Episomal Expression of Truncated Listeriolysin O in LmddA-LLO–E7 Vaccine Enhances Antitumor Efficacy by Preferentially Inducing Expansions of CD4<sup>+</sup> FoxP3<sup>+</sup> and CD8<sup>+</sup> T Cells

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Abstract
Studies have shown that Listeria monocytogenes (Lm)–based vaccine expressing a fusion protein comprising truncated listeriolysin O (LLO) and human papilloma virus (HPV) E7 protein (Lm–LLO–E7) induces a decrease in regulatory T cells (Treg) and complete regression of established, transplanted HPV-TC-1 tumors in mice. However, how the Lm-based vaccine causes a decrease in Tregs remains unclear. Using a highly attenuated Lm dal dat ΔactA strain (LmddA)–based vaccine, we report here that the vector LmddA was sufficient to induce a decrease in the proportion of Tregs by preferentially expanding CD4<sup>+</sup> FoxP3<sup>+</sup> T cells and CD8<sup>+</sup> T cells by a mechanism dependent on and directly mediated by LLO. Episomal expression of a nonhemolytic truncated LLO in Lm (LmddA-LLO) significantly augmented the expansion, thus further decreasing Treg frequency. Although adoptive transfer of Tregs compromised the antitumor efficacy of the LmddA-LLO–E7 vaccine, a combination of LmddA-LLO and an Lm-based vaccine expressing E7 protein (Lm–E7) induced complete regression against established TC-1 tumors. An engineered LLO-minus Lm expressing perfringolysin O (PFO) that enables the recombinant bacteria to exit the phagolysosome without LLO confirmed that the adjuvant effect was dependent on LLO. These results suggest that LLO may serve as a promising adjuvant by preferentially inducing the expansions of CD4<sup>+</sup> FoxP3<sup>+</sup> T cells and CD8<sup>+</sup> T cells, thus reducing the ratio of Tregs to CD4<sup>+</sup> T cells and to CD8<sup>+</sup> T cells favoring immune responses to eradicate tumor.

Introduction
Listeria monocytogenes (Lm) is a gram-positive facultative intracellular pathogen that causes listeriosis (1). Upon invading a host cell, Lm can escape from the phagolysosome by producing a pore-forming protein, listeriolysin O (LLO), which lyses the vesicular membrane, allowing Lm to enter the cytoplasm, in which it replicates and spreads to adjacent cells mediated by the mobility of actin-polymerizing protein (ActA; ref. 2). In the cytoplasm, Lm-secreted proteins are degraded by the proteasome and processed into peptides that associate with MHC class I molecules in the endoplasmic reticulum (3). This unique characteristic makes it an attractive cancer vaccine vector in that Lm-expressed tumor antigens can be presented with MHC class I molecules to activate tumor-specific cytotoxic T lymphocytes (CTL). Attenuated Lm strains have been generated and developed as cancer vaccine vectors delivering tumor antigens or tumor-associated antigens as immunogens to treat various types of cancer (4–10).

Human papillomavirus (HPV) infection is associated with most cervical cancer, and HPV strain 16 (HPV-16) is detected in about half of cervical cancer cases worldwide (11). Constitutive expression of HPV-16 E6 and E7 viral proteins in infected cells disrupts the cell cycle and induces malignant proliferation (12). Although prophylactic HPV vaccines are effective against HPV infection and development of cervical intraepithelial neoplasia (13), a therapeutic vaccine for advanced-stage cervical cancer is still being developed. Progress has been made in the construction of an Lm–LLO–E7 vaccine, a live-attenuated Lm-based vector producing and secreting a fusion protein comprising a truncated LLO and full-length E7 antigen. The Lm–LLO–E7 vaccine induced complete regression of established HPV-immortalized TC-1 tumors in mice (14). CD8<sup>+</sup> T cells have a critical role in the antitumor activity induced by Lm–LLO–E7, as depletion of CD8<sup>+</sup> T cells before vaccination abrogated the inhibition of tumor growth (14). Lm–LLO–E7 vaccine has been shown to decrease regulatory T cells (Treg) in mouse spleens and tumors (15). Tregs, identified as CD4<sup>+</sup> FoxP3<sup>+</sup> (or CD4<sup>+</sup> CD25<sup>+</sup> when it was first discovered), are a small population of T cells that suppress immunity. An...
effective immunotherapy must overcome the Treg obstacle to trigger helpful immune responses. It is conceivable that the Lm-LLO–E7–induced reduction of Tregs contributes to its antitumor effect, but how the Lm-LLO–E7 vaccine induces Treg decrease remains unclear. Studies toward identifying the mechanism by which Lm-LLO–E7 causes Treg reduction may lead to further improvement of its antitumor efficacy, such as the development of novel therapeutic strategies to manipulate Tregs.

