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Recombinant *Listeria* Vaccines Containing PEST Sequences Are Potent Immune Adjuvants for the Tumor-Associated Antigen Human Papillomavirus-16 E7

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Abstract

Previous work in our laboratory has established that the fusion of tumor-associated antigens to a truncated form of the *Listeria monocytogenes* virulence factor listeriolysin O (LLO) enhances the immunogenicity and antitumor efficacy of the tumor antigen when delivered by *Listeria* or by vaccinia. LLO contains a PEST sequence at the NH_2 terminus. These sequences, which are found in eukaryotic proteins with a short cellular half-life, target proteins for degradation in the ubiquitin-proteosome pathway. To investigate whether the enhanced immunogenicity conferred by LLO is due to the PEST sequence, we constructed new *Listeria* recombinants that expressed the HPV-16 E7 antigen fused to LLO, which either contained or had been deleted of this sequence. We then compared the antitumor efficacy of this set of vectors and found that *Listeria* expressing the fusion protein LLO-E7 or PEST-E7 were effective at regressing established macroscopic HPV-16 immortalized tumors in syngeneic mice. In contrast, *Listeria* recombinants expressing E7 alone or E7 fused to LLO from which the PEST sequence had been genetically removed could only slow tumor growth. Because $CD8^+$ T cell epitopes are generated in the ubiquitin-proteosome pathway, we also investigated the ability of the vaccines to induce E7-specific $CD8^+$ T cells in the spleen and to generate E7-specific tumor-infiltrating lymphocytes. A strong correlation was observed between $CD8^+$ T-cell induction and tumor homing and the antitumor efficacy of the *Listeria*-E7 vaccines. These findings suggest a strategy for the augmentation of tumor antigen-based immunotherapeutic strategies that may be broadly applicable.

Introduction

Listeria monocytogenes is an intracellular pathogen that induces potent cellular immune responses because of its unusual life cycle (1). Upon phagocytosis by an antigen-presenting cell, the bacteria are able to escape into the cytoplasm of the cell by perforating the phagolysosome. Once in the cytoplasm, bacterial proteins are effectively presented to cytotoxic T lymphocytes, thus initiating a cellular immune response against bacterial antigens. By engineering recombinant *Listeria* vaccines to secrete not only bacterial antigens but also tumor antigens, immune responses have been generated sufficient to cause the regression of established tumors in animal models (1–5).

In a previous study, two *Listeria* strains were engineered to secrete a tumor antigen associated with human papilloma virus (HPV) (4). The most effective of the two vaccines secreted the tumor antigen as a fusion with another protein, listeriolysin O (LLO). LLO is a hemolytic protein encoded by the *hly* gene that leads to perforation of the phagolysosome (6). However, the form of LLO included in the fusion was a truncated, nonhemolytic form, so the reason for enhanced efficacy was not due to increased virulence of the recombinant strain.

A possible reason for the enhanced efficacy may be the presence of a 19-amino acid sequence within LLO called a PEST sequence. PEST regions (P, proline; E, glutamic acid; S, serine; T, threonine) are hydrophilic amino acid sequences that reside near the NH_2 or COOH termini of certain enzymes. They are thought to target proteins for rapid degradation by the cellular proteasome. It has been shown that the PEST region of LLO is vital for the survival of *Listeria* in the host because it causes the rapid degradation of LLO itself before it damages the critical host cell (7). The presence of the PEST region in our vaccine may enhance its efficacy by causing rapid degradation and presentation of the tumor antigen by antigen-presenting cells.

It is our hypothesis that the PEST region of LLO is crucial to the success of our recombinant HPV vaccines. To test this, three new vaccines were designed that express the HPV tumor antigen in different combinations: E7 with the PEST region, E7 without the PEST region but with the rest of LLO, or E7 alone. Fig. 1 depicts the fusion proteins expressed and secreted by each of these three strains compared with Lm-LLO-E7. Our results indicate that the inclusion of the PEST region has dramatic effects on the efficacy of our vaccines. This result has broad implications for the augmentation of live and protein antigen-based immunotherapeutic strategies.

Materials and Methods

Mice. Six- to 8-week-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Animals were cared for and used in accordance with protocols approved by the Animal Care and Use Committee of The University of Pennsylvania (Philadelphia, PA).

