Episomal expression of truncated listeriolysin O in LmddA-LLO-E7 vaccine enhances antitumor efficacy by preferentially inducing expansions of CD4+FoxP3− and CD8+ T cells

Zhisong Chen, Laurent Ozbun, Namju Chong, Anu Wallecha, Jay A. Berzofsky, and Samir N. Khleif

Authors’ Affiliation:
Zhisong Chen, Laurent Ozbun, Namju Chong, Jay A. Berzofsky
Vaccine Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD 20892
Anu Wallecha
Advaxis Inc., Princeton, NJ 08540
Samir N. Khleif
GRU Cancer Center, Georgia Regents University, Augusta, GA 30912

Corresponding Authors:
Samir N. Khleif, Cancer Center, Georgia Regents University, 1410 Laney Walker Boulevard, CN-2133, Augusta, GA 30912. Phone: 706-721-0570; Fax: 706-721-8787; E-mail: skhleif@gru.edu
Jay A. Berzofsky, Vaccine Branch, Center for Cancer Research, National Cancer Institute, NIH, Bldg. 41–Rm D702D, 41 Medlars Drive, Bethesda, MD 20892. Phone: 301-496-6874; Fax: 301-480-0681; E-mail: berzofsk@helix.nih.gov
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) induced 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^+FoxP3^- T cells and CD8^+ 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. While 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 from 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^+FoxP3^- T cells and CD8^+ T cells, thus reducing the ratio of Tregs to CD4^+FoxP3^- T cells and to CD8^+ 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, where it replicates and spreads to adjacent cells mediated by the mobility of actin-polymerizing protein (ActA) (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 (TAA) as immunogens to treat various types of cancer (4-10).

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). While prophylactic HPV vaccines are effective against HPV infection and development of cervical intraepithelial neoplasia (CIN) (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⁺ T cells have a critical role in the antitumor activity induced by Lm-LLO-E7, as depletion of CD8⁺ T cells prior to 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⁺FoxP3⁺ (or CD4⁺CD25⁺ when it was first discovered), are a small population of T cells that suppresses 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 contribute 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. While 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, 6-8 weeks old (unless stated otherwise), were purchased from Frederick National Laboratory for Cancer Research (FNLCR). Mice were housed in the Animal Facility of National Cancer Institute, Bethesda. Protocols for use of experimental mice were approved by the Animal Care and Use Committee at National Institutes of Health.

**Cell line**

TC-1 cell line, a generous gift from Professor TC Wu at Johns Hopkins University, 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 µM 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 (Princeton, NJ). 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. LmddA-LLO-E7 strain was constructed by transformation of LmddA with the pTV3 plasmid (18) after deletion of prfA, and the chloramphenicol resistance gene in the plasmid (17). Expression and secretion of the LLO-E7 fusion protein was confirmed in culture supernatants of the LmddA-LLO-E7 strain by Western blotting as previously described (14). Construction of LmddA-LLO control strain was similar as that of LmddA-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 (San Diego, CA). FoxP3-FITC (FJK-16s) was from eBioscience (San Diego, CA). H-2Db tetramers loaded with the E7 peptide (RAHYNIVTF) was kindly provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility and the National Institutes of Health AIDS Research and Reference Reagent Program. CountBright™ absolute counting beads were from Life Technologies (Grand Island, NY).

Tumor inoculation and mouse vaccination

TC-1 cells (10^5 cells/mouse) were implanted s.c. in the right flank of mice on day 0. On day 10, when tumors were 5-6 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-2D^b E7 tetramer-APC for 30 min. Cells were fixed, permeabilized, and stained with FoxP3-FITC overnight. Cells were analyzed by flow cytometry. A lymphocyte gate was set where Tregs were identified as CD4^+FoxP3^+. CountBright™ absolute counting beads were added for counting absolute cell numbers.

Adoptive transfer of CD4^+CD25^+ Tregs

CD4^+CD25^+ T cells were isolated from mouse spleens by Dynal® CD4^+CD25^+ Treg Kit (Life Technologies, Grand Island, NY). Cells were injected i.v. into TC-1 tumor-bearing mice at day 9 post tumor cell inoculation. One day after Treg transfer, mice were immunized i.p. with LmddA-LLO-E7 (0.1 LD50) twice at one week interval. Tumor growth was monitored.