Here, we describe the development of LmddA-LLO–E7, an improved attenuated Lm-based vaccine that decreased Treg frequency but not its absolute cell number. Specifically, LmddA-LLO–E7 preferentially induced the expansions of CD4–FoxP3– T cells and CD8+ T cells, thus effectively decreasing the proportion of CD4+FoxP3+ T cells by dilution. We found that the LmddA vector was able to induce CD4+FoxP3+ T-cell and CD8+ T-cell expansions, but the addition of episomal expression of a truncated LLO dramatically enhanced such an expansion, thus further decreasing the percentage of CD4+ FoxP3+ T cells. Lm-induced CD4+FoxP3+ T-cell and CD8+ T-cell expansions were dependent on and directly mediated by LLO. Although enhancement of the expansions of CD4+ FoxP3+ T cells and CD8+ T cells by the combination of LmddA-LLO and Lm–E7 induced complete regression of established TC-1 tumors, adoptive transfer of CD4+CD25+ Tregs compromised LmddA-LLO–E7 antitumor efficacy, suggesting that different T-cell subsets and their balance are critical and can affect the outcome of immunotherapy.

Materials and Methods

Mice

C57BL/6 mice, female, 6- to 8-weeks-old (unless stated otherwise), were purchased from the Frederick National Laboratory for Cancer Research (Frederick, MD). Mice were housed in the Animal Facility of the National Cancer Institute (Bethesda, MD). Protocols for use of experimental mice were approved by the Animal Care and Use Committee at the NIH.

Cell line

The TC-1 cell line, a generous gift from Professor T.C. Wu at Johns Hopkins University (Baltimore, MD), was generated by the transformation of primary lung epithelial cells from C57BL/6 mice with HPV-16 E6 and E7 and activated ras oncogene (16). TC-1 cells were tested to be mycoplasma free; no other authentication was performed. The cells were grown in RPMI-1640, supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/mL nonessential amino acids, and 0.4 mg/mL G418 at 37°C with 5% CO2.

L. monocytogenes strains

LmddA-LLO–E7 and the corresponding controls LmddA-LLO and LmddA were generated in Advaxis Inc. The dal dat ΔactA strain (LmddA) was constructed from the dal dat strain, which is based on Lm wild-type strain 10403S (17). With dal, dat, and actA mutated, Lmdda is highly attenuated. The LmddA-LLO–E7 strain was constructed by the transformation of Lmdda with the pTV3 plasmid (18) after deletion of prfA and the chloramphenicol-resistant gene in the plasmid (17). Expression and secretion of the LLO–E7 fusion protein were confirmed in culture supernatants of the LmdA-LLO–E7 strain by Western blot analysis as previously described (14). Construction of the LmdA-LLO control strain was similar to that of the LmdA-LLO–E7 strain, but both prfA and E7 were deleted in the pTV3 plasmid. Lm wild-type strain 10403S and mutant strains Δhly, Δhly::pfo, and hly::Tn917–lac (pAM401-hly) were kindly provided by Dr. D. Portnoy (University of California, Berkeley, CA). Lm–E7 strain, in which the full-length E7 gene was integrated into the Lm chromosome, was kindly provided by Dr. Y. Paterson (University of Pennsylvania, Philadelphia, PA). The strain hly::Tn917–lac is a nonhemolytic mutant of wild-type Lm, in which the Tn917–lac fusion gene is inserted into the hly gene (the gene encoding LLO) to disrupt LLO hemolytic activity. When this mutant hly::Tn917–lac strain is transfected with plasmid pAM401-hly expressing LLO, it regains hemolytic activity. Bacteria were cultured in brain–heart infusion medium plus streptomycin (100 μg/mL) with or without d-alanine (100 μg/mL).