Cell Line. The TC-1 cell line, a generous gift from Dr. T. C. Wu (Johns Hopkins University School of Medicine, Baltimore, MD) is a lung epithelial cell immortalized with HPV-16 E6 and E7 and transformed with the c-*Ha-ras* oncogene (8). TC-1 was grown in RPMI 1640 supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 50 μmol/L 2-ME, 400 μg/mL G418, and 10% National Collection Type Culture-109 medium at 37°C with 10% CO_2.

***L. monocytogenes* Strains and Propagation.** The *Listeria* strain Lm-LLO-E7 has been described in detail previously (4). In short, it is a recombinant bacterial strain that secretes E7 as a fusion protein joined to a nonhemolytic LLO via an episomal expression system. The fusion gene contains the *hly* promoter, the *hly* gene encoding the first 441 amino acids of LLO (including the signal sequence and the PEST region), the *E7* gene, and the *Listeria* pluripotential transcription factor *prfA*. Only the first 441 amino acids of LLO are included so that the COOH terminus hemolytic domain is deleted.

Lm-PEST-E7 is a *Listeria* strain identical to Lm-LLO-E7 except that it contains only the promoter and PEST sequence of the *hly* gene. This includes the first 50 amino acids of LLO. It was constructed as follows:

First, the *hly* promoter and PEST regions were fused to the full-length *E7*

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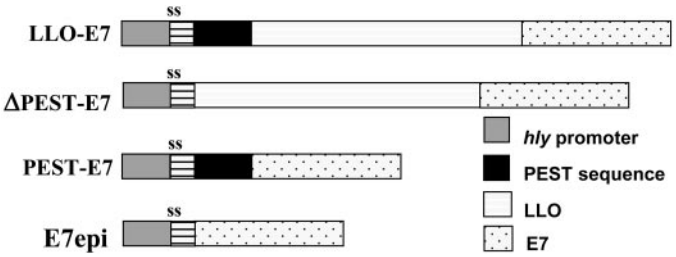

Fig. 1. A schematic representation of the plasmid inserts used to create four *L. monocytogenes* vaccines. As shown, the Lm-LLO-E7 insert contains all of the *Listeria* genes used to optimize our vaccines. It contains the *hly* promoter, the first 1.3 kb of the *hly* gene (which encodes the protein LLO), and the HPV-16 E7 gene. The first 1.3 kb of *hly* includes the signal sequence (*ss*) and the PEST region as shown. Lm-PEST-E7 includes the *hly* promoter, the signal sequence, and PEST and E7 sequences but excludes the remainder of the truncated LLO gene. Lm-ΔPEST-E7 excludes the PEST region, but contains the *hly* promoter, the signal sequence, E7, and the remainder of the truncated LLO. Lm-$E7_{epi}$ has only the *hly* promoter, the signal sequence, and E7.

gene. This was accomplished with SOEing PCR techniques (gene splicing by overlap extension). The *E7* gene and the *hly*-PEST gene fragment were amplified from the plasmid pGG-55, which contains the first 441 amino acids of LLO, and spliced together by conventional PCR techniques. To create a final plasmid, pVS16.5, the *hly*-PEST-E7 fragment and the *L. monocytogenes* transcription factor *prfA* were cloned into the plasmid pAM401, which includes a chloramphenicol resistance gene for selection *in vitro* (9). The *prfA* gene fragment was generated as described previously (4). After ligation, the resultant plasmid was then used to transform XFL-7, a *prfA*-negative strain of *Listeria* (a gift from Dr. Jeffery Miller, University of California, Los Angeles, CA), so that bacteria that retain the plasmid are selected *in vivo*. This recombinant *Listeria* strain was termed Lm-PEST-E7.

Lm-ΔPEST-E7 is a recombinant *Listeria* strain that is identical to Lm-LLO-E7 except that it lacks the PEST sequence. It was made essentially as described for Lm-PEST-E7 except that the episomal expression system was constructed using primers designed to remove the PEST-containing region (bp 333–387) from the *hly*-E7 fusion gene.

Lm-$E7_{epi}$ is a recombinant strain that secretes E7 without the PEST region or LLO. The plasmid used to transform this strain contains a gene fragment of the *hly* promoter and signal sequence fused to the E7 gene. This construct differs from the original Lm-E7, which expressed a single copy of the E7 gene integrated into the chromosome (4). Lm-$E7_{epi}$ is completely isogenic to Lm-LLO-E7, Lm-PEST-E7, and Lm-ΔPEST-E7 except for the form of the E7 antigen expressed.

