5 mg of anti-TGF-β mAb (2G7; open symbols) on days 6, 7, 8 10, 12, 14, 16, 18, and 20. Control mice were not treated with Ab (closed symbols). The C57BL/6 mice (eight per group) were treated with 0.1 LD_{50} Lm-E7 (diamonds) or Lm-LLO-E7 (circles) on day 7 (diamonds) or were left untreated (triangles). Mice were sacrificed when tumor diameter reached approximately 2.0 cm. Tumor measurements for each time point are shown only for surviving mice. Depicted is one of two similar experiments.

and boosted donor C57BL/6 mice with Lm-E7 or the control, Lm-Gag. Splenocytes were harvested from the donor mice and enriched for $CD4^+$ T cells. These $CD4^+$-enriched splenocytes were transferred (10^7 cells/recipient mouse) to TC-1-bearing mice that had been treated with Lm-LLO-E7. While transfer of $CD4^+$ splenocytes from Lm-Gag-immunized donor mice had no effect on Lm-LLO-E7 immunotherapy, $CD4^+$ cells from Lm-E7-immunized donors largely abrogated the anti-TC-1 immune response, with two of eight mice tumor free compared with six of eight tumor free in the other Lm-LLO-E7-treated groups (Fig. 11). These results demonstrate that Lm-E7 not only fails to induce a potent Th cell response, but instead induces a $CD4^+$-suppressive population capable of disrupting the anti-TC-1 immune response induced by Lm-LLO-E7.

Discussion

Lm-E7 and Lm-LLO-E7 both secrete the HPV-16 E7 protein. However, they differ in several potentially important aspects. They are constructed from different parental strains, Lm-E7 from wild-type 10403S and Lm-LLO-E7 from the 10403S-derived, *prfA*$^-$ strain, XFL-7. *PrfA* is a pluripotential transcription factor that regulates the expression of the majority of the *L. monocytogenes* virulence genes, including *hly*, the promoter that drives the expression of LLO-E7 and E7. Loss of the plasmid pGG-55 would render Lm-LLO-E7 avirulent in vivo. Since each copy of pGG-55 in Lm-LLO-E7 will contain the *prfA* gene, the increased expression of this transcription factor could potentially increase the expression of the LLO-E7 fusion by Lm-LLO-E7 compared with Lm-E7. We have shown (Fig. 2) that Lm-LLO-E7 does not secrete more E7 than Lm-E7 when cultured in vitro. However, since the expression of many of the *PrfA*-regulated *L. monocytogenes* virulence factors is up-regulated in vivo, it is possible that Lm-LLO-E7 expresses more of the LLO-E7 fusion protein in vivo than does Lm-E7. It is also conceivable that multiple copies of *prfA* could increase the virulence of Lm-LLO-E7, thereby providing a higher Ag load and longer Ag exposure to responding T cells. We have evidence that this is not the case, since Lm-LLO-E7 has an approximately 10-fold higher LD_{50} than Lm-E7. Therefore, neither expression level of E7 nor the virulence increase resulting from multiple copies of *prfA* appears to explain the difference in anti-tumor immune responses provoked by the two recombinant bacteria.