Reagents

Fluorescence-conjugated anti-mouse antibodies CD4-PerCP-Cy5.5 (GK1.5) and CD8-Brilliant Violet 421 (53-6.7) were from BioLegend. FoxP3-FITC (FJK-16s) was from ebioscience. H-2Db tetramers loaded with the E7 peptide (RAHYNIVTF) were kindly provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility and the NIH AIDS Research and Reference Reagent Program. CountBright absolute counting beads were from Life Technologies.

Tumor inoculation and mouse vaccination

TC-1 cells (105 cells/mouse) were implanted subcutaneously in the right flank of mice on day 0. On day 10, when tumors were 5 to 6 mm in diameter, mice were injected i.p. with LmddA-LLO–E7 vaccine or corresponding controls at a dose of 0.1 LD50. Vaccination was boosted on day 17. Tumors were measured twice a week using an electronic caliper, and tumor size was calculated by the formula: length × width × width/2. Mice were euthanized when tumors reached 2.0 cm in diameter.

Flow cytometry

Mouse splenocytes or cells harvested from tumors were stained with CD4-PerCP-Cy5.5, CD8-Brilliant Violet 421, and H-2Db E7 tetramer–allophycocyanin (APC) for 30 minutes. Cells were fixed, permeabilized, and stained with FoxP3–FITC overnight. Cells were analyzed by flow cytometry. A lymphocyte gate was set in which Tregs were identified as CD4+FoxP3+. CountBright absolute counting beads were added for counting absolute cell numbers.

Adaptive transfer of CD4+CD25+ Tregs

CD4+CD25+ T cells were isolated from mouse spleens by the Dynal CD4+CD25+ Treg Kit (Life Technologies). Cells were injected i.v. into TC-1–tumor–bearing mice at day 9 after tumor cell inoculation. One day after Treg transfer, mice were immunized i.p. with LmddA-LLO–E7 (0.1 LD50) twice at 1-week interval. Tumor growth was monitored.
Statistical analysis
The data were analyzed using the nonparametric Mann-Whitney test. Statistical significance was determined at $P < 0.05$.

Results
LmddA-LLO–E7 induces regression of established TC-1 tumors accompanied by a decrease in Treg frequency
It was reported previously that an Lm-based vaccine, Lm-LLO–E7, which comprises a fusion protein of LLO–E7 and PrfA expressed episomally in a proA-negative strain of Listeria XFL-7, induced complete regression of established TC-1 tumors (14). Here, we investigated the antitumor activity of another highly attenuated Lm-based vaccine, LmdddA-LLO–E7, which produces the fusion protein LLO–E7 by a plasmid in a dal, dat, and actA mutated Lm strain (17). LmdddA-LLO–E7 is safer and even more attenuated compared with Lm-LLO–E7, because the chloramphenicol resistance gene and PrfA have been removed from the plasmid. We found that similar to Lm-LLO–E7 (14), LmdddA-LLO–E7 significantly inhibited the growth of established TC-1 tumors (Fig. 1A and B and Supplementary Fig. S1). Tumors completely regressed in approximately 40% of TC-1 tumor-bearing mice after two vaccinations with LmdddA-LLO–E7 (Fig. 1B and Supplementary Fig. S1). Except for one mouse that relapsed and died at 3 months, mice that showed tumor regression (33% of total animals) survived at least 6 months without relapse (Fig. 1C). Although Lm–E7 slowed the growth of TC-1 tumors, it failed to induce complete tumor regression (Fig. 1A and B and Supplementary Fig. S1). LmdddA-LLO (without E7) was unable to significantly inhibit TC-1 tumor growth (Fig. 1A and B and Supplementary Fig. S1), suggesting that innate immune response is not sufficient to eradicate TC-1 tumor cells. LmdddA-LLO–E7 and Lm–E7 induced similar H-2D* E7-tetramer+ CD8+ T-cell response in the spleen (Supplementary Fig. S2A top, S2B, and S2D), which was consistent with previous finding (14). We then analyzed CD4+ FoxP3+ Tregs. Unexpectedly, we found that LmdddA-LLO–E7, Lm–E7, and LmdddA-LLO all significantly decreased Treg frequency in spleens and more dramatically in tumors compared with PBS control, although LmdddA-LLO–E7 and LmdddA-LLO decreased the frequency more than did Lm–E7 (Fig. 1D–H). We note that a previous report found that Lm–E7 was unable to decrease Tregs (15); whether the differences in these models account for the different findings are not clear.

