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The Ability of Two Listeria monocytogenes Vaccines Targeting Human Papillomavirus-16 E7 to Induce an Antitumor Response Correlates with Myeloid Dendritic Cell Function

Xiaohui Peng, S. Farzana Hussain, and Yvonne Paterson

Previous work from our laboratory has shown that Lm-LLO-E7 induces complete regression of ~75% of established TC-1 tumors, whereas Lm-E7 only slows the growth of such tumors. In this study, we examine the effects of Lm-LLO-E7 vs Lm-E7 on APCs. We hypothesize that the difference in antitumor efficacy of the two vaccines is due to the ability of each of these vectors to render immature dendritic cells (DCs) effective APCs in terms of MHC class II or costimulatory molecule expression. We also examine the ability of these vectors to stimulate cytokine production by DCs. Both vectors induced IL-12 and TNF-α, but only Lm-LLO-E7 induced IL-2 production by DCs. Lm-LLO-E7 also induced significantly higher levels of MHC class II molecules, CD40, and B7 costimulatory molecules (CD86, B7-H1, and B7-DC) on DCs than Lm-E7. Interestingly, a shift of CD11c+ cells from CD86low to CD86high is observed post-Lm-LLO-E7 infection. A similar shift is also observed for B7-H1 and B7-DC molecules. Moreover, Lm-LLO-E7, but not Lm-E7-pulsed DCs, stimulate naive T cell proliferation. These results indicate that Lm-LLO-E7 is more effective than Lm-E7 at inducing DC maturation. This effect is independent of the E7 Ag, because Lm-LLO-NP, and a mixture of Lm-LLO-NP and Lm-E7 induce the same changes in DC phenotype as Lm-LLO-E7. Taken together, the changes in DC expression correlate well with the differences in antitumor efficacy between these two vaccines.


Human papillomavirus (HPV) is a double-stranded virus that infects skin and mucosal epithelial tissues. Currently, over 100 genetically different HPV types have been isolated from humans. More than 90% of cervical cancers are associated with the HPV-16 genotype (1–3). HPV-16 early gene products, E6 and E7, are sufficient to immortalize human and rodent cells (4, 5). These viral proteins are constitutively expressed by HPV-associated cervical cancer (6) and are thus ideal target Ags for cancer immunotherapy.

Listeria monocytogenes, a Gram-positive, facultative intracellular bacterium, is taken up by phagocytosis into APCs. It has evolved to escape the phagocytic compartment and enter the host cell cytosol by secreting a membrane-active virulence factor, listeriolysin O (LLO) (7). It is known that L. monocytogenes serves as a vaccine vector to deliver a foreign Ag to the cellular immune system (8–10). We have developed two L. monocytogenes-based cancer vaccines that induce immunity to the HPV-16 oncoprotein E7, Lm-E7 and Lm-LLO-E7 (11). Lm-E7 was generated by inserting the E7 gene in the L. monocytogenes chromosome, whereas Lm-LLO-E7 was generated with a multicopy episomal expression system. Lm-E7 secretes recombinant protein E7, but Lm-LLO-E7 secretes a fusion protein consisting of a truncated, non-membrane-
activation. Recent studies have demonstrated the potential use of DCs that are modified to carry tumor Ags in cancer vaccines (20). DC-based vaccines including DCs loaded with E7 peptide or proteins, DCs fused with tumor cells, and DCs transduced with the E7 gene or viral vectors have been used in cervical cancer models (21–24).

To date, no study has reported using L. monocytogenes as a vehicle to load tumor Ag in DC-based vaccines. In this study, we show that Lm-LLO-E7 induced significantly higher levels of MHC class II, CD40, and B7 costimulatory molecules on DCs than Lm-E7. Thus, the antitumor effectiveness of these two E7-expressing recombinant L. monocytogenes strains and propagation

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 cell has been immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene (25). TC-1 tumor cells express low levels of E6 and E7 and are highly tumorigenic. TC-1 cells were grown in RPMI 1640 medium, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM non-essential amino acids, 1 mM sodium pyruvate, and 50 μM 2-ME at 37°C with 10% CO2.