All recombinant strains were grown in brain heart infusion medium with chloramphenicol (20 μg/mL). Bacteria were frozen in aliquots at −80°C.

Western Blotting. *Listeria* strains were grown in Luri Bertoni medium at 37°C and were harvested at the same OD measured at 600 nm. The cells were pelleted, and the supernatants were trichloroacetic acid precipitated and resuspended in 0.1 N NaOH + 2% SDS. The samples were diluted 1:200, and the OD_{280} was measured. Identical amounts of total resuspended protein as calculated from the OD_{280} readings were mixed with NuPAGE 4× sample buffer and separated on 4 to 20% Tris-glycine SDS PAGE gels (Invitrogen, Carlsbad, CA). The gels were transferred to a polyvinylidene difluoride membrane using a Bio-Rad semi-dry transfer cell (Bio-Rad Laboratories, Hercules, CA) and probed with an anti-E7 monoclonal antibody (Zymed, San Diego, CA) as recommended by the manufacturer. Horseradish peroxidase–conjugated anti-mouse antibody (Amersham Biosciences, Piscataway, NJ) was used for detection of bound anti-E7 antibody. Blots were developed with SuperSignal WestDura chemiluminescent substrate (Pierce, Rockford, IL) and visualized using a CCD camera.

Tumor Regression Studies. Six- to 8-week-old C57BL/6 mice (Charles River) received 2×10^5 TC-1 cells subcutaneously on the left flank. One week after tumor inoculation, the tumors had reached a palpable size, 4 to 5 mm in diameter. Groups of eight mice were then treated with 0.1 LD_{50} intraperitoneal Lm-LLO-E7, Lm- $E7_{epi}$, Lm-PEST-E7, and Lm-ΔPEST-E7 on days 7 and 14, or the mice were left untreated. The longest and shortest surface diameters of the tumors were measured with calipers. 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 in compliance with our Institutional Animal Care and Use Committee protocols. Tumor measurements for each time point are shown only for surviving mice. For comparison of tumor diameters, the means and *SD*s were calculated for each treatment group at day 28, and statistical significance was calculated using the Student's *t* test. $P < 0.05$ was considered significant.

Antigen-Specific $CD8^+$ T-Cell Analyses in Tumors and Spleens of Vaccinated Mice. C57BL/6 mice received 2×10^5 TC-1 cells each mixed with basement membrane matrix (Matrigel; Collaborative Biomedical Products, Bedford, MA). The cells were suspended in 100 μL of PBS then mixed with 400 μL of Matrigel. This solution was kept on ice until subcutaneous administration in the left flank was performed. Seven days later, the mice were immunized with 0.1 LD_{50} of the various vaccines and boosted with the same dose 7 days later. Tumors and spleens were then harvested 7 days later. Tumors were digested with collagenase P (2 mg/mL; Roche Applied Science, Indianapolis, IN) and DNase (1 mg/mL; Sigma Chemical Co., St. Louis, MO) for 1 hour at 37°C. Cells were isolated from the tumor digests using a 100-μm cell strainer (BD Biosciences PharMingen, San Diego, CA). Three-color flow cytometry for CD8β.2 (fluorescein isothiocyanate–conjugated antibody; BD Biosciences PharMingen), CD62L (APC-conjugated antibody, BD Biosciences PharMingen), and the tetramer of the H-$2D^b$-restricted immunodominant E-7 epitope in the C57BL/6 mouse (RAHYNIVTF) was performed using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences PharMingen). The E7/D^b tetramer was made by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility at Emory University through the NIH AIDS Research and Reference Reagent Program. Cells were analyzed by comparing tetramer$^+$, $CD8^+$, and $CD62L^{low}$ cells within the spleen and the tumor generated by the four vaccines.