Statistics

The data were analyzed using the nonparametric Mann-Whitney test. 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 a Lm-based vaccine, Lm-LLO-E7, which comprises a fusion protein of LLO-E7 and PrfA expressed episomally in a prfA 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, LmddA-LLO-E7, which produces the fusion protein LLO-E7 by a plasmid in a *dal*, *dat*, and *actA* mutated Lm strain (17). LmddA-LLO-E7 is safer and even more attenuated compared to Lm-LLO-E7, since the chloramphenicol resistance gene and *PrfA* have been removed from the plasmid. We found that similar to Lm-LLO-E7 (14), LmddA-LLO-E7 significantly inhibited the growth of established TC-1 tumors (Fig. 1, A and B, Fig. S1). Tumors completely regressed in approximately 40% of TC-1 tumor-bearing mice after two vaccinations with LmddA-LLO-E7 (Fig. 1B and 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. 1, A and B and Fig. S1). LmddA-LLO (without E7) was unable to significantly inhibit TC-1 tumor growth (Fig. 1, A and B and Fig. S1), suggesting that innate immune response is not sufficient to eradicate TC-1 tumor cells. LmddA-LLO-E7 and Lm-E7 induced similar H-2D^b^ E7-tetramer^+^CD8^+^ T-cell response in the spleen (Fig S2, A-upper panel, B, and D), which was consistent with previous finding (14). We then analyzed CD4^+^FoxP3^+^ Tregs. Unexpectedly, we found that LmddA-LLO-E7, Lm-E7, and LmddA-LLO, all significantly decreased Treg frequency in spleens and more dramatically in tumors compared to PBS control, although LmddA-LLO-E7 and LmddA-LLO decreased the frequency more than did Lm-E7 (Fig. 1, D-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. 1, D-H). This observation suggests that Lm might be able to decrease Treg frequency. Indeed, both Lmdda, the vector control for Lmdda-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 Lmdda-LLO-E7, Lmdda-LLO, Lmdda, Lm-E7, or Lm (10403S). As shown in Fig 3, surprisingly, we found that Lmdda 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 Lmdda-LLO and Lmdda-LLO-E7 further increased the numbers of CD4↑FoxP3↓ T cells and the Lmdda-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 of 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 cytolysin by which Lm can escape from a host cell phagosomal vacuole into the cytoplasm (19). Since LmddA-LLO-E7, Lm-E7 and their respective controls produce LLO, we used a 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 Δhly 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). Perfringolysin O, produced by *Clostridium perfringens*, is 43% identical in amino acids with LLO and can also lyse the vacuolar membrane (21). The *pfo* gene, encoding PFO under the control of *hly* promoter, was recombined into the chromosome of the Δhly strain to form Δhly::pfo strain (20). Although Δhly::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 Δhly::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 Δhly and Δhly::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 a LLO-expressing plasmid pAM401-hly, induced expansions of mouse splenic CD4+FoxP3− T cells and CD8+ T cells (Fig. 4A). These results suggest that expansions of CD4+FoxP3− T cells and CD8+ T cells are directly mediated by LLO independent of the hemolytic activity. Since Lm did not induce CD4+FoxP3+ T-cell expansion significantly, Lm-induced Treg decrease in frequency resulted from the increase of CD4+FoxP3− T cells and CD8+ T cells (Fig. 4, A–D).

**Episomal expression of a truncated LLO in LmddA induces expansion of CD4+FoxP3− T cells and CD8+ T cells to a higher level**

We compared LmddA and LmddA-LLO to assess the effect of episomal expression of a truncated LLO on T-cell proliferation in healthy, tumor-free mice. We found that LmddA 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 LmddA-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 LmddA or LmddA-LLO infection (Fig 5A). The administration of LmddA-LLO resulted in a significant decrease of Treg proportion compared to that in PBS control (Fig. 5, B–D). We also examined the cell proliferation marker Ki-67 in these cells. LmddA increased the frequency and absolute number of Ki-67+CD4+FoxP3− T cells and Ki-67+CD8+ T cells, and LmddA-LLO increased the numbers of these cells to a greater extent (Fig.
5, E-G). The level of Ki-67 expression in CD4^+FoxP3^- T cells and CD8^+ T cells was also increased accordingly (Fig. 5H). In contrast, the frequency and absolute number of Ki-67^-CD4^+FoxP3^+ T cells and Ki-67 expression in CD4^+FoxP3^- T cells was not markedly changed, indicating that LmddA and LmddA-LLO did not induce their proliferation.