Lm is sufficient to induce decrease of Treg frequency
Initially, we suspected that the decrease of Treg frequency was mediated by the truncated LLO. However, Lm–E7 did not express truncated LLO but was able to decrease Treg frequency (Fig. 1D–H). This observation suggests that Lm might be able to decrease Treg frequency. Indeed, both LmdddA, the vector control for LmdddA-LLO–E7, and 10403S, a wild-type Lm strain and the vector control for Lm–E7, significantly decreased Treg frequency in spleens and more so in tumors (Fig. 2).

Lm decreases Treg frequency by preferentially inducing CD4+ FoxP3+ T-cell and CD8+ T-cell expansions
A relative Treg frequency (proportion of total T cells) is determined by the numbers of Tregs, CD4+ FoxP3+ T cells, and CD8+ T cells. To investigate how Lm decreases Treg frequency, we quantified the numbers of CD4+ FoxP3+ Tregs, CD4+ FoxP3+ T cells, and CD8+ T cells in TC-1 tumor-bearing mice treated with either LmdddA-LLO–E7, LmdddA-LLO, LmdddA, Lm–E7, or Lm (10403S). As shown in Fig. 3, surprisingly, we found that LmdddA did not markedly change the number of CD4+ FoxP3+ T cells in the tumors. It actually increased CD4+ FoxP3+ T cells and CD8+ T cells, thus decreasing Treg frequency proportionately. Episomal expression of a truncated LLO in LmdddA-LLO and LmdddA-LLO–E7 further increased the numbers of CD4+ FoxP3+ T cells and the LmdddA-LLO–E7 also increased the numbers of CD8+ T cells, thus further decreasing the frequency of CD4+ FoxP3+ T cells. Wild-type Lm 10403S also induced an increase in CD4+ FoxP3+ T cells and CD8+ T cells while not significantly changing CD4+ FoxP3+ T-cell number. Lm–LLO–E7 significantly increased the density of CD4+ FoxP3+ T cells and CD8+ T cells in tumors. These results demonstrate that Lm preferentially induces CD4+ FoxP3+ T-cell expansion and to a lesser extent CD8+ T-cell expansion resulting in a decrease in CD4+ FoxP3+ T-cell frequency.

Lm-induced expansion of CD4+ FoxP3+ T cells and CD8+ T cells is dependent on and mediated by LLO
LLO, encoded by the hly gene, is a pore-forming cytolsin by which Lm can escape from a host cell phagosomal vacuole into the cytoplasm (19). Because LmdddA-LLO–E7, Lm–E7, and their respective controls produce LLO, we used an LLO-deficient Lm-mutant derived from 10403S, in which the hly gene is deleted using a shuttle vector followed by homologous recombination (20), to study whether LLO plays a role in inducing the expansion of CD4+ FoxP3+ T cells and CD8+ T cells. We found that hly Lm was unable to increase CD4+ FoxP3+ T cells and CD8+ T cells in the spleen of mice on day 7 after a single administration (Fig. 4A), indicating that induction of expansion of CD4+ FoxP3+ T cells and CD8+ T cells is dependent on LLO. This dependence could be either a direct effect of LLO on the immune cells or a requirement for the bacteria to escape the phagolysosome. To address this question, we studied an Lm strain with LLO replaced by perfringolysin O (PFO). PFO, produced by Clostridium perfringens, is 43% identical in amino acids with LLO and can also lyse the vacular membrane (21). The pfo gene, encoding PFO under the control of hly promoter, was recombined into the chromosome of the hly strain to form hly::pfo strain (20). Although hly::pfo was able to escape from phagocytosis into the cytoplasm (20), it was unable to increase the numbers of CD4+ FoxP3+ T cells and CD8+ T cells in the mouse spleen (Fig. 4A). A limitation of hly::pfo control is that PFO is toxic to the infected host cell when secreted by Lm and does not allow productive intracellular replication of Lm within the infected cells (22). Thus, a mutant of PFO that allows for an effective intracellular replication in the context of an Lm infection might be a more appropriate control in the experiment (22). Different from the hly and hly::pfo strains, hly::Tn917–lac (pAM401-hly), a nonhemolytic Tn917–lac mutant of wild-type Lm (in which the Tn917–lac fusion gene is inserted into the hly gene to disrupt LLO hemolytic activity) transformed with an LLO-expressing plasmid pAM401-hly,
induced expansions of mouse splenic CD4<sup>+</sup>FoxP3<sup>+</sup>/C<sup>0</sup> T cells and CD8<sup>+</sup> T cells (Fig. 4A). These results suggest that expansions of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells and CD8<sup>+</sup> T cells are directly mediated by LLO independently of the hemolytic activity. Because Lm did not induce CD4<sup>+</sup>FoxP3<sup>+</sup> T-cell expansion significantly, Lm-induced Treg decrease in frequency resulted from the increase of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells and CD8<sup>+</sup> T cells (Fig. 4A–D).