L. monocytogenes strains and propagation

The Listeria strains, Lm-LLO-E7 (which expresses a hly-E7 fusion gene episomally), Lm-E7 (which has a single-copy E7 gene cassette integrated in the Listeria genome), and wild-type Listeria 10403S, have been previously described (11). Briefly, E7 was ligated into the expression system, pGG-55, which is modeled on pDP-2028 (26). The hly-E7 fusion gene and pffa 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, including the signal sequence, which is joined by the XhoI 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, the hemolytic activity of LLO in the fusion protein has been removed. The recombinant bacteria retain hemolytic ability through the transcription of the endogenous hly gene on the chromosome. The potential transcription factor pffa is also included on pGG-55. By transforming a pffa-negative strain of Listeria, XFL-7 (a kind gift from Dr. H. Shen (University of Pennsylvania, Philadelphia, PA)), with pGG-55, we select for the retention of the plasmid in vivo. 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 (11). Lm-LLO-NP, also known as DPL208 (26), and Lm-Gag (27) were used as controls in this study. Their construction has also been described previously (26, 27).

Bacteria were grown in brain heart infusion medium with (Lm-LLO-E7 and Lm-LLO-NP) or without (Lm-E7 and Lm-Gag) chloramphenicol (25 μg/ml). Bacteria were frozen in aliquots at −80°C.

Preparation of bone marrow-derived DCs

Bone marrow was collected from the femurs of C57BL/6 mice at 6–8 wk of age. Bone marrow cells from five mice were pooled, and cells were cultured in RPMI 1640 medium containing 10% FCS and 100 U/ml penicillin/streptomycin in 100 × 15-mm petri dishes. After 2-h incubation at 37°C in 10% CO2, nonadherent cells were removed with washing with warm medium. The remaining adherent cells were collected by scraping with a sterile cell scrapper. After washing, the cells were adjusted to 0.5 × 10⁶/ml, and were placed in a 24-well plate with 20 ng/ml recombinant murine GM-CSF (R&D Systems, Minneapolis, MN). The medium was changed every 2–3 days. After 7 days of culture, nonadherent cells were collected, washed, and used in the experiments. We examined the phenotype of the DCs obtained using this protocol by FACS analysis. Fig. 1A shows the R1*R2 gate that was used for this and for all other experiments. This gate excluded dead cells (7-aminoactinomycin D (7AAD)-positive cells). Fig. 1B shows that naive DCs cultured in vitro in the presence of GM-CSF belong to the CD8α−CD11c+ subset (myeloid DCs).

Infection of DCs with Listeria recombinants

Bone marrow-derived DCs (10⁶/ml) were cultured with 10⁷ CFU/ml or 10⁹ CFU/ml Lm-E7, Lm-LLO-E7, Lm-LLO-NP, or wild-type 10403S in RPMI 1640 medium without antibiotics in a 24-well plate. After 1-h incubation, 50 μg/ml gentamicin was added to kill the remaining extracellular bacteria outside the cells. After 30 min, cells were washed and cultured in antibiotic-free medium for either 4 or 24 h as indicated in the figures. Cells were then harvested and washed two times with PBS before they were used in the experiments. In some experiments, cell-free supernatants were collected at 4 or 24 h for cytokine ELISA.

Flow cytometry

DCs were harvested at 4 or 24 h as described above. Cells were stained with PE-labeled mAbs specific for mouse CD11c, B7-H1, B7-DC, or FITC-labeled mAb specific for mouse CD80, CD86, MHC class II, CD40, and CD11c Ags. Isotype-matched mouse IgG was used as a negative control. mAbs for B7-H1 and B7-DC were purchased from eBioscience (San Diego, CA). All other mAbs were purchased from BD PharMingen (San Diego, CA). Cells were incubated with mAbs for 30 min at 4°C in the dark.

![FIGURE 1. DCs cultured with recombinant mouse GM-CSF belong to the CD8α−CD11c+ subset. Preparation of bone marrow-derived DCs was described in Materials and Methods. After 7-day culture with GM-CSF, naive DCs were stained with anti-MHC class II–FITC, anti-CD11c–PE, and anti-CD8α–allophycocyanin. Isotype-matched mouse IgG was used as a negative control. Ten microliters of 7AAD was added to all samples 10 min before cells were analyzed on a FACS flow cytometer. A, R1 was gated on bigger cells on forward-side scatter, and this population was further gated for CD11c−CD8α−CD11c− and CD11c−CD8α−CD11c− as appropriate. B, Dot plots are from a single experiment and are representative for three experiments with similar results.](http://www.jimmunol.org/doi/fig/1)
Following two washes with PBS, 10 μl of 7AAD (Beckman Coulter, Marseille, France) was added 10 min before cells were analyzed on a FACS flow cytometer.