**The combination of Lm-E7 and LmddA-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. Since LmddA-LLO induced CD4^+FoxP3^- T-cell and CD8^+ T-cell expansion (Fig. 3 and Fig. 5A), it is conceivable that the antitumor effect of Lm-E7 may be improved in the presence of LmddA-LLO. Indeed, the combination of Lm-E7 and LmddA-LLO induced nearly complete regression of established TC-1 tumors (Fig. 6, A-C), and prolonged survival of about 20% of the mice (Fig. 6C). In contrast, 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. 6, E-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. 1, D-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 naïve 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. 7, A and B). However, in mice given Tregs, LmddA-LLO-E7 was unable to significantly inhibit TC-1 tumor growth (Fig. 7, A and B). Mice receiving Tregs showed a slight increase of Treg number in the spleens but higher increase in tumors (Fig. 7, F 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 to those in the LmddA-LLO-E7 control, indicating adoptive transfer of Tregs inhibits CD4⁺FoxP3⁻ T-cell and CD8⁺ T-cell expansion (Fig 7, F and G). These together resulted in the increase of Treg frequency in the Treg-recipient mice (Fig. 7, C-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. 1, A-C, Fig. S1, Fig. S2, on September 29, 2015. © 2014 American Association for Cancer Research. cancerimmunolres.aacrjournals.org Downloaded from
and (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. 1, A-C and 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 to that in tumor-bearing mice treated with PBS control (Fig. 1, D-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 while the latter induced a much smaller increase (Fig. 3). This explains how LmddA-LLO-E7 decreased Treg percentage to a greater degree than did Lm-E7 (Fig. 1, D-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 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- T cells and CD8+ T cells. Indeed, not only is LLO necessary for *L. monocytogenes* to escape from the phagolysosome but it also directly induces the expansions of CD4+FoxP3- T cells and CD8+ T cells, as neither an LLO-minus (Δhly) *L. monocytogenes* strain nor Δhly::pfo, an LLO-minus
strain expressing PFO that enables Lm to enter the cytoplasm, was able to induce the proliferation of CD4+FoxP3- T cells and CD8+ T cells. Transformation of a nonhemolytic LLO mutant Lm strain with an LLO-expressing plasmid restored CD4+FoxP3- T-cell and CD8+ T-cell expansions (Fig. 4). LLO-mediated induction of CD4+FoxP3- T-cell and 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- T cells and CD8+ T cells (Fig. 5). Although the expansion of both CD4+ T-cell and CD8+ T-cell responses by LLO appears to be an antigen-non-specific 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) (23-25).

The antitumor effect of LmddA-LLO-E7 may derive from its ability to induce a significant increase in CD4+FoxP3- T cells and CD8+ T cells. In contrast, the inability of Lm-E7 to induce marked increase in CD4+FoxP3- T cells and 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- T cells and CD8+ T cells compared to 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 (Fig S3), implying that the truncated LLO was necessary. Our data indicate that the LmddA-LLO-E7-induced decrease in Treg frequency is the consequence of an increase in the numbers of CD4+FoxP3- T cells and CD8+ T cells. The ratio of Tregs to CD4+FoxP3- 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- 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− T cells and CD8+ T cells and consequently compromised the vaccine’s antitumor efficacy (Fig. 7).

It is noteworthy that besides preferentially inducing the expansion of CD4+FoxP3− T cells and CD8+ T cells, truncated non-hemolytic 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 (Figure 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 (26). 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 due only to its inability to effectively expand CD4+ FoxP3− T cells and CD8+ T cells but also to its inefficient secretion of antigens from Lm in 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 vaccine’s antitumor activity. Our results suggest that many factors, including threshold levels of antigen-specific CTLs and non-tumor 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.
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Disclosure of Potential Conflicts of Interest

A. Wallecha is employed at Advaxis Inc and thus has ownership and financial interest. J.A. Berzofsky has received financial support from Advaxis Inc. No potential conflicts of interest were disclosed by the other authors.
Authors' Contributions

Conception and design: Z. Chen, J.A. Berzofsky, S.N. Khleif

Development of methodology: Z. Chen, L. Ozbun, N. Chong, A. Wallecha

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):
Z. Chen, L. Ozbun, N. Chong, A. Wallecha

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):
Z. Chen, L. Ozbun, N. Chong, A. Wallecha