Figure 1. LmddA-LLO-E7 induces regression of established TC-1 tumors accompanied by Treg frequency decrease. C57BL/6 mice were inoculated s.c. with 1 × 10<sup>3</sup> TC-1 tumor cells each, and immunized i.p. with 0.1 LD<sub>50</sub> LmddA-LLO-E7 (1 × 10<sup>7</sup> CFU), Lm-E7 (1 × 10<sup>5</sup> CFU), or LmddA-LLO (1 × 10<sup>5</sup> CFU) in PBS (100 µL) on days 10 and 17 after tumor challenge. Tumors were measured twice a week using an electronic caliper. Tumor volumes were calculated by the formula, length × width × width/2. Mice were sacrificed when tumor diameter reached approximately 2.0 cm or on day 24 for flow cytometric analysis. A, average tumor volume from days 10 to 24. B, tumor volume on day 24. C, survival percentage over time. D, flow cytometric profile of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells in total CD4<sup>+</sup> T cells. E, the percentage of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells relative to total CD4<sup>+</sup> T cells in the spleen. F, ratio of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells to CD8<sup>+</sup> T cells in the spleen. G, percentage of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells relative to total CD4<sup>+</sup> T cells in the tumor. H, ratio of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells to CD8<sup>+</sup> T cells in the tumor. Data, mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Mann-Whitney test). Data are from three independent experiments (A and B) and are representative of three independent experiments (C–H).
Episomal expression of a truncated LLO in LmdA induces expansion of CD4\(^+\)FoxP3\(^-\) T cells and CD8\(^+\) T cells to a higher level

We compared LmdA and LmdA-LLO to assess the effect of episomal expression of a truncated LLO on T-cell proliferation in healthy, tumor-free mice. We found that LmdA induced a slight increase in the numbers of CD4\(^+\)FoxP3\(^-\) T cells and CD8\(^+\) T cells in the spleens of mice at day 7 after a single administration, and LmdA-LLO induced the increase of these T cells to a higher level (Fig. 5A). In contrast, the number of CD4\(^+\)FoxP3\(^+\) T cells was not changed significantly after either LmdA or LmdA-LLO infection (Fig. 5A). The administration of LmdA-LLO resulted in a significant decrease of Treg proportion compared with that in PBS control (Fig. 5B–D). We also examined the cell proliferation marker Ki67 in these cells. LmdA increased the frequency and absolute number of Ki67\(^+\)CD4\(^+\)FoxP3\(^-\) T cells and Ki67\(^+\)CD8\(^+\) T cells, and LmdA-LLO increased the numbers of these cells to a greater extent (Fig. 5E–G). The level of Ki67 expression in CD4\(^+\) FoxP3\(^-\) T cells and CD8\(^+\) T cells was also increased accordingly (Fig. 5H). In contrast, the frequency and absolute number of Ki67\(^+\)CD4\(^+\)FoxP3\(^+\) T cells and Ki67\(^+\) expression in CD4\(^+\) FoxP3\(^+\) T cells were not markedly changed, indicating that LmdA and LmdA-LLO did not induce their proliferation.