**Intracellular staining for retinoblastoma protein (Rb)**

DCs were harvested at 4 or 24 h as described above. Cells were permeabilized with 70% cold ethanol for 10 min and stained with PE-labeled anti-Rb mAb (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 4°C. Isotype matched PE-labeled mAb specific for mouse CD11c, or FITC-labeled mAb specific for mouse CD80, CD86, or MHC class II. Isotype-matched mouse IgG was used as a negative control. Ten microliters of 7AAD was added to all samples 10 min before cells were analyzed on a FACS flow cytometer. Dot plots are from a single experiment and are representative for three experiments with similar results.

**Measurement of tumor growth**

Tumors were measured every 3 days with calipers spanning the shortest and longest surface diameters. A tumor with a diameter of 20 mm was considered a tumor-free size. Mice were sacrificed when the tumor diameter reached >20 mm.

**Cytokine ELISA**

Cell-free supernatants were collected at 4 or 24 h as described above and frozen at −80°C until determination of cytokine levels by sandwich ELISA. Mouse IL-2, IL-12, TNF-α, and IL-10 ELISA sets were purchased from BD PharMingen. Brieﬂy, samples were incubated for 2 h at room temperature on plates coated with mAb specific for the cytokine. The plates were washed with PBS containing Tween 20 before the addition of biotinylated secondary Ab for 1 h. The plates were washed, and a 1/250 dilution of avidin-HRP was added for 60 min. After thorough washing, 100 μl of ABTS was added, and the plates were incubated at room temperature for ~30 min. OD405 was determined using an automated microplate reader. Cytokine concentrations were calculated from standard curves developed for each cytokine.

**CFSE staining and proliferation assay**

Splenocytes were harvested from C57BL/6 mice, and CD3+ T cells were isolated using a mouse T cell enrichment column kit (R&D Systems) with a purity of >70% CD3+ T cells. Cells were washed in PBS and resuspended in serum-free medium at 1×10^6 cells/ml, and CFSE (Molecular Probes, Eugene, OR) was added at a final concentration of 0.5 μmol/ml. Cell suspension was agitated at room temperature for 8 min, and the reaction was quenched by adding an equal volume of FCS for 1 min at room temperature. Cells were washed and counted, and 75,000 cells/well were incubated with appropriate stimuli (medium alone, anti-CD3 Ab (BD PharMingen), uninfected DCs, Lm-E7-pulsed DCs, or Lm-LLO-E7-pulsed DCs) in a 96-well plate for 3 days at 37°C. Cells were analyzed for proliferation by determining halving of CFSE fluorescence intensity per each cell division using flow cytometry.

**Statistics**

Statistical analyses were done with Student’s t test. Values of p < 0.05 were considered significant.

**Results**

Lm-LLO-E7 up-regulates MHC class II molecules and B7 family members (CD80, CD86, B7-H1, B7-DC) on bone marrow-derived DCs

To analyze the expression of cosstimulatory molecules on DCs infected with two Listeria cancer vaccines, bone marrow-derived DCs were cultured with either medium alone as uninfected DCs or Listeria strains, including Lm-E7, Lm-LLO-E7, and wild-type Listeria 10403S, at multiplicities of infection (MOIs) of 10 and 1000, as described in Materials and Methods. After 4 or 24 h of infection, surface molecules on DCs were measured by flow cytometry.