Results

Construction of *L. monocytogenes* Strains with and without the PEST Region of Listeriolysin O. We have made three new *L. monocytogenes* strains to test the hypothesis that the PEST amino acid sequence is critical for the efficacy of our *Listeria*-based vaccines. The PEST-like sequence of LLO is 19 amino acids long and resides near the NH_2 terminus (7). The new constructs are based on the previously described recombinant *Listeria* strain Lm-LLO-E7 (4), which secretes the HPV-16 protein E7 as a fusion protein with the *Listeria* virulence factor LLO. Similar to Lm-LLO-E7, the new constructs are based on an episomal expression system, but the E7 gene is now linked to different, smaller portions of *hly*, the gene which encodes LLO. The episomal expression system relies on chloramphenicol resistance for *in vitro* selection and the expression of *prfA* for *in vivo* selection (4). The gene insert used to create the Lm-PEST-E7 vaccine contains E7 fused to only the PEST region and the signal sequence of LLO. Lm-ΔPEST-E7 contains E7 linked to most of the LLO gene except the PEST sequence. Lm-$E7_{epi}$ contains the E7 gene fused only to the signal sequence of LLO (Fig. 1). All of these recombinant strains retain the hemolytic activity conferred by the chromosomal copy of *hly*, which has not been altered.

Secretion of the fusion proteins was confirmed by Western blot. Although the strains are isogenic, except for the size and/or sequence of the *hly* fusion gene, the fusion protein was expressed at different levels. The highest level was shown by Lm-ΔPEST-E7, followed by Lm-LLO-E7 and Lm-PEST-E7, and the lowest level was expressed by Lm-E7.

Vaccines Containing PEST Sequences Cause Effective Tumor Regression. Lm-LLO-E7, Lm-PEST-E7, Lm-ΔPEST-E7, and Lm-$E7_{epi}$ were compared for their ability to cause regression of tumors that express HPV-16 E7 protein. Subcutaneous tumors were established on the left flank of 40 C57BL/6 mice with the cell line TC-1. TC-1 is immortalized with HPV-16 E6 and E7 (8). After tumors had reached a palpable size of 4 to 5 mm, mice were divided into five groups of eight mice. Each of the groups was treated with one of four recombinant *L. monocytogenes* vaccines, and one group of mice was left untreated. The previously described PEST-containing vaccine,

Lm-LLO-E7, successfully caused the regression of established tumors in five of eight cases. Administration of Lm-PEST-E7 also led to regression of established tumors in three of eight cases. There was no statistical difference between the average tumor size of mice treated with Lm-PEST-E7 or Lm-LLO-E7 at any time point. However, the vaccines that expressed E7 without the PEST sequences, Lm-ΔPEST-E7 and Lm-$E7_{epi}$, failed to cause tumor regression in all mice except one (Fig. 2*A*). This was representative of two experiments. For statistical analysis, the mean tumor sizes at day 28 were compared for the two experiments. A statistically significant difference in tumor sizes was seen between those tumors treated with PEST constructs (Lm-LLO-E7 or Lm-PEST-E7) and those treated without PEST regions (Lm-$E7_{epi}$ or Lm-ΔPEST-E7; $P = 0.001$, Student's t test; Fig. 2*B*).

Vaccines Containing PEST Sequences Cause Increased Percentages of Antigen-Specific $CD8^+$ Lymphocytes within the Spleen. The vaccines were administered to tumor-bearing mice to compare the levels of E7-specific lymphocytes generated by the vaccine in the spleen. Mice were treated on days 7 and 14 with 0.1 LD_{50} of the four vaccines. Spleens were harvested on day 21 and stained with antibodies to CD62L, CD8, and the E7/D^b tetramer. Lm-$E7_{epi}$ and Lm-ΔPEST-E7 induced similar low levels of E7 tetramer–positive activated $CD8^+$ T cells in the spleen. Lm-PEST-E7 induced approximately five times more and Lm-LLO-E7 induced approximately 15 times more (Fig. 3*A*). Thus, increased percentages of tetramer-positive splenocytes were seen in mice vaccinated with PEST-containing vaccines. This result was reproducible over three experiments performed on different occasions. The mean and SE for data obtained from the three experiments are depicted in Fig. 3*B*. The average values for the number of tetramer-positive $CD8^+$ cells induced was statistically higher for Lm-LLO-E7 and Lm-PEST-E7 than for Lm-Δ-PEST-E7 and Lm-$E7_{epi}$ ($P < 0.05$) by Student's t test.