The combination of Lm–E7 and LmdA-LLO induces regression of established TC-1 tumors

The Lm–E7 vaccine alone did not induce much expansion of CD4\(^+\)FoxP3\(^-\) T cells and CD8\(^+\) T cells (Fig. 3). This may account for its failure in the induction of TC-1 tumor regression. Because LmdA-LLO induced CD4\(^+\)FoxP3\(^-\) T-cell and CD8\(^+\) T-cell expansions (Figs. 3 and 5A), it is conceivable that the antitumor effect of Lm–E7 may be improved in the presence of LmdA-LLO. Indeed, the combination of Lm–E7 and LmdA-LLO induced nearly complete regression of established TC-1 tumors (Fig. 6A–C).
and prolonged survival of about 20% of the mice (Fig. 6C). In contrast, the addition of LmddA failed to augment Lm–E7-induced antitumor activity (Supplementary Fig. S3), indicating that endogenous LLO produced by the LmddA vector is not sufficient to enhance antitumor efficacy of the Lm–E7 vaccine. As expected, CD4+ FoxP3+ T-cell and CD8+ T-cell numbers were significantly increased in the spleens of mice that received combination treatment compared with those in mice treated with Lm–E7 or PBS (Fig. 6D). Again, because the number of CD4+ FoxP3+ T cells was relatively unchanged, the increase of CD4+ FoxP3+ T-cell and CD8+ T-cell numbers to a higher level induced by the combined Lm–E7 and LmddA-LLO treatment resulted in a greater decrease in the CD4+ FoxP3+ T-cell proportion (Fig. 6E–G).

Adoptive transfer of Tregs compromises the antitumor efficacy of LmddA-LLO–E7 against established TC-1 tumors

LmddA-LLO–E7 did not significantly change Treg numbers, although it decreased Treg frequency (Fig. 1D–H). The ratio of Tregs to CD4+ FoxP3+ T cells or to CD8+ T cells has been a well-accepted parameter to determine Treg-suppressive ability. To determine whether the Treg proportion has any impact on the antitumor efficacy of LmddA-LLO–E7, we isolated CD4+ CD25+ Tregs from naive C57BL/6 mice and injected them i.v. into TC-1 tumor–bearing mice followed by LmddA-LLO–E7 vaccination. LmddA-LLO–E7 significantly inhibited TC-1 tumor growth in the mice without adoptive transfer of Tregs (Fig. 7A and B). However, in mice given Tregs, LmddA-LLO–E7 was unable to significantly inhibit TC-1 tumor growth (Fig. 7A and B). Mice receiving Tregs showed a slight increase of Treg number in the spleens but higher increase in tumors (Fig. 7F and G). On the other hand, mice that received Tregs had fewer CD4+ FoxP3+ T cells and CD8+ T cells after being vaccinated with LmddA-LLO–E7 compared with those in the LmddA-LLO–E7 control, indicating that adoptive transfer of Tregs inhibits CD4+ FoxP3+ T-cell and CD8+ T-cell expansions (Fig. 7F and G). These together resulted in the increase of Treg frequency in the Treg-recipient mice (Fig. 7C–E).

Discussion

It is well known that tumor antigen–specific CTLs play dominant roles in killing tumor cells, and Lm, an intracellular bacteria, can deliver antigens associated with MHC class I molecules to activate CTLs. However, it is unclear why two Lm-based vaccines, Lm-LLO–E7 and Lm–E7, induced similar levels of HPV E7–specific CTLs in the spleens but exhibited distinct antitumor activity, with the former inducing a much stronger antitumor effect (Fig. 1A–C and Supplementary Figs. S1 and S2; ref. 14). CD8+ T cells are known to participate in killing tumor cells, as their depletion abrogated Lm-LLO–E7-induced tumor regression (14). It is also clear that a certain level of tumor antigen–specific CTLs is necessary for killing tumor cells, as LmddA-LLO, which lacks E7 expression, was unable to significantly inhibit TC-1 tumor growth (Fig. 1A–C and Supplementary Fig. S1). It has been proposed that Lm–E7 induced an increase of Tregs to suppress the host immune response, thus compromising host antitumor immunity (15). However, we found that both Lm–E7 and LmddA-LLO–E7 decreased Treg frequency in a TC-1 tumor model compared with that in tumor-bearing mice treated with PBS control (Fig. 1D–H). Furthermore, we found that neither Lm–E7 nor LmddA-LLO–E7 significantly increased the total number of Tregs in TC-1 tumors after vaccination (Fig. 3).