Writing, review, and/or revision of the manuscript: Z. Chen, L. Ozbun, Berzofsky, S.N. Khleif
References


Figure Legends

**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 1×10⁵ TC-1 tumor cells each, and immunized i.p. with 0.1 LD₅₀ LmddA-LLO-E7 (1×10⁸ CFU), Lm-E7 (1×10⁶ CFU), or LmddA-LLO (1×10⁸ CFU) in PBS (100 µl) on day 10 and day 17 post 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 day 10 to day 24. (B) Tumor volume on day 24. (C) Survival percentage over time. (D) Flow cytometric profile of CD4⁺FoxP3⁺ T cells in total CD4⁺ T cells. (E) Percentage of CD4⁺FoxP3⁺ T cells relative to total CD4⁺ T cells in the spleen. (F) Ratio of CD4⁺FoxP3⁺ T cells to CD8⁺ T cells in the spleen. (G) Percentage of CD4⁺FoxP3⁺ T cells relative to total CD4⁺ T cells in the tumor. (H) Ratio of CD4⁺FoxP3⁺ T cells to CD8⁺ T cells in the tumor. Data are presented as Mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 (Mann-Whitney test). Data are from 3 independent experiments (A and B) and are representative of 3 independent experiments (C-H).

**Figure 2.** *L. monocytogenes* is sufficient to induce a decrease in Treg frequency. C57BL/6 mice were inoculated s.c. with 1×10⁵ TC-1 tumor cells each, and immunized i.p. with 0.1 LD₅₀ LmddA (1×10⁸ CFU) or 0.5 LD₅₀ wild-type Lm 10403S (1×10⁴ CFU) in PBS (100 µl) on day 10 and day 17 post tumor challenge. Mice were sacrificed at day 24 and lymphocytes isolated from the spleens and tumors were analyzed by flow cytometry. (A) Flow cytometric profile of CD4⁺FoxP3⁺ T cells in total CD4⁺ T cells. (B) Percentage of CD4⁺FoxP3⁺ T cells relative to total CD4⁺ T cells.
total CD4$$^+$$ T cells in the spleen. (C) Ratio of CD4$$^+$$FoxP3$$^+$$ T cells to CD8$$^+$$ T cells in the spleen. (D) Percentage of CD4$$^+$$FoxP3$$^+$$ T cells relative to total CD4$$^+$$ T cells in the tumor. (E) Ratio of CD4$$^+$$FoxP3$$^+$$ T cells to CD8$$^+$$ T cells in the tumor. Data are presented as Mean ± SEM. *$$P < 0.05$$, **$$P < 0.01$$, and ***$$P < 0.001$$ (Mann-Whitney test). Data are representative of 3 independent experiments.

Figure 3. *L. monocytogenes* decreases Treg frequency by preferentially inducing CD4$$^+$$FoxP3$$^-$$ T-cell and CD8$$^+$$ T-cell expansions. C57BL/6 mice were inoculated s.c. with 1$$\times$$10$$^5$$ TC-1 tumor cells each, and immunized i.p. with 0.1 LD50 LmddA-LLO-E7 (1$$\times$$10$$^8$$ CFU), LmddA-LLO (1$$\times$$10$$^8$$ CFU), LmddA (1$$\times$$10$$^8$$ CFU), Lm-E7 (1$$\times$$10$$^6$$ CFU), or 0.5 LD50 wild-type Lm 10403S (1$$\times$$10$$^4$$ CFU) in PBS (100 µl) on day 10 and day 17 post tumor challenge. Mice were sacrificed at day 24 and lymphocytes isolated from the tumors were analyzed by flow cytometry. Data are presented as (Mean ± SEM). n= 3-10. *$$P < 0.05$$, **$$P < 0.01$$ (Mann-Whitney test). Data are representative of 3 independent experiments.

Figure 4. *L. monocytogenes*-induced expansions of CD4$$^+$$FoxP3$$^-$$ T cells and CD8$$^+$$ T cells are dependent on and mediated by LLO. C57BL/6 mice were injected i.p. with 1$$\times$$10$$^4$$ CFU 10403S, Δhly, Δhly::pfo, or hly::Tn917-lac (pAM401-hly) in PBS (100 µl). Mice were sacrificed on day 7 post injection and lymphocytes isolated from the spleens were analyzed by flow cytometry. (A) T cell numbers in the spleen. (B) Flow cytometric prolife of CD4$$^+$$FoxP3$$^+$$ T cells in total CD4$$^+$$ T cells. (C) Percentage of CD4$$^+$$FoxP3$$^+$$ T cells relative to total CD4$$^+$$ T cells. (D) Ratio of
CD4⁺FoxP3⁺ T cells to CD8⁺ T cells. *P < 0.05 (Mann-Whitney test). Data are representative of 3 independent experiments.