**FIGURE 2.** Lm-LLO-E7 up-regulates the expression levels of MHC class II molecules and CD86 costimulatory molecules on bone marrow-derived DC. Bone marrow-derived DCs (10^6/ml) were cultured with 10^9 CFU/ml Lm-E7, Lm-LLO-E7, or wild-type Listeria 10403S, or left untreated. After 1-h incubation, 50 μg/ml gentamicin was added to kill the remaining extracellular bacteria. Cell culture continued at 37°C for 24 h. Cells were stained with either PE-labeled mAbs specific for mouse CD11c, or FITC-labeled mAb specific for mouse CD80, CD86, or MHC class II. Isotype-matched mouse IgG was used as a negative control. Ten microliters of 7AAD was added to all samples 10 min before cells were analyzed on a FACS flow cytometer. Dot plots are from a single experiment and are representative for three experiments with similar results.
Four-hour infection by any of the *Listeria* strains did not significantly change the CD80 and MHC class II expression level on DCs. The level of CD86 was slightly increased by both Lm-E7 and Lm-LLO-E7, but no difference was seen between Lm-E7 and Lm-LLO-E7 (data not shown). When DCs were infected with *Listeria* at a MOI of 1000 and cultured for 24 h, all three strains enhanced CD86 expression on DCs compared with the level of CD86 expressed on uninfected cells (15%). However, Lm-LLO-E7 strongly enhanced CD86 expression (86%) (Fig. 2D) compared with Lm-E7 (40%) (C) or 10403S (67%) (B). Interestingly, a shift of CD11c+ cells from CD86low (gate R5) to CD86high (gate R6) was observed post-*Listeria* infection (Fig. 2). Both E7-expressing recombinants slightly increased CD80 expression level at 24-h culture. However, a significant shift from dull CD80 to bright CD80 was not observed. MHC class II expression was also enhanced in the Lm-LLO-E7-infected group after 24-h culture. Sixty-five percent of DCs were MHC class II+ in the Lm-LLO-E7-infected group, whereas similar levels (10–18%) were expressed on the uninfected, Lm-E7-, and 10403S-infected groups.

Besides CD80 and CD86, we also analyzed other members of the B7 costimulatory molecule family, B7-H1 and B7-DC, under similar experimental conditions. As shown in Fig. 3, B7 expression...
was gated as B7-H1\textsuperscript{high} (R3) and B7-DC\textsuperscript{high} (R9). Lm-LLO-E7 up-regulated B7-H1\textsuperscript{high} (63%) compared with uninfected DCs (34%). However, Lm-E7 and 10403S down-regulated the levels of B7-H1\textsuperscript{high} (11 and 1%). Similarly, B7-DC\textsuperscript{high} was up-regulated by Lm-LLO-E7 infection (85%) compared with uninfected DCs (67%). However, Lm-E7 and 10403S infection did not change the level of expression of B7-DC.

When a lower dose of Listeria (MOI, 10) was used, no significant difference was seen between Lm-E7 and Lm-LLO-E7 regarding the expression level of CD80, CD86, B7-H1, and B7-DC (data not shown).

**Lm-LLO-E7 up-regulates CD40 expression on bone marrow-derived DCs**

Next, we analyzed the expression level of the CD40 molecule on DCs. It has been shown that mature DCs express higher levels of CD40 than immature DCs. In Fig. 4, Lm-LLO-E7 (83%) and Lm-E7 infection (81%) equally up-regulated the CD40 expression level at an MOI of 10. When DCs were infected with Listeria at an MOI of 1000, Lm-LLO-E7 radically increased the expression level of the CD40 molecule (46%) compared with Lm-E7 (26%) and uninfected DCs (19%). Taken together, our data demonstrated that Lm-LLO-E7 induces more efficient DC maturation than Lm-E7.

**Lm-LLO-E7 induces IL-2 production by bone marrow-derived DCs**

Experiments were conducted to determine the cytokine levels produced by DCs pulsed with *L. monocytogenes* vaccines. Bone marrow-derived DCs (10\textsuperscript{6}/ml) were cultured with 10\textsuperscript{9} CFU/ml Lm-E7, Lm-LLO-E7, or wild-type *Listeria* 10403S or left untreated in RPMI 1640 medium without antibiotics at 37°C. After 1-h incubation, 50 µg/ml gentamicin was added to kill the remaining bacteria outside the cells. Cell culture continued at 37°C for either 4 or 24 h. Cell-free supernatants were collected. The levels of IL-2, IL-12, TNF-α, and IL-10 in the supernatants were determined by ELISA. No IL-10 was detected in the supernatants from any of the cultures (data not shown). As shown in Fig. 5, uninfected DCs also did not produce any of the other cytokines tested. Interestingly, Lm-LLO-E7 induced significant levels of IL-2 by DCs at either 4- or 24-h culture compared with Lm-E7 (Fig. 5A). However, Lm-E7 infection resulted in higher levels of IL-12 (Fig. 5B) and TNF-α (C) than Lm-LLO-E7 at 4 h. Although wild-type 10403S induced large amounts of TNF-α and IL-12 after 4 h, it did not induce any detectable IL-2 in the supernatants.