In fact, we found that a major difference in vaccine efficacy between LmddA-LLO–E7 and Lm–E7 is that the former was able to induce a marked increase in CD4+ FoxP3+ T cells and CD8+ T cells, whereas the latter induced a much smaller increase (Fig. 3). This explains how LmddA-LLO–E7 decreased the Treg percentage to a greater degree than did Lm–E7 (Fig. 1D–H). We observed that the Lm vector alone was sufficient to increase CD4+ FoxP3+ T-cell and CD8+ T-cell numbers. However, with episomal expression of a truncated LLO, Lm increased the numbers of CD4+ FoxP3+ T cells and CD8+ T cells, which results in the decreased Treg frequency (Fig. 7C–E).
T cells to a higher level, thus decreasing Treg frequency even further by dilution, but there was no change in the absolute number of Tregs (Fig. 5). Thus, it is conceivable that LLO may play a critical role in inducing increases of CD4\(^+\)FoxP3\(^+\)/CD8\(^+\) T cells. Indeed, not only is LLO necessary for Lm to escape from the phagolysosome, but it also directly induces the expansions of CD4\(^+\)FoxP3\(^+\)/CD8\(^+\) T cells, as neither an LLO-minus (\(\Delta hly\)) Lm strain nor \(\Delta hly::pfo\), an LLO-minus strain expressing PFO that enables Lm to enter the cytoplasm, was able to induce the proliferation of CD4\(^+\)FoxP3\(^+\)/CD8\(^+\) T cells. Transformation of a nonhemolytic LLO-mutant Lm strain with an LLO-expressing plasmid restored CD4\(^+\)FoxP3\(^+\)/CD8\(^+\) T-cell expansions (Fig. 4). LLO-mediated induction of CD4\(^+\)FoxP3\(^+\)/CD8\(^+\) T-cell expansions is not related to its hemolytic activity, as episomal expression of a nonhemolytic-truncated LLO (\(hly::Tn917-lac\)) in LmddA greatly augmented the expansions of CD4\(^+\)FoxP3\(^+\)/CD8\(^+\) T cells (Fig. 5). Although the expansion of both CD4\(^+\) T-cell and CD8\(^+\) T-cell responses by LLO seems to be an antigen-nonspecific adjuvant effect, LLO may also contain immunodominant epitopes of these two cell types. Indeed, early studies have identified that LLO bears two CD4\(^+\)T-cell epitopes (residues 189–201 and residues 215–226) and one CD8\(^+\)T-cell epitope (residues 91–99; refs. 23–25). The antitumor effect of LmddA-LLO–E7 may derive from its ability to induce a significant increase in CD4\(^+\)FoxP3\(^+\)/CD8\(^+\) T cells and CD8\(^+\) T cells. In contrast, the inability of Lm–E7 to induce a marked increase in CD4\(^+\)FoxP3\(^+\)/CD8\(^+\) T cells may account for its inefficiency in eradicating tumors, as the combination of Lm–E7 and LmddA-LLO, which dramatically increased CD4\(^+\)FoxP3\(^+\)/CD8\(^+\) T cells compared with that by Lm–E7 alone, induced nearly complete regression of established TC-1 tumors (Fig. 6). As a control, LmddA (without the LLO plasmid) in combination with Lm–E7 did not show this effect (Supplementary Fig. S3), implying that the truncated LLO was necessary. Our data indicate that the