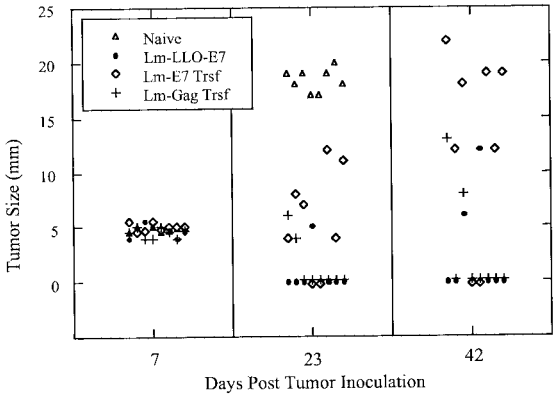

FIGURE 11. The transfer of $CD4^+$ cells from Lm-E7-immunized mice abrogates the effect of Lm-LLO-E7 immunotherapy. C57BL/6 mice (eight per group) received 2×10^5 TC-1 cells on the left flank. Mice were treated with 0.1 LD_{50} Lm-LLO-E7 (circles, diamonds, and crosses) on day 7 or were left untreated (triangles). $CD4^+$-enriched splenocytes (10^7/mouse) from mice immunized and boosted 1 wk later with 0.1 LD_{50} Lm-E7 (diamonds) or Lm-Gag (crosses) were transferred on day 9 to Lm-LLO-E7-treated, tumor-bearing mice. Mice were sacrificed when tumor diameter reached approximately 2.0 cm. Tumor measurements for each time point are shown only for surviving mice. Depicted is one of two similar experiments.

Lm-LLO-E7 and Lm-E7 express and secrete E7 in different forms. Lm-LLO-E7 expresses a large fusion protein made of a nonhemolytic LLO joined at its C-terminus to E7. In contrast, Lm-E7 expresses E7 preceded only by the LLO signal sequence and the first several amino acids of LLO. The listerial Ag, LLO, is very efficiently processed and presented to the immune system via MHC class I (34). LLO has been shown to be the dominant target of $CD8^+$ T cells in mice immunized with wild-type *L. monocytogenes* (35). Work with another secreted listerial protein, p60, shows that manipulation of the N-terminus amino acid can drastically effect the processing and presentation of this secreted Ag, an example of the N-end rule (36). However, we designed Lm-E7 to ensure the efficient processing of the LLO signal sequence by including the first several amino acids of LLO after the signal sequence and preceding E7. Therefore, since both constructs encode the same N-terminus for the recombinant E7 protein, the N-end rule does not explain the vast difference in anti-tumor immunity induced by the two strains. Since the hemolytic domain of LLO was deleted from the LLO-E7 fusion protein, it is also not the case that the LLO portion of the LLO-E7 fusion protein alters the location or distribution of the protein by virtue of the hemolytic activity of LLO. It is, however, possible that a domain present in the LLO-E7 fusion protein that is absent in the E7 protein may influence the processing and presentation of LLO-E7 in such a way that it induces a superior anti-tumor response.

The experiments we have described in this paper demonstrate that $CD8^+$ T cell responses to the immunodominant peptide RA HYNIVTF are induced by both Lm-E7 and Lm-LLO-E7 to similar levels. However, it is possible that the form of the Ag may be influencing the emergence of subdominant epitopes in the animals immunized by the fusion protein. We do not believe that this is the case because the level of CTL activity in the spleen of mice immunized by Lm-LLO-E7, as measured by ^{51}Cr release assays (see Figs. 3–5), is identical for RAHYNIVTF-pulsed targets and for tumors expressing E7 as an endogenous Ag. However, such assays are only crude indicators of the precursor frequency of epitope-specific CTL. Therefore, we have not ruled out that Lm-LLO-E7 allows the emergence of cryptic epitopes within the E7 sequence.

Lm-LLO-E7 and Lm-E7 induce greatly different TC-1-specific proliferative responses. The proliferation assay shown in Fig. 5 demonstrates a very considerable difference in this functional quality of the Lm-LLO-E7- and Lm-E7-induced responses. The proliferative response of Lm-LLO-E7-immunized splenocytes in response to irradiated TC-1 cells demonstrates that the E7 response induced by Lm-LLO-E7 can be recalled by endogenous levels of E7. Proliferation in response to an exogenous source of Ag is a conventional measure of Ag-specific T cell help. Since Lm-E7 fails to elicit a TC-1-specific proliferative response, this suggests poor induction of an E7-specific, $CD4^+$ T cell response by Lm-E7.