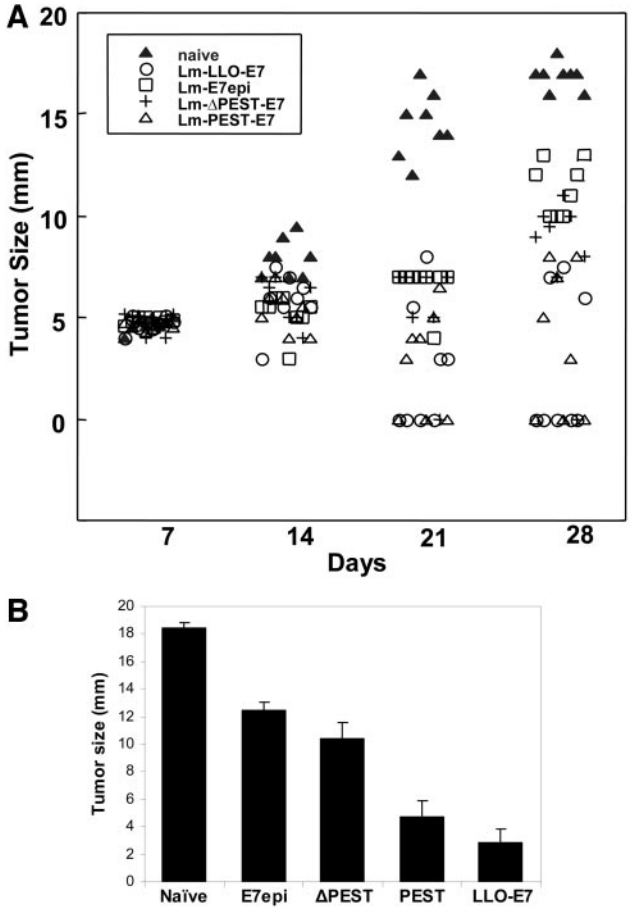

Fig. 2. *A*. *Listeria* constructs containing PEST regions lead to greater tumor regression. C57BL/6 mice (eight per group) received 1×10^5 TC-1 cells by subcutaneous injection in the left flank. Mice were treated on days 7 and 14 after tumor challenge with 0.1 LD_{50} Lm-LLO-E7, Lm-PEST-E7, Lm-ΔPEST-E7, or Lm-$E7_{epi}$, or they were left untreated. The average tumor diameter is shown for each mouse. Tumor measurements for each time point are shown only for surviving mice; mice were sacrificed if their tumors reached 20 mm. On day 28, five of eight mice are tumor-free in the Lm-LLO-E7 group, and three of eight mice are tumor-free in the Lm-PEST-E7 group. These data are representative of two similar experiments. *B*, average tumor size in mice treated with *Listeria* vaccines. This graph depicts the average tumor sizes at day 28 post tumor challenge. There is a statistically significant difference between the Lm-PEST-E7 group and the Lm -ΔPEST-E7 group ($P = 0.004$, Student's t test). Depicted is the average of two experiments.