**L. monocytogenes down-regulates the level of Rb in DCs**

It is known that the HPV-16 E7 protein plays a critical role in down-regulating the function of Rb. Although Rb negatively regulates cell cycle progression from G0 through to G1 and into S phase, Zhu et al. (28) have shown that Rb is also required for the expression of the MHC class II molecule. We therefore hypothesized that Lm-E7 might differentially activate Rb protein in the DCs when compared with Lm-LLO-E7 via the E7-Rb interaction, resulting in the differential expression of MHC class II molecules on the DCs. To test this hypothesis, DCs were infected with Lm-E7 or Lm-LLO-E7 at an MOI of 1000. Four hours later, DCs were permeabilized with 70% cold ethanol and stained with PE-labeled anti-Rb mAb or isotype-matched control mAb for FACS
Lm-LLO-E7 is down-regulated compared with the data were tabulated in Fig. 8, with 4.3% of the T cells exposed to B, uninfected DC). Interestingly, whereas A T cells underwent three cycles of cell division in contrast to the T A CD3 Ab via the TCR (Fig. 8, anti-CD3 Ab), the majority of the show that, in the control T cell group stimulated with soluble anti-

Our results indicate that the LLO fusion proteins LLO-NP and LLO-E7 may be preventing Rb down-regulation by Listeria.

**Lm-LLO-NP induces the same changes in DC phenotype as the Lm-LLO-E7**

To address the question whether DC maturation by Lm-LLO-E7 is conferred by the LLO fusion protein, we tested the ability of the vector control Lm-LLO-NP to up-regulate CD86 and MHC class II. As shown in Fig. 7, Lm-LLO-NP induces similar changes in MHC class II and CD86 expression on DCs as Lm-LLO-E7. A mixture of Lm-LLO-NP and Lm-E7 (Fig. 7E) also induces the same change in DC phenotype as Lm-LLO-E7, confirming that its effects are independent of the E7 molecule.

**Lm-LLO-E7-infected DCs alone induce CD8 T cell proliferation in vitro**

To further characterize the functional activity of Lm-E7-infected DCs vs Lm-LLO-E7-infected DCs, these cells were analyzed for their ability to stimulate proliferation of T cells in vitro. Bone marrow DCs were infected for 4 h with either Lm-E7 or Lm-LLO-E7 or left uninfected and then incubated with CFSE-labeled CD3+ T cells for 3 days. Proliferation of the CD8 T cell effector subset was assessed by flow cytometric analysis of CFSE-labeled CD8+ T cells, using decreases or halving of CFSE fluorescence intensity to indicate corresponding cell division cycles. Our results show that, in the control T cell group stimulated with soluble anti-CD3 Ab via the TCR (Fig. 8A, anti-CD3 Ab), the majority of the T cells underwent three cycles of cell division in contrast to the T cells stimulated with uninfected DCs alone, which did not show any proliferation (Fig. 8A, uninfected DC). Interestingly, whereas the Lm-E7-infected DCs were also unable to induce any proliferation of the responding T cells (Fig. 8A, DC + Lm-E7), the Lm-LLO-E7-infected DCs were able to induce three cell divisions (similar to the positive control group) in a small but significant population of CD8+ T cells (Fig. 8A, DC + Lm-LLO-E7). The data were tabulated in Fig. 8B, with 4.3% of the T cells exposed to Lm-LLO-E7 DCs undergoing cell division as compared with the Lm-E7 group where all the T cells remained undivided. These results indicate that the Lm-LLO-E7-infected DCs alone are capable of stimulating a naive CD8+ T cell-proliferative response in vitro.

