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ORIGINAL ARTICLE

Targeting tumor vasculature with novel *Listeria*-based vaccines directed against CD105

Laurence M. Wood · Zhen-Kun Pan · Patrick Guirnalda · Peter Tsai · Matthew Seavey · Yvonne Paterson

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Abstract The FDA approval of bevacizumab (Avastin®, Genentech/Roche), a monoclonal antibody raised against human VEGF-A, as second-line therapy for colon and lung carcinoma validated the approach of targeting human tumors with angiogenesis inhibitors. While the VEGF/VEGFR pathway is a viable target for anti-angiogenesis tumor therapy, additional targets involved in tumor neovascularization have been identified. One promising target present specifically on tumor vasculature is endoglin (CD105), a member of the TGF-β receptor complex expressed on vascular endothelium and believed to play a role in angiogenesis. Monoclonal antibody therapy and preventive vaccination against CD105 has met with some success in controlling tumor growth. This report describes the in vivo proof-of-concept studies for two novel therapeutic vaccines, Lm-LLO-CD105A and Lm-LLO-CD105B, directed against CD105 as a strategy to target neovascularization of established tumors. *Listeria*-based vaccines directed against CD105 lead to therapeutic responses against primary and metastatic tumors in the 4T1-Luc and NT-2 mouse models of breast cancer. In a mouse model for autochthonous Her-2/neu-driven breast cancer, Lm-LLO-CD105A vaccination prevented tumor incidence in 20% of mice by week 58 after birth while all control mice developed tumors by week 40. In comparison with previous *Listeria*-based vaccines targeting tumor vasculature, Lm-LLO-CD105A and Lm-LLO-CD105B demonstrated equivalent or superior efficacy against two transplantable mouse models of breast cancer. Support is provided for epitope spreading to endogenous tumor antigens and reduction in tumor vascularity after vaccination with *Listeria*-based CD105 vaccines. Reported here, these CD105 therapeutic vaccines are highly effective in stimulating anti-angiogenesis and anti-tumor immune responses leading to therapeutic efficacy against primary and metastatic breast cancer.

Keywords Endoglin · CD105 · Breast cancer · Vasculature · *Listeria monocytogenes* · Vaccine

Laurence M. Wood and Zhen-Kun Pan contributed equally to this manuscript.

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L. M. Wood · Z.-K. Pan · P. Guirnalda · P. Tsai · M. Seavey · Y. Paterson (✉)
Department of Microbiology, University of Pennsylvania, 323 Johnson Pavilion, Philadelphia, PA 19104, USA
e-mail: yvonne@mail.med.upenn.edu

Present Address:
M. Seavey
Cephalon, Inc., 145 Brandywine Pkwy, Rm B311/305, West Chester, PA 19380-4245, USA

Introduction

Tumor-associated neovascularization was first documented as early as the 1940s [1, 2]. However, it was not until the work by Dr. Judah Folkman in the early 1970s that targeting this neovascularization, or "angiogenesis" as termed by Folkman, was postulated as a viable strategy for cancer therapy [3, 4]. Development of anti-angiogenesis therapies over the next few decades culminated in the first commercially available angiogenesis inhibitor in 2004, bevacizumab (Avastin®, Genentech/Roche), a monoclonal antibody directed against human VEGF-A, as a second-line treatment for colon cancer [5]. While passive immunotherapy with monoclonal antibodies is an established strategy in

anti-angiogenesis therapy, evidence from preclinical studies suggests that active immunotherapy is also a promising and effective alternative [6–9]. Previous work by Seavey et al. and others demonstrates that tumor vasculature-associated proteins such as VEGFR2 can be effectively targeted by CTL-based active immunotherapy that leads to tumor regression in mouse models of breast cancer [6, 8]. Recent studies offer CD105 as a promising new target for anti-angiogenesis CTL-based active immunotherapy [9, 10].

CD105 is a large transmembrane glycoprotein that plays a crucial role in TGF-β signaling and angiogenesis [11–16]. As a member of the TGF-β receptor complex, it binds TGF-β1 and β3 and forms a heterodimeric complex with TGFβ-RII to facilitate signal transduction [17, 18]. Expression of CD105 is enhanced upon TGF-β stimulation and exposure to hypoxic conditions, such as those found in tumors [19]. The highest expression of CD105 is found on vascular endothelial cells, and studies suggest that the angiogenic properties of TGF-β are dependent on CD105 expression [14, 15]. The importance of CD105 in angiogenesis is highlighted in observations found with CD105 knockout mice [12, 13, 20, 21]. In the absence of CD105, maturation of cardiac tissue vasculature is impaired, resulting in early embryonic death [12]. While the evidence is clear that CD105 is crucial for normal vascular development, there is a growing body of evidence that CD105 also plays an additional role in tumorigenesis [22–25].

The importance of neovascularization during tumorigenesis is well characterized, and CD105 is believed to be one of the factors involved in this process [22–26]. As a prognostic factor, CD105 protein can be detected in the serum of tumor-bearing patients and the levels of serum CD105 increase with metastatic severity [27, 28]. Additionally, immunohistochemical analyses suggest that CD105 is highly expressed in the vascular endothelium of tumor tissue while it is barely detectable in normal tissues [29–31]. In addition, tumor-specific expression of CD105 within vascular capillary beds has previously been utilized in several therapeutic strategies [9, 10, 32–34]. CD105-specific monoclonal antibodies fused to either a radioactive isotope or a toxic compound, can target tumor vasculature specifically and were shown to inhibit tumor growth in preclinical mouse models of breast cancer [32–34]. Previously reported studies suggest that CD105 can even be targeted in a CTL-mediated tumor immunotherapy approach. Preventive vaccination with a cDNA encoding for CD105 resulted in reduced neovascularization and a reduced tumor burden in a mouse model for breast cancer [9, 10]. These approaches strongly support a case for the use of a CD105-directed vaccine to target the tumor vasculature.