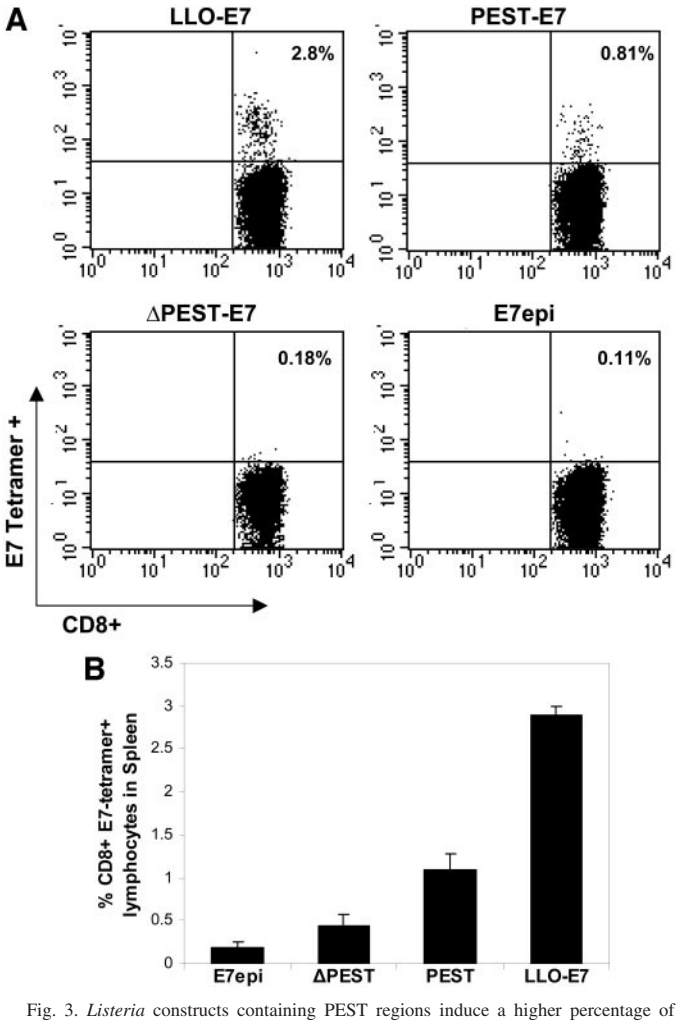

Fig. 3. *Listeria* constructs containing PEST regions induce a higher percentage of E7-specific lymphocytes in the spleen. Spleens were harvested from two or three mice 14 days after the first vaccination and 7 days after a booster vaccination. Pooled splenocytes were stained with anti-CD8 and anti-CD62L antibody and with E7/D^b tetramers. *A*, data from one experiment. These data are representative of three similar experiments. *B*, average and SE of data from all 3 experiments.

Vaccines Containing PEST Sequences Cause Increased Percentages of Antigen-Specific Tumor-Infiltrating Lymphocytes. We have previously observed that there is a better correlation between antitumor efficacy and the number of tumor-infiltrating antigen-specific $CD8^+$ T cells than with the numbers induced in peripheral lymphoid organs (10). Therefore, the vaccines were administered to tumor-bearing mice to compare the levels of E7-specific lymphocytes generated by the vaccine within the tumor. Mice were treated on days 7 and 14 with 0.1 LD_{50} of the four vaccines. Tumors were harvested on day 21 and stained with antibodies to CD62L, CD8, and with the E7/D^b tetramer. An increased percentage of tetramer-positive lym-

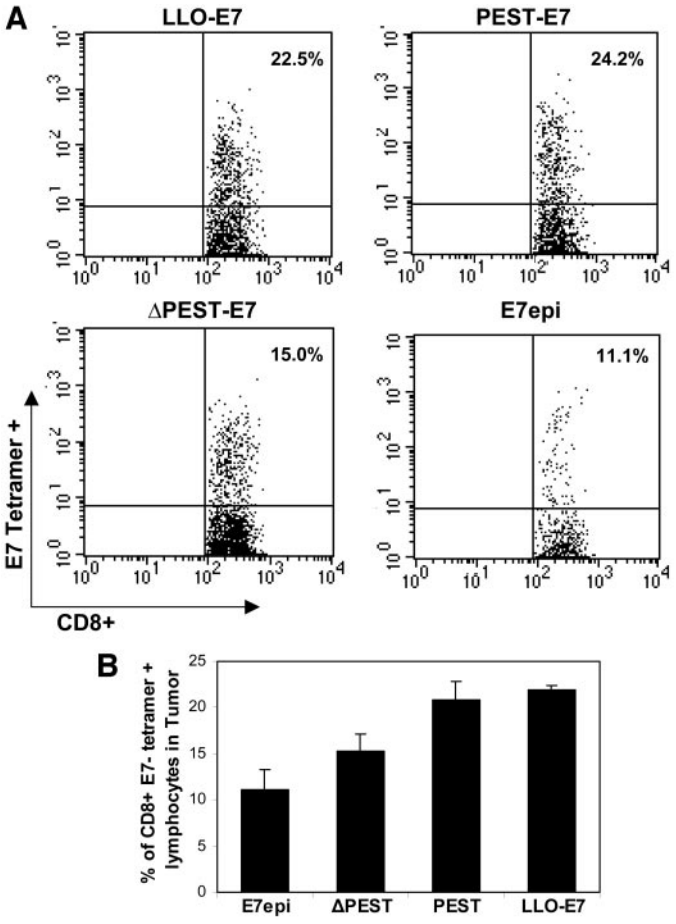

Fig. 4. *Listeria* constructs containing PEST regions induce a higher percentage of E7-specific lymphocytes within the tumor. Tumors were harvested from two or three mice 14 days after the first vaccination and 7 days after the boost. Pooled tumor cells were stained with anti-CD8 and anti-CD62L antibody and with E7/D^b tetramers. *A*, representative data from one experiment vaccinated with the various constructs. These data are representative of three similar experiments. *B*, average and SE of data from all three experiments.

phocytes within the tumor were seen in mice vaccinated with PEST-containing vaccines Lm-LLO-E7 and Lm-PEST-E7 (Fig. 4*A*). This result was reproducible over three experiments. The mean and SE for the three experiments are depicted in Fig. 4*B*. The average values for the number of tetramer-positive $CD8^+$ TILs induced was statistically higher for Lm-LLO-E7 than for Lm-Δ-PEST-E7 and Lm-$E7_{epi}$ ($P < 0.05$) by Student's *t* test. The difference between Lm-PEST-E7 and either Lm-Δ-PEST-E7 or Lm-$E7_{epi}$ did not achieve statistical significance between the three experiments ($P < 0.1$), but the same trend was observed in each experiment. It is of interest that even though the number of E7-specific $CD8^+$ T cells actually induced in the spleen was lower in mice immunized with Lm-PEST-E7 than in mice that received Lm-LLO-E7, very similar numbers of E7-specific $CD8^+$ TILS were seen in mice that received either vaccine (about 22%).

Discussion

An important goal of tumor immunotherapeutic strategies is the optimal presentation of tumor antigens so that a rapid and potent immune response against the tumor is achieved. Recombinant *L. monocytogenes* vaccines that express tumor antigens have been shown to lead to the generation of tumor-specific T-lymphocytes and tumor regression in mouse models (1–5). In the present study, we have determined that the fusion of the antigen to a PEST sequence is critical for the efficacy of these vaccines. PEST regions are thought to target proteins for rapid degradation (7, 11–17). A study that compared the amino acid sequences of 10 proteins with short intracellular half-lives found regions rich in proline, glutamic acid, serine, and threonine in all 10 proteins (11). Subsequent studies have suggested that PEST regions are recognized and bound by components of the ubiquitin proteolytic pathway (18) and that the 26S proteasome is involved in the rapid degradation of PEST proteins (14).

Our results indicate that the fusion of a PEST sequence to the tumor antigen E7 is essential for the efficacy of the vaccine. The two constructs that express the PEST sequence within the fusion protein generated more E7-specific lymphocytes and cured more mice of their tumors than those constructs that do not contain the PEST region. It is unlikely that this difference is due to different levels of antigen expression by the four constructs, because expression of the fusion protein *in vitro* was highest for Lm-Δ-PEST-E7. Instead, we propose that the PEST region facilitates protein degradation and enhances antigen presentation to T-lymphocytes. These results have important implications for cancer vaccine design in general. Many tumor immunotherapeutic strategies currently under investigation use proteins to generate immune responses to tumor antigens. Often this strategy does not generate sufficient immune responses without an adjuvant such as CpG DNA or bacterial cell wall components (19, 20). Addition of a PEST region may improve not only these strategies, but also other live recombinant strategies such as vaccinia-based vaccination. Indeed, we have previously shown that the LLO-E7 fusion protein shows enhanced immunogenicity and antitumor efficacy compared with other forms of the E7 antigen when delivered by recombinant vaccinia virus (10).

Additional studies to examine this phenomenon should be considered, including *in vitro* degradation assays of translated fusion proteins and immunofluorescence studies to localize the proteins within an infected cell. It will also be important to determine whether the effect is due to more rapid degradation of the PEST fusion constructs, as opposed to an increase in the total amount of protein degraded by the proteasome.

Within our model, antigen presentation also occurs in the absence of PEST, because Lm-ΔPEST-E7 also caused some tumor regression and generation of E7-specific lymphocytes. Furthermore, there was a slight difference in efficacy between Lm-LLO-E7 and Lm-PEST-E7 and between Lm-ΔPEST-E7 and Lm-$E7_{epi}$. In both cases, the vaccine that contained LLO sequences was superior. The question of whether the presence of other LLO sequences also promotes antigen presentation is unknown. One hypothesis is that the size of the fusion protein plays a role in the ability to process it. In this case, the larger protein was slightly more effective regardless of the presence or absence of PEST. There are also other peptide motifs known to target proteins for rapid degradation, including KFERQ motifs of Rnase A (21) and the cyclin destruction box from cyclin B (22), among others (23). Studies to determine whether there is a critical portion of LLO besides PEST are warranted.

In summary, our results indicate that the PEST sequence of amino acids enhances the efficacy of E7-specific *L. monocytogenes* vaccines. This result has broad implications for the augmentation of live and protein antigen-based immunotherapeutic strategies.

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