**DCs infected with Lm-LLO-E7 elicit antitumor immunity**

To compare the in vivo antitumor efficacy of Lm-E7- and Lm-LLO-E7-infected DCs, we examined the impact of s.c. immunization with Lm-E7- and Lm-LLO-E7-pulsed DCs on established 5-mm TC-1 tumors. In contrast with our published results with Lm-LLO-E7 (11), neither treatment eliminated established TC-1 (data not shown), indicating that Lm-LLO-E7 is a more effective tumor immunotherapeutic than pulsed DCs. Thus, to compare the in vivo antitumor capabilities of Lm-E7- and Lm-LLO-E7-pulsed DCs, we turned to using the less stringent prophylactic model. C57BL/6 mice were injected s.c. on the left flank with 2 x 10^5 TC-1 tumor cells together with 1 x 10^8 DCs either unpulsed or pulsed with Lm-LLO-E7 or Lm-E7. Mice receiving TC-1 cells only developed tumors at day 7. Unpulsed DCs significantly slowed TC-1 tumor growth, all of the mice developed tumors by day 21. In contrast, for the mice receiving Lm-LLO-E7-pulsed DCs, 75% of mice remained tumor-free 42 days after TC-1 challenge, whereas only 37.5% of mice receiving Lm-E7-pulsed DCs remained tumor free (Fig. 9). These data indicate that Lm-LLO-E7-pulsed DCs have better in vivo antitumor efficacy than Lm-E7-pulsed DCs.

**Discussion**

In the murine system, at least two DC subsets have been identified on the basis of their differential CD8α expression. CD8α+ DCs and CD8α− DCs were originally considered as lymphoid and myeloid, respectively, because it was thought that they were generated from different precursors (12). However, recent studies demonstrated that both CD8α− and CD8α+ DCs can be generated from the same myeloid or lymphoid precursors (29–31). When bone marrow cells were cultured with GM-CSF for 7 days, the phenotype of cells was CD8α−CD11c+. Our data indicate that GM-CSF expands myeloid DC development in vitro. This finding is consistent with the results shown in vivo, which demonstrated that treatment of mice with pegylated GM-CSF preferentially expands the CD8α− myeloid DCs in the spleen (32, 33).

**FIGURE 6.** E7-Rb interaction is not involved in the differential up-regulation of MHC class II molecules by Lm-LLO-E7. DCs were infected with Lm-LLO-E7 or Lm-E7 at an MOI of 1000. After 4 h, cells were harvested and permeabilized with 70% cold ethanol for 10 min and stained with PE-labeled anti-Rb mAb for 30 min at 4°C. Isotype-matched mAb was used as a control. Cells were analyzed on a FACS flow cytometer. To find the difference between the isotype- and Rb-stained samples, the histograms were analyzed using Kolmogorov-Smirnov statistics, where D is the greatest difference between the two curves. In this study, p < 0.001 is the probability of D being as large as it is, given that the two selected histograms are from the same population.
In this study, we have examined the effects of Lm-LLO-E7 vs Lm-E7 on myeloid DCs. We first assessed the expression of surface molecules on DCs. MHC class II molecules were strongly up-regulated post-Lm-LLO-E7 infection. Lm-LLO-E7 also enhanced costimulatory molecules such as CD80, CD86, and CD40, indicating maturation of infected DCs. In contrast, Lm-E7 down-regulated the level of MHC class II molecule. We excluded the possibility that the up-regulation of MHC class II and costimulatory molecules by Lm-LLO-E7 was due to a more rapid intracellular bacterial growth, because both recombinants have a similar growth curve in bone marrow-derived DCs (data not shown). It is also not likely that this effect is due to the ability of listeriolsin to dissolve endosomal membranes, because the fusion protein is constructed with a truncated version of LLO that has lost the domain responsible for the lytic activity.