**Figure 5.** Episomal expression of a truncated LLO in LmddA induces expansion of CD4⁺FoxP3⁺ T cells and CD8⁺ T cells to a higher level. C57BL/6 mice were injected i.p. with 1×10⁸ CFU LmddA or LmddA-LLO in PBS (100 µl). Mice were sacrificed on day 7 post injection and lymphocytes isolated from the spleens were analyzed by flow cytometry. (A) T cell numbers in the spleen. (B) Flow cytometric prolife of CD4⁺FoxP3⁺ T cells in total CD4⁺ T cells. (C) Percentage of CD4⁺FoxP3⁺ T cells relative to total CD4⁺ T cells. (D) Ratio of CD4⁺FoxP3⁺ T cells to CD8⁺ T cells. (E) Flow cytometric prolife of Ki-67⁺ T cells. (F) Percentage of Ki-67⁺ T cells. (G) Fluorescent intensity of Ki-67⁺ T cells. Data are presented as Mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 (Mann-Whitney test). Data are representative of 3 independent experiments.

**Figure 6.** Combination of Lm-E7 and LmddA-LLO induces regression of established TC-1 tumors. C57BL/6 mice were inoculated s.c. with 1×10⁵ TC-1 tumor cells each, and immunized i.p. with 0.05 LD50 Lm-E7 (5×10⁵ CFU), 0.05 LD50 LmddA-LLO (5×10⁷ CFU), 0.05 LD50 Lm-E7 plus 0.05 LD50 LmddA-LLO in PBS (100 µl) on day 10 and day 17 post tumor challenge. Tumors were measured twice a week using an electronic caliper and tumor volumes were calculated by the formula: length × width × width /2. Mice were observed for survival or sacrificed on day 24 and lymphocytes isolated from the spleens were analyzed by flow cytometry. (A) Average tumor volume from day 10 to day 24. (B) Tumor volume on day 24. (C) Survival
percentage over time. (D) T-cell number in the spleen. (E) Flow cytometric prolife of CD4⁺FoxP3⁺ T cells in total CD4⁺ T cells. (F) Percentage of CD4⁺FoxP3⁺ T cells relative to total CD4⁺ T cells. (G) Ratio of CD4⁺FoxP3⁺ T cells to CD8⁺ T cells. Data are presented as Mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 (Mann-Whitney test). Data are representative of 2 independent experiments.

**Figure 7.** Adoptive transfer of Tregs compromises the antitumor efficacy of LmddA-LLO-E7 against established TC-1 tumors. C57BL/6 mice (11 weeks old) were injected s.c. with 1×10⁵ TC-1 tumor cells each, and i.v. with CD4⁺CD25⁺ Tregs (1×10⁶ cells/each) on day 9 post tumor challenge. Mice were immunized i.p. with 0.1 LD50 LmddA-LLO-E7 (1×10⁸ CFU) in PBS (100 µl) on day 10 and day 17 post tumor challenge. Tumors were measured twice a week using an electronic caliper and tumor volumes were calculated by the formula: length × width × width /2. Mice were sacrificed on day 24 and lymphocytes isolated from the spleens were analyzed by flow cytometry. (A) Average tumor volume from day 10 to day 24. (B) Tumor volume on day 24. (C) Flow cytometric prolife of CD4⁺FoxP3⁺ T cells in total CD4⁺ T cells. (D) Percentage of CD4⁺FoxP3⁺ T cells relative to total CD4⁺ T cells in the spleen. (E) Percentage of CD4⁺FoxP3⁺ T cells relative to total CD4⁺ T cells in the tumor. (F) T-cell number in the spleen. (G) T-cell number per million tumor cells. Data are presented as Mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 (Mann-Whitney test). Data are representative of 2 independent experiments.
Figure 1
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Episomal expression of a truncated listeriolyisin O in LmddA-LLO-E7 vaccine enhances anti-tumor efficacy by preferentially inducing CD4+FoxP3- T cell and CD8+ T cell expansion

Zhisong Chen, Laurent Ozbun, Namju Chong, et al.

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