It is also possible that the inclusion of the LLO fusion and/or the lesser virulence of Lm-LLO-E7 effectively promotes a potent $CD4^+$, E7-specific T cell response. The majority of phagocytosed *L. monocytogenes* are killed and degraded in the phagosomal-lysosomal compartment (37). Peptides generated by phagocytosis and degradation in the phagosomal-lysosomal compartment can be efficiently presented by MHC class II molecules to $CD4^+$ T cells. The *hly* gene (that encodes for the LLO hemolysin) is up-regulated in the phagosome (38). Since they use the same promoter, the LLO-E7 fusion protein and E7 are also probably up-regulated, while Lm-LLO-E7 and Lm-E7 are in the phagosome. Nevertheless, it also may be that by including LLO in a fusion to E7 we

have altered the processing of E7 in the phagosomal compartment compared with the E7 secreted by Lm-E7. The fact that Lm-LLO-E7 is less virulent than Lm-E7 and can be given in higher quantities may also influence the $CD4^+$ T cell response, since the initial Ag load is approximately 10-fold larger than that for Lm-E7. Therefore, with the combination of a higher early Ag load and more efficient processing and presentation, Lm-LLO-E7 may induce a potent $CD4^+$ T cell response, whereas Lm-E7 does not.

It should be noted that the lack of E7-specific Th cell responses in mice immunized with Lm-E7 did not impact on the ability of this vaccine to induce conventional E7-specific CTL responses (Fig. 4). This is not surprising, since Th1 responses are undoubtedly provided in abundance by responses to listerial Ags expressed by the vector. However, E7-specific $CD4^+$ effector cells clearly play a very important role in competent anti-tumor immunity, as was demonstrated by the depletion experiments shown in Fig. 8. Thus, the efficacy of rLm vaccines engineered to express E7 appears to correlate with the type of $CD4^+$ T cell immunity induced by these vectors.

Finally, the role of $CD4^+$ T cells in the regression of TC-1 seems to be complex. Depletion of $CD4^+$ cells in TC-1-bearing mice treated with Lm-LLO-E7 predictably diminishes the ability of these mice to reject their tumors. We have shown similar results in the influenza NP model, where depletion of $CD4^+$ cells weakened, but did not completely abrogate, the anti-tumor response (11). It is likely that these $CD4^+$ T cells supply some of the IFN-γ that is necessary for tumor regression (39). However, depletion of $CD4^+$ T cells in mice treated with Lm-E7 improved the anti-TC-1 response (Fig. 8). This suggests that the $CD4^+$ T cell response induced by Lm-E7 treatment is detrimental to the overall anti-tumor response. The adoptive transfer data (Fig. 11) suggest that Lm-E7 elicits $CD4^+$ T cells that actively respond with a suppressive phenotype and that the deletion of the $CD4^+$ compartment removes this suppression. The lack of an in vitro proliferative response from splenocytes isolated from Lm-E7-treated mice supports the suppression hypothesis, since $CD4^+$, $CD25^+$-suppressive T cells, as their designation suggests, fail to elicit proliferative responses to Ag-specific stimulation (29). We and others have also shown that depletion of $CD25^+$ cells improves tumor-specific immune responses (30) (Figs. 3–11). The $CD4^+$ T cell suppression is probably mediated at least in part by the immune suppressive action of TGF-β (Fig. 10). Studies are currently underway to determine the exact mechanism of this suppression.

Our results demonstrate that Lm-LLO-E7 is capable of inducing a potent anti-tumor response that targets the HPV E7 protein. As this protein is present in HPV-16-infected cells and in most cervical tumors, Lm-LLO-E7 may prove to be an effective immunotherapeutic in humans. We have also shown that CTL induction alone is a poor indicator for effectiveness of an anti-tumor vaccine. Ag-specific inhibition of tumor growth may be a better indicator of potential clinical effectiveness. It has been noted that there are no reliable surrogate immune markers for anti-tumor efficacy (40). A positive correlation between an in vitro measurable immune parameter and in vivo tumor regression would provide a powerful catalyst to rationally designed immunotherapy.

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