This report describes the successful targeting of the neovascularization of established tumors by two *Listeria*-based vaccines directed against CD105. Each vaccine, Lm-LLO-CD105A and Lm-LLO-CD105B, is effective in halting tumor growth in two separate mouse models of breast cancer, while Lm-LLO-CD105A significantly delays time to tumor progression in an autochthonous mouse mammary tumor model. In comparison with previous *Listeria*-based vaccines targeting antigens present in tumor vasculature, the *Listeria*-based CD105 vaccines are either similar or superior in efficacy to each in treating established NT-2 and 4T1-Luc tumors. Vaccination against CD105 also resulted in secondary tumor-specific CTL responses in a process known as epitope spreading. The resulting impact on tumor growth and neovascularization demonstrate that *Listeria*-based vaccines are effective for targeting CD105 expression on tumor vasculature in established tumors.

Materials and methods

Mice

Balb/c female mice (6–8 week old) were purchased from Charles River Laboratories, and FVB/NJ female mice (6–8 week old) were purchased from Jackson Laboratories. A rat Her-2/neu-transgenic mouse strain in the FVB/N background [35] used for prevention studies as a model for autochthonous breast tumor formation was housed and bred at the animal core facility at the University of Pennsylvania. All mouse experiments were performed in accordance with the regulations of the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Construction of Lm-LLO-CD105A and Lm-LLO-CD105B

To construct the *Listeria*-based CD105 vaccines, mRNA was purified from 4T1-Luc cells and converted into cDNA. The regions of CD105 incorporated into each vaccine, as illustrated in Fig. 1a, were amplified from the total cDNA pool with the following primers: Lm-LLO-CD105A, (F) 5′-TAAT-CTCGAG-TATAGCTTTGTACCCACA-3′; Lm-LLO-CD105A (R) 5′-ATTA-ACTAGT-CAGGGAG ACATTGCTGAC-3′; Lm-LLO-CD105B (F), 5′-TAAT-CT CGAG-CAGCCAAAGTGTGGCAA; Lm-LLO-CD105B (R), 5′-ATTA-CCCGGG-GGCACCAAAGGTGATACC. XhoI sequences underlined for forward primers, and SpeI (ACTAGT) and SmaI (CCCGGG) sequence underlined for reverse primers. Each fragment amplicon was restriction-enzyme digested and ligated into the *Listeria* expression plasmid, pGG34. Each sequence was genetically fused downstream to the gene encoding truncated Listeriolysin O (tLLO) under the control of the *hly* promoter. Subsequently, each construct, pGG34-LLO-CD105A and pGG34-LLO-CD105B, was electroporated into the attenuated *Listeria*

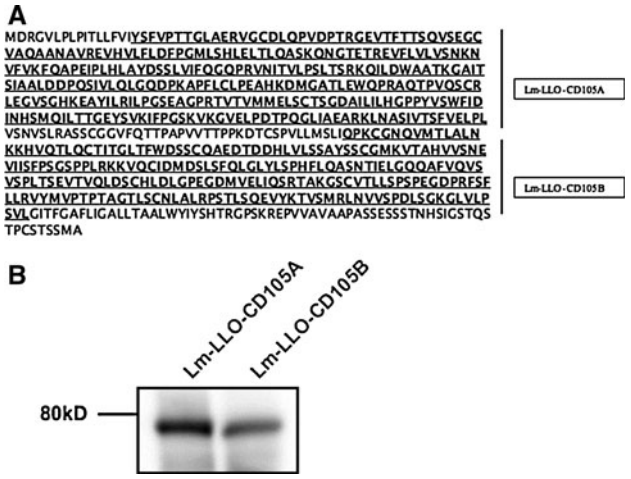

Fig. 1 Construction of *Listeria*-based CD105 Vaccines. **a** Depiction of the sequences from mouse endoglin (NP_031958.2), CD105, that are fused to the C-terminal end of truncated LLO for secretion by each attenuated *Listeria monocytogenes* vaccine. **b** Western blot analysis of secreted proteins by Lm-LLO-CD105A and Lm-CD105B was performed with a polyclonal antibody against the N-terminal region of Listeriolysin O to determine proper expression and secretion of the tLLO-CD105 fusion proteins

monocytogenes (Lm) strain, XFL7, and plasmid-containing colonies were selected for resistance on BHI/chloramphenicol plates. To confirm proper construction of Lm-LLO-CD105A and Lm-LLO-CD105B, each vaccine was grown in BHI/chloramphenicol selection media and secreted proteins were precipitated with trichloroacetic acid. After boiling in SDS sample buffer, secreted proteins were subject to SDS–PAGE analysis and transferred to a PVDF membrane. Western analysis on the membrane was performed with anti-PEST antibody (specific for the N-terminus of Listeriolysin O) to confirm secretion of tLLO-CD105 fusion proteins. The isogenic *Listeria* vaccine, Lm-LLO-NYESO-1, was administered in all experiments to provide a *Listeria* control that produced and secreted an antigen not found in the mouse models of breast cancer used in this manuscript. Additionally, Lm-LLO-NYESO-1 administration has little or no impact on tumor burden in each of these tumor models in previous studies [6].

Tumor load study

Two mouse breast tumor cell lines were used in these studies. The FVB/N syngeneic NT-2 tumor cell line was developed from a spontaneously occurring mammary tumor in a FVB/N HER-2/neu-transgenic mouse [36]. The 4T1 tumor cell line is a 6-thioguanine-resistant cell line, which was derived from a mouse mammary tumor virus (MMTV)-induced spontaneous mammary carcinoma [37]. It was transduced with luciferase and kindly made available to us by Dr. Ellen Pure, The Wistar Institute [38]. FVB/N mice were implanted subcutaneously in the right flank with 10^6 NT-2 tumor cells. Subsequently, mice were immunized intraperitoneally with 2×10^8 colony-forming units (CFU) of Lm-LLO-CD105A, Lm-LLO-CD105B, or a control Lm vaccine, Lm-LLO-NYESO-1, (8 mice/group) on day 4, 11, and 18. Tumor tissue and spleens were removed on day 56 for tumor-specific tetramer analysis. Balb/c mice were implanted with 2×10^5 4T1-Luc tumor cells in the mammary fat pad. Subsequently, mice were vaccinated intraperitoneally with 2×10^8 CFU of Lm-LLO-CD105A, Lm-LLO-CD105B, or control Lm (8 mice/group) on day 4, 11, and 18. Tumor tissue and spleens were removed on day 32 for tumor-specific tetramer analysis. Additionally, lungs were removed for analysis of 4T1-Luc natural lung metastasis by light microscopy. Tumor volume was calculated as (tumor diameter)^3/2.

Tetramer analysis

Spleens from each vaccination group were pooled and processed for HIV gag, gp70, and Her-2/neu-specific T cells by staining for $CD62L^-$, $CD11b^-$, $CD8\alpha^+$, and $tetramer^+$ cells.

Additionally, tumors from each group were manually dissociated into single-cell suspensions, pooled, and Ficoll-purified to remove dead cells and cellular debris by excluding the low-density fraction after centrifugation. The purified tumor cell suspension was then processed in triplicate for HIV gag, gp70, and Her-2/neu-specific T cells by staining for $CD62L^-$, $CD11b^-$, $CD8\alpha^+$, and $tetramer^+$ cells. All samples were processed using a FACSCalibur flow cytometer with CellQuest software (Becton–Dickinson, Mountain View, CA). The HIV-gag tetramer is specific for the $H\text{-}2K^d$ CTL epitope (AMQMLKETI), the gp70 tetramer is specific for the $H\text{-}2L^d$ CTL epitope (SPSYVYHQF), and the Her-2/neu-specific tetramers are specific for the $H\text{-}2K^d$ epitopes Her-2-EC1 (PYNYLSTEV), Her-2-EC2 (LFRNPHQALL), and Her-2-IC1 (PYVSRLLGI) [39]. Tetramers were provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility and used at a 1:200 dilution.

Lung surface metastases

Lungs from the naïve group ($n = 5$) and each vaccinated group, control Lm ($n = 7$), Lm-LLO-CD105A ($n = 7$), and Lm-LLO-CD105B ($n = 6$), were removed from the 4T1-Luc tumor load study on day 32. Lung surface metastatic nodules per lung were then counted with a Nikon SMZ1B Zoom Stereomicroscope attached to a Fostec 8375 Illuminator and Ringlight. The data are reported as the number of lung surface metastases per animal.

Quantitative 4T1-Luc metastases luciferase assay

Balb/c mice were implanted with 2×10^5 4T1-Luc tumor cells in the mammary fat pad and subsequently immunized intraperitoneally with 2×10^8 CFUs of each vaccine. Lungs were removed on day 46, and lung tissue was manually separated into a single-cell suspension. After removal of red blood cells with ACK lysing buffer, remaining cells were resuspended in 100 µl of PBS and added to a single well of a 96-well black polystyrene assay plate. Subsequently, 100 µl of Bright-Glo Luciferase reagent was added to each well, and the plate was incubated at room temperature for 5 min. After incubation, the plate was read in a Luminoskan Ascent Reader (Thermo Scientific, Waltham, MA) and arbitrary light units collected. To approximate the number of metastatic tumor cells in lung tissue, a standard curve of 4T1-Luc tumor cells was created and used to calculate the approximate number 4T1-Luc tumor cells in each lung.

CD31 mRNA expression

At the end of the experiment, tumors from each group were excised, placed in RNAlater solution (Ambion, Austin, TX) and stored at 4°C. RNA was extracted from the tumor tissue with Qiagen RNeasy plus mini kits (Qiagen, Valencia, CA), and total RNA was reverse-transcribed into cDNA using Applied Biosystems High Capacity cDNA reverse transcription kit (Applied Biosystems, Forest City, CA). cDNA from each tumor was then subjected to qPCR analysis for expression of the vascular marker, CD31. The primers used for CD31 expression are as follows: qCD31. FORWARD 5′-CAGGACCAGGTGTTAGTGTT-3′ and qCD31.REVERSE 5′-ACTCCTGATGGGTTCTGACT-3′. Amplification of 18S ribosomal RNA was used for standardization of all samples.

Comparison of Anti-angiogenesis *Listeria*-based Vaccines with the NT-2 tumor model

FVB/N mice were implanted subcutaneously in the right flank with 10^6 NT-2 tumor cells. Subsequently, after establishment of the primary tumors, mice were immunized intraperitoneally with *Listeria* -based vaccines (8 mice/group) at day 7, 14, and 21. All *Listeria*-based vaccines were given at 2×10^8 CFU/mouse except for Lm-LLO-HMWMAA-C (5×10^7 CFU/mouse) and Lm-LLO-FLK-I1 (2×10^7 CFU/mouse) due to their increased virulence. Tumor size was measured by calipers twice weekly throughout the course of the experiment. Tumor volume was calculated as (tumor diameter)^3/2.

Comparison of anti-angiogenesis *Listeria*-based vaccines with the 4T1-Luc tumor model

Balb/c mice were implanted with 2×10^5 4T1-Luc tumor cells in the mammary fat pad. Subsequently, after establishment of the primary tumors, mice were immunized intraperitoneally with *Listeria*-based vaccines (8 mice/group) on day 14, 21, and 28. All *Listeria*-based vaccines were given at 2×10^8 CFU/mouse except for Lm-LLO-HMWMAA-C (5×10^7 CFU/mouse) and Lm-LLO-FLK-IC1 (2×10^7 CFU/mouse) due to their increased virulence. Tumor size was measured by calipers twice weekly throughout the course of the experiment. Tumor and lung tissue was removed at experimental end on day 46. The tumor tissues were analyzed for CD31 mRNA expression by qPCR, and the lung tissues were analyzed for 4T1-Luc natural lung metastasis by light microscopy and luciferase activity. Tumor volume was calculated as (tumor diameter)^3/2.

FVB/N Her-2/neu-transgenic autochthonous tumor study

The FVB/N Her-2/neu-transgenic mouse expresses the rat HER-2/neu proto-oncogene under the control of the mouse mammary tumor virus (MMTV) promoter as a result of which female mice develop autochthonous mammary tumors between 4 and 9 months of age [35, 40]. Six-week-old FVB/N Her-2/neu-transgenic mice were immunized intraperitoneally every 3 weeks until 21 weeks of age with 2×10^8 CFUs of Lm-LLO-CD105A ($n = 15$), Lm-LLO-CD105B ($n = 15$), and Lm-LLO-NYESO-1 ($n = 15$) as a control vaccine together with untreated ($n = 20$) as a naïve control. After week 21, mice were monitored weekly for appearance of autochthonous mammary tumors.

Hemoglobin assay

FVB/N mice were implanted with 10^6 NT-2 tumor cells mixed with Matrigel (100 µl 10^6 NT-2 cells + 400 µl Matrigel) subcutaneously in the right flank (4 mice/group) and were immunized intraperitoneally on day 7, 14, and 21 with 2×10^8 CFU of Lm-LLO-CD105A, Lm-LLO-CD105B, or Lm-LLO-NYESO-1 as a vaccine control along with a group of untreated mice as a naïve control. Matrigel plugs were removed on day 34 and added to 10 ml of RF10 medium. The resulting cell suspensions were centrifuged, and 1 mL of ACK lysing buffer was added to the pellet and incubated at room temperature for 5 min. The RBC lysates were transferred to microcentrifuge tubes and stored at −80°C. Balb/c mice were implanted with 2×10^5 4T1-Luc cells mixed with Matrigel (100 µl 2×10^5 4T1-Luc cells + 400 µl Matrigel) subcutaneously in the mammary

fat pad. Mice were subsequently immunized intraperitoneally on day 4 and 11 after tumor implantation with 2×10^8 CFU of Lm-LLO-CD105A, Lm-LLO-CD105B, or Lm-LLO-NYESO-1 as a vaccine control along with a group of untreated mice as a naïve control. Matrigel plugs were removed on day 20 and processed as described earlier. Hemoglobin content was measured by the Enzo Hemoglobin Detection Kit according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY).

ELISpot analysis

The 96-well filtration plates (Millipore, Bedford, MA) were coated with 15 μg/ml of rat anti-mouse IFN-γ antibody (clone AN18, MABTECH, Mariemont, OH) in 100 μl of PBS. After overnight incubation at 4°C, the wells were washed and blocked with culture medium containing 10% fetal bovine serum. For Supplemental Figure 1B, CD8-enriched splenocytes were pooled from each experimental group ($n = 3$) and added to wells along with irradiated splenocytes containing either media alone, media with rIL-2 (5 U/mL), or media with rIL-2 and one of the following CD105 peptides at 2 μg/ml: CD105a-1 (DLQPVDPTRGEVTFTTSQVS), CD105a-2 (ETREVFLVLVSNKNVFVKFQ), CD105a-3 (EARKLNASIVTSFVELPL), CD105a-4 (AATKGAITSIAALDDPQSIV), CD105a-5 (EGVSGHKEAYILRILPGSEA). After 78 h of incubation at 37°C and 5% CO_2, remaining cells were transferred to the antibody-coated plates for overnight incubation. Subsequently, the plate was washed followed by incubation with 1 μg/ml biotinylated IFN-γ antibody (clone R4-6A2, MABTECH, Mariemont, OH) in 100 μl PBS at 4°C overnight. After washing, 100 μl of 1:100 streptavidin–horseradish peroxidase in PBS was added and incubated for 1 h at room temperature. Spots were developed by adding 100 μl of substrate after washing and incubating at room temperature for 15 min. Color development was stopped by washing extensively in tap water, and spot-forming cells (SFC) were counted on an ELISpot reader.

Statistical analyses

One-tailed Student's *t*-tests were performed comparing Lm-LLO-CD105A and Lm-LLO-CD105B vaccination to the control Lm vaccination, Lm-LLO-NYESO-1, for all tumor load study, tetramer analysis, luciferase activity, CD31 mRNA expression, and hemoglobin concentration data except Figs. 3 and 4, wherein a two-tailed Student's *t*-test was performed using GraphPad Prism version 4.0a for Macintosh (San Diego, CA, www.graphpad.com). For Fig. 2b, a log rank test was performed to determine significance between control Lm and each of the CD105 vaccines. Significant *P* values for all comparisons are depicted in figures as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and n.s. = not significant.

Results

To determine whether an anti-tumor immune response can be generated against CD105, two relatively small regions of the gene were identified to fuse to our adjuvant protein, truncated LLO, for insertion into the vaccine vector (Fig. 1a, regions included in each vaccine are underlined). The rationale for this strategy was based on previous observations that attenuated *Listeria*-based vectors have difficulty in secreting large recombinant proteins (data not shown). Additionally, initial attempts to construct a CD105 vaccine using a single large fragment of the gene resulted in poor secretion of the recombinant tLLO-CD105 protein (data not shown). The two regions chosen from CD105 to place into the vaccines were selected to include a number of predicted epitopes and to exclude hydrophobic regions of the protein that could impede the ability of the bacterium to secrete the antigen. The region of CD105 in the Lm-LLO-CD105A vaccine contains at least three predicted H-$2K^d$ epitopes. Additionally, the region placed into the Lm-LLO-CD105B vaccine contains at least two predicted H-$2K^d$ epitopes. Western analysis of secreted proteins by each vaccine (Fig. 1b) demonstrated efficient production and secretion of LLO N-terminal-containing proteins at the proper sizes for tLLO-CD105A and tLLO-CD105B. However, while cultures contained equivalent CFUs of each attenuated *Listeria*-based vaccine (data not shown), there is relatively more secretion of antigen by Lm-LLO-CD105A in comparison with Lm-LLO-CD105B possibly due to increased hydrophobicity of the tLLO-CD105B fragment (Fig. 1b). These results taken in total confirm that Lm-LLO-CD105A and Lm-LLO-CD105B each efficiently produce and secrete their respective recombinant fusion proteins. Efficient secretion of the recombinant antigenic proteins from attenuated *Listeria* vaccines is important for inducing the CTL-mediated responses vital to anti-tumor immunity [41].

In order to test the efficacy of our CD105 vaccines in controlling tumor growth, each vaccine has been tested in three separate models for mouse breast cancer. The first tumor model is the implantable tumor cell line NT-2 derived from the Her-2/neu-transgenic mouse [36]. After implantation into the hind flank of syngeneic FVB/N mice, mice were subsequently vaccinated followed by 2-weekly boosts with each CD105 vaccine or a control vaccine and tumor volume monitored throughout. As seen in Fig. 2a, vaccination with either CD105 vaccine resulted in primary tumor stasis and a significantly smaller tumor volume at experiment end in comparison with control Lm vaccination. The second mouse breast tumor model used in our study

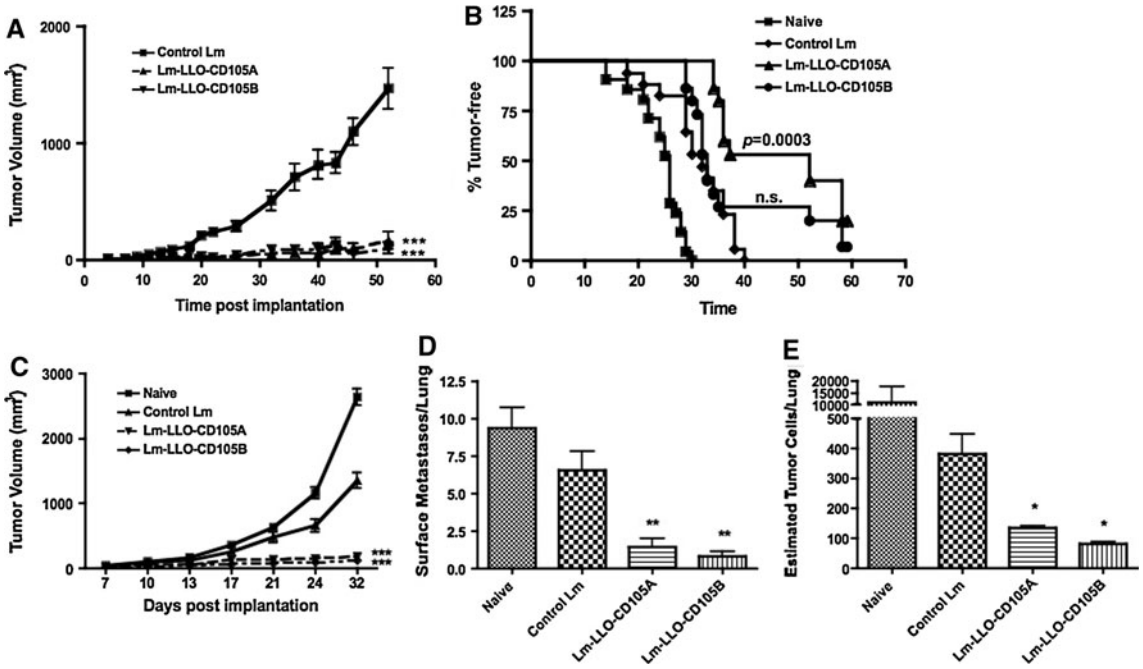

Fig. 2 Therapeutic impact on primary and metastatic breast tumors by CD105 vaccines. **a** Tumor load study demonstrating significant therapeutic efficacy against implanted NT-2 mouse mammary tumor cells by *Listeria*-based CD105 vaccines in comparison with control vaccination. **b** Prevention of spontaneous Her-2/neu-transgenic mammary tumor formation by Lm-LLO-CD105A and Lm-LLO-CD105B vaccination. Percent of tumor-free Her-2/neu-transgenic mice per treatment group ($n = 15$) is depicted up to week 58 after birth. **c** Tumor load study demonstrating significant therapeutic efficacy against implanted 4T1-Luc mouse mammary tumor cells by *Listeria*-based CD105 vaccines in comparison with control vaccination. **d** Significant reduction in 4T1-Luc lung surface metastases after vaccination by *Listeria*-based CD105 vaccines in comparison with control vaccination. **e** Reduced luciferase activity in the lungs of 4T1-Luc tumor-bearing mice after vaccination with Lm-LLO-CD105A and Lm-CD105B. Number of 4T1-Luc tumor cells in each lung was estimated against a standard curve of luciferase activity with lysate from 4T1-Luc cells grown in culture

utilizes a Her-2/neu-transgenic mouse, wherein the neu oncogene is transcriptionally expressed under the MMTV promoter [35]. In this model, mice are vaccinated every 3 weeks starting at week 6 and ending at week 21 and subsequently monitored for tumor incidence thereafter. While all of the unvaccinated naïve mice and control-vaccinated mice developed autochthonous tumors by week 40, each group of mice vaccinated with Lm-LLO-CD105A and Lm-LLO-CD105B contained tumor-free mice at week 58, 20, and 6.7%, respectively (Fig. 2b). In fact, Lm-LLO-CD105A vaccination significantly increased median time to tumor incidence in comparison with control Lm vaccination, 52 days versus 32 days, respectively ($P = 0.0003$). The third mouse breast tumor model used in this study is the aggressive 4T1-Luc tumor cell line. Unlike NT-2, if implanted in the mammary tissue, 4T1-Luc will naturally metastasize to organs such as the lungs, spleen, and brain with noticeable metastases present within 3 weeks post-implantation [37, 38]. Lm-LLO-CD105A and B both significantly control 4T1-Luc primary tumor growth as compared with unvaccinated and control-vaccinated mice (Fig. 2c). Additionally, the number of visible surface metastases on the lungs of Lm-LLO-CD105A and B is significantly reduced as compared with each control at the termination of the experiment on day 32 post-tumor implantation (Fig. 2d). As an additional measure of 4T1-Luc metastases in the lungs of vaccinated mice, luciferase activity was also measured in lung lysates. Luciferase activity in the lungs of CD105-vaccinated mice was significantly reduced in comparison with control mice suggesting reduced metastases in that organ (Fig. 2e). These results demonstrate that *Listeria*-based vaccines targeting CD105 can be highly effective immunotherapeutics in controlling primary as well as metastatic breast tumor burden.

To assess whether CD105 is a superior target for anti-vasculature tumor immunotherapy, we compared the effectiveness of each Lm-LLO-CD105 vaccine against previously characterized anti-vasculature *Listeria*-based vaccines. The previously characterized anti-angiogenesis vaccines target either the pericyte-specific protein HMW-MAA, Lm-LLO-HMWMAA-C, or two regions of the vascular endothelial growth factor receptor 2, Lm-LLO-FLK-I1 and Lm-LLO-FLK-E2 [6, 7]. Tumor load studies were performed with each of the implanted mouse breast tumor models, NT-2 and 4T1-Luc, and the efficacy of each vaccine determined by their ability to control growth of established primary tumors and limit metastatic spread. While each of the previous anti-angiogenesis vaccines is more

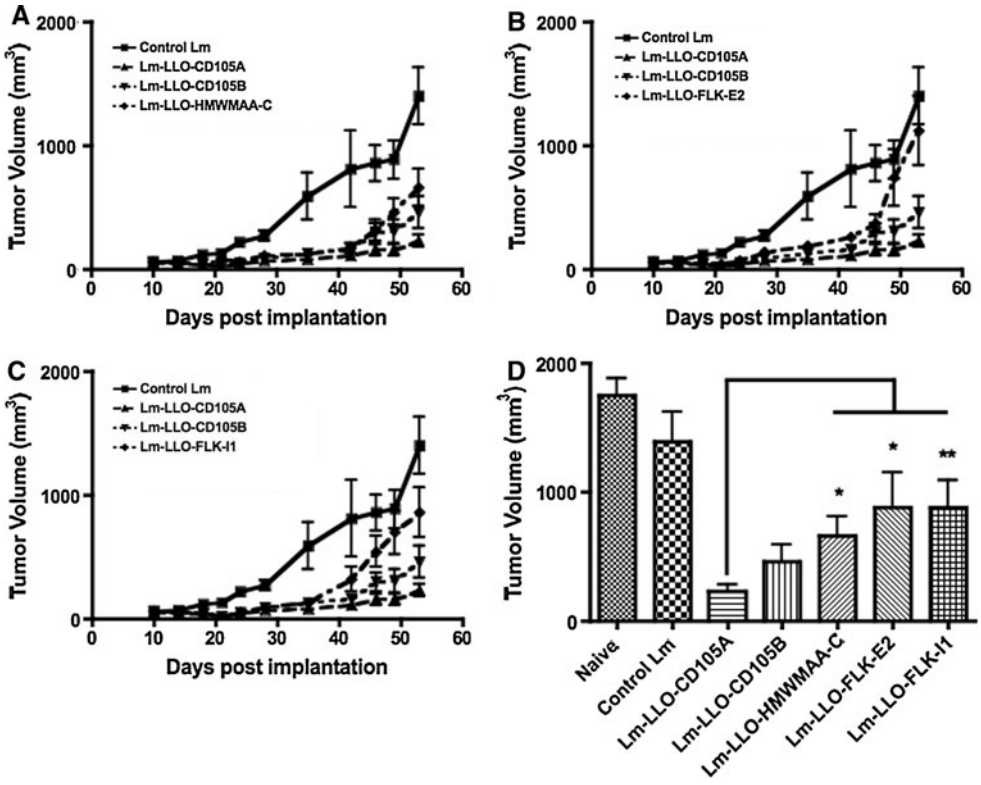

Fig. 3 Comparison of *Listeria*-based anti-angiogenesis vaccines for therapeutic efficacy against NT-2 primary tumors. To determine the efficacy of *Listeria*-based CD105 vaccines in comparison with previous *Listeria*-based anti-angiogenesis vaccines, mice were implanted with NT-2 tumor cells and subsequently vaccinated with each of the vaccines**.** Comparative tumor load study demonstrating therapeutic efficacy against implanted NT-2 primary tumors after vaccination with control Lm, Lm-LLO-CD105A, Lm-LLO-CD105B, and **a** Lm-LLO-HMWMAA-C, **b** Lm-LLO-FLK-E2, or **c** Lm-LLO-FLK-I1. The experiment is depicted in three sections to facilitate visual comparisons between each vaccine in comparison with Lm-LLO-CD105A and Lm-LLO-CD105B. **d** NT-2 primary tumor volume at the conclusion of experiment depicted in **a**, **b**, and **c**

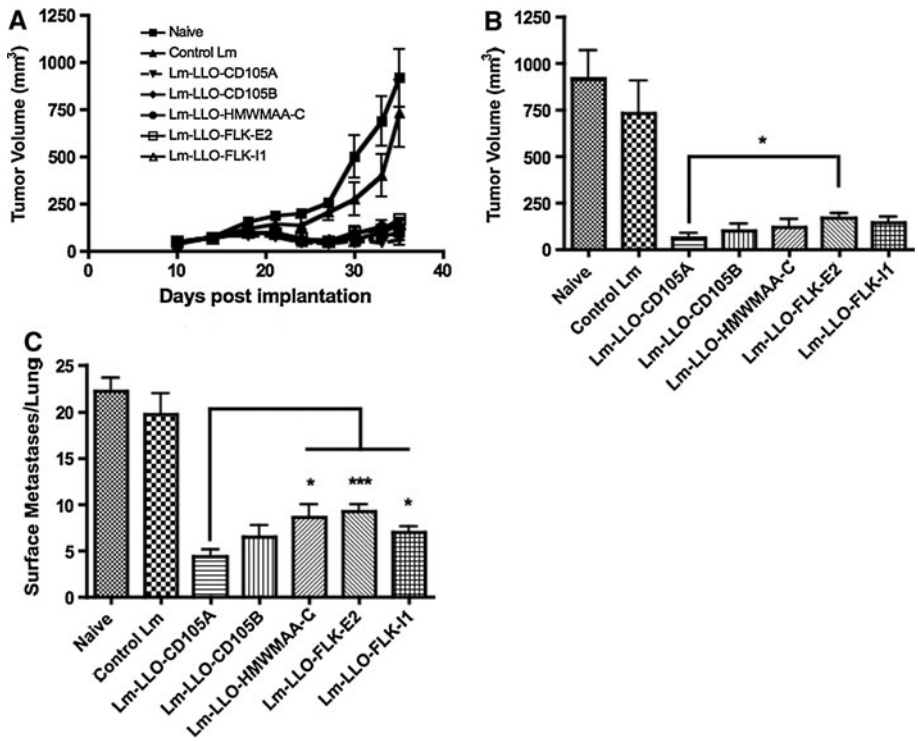

Fig. 4 Comparison of *Listeria*-based anti-angiogenesis vaccines for therapeutic efficacy against 4T1-Luc primary and metastatic tumors. **a** Comparative tumor load study demonstrating tumor volume of mammary tissue implanted 4T1-Luc tumors after vaccination with control Lm, Lm-LLO-CD105A, Lm-LLO-CD105B, Lm-LLO-HMWMAA-C, Lm-LLO-FLK-E2, and Lm-LLO-FLK-I1. **b** 4T1-Luc primary tumor volume at the conclusion of experiment depicted in **a**. **c** 4T1-Luc lung surface metastases after vaccination by *Listeria*-based anti-angiogenesis vaccines

effective than control vaccination at reducing the growth of implanted NT-2 tumors, both vaccines targeting CD105 appear to be at least as effective therapeutically (Fig. 3a, b, c). In fact, Lm-LLO-CD105A vaccination performed significantly better at reducing tumor volume at experiment end on day 53 versus the other anti-angiogenesis vaccines, Lm-LLO-HMWMAA-C ($P = 0.0156$), Lm-LLO-FLK-E2 ($P = 0.0255$), and Lm-LLO-FLK-I1 ($P = 0.008$) (Fig. 3d). Similar results are obtained for each of these vaccines when compared in the 4T1-Luc tumor model (Fig. 4a). After

allowing for establishment of 4T1-Luc primary tumors in the mammary tissue and subsequent vaccinations, both CD105 vaccines perform just as effectively at reducing primary tumor volume at experiment end as previous vaccines and, in comparison with Lm-LLO-FLK-E2, Lm-LLO-CD105A was significantly more effective ($P = 0.015$) (Fig. 4b). While the control Lm vaccine significantly reduced tumor volume against less established 4T1-Luc tumors (Fig. 2c, $P < 0.0001$) as expected based on previously published findings [42], no significant impact was found against more established tumors (Fig. 4b). In addition to tumor volume, Lm-LLO-CD105A vaccination was also significantly superior to each of the previous anti-angiogenesis vaccines at reducing the number of lung surface metastases in 4T1-Luc tumor-implanted mice (Fig. 4c). These results suggest that each *Listeria*-based CD105 vaccine is just as effective if not superior to previous *Listeria*-based anti-angiogenesis vaccines at reducing primary and metastatic tumor burden in mouse models of breast cancer.

Due to the superior anti-tumor efficacy of Lm-LLO-CD105A, a preliminary analysis was performed to determine the generation of a CD105-specific immune response by Lm-LLO-CD105A. Regions with predicted CTL epitopes in the fragment contained within Lm-LLO-CD105A were synthesized as 20-mer peptides (Supplemental Fig. 1a). Pooled splenocytes from either Lm-LLO-CD105A-vaccinated mice or naive mice were enriched for $CD8^+$ T cells and co-cultured with irradiated splenocytes loaded with the 20-mer CD105 peptides, cd105a-1 though 5, and then assayed for IFN-γ production by ELISpot analysis after 78 h. Stimulation with two of the peptides, cd105a-4 and cd105a-5, along with the positive control, Listeriolysin O (LLO) H-$2K^d$ CTL epitope peptide, resulted in an increase in IFN-γ producing spot-forming cells only in the splenocytes from Lm-LLO-CD105A-vaccinated mice (Supplemental Fig. 1b). While this suggests that vaccination with Lm-LLO-CD105A can generate CD105-specific immune responses, further work will be needed in order to confirm and characterize the specific CTL epitope(s) involved.

As previous work has demonstrated, effective immunotherapy targeting the tumor vasculature is dependent upon epitope spreading [6]. Epitope spreading is a phenomenon wherein processing of dead tumor cells leads to secondary tumor-specific $CD8^+$ T cell responses. To determine whether Lm-LLO-CD105A and Lm-LLO-CD105B are inducing epitope spreading in tumor-bearing mice, tetramer analysis was performed by flow cytometry to inspect mice for stimulation of $CD8^+$ T cells specific for two endogenous breast tumor antigens, Her-2/neu and gp70. Gp70 is an endogenous ecotropic murine leukemia provirus gene product that is expressed on a wide variety of murine tumors [43 and Beatty and Paterson, unpublished observations]. In the spleens of NT-2 tumor-bearing CD105-vaccinated mice, significant stimulation of CTLs specific for two separate epitopes of Her-2/neu, EC1 and EC2, was observed in comparison with naïve and control-vaccinated mice (Fig. 5a). Similarly, inspection of NT-2 tumor-infiltrating CD8 + T cells demonstrates significant increases in the percentage of CTLs specific for three separate epitopes of Her-2/neu in comparison with control mice (Fig. 5b). The same study was performed in 4T1-Luc tumor-bearing mice after vaccination. While the spleens of CD105-vaccinated mice only show significant increases in the percentage of $CD8^+$ T cells specific for one epitope of Her-2/neu (Fig. 5c), the tumors of these mice have significantly increased percentages of $CD8^+$ T cells specific for of all three Her-2/neu epitopes inspected and for gp70 (Fig. 5d). Taken together these results demonstrates that Lm-LLO-CD105A and B can significantly stimulate epitope spreading to endogenous tumor antigens that may partially explain their effectiveness in each of these tumor models.

To determine whether the anti-tumor efficacy of each of our CD105 vaccines is due to the ability of each vaccine to disrupt tumor vasculature, expression of the vascular endothelium-specific protein, CD31, was assayed as a determination of vascular coverage [44]. After implantation and subsequent vaccination, NT-2 and 4T1-Luc tumors were excised and total tumor mRNA purified. After cDNA conversion, qPCR analysis was performed to determine the impact of each vaccine on tumor-specific expression of CD31. In comparison with control vaccination, Lm-LLO-CD105A and B each significantly reduced tumor-specific expression of CD31 in both the NT-2 (Fig. 6a) and the 4T1-Luc (Fig. 6b) tumor models. As an additional measure of tumor vascularization, the hemoglobin concentration of tumor red blood cell lysate was assayed. In the NT-2 tumor model, Lm-LLO-CD105A vaccination resulted in significant reduction in the concentration of tumor-specific hemoglobin