Figure 4. Lm-induced expansions of CD4\(^+\)FoxP3\(^+\)/CD8\(^+\) T cells are dependent on and mediated by LLO. C57BL/6 mice were injected i.p. with 1 \(\times\) 10\(^5\) CFU 10403S, \(\Delta hly\), \(\Delta hly::pfo\), or \(hly::Tn917-lac\) (pAM401-hly) in PBS (100 \(\mu\)L). Mice were sacrificed on day 7 after injection, and lymphocytes isolated from the spleens were analyzed by flow cytometry. A, T-cell numbers in the spleen. B, flow cytometric profile of CD4\(^+\)FoxP3\(^+\)/CD8\(^+\) T cells in total CD4\(^+\)/CD8\(^+\) T cells. C, percentage of CD4\(^+\)FoxP3\(^+\)/CD8\(^+\) T cells relative to total CD4\(^+\)/CD8\(^+\) T cells. D, ratio of CD4\(^+\)FoxP3\(^+\)/CD8\(^+\) T cells; *, \(P < 0.05\); **, \(P < 0.01\); and ***, \(P < 0.001\) (Mann–Whitney test). Data are representative of three independent experiments.
LmddA-LLO–E7-induced decrease in Treg frequency is the consequence of an increase in the numbers of CD4+FoxP3+/CXCR3− T cells and CD8+ T cells. The ratio of Tregs to CD4+FoxP3+/CXCR3− T cells or to CD8+ T cells is critical, at least in an in vitro Treg-suppression assay, to suppress the function of CD4+FoxP3+/CXCR3− T cells and CD8+ T cells. Indeed, we found that increasing the Treg ratio in vivo by adoptive transfer of Tregs into tumor-bearing mice followed by LmddA-LLO–E7 vaccination inhibited the expansion of CD4+FoxP3+/CXCR3− T cells and CD8+ T cells and consequently compromised the antitumor efficacy of the vaccine (Fig. 7).

It is noteworthy that besides preferentially inducing the expansion of CD4+FoxP3+/CXCR3− T cells and CD8+ T cells, truncated nonhemolytic LLO may make other contributions to improving the antitumor efficacy of the LmddA-LLO–E7 vaccine. We noticed that although Lm–E7 and LmddA-LLO–E7 induced similar expansion of E7-specific CD8+ T cells in the spleen, this is not the case in the tumor. With episomal expression of the truncated LLO (LmddA-LLO–E7), more E7-specific CD8+ T cells were induced in the tumor (Supplementary Fig. S2E) than by Lm–E7. We found that LmddA-LLO–E7 upregulated the expression of chemokine receptors CCR5 and CXCR3 on CD4+...
FoxP3+ T cells and CD8+ T cells, but not on CD4+FoxP3+ T cells (unpublished data). CCR5 and CXCR3 are crucial for Th1 and CD8+ T-cell trafficking (25). Our results suggest that LLO may induce CD4+FoxP3+ T-cell and CD8+ T-cell migration to the tumor microenvironment through upregulation of CCR5 and CXCR3. In addition, it is known that truncated LLO is required for the efficient secretion of antigens from Lm (27), and antigens that are not secreted from the Lm vector induced
less effective antigen-specific antitumor immunity (28). Hence, the lack of potent antitumor activity of the Lm–E7 vector might not be only due to its inability to effectively expand CD4⁺ FoxP3⁺ T cells and CD8⁺ T cells but also due to its inefficient secretion of antigens from Lm in the context of an infected APC, resulting in the priming of an ineffective antigen-specific T-cell response.

In summary, we have provided evidence demonstrating that episomal expression of a nonhemolytic truncated LLO in an LmddA-LLO–E7 vaccine preferentially induced the expansions of CD4⁺ FoxP3⁺ T cells and CD8⁺ T cells, thus enhancing the antitumor activity the vaccine. Our results suggest that many factors, including threshold levels of antigen-specific CTLs and nontumor antigen-specific CD4⁺ FoxP3⁺ T cells and CD8⁺
T cells, and a decreased Treg proportion, are needed to trigger an effective antitumor immune response. Our study indicates that LLO may be a promising vaccine adjuvant in that it preferentially induces the expansion of CD4+ FoxP3+ T cells and CD8+ T cells, thus decreasing Treg frequency and favoring immune responses to kill tumor. Our future studies will aim to investigate how LLO preferentially induces the expansion of protective immune cells without changing the absolute number of immunosuppressive CD4+ FoxP3+ T cells.

Disclosure of Potential Conflicts of Interest
A. Wallecha has ownership interest (including patents) in Advaxis Inc. JA Berzofsky reports receiving a commercial research grant from Advaxis. No potential conflicts of interest were disclosed by the other authors.

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Episomal Expression of Truncated Listeriolyasin O in LmddA-LLO - E7 Vaccine Enhances Antitumor Efficacy by Preferentially Inducing Expansions of CD4+FoxP3− and CD8+ T Cells

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