Why do these two vaccines have a different impact on the level of MHC class II molecules? One possibility is the interaction between the E7 protein, delivered by the vaccine vectors, with the Rb in the DC. It has been shown that the Rb family of pocket proteins negatively regulates progression from G0 through to G1 and into S phase (34). Upon infection with HPV-16, the viral E7 protein neutralizes this cell cycle control pathway mediated by Rb, by binding to it and inducing degradation of Rb (35). Interestingly, Eason et al. (36) have also shown MHC class II molecule expression was not induced in Rb-deficient mice, demonstrating that Rb plays a role in inducibility of MHC class II genes. We hypothesized that perhaps the two vaccines, Lm-E7 and Lm-LLO-E7, have differing impacts on the Rb molecule, thereby resulting in differential up-regulation of MHC class II molecules on the infected DCs. Fig. 6 demonstrated that both Lm-LLO-E7 and Lm-E7 decreased Rb levels, and comparable results were also obtained with Listeria vector controls, Lm-LLO-NP and Lm-Gag, that did not target the E7 protein. However, Lm-E7 and Lm-Gag did have a more pronounced effect on cellular Rb levels than did Lm-LLO-E7 and Lm-LLO-NP. Thus, decreases in Rb in the infected cells appear to be due to bacterial factors rather than by an E7-mediated Rb interaction.

To further investigate whether the up-regulation of costimulatory molecules on DCs by Lm-LLO-E7 is independent of the E7 molecule, we examined the ability of a vector control Lm-LLO-NP to mature DCs (Fig. 7) and found that it is similar to Lm-LLO-E7. Indeed, even a mixture of Lm-LLO-NP and Lm-E7 could induce similar changes in DC phenotype as Lm-LLO-E7. This suggests that the overexpression of the truncated nonhemolytic LLO by the multicopy plasmid in Lm-LLO-E7 and Lm-LLO-NP may facilitate the maturation of DCs and the up-regulation of costimulatory molecules. This may explain why the higher MOI of 1000 is more effective at stimulating DCs than an MOI of 10, because a higher level of LLO fusion protein could be rapidly accumulated with higher levels of infection. Our laboratory is further investigating these possible adjuvant properties of LLO.

In addition to CD80 and CD86, we also assessed the up-regulation of two newly defined B7 family members, B7-H1 and B7-DC. B7-DC has been shown to have potent costimulatory properties for T cells (15). The precise function of B7-H1 and B7-DC is still controversial, and both stimulatory and regulatory properties have been assigned to their ligation (15–18). Our data showed that the modulation of B7-H1 and B7-DC by Listeria is similar to CD80 and CD86. In addition, all four B7 analogues were preferentially up-regulated by Lm-LLO-E7, the effective cancer immunotherapeutic. This indicates that B7-H1 and B7-DC might play a similar role to CD80 and CD86 in T cell costimulation.

DCs not only express costimulatory molecules, but they also secrete cytokines upon infection. By using highly purified CD8α− DCs, we were able to demonstrate the cytokine profile secreted by myeloid DCs; as expected, IL-12 and TNF-α were detected in DC cultures infected with all three Listeria strains. IL-12 secretion was detected at 4-h culture. However, the level of IL-12 was decreased in the 24-h cultures. A possible explanation might be that IL-12 in
the supernatants was consumed by DCs. Indeed, a study by Grohmann et al. (37) demonstrated that IL-12 acts selectively on CD8\(^+\)/H9251/H11002/DCs to enhance presentation of a tumor peptide in vivo. Unexpectedly, Lm-E7 infection resulted in a higher level of IL-12 production than Lm-LLO-E7. The higher IL-12 induction by Lm-E7 might suppress the antitumor response of this vaccine, because several studies have demonstrated that IL-12 enhances cellular immune responses to vaccination only after a period of suppression (38, 39) mediated by IFN-\(\gamma\)/H9253/H1002/induction of NO (38).

Increased levels of TNF-\(\alpha\)/H9251/H149/production were observed in Lm-E7 at 4 h. In contrast, TNF-\(\alpha\)/H9251/H149/levels were similar in Lm-E7 vs Lm-LLO-E7 at 24 h. However, both vaccines were much less effective than 10403S in inducing TNF-\(\alpha\) after 24-h culture.

Interestingly, Lm-LLO-E7 induced a significant amount of IL-2 after 4-h infection, and IL-2 accumulated in the supernatants. By contrast, Lm-E7 and wild-type 10403S did not induce any IL-2. This finding is particularly interesting, because IL-2 produced by DCs early in infection might play a role in activating naïve T cells. Granucci et al. (19) also observed the induction of IL-2 mRNA and induction of IL-2 protein in culture supernatants 2 h after Gram-negative bacteria encounter. They compared the ability of wild-type and IL-2\(^{-/-}\)/ DCs to stimulate T cells in primary MLRs. The ability of IL-2\(^{-/-}\)/ DCs to induce both CD4\(^+\) and CD8\(^+\)/T cell proliferation was severely impaired, which indicated that IL-2 is a key cytokine in conferring the unique T cell stimulatory capacity to DCs. We also examined whether Lm-LLO-E7-pulsed DCs were more effective than Lm-E7-pulsed DCs at priming naïve T cells in vitro (Fig. 8). Despite the very low frequency of T cells with a single Ag specificity in the naïve peripheral T cell repertoire, we readily detected T cell proliferation after stimulation with Lm-LLO-E7-pulsed DCs but not with Lm-E7-pulsed DCs. These data indicate that Lm-LLO-E7-pulsed DCs are more functional than Lm-E7-pulsed DCs. Finally, to verify whether the changes in DC expression we observed are responsible for the differences in antitumor efficacy between these two vaccines, C57BL/6 mice were

![Figure 8](https://www.jimmunol.org/)

**FIGURE 8.** Lm-LLO-E7-pulsed DCs induce CD8 T cell proliferation in vitro. Bone marrow DCs from C57BL/6 mice were left uninfected or pulsed with Lm-E7 (1:1000) or Lm-LLO-E7 (1:1000) for 4 h. CD3\(^+\)/H11001/T cells were isolated from splenocytes from naïve C57BL/6 mice and labeled with CFSE. A total of 75,000 CFSE-labeled CD3\(^+\)/T cells were incubated in a 96-well plate at 37°C for 3 days with either soluble anti-CD3 Ab (5 \(\mu\)g/ml) and APC (anti-CD3 Ab), 150,000 uninfected DCs (uninfected DC), 150,000 DCs pulsed with Lm-E7 (DC + Lm-E7), or 150,000 DCs pulsed with Lm-LLO-E7 (DC + Lm-LLO-E7). Cells were then washed and labeled with anti-CD8 Ab and examined by FACS. A, Cells from each group were gated on CD8\(^+\)/T cells and analyzed for dilution of CFSE stain. B, Each cell division as determined by CFSE dilution from the histogram analysis in A was gated, and the percentage of cells in each population was tabulated.

![Figure 9](https://www.jimmunol.org/)

**FIGURE 9.** Lm-LLO-E7 enhances in vivo antitumor response. Bone marrow-derived DCs (10\(^6\)/ml) were pulsed with 10\(^9\) CFU/ml Lm-E7 or Lm-LLO-E7 in RPMI 1640 medium without antibiotics. After 1-h incubation, 50 \(\mu\)g/ml gentamicin was added. After 30 min, cells were washed and cultured in antibiotic-free medium for 4 h. After washing twice with PBS, 1 \(\times\) 10\(^5\) pulsed DCs or unpulsed DCs or PBS alone were mixed with 2 \(\times\) 10\(^5\) TC-1 cells and injected s.c. on the left flank of C57BL/6 mice.
injected s.c. on the left flank with 2 × 10^5 TC-1 cells together with 1 × 10^5 DCs, either unpulsed or pulsed with Lm-E7 or Lm-LLO-E7. Before injection, DCs were treated with either of the two Listeria recombinants for 4 h at an MOI of 1000 in vitro. Fig. 9 showed that unpulsed DCs significantly slowed tumor growth. This is probably because DCs can acquire E7 Ag from TC-1 cells and induce an E7-specific immune response against TC-1 cells. When DCs pulsed with Lm-LLO-E7 were injected into mice, 75% of mice remained tumor free. However, only 37.5% of mice receiving Lm-E7-pulsed DCs remained tumor free. These data indicate that Lm-LLO-E7 renders DCs better APCs, which in turn induce a better antitumor response.

In conclusion, our study demonstrates that Listeria infection induces a rapid and effective phenotypic and functional maturation of myeloid DCs. Lm-LLO-E7 is more effective than Lm-E7 at inducing DC maturation. Furthermore, enhanced DC maturation by Lm-LLO-E7 induces better in vitro T cell proliferation and in vivo antitumor immunity. It will be of interest to determine whether the strong correlation that we have measured between whether the strong correlation that we have measured between Lm-LLO-E7 and CD8+ T cell immunity that correlates with their ability to induce regression of established tumors immortalized by HPV-16. J. Immunol. 167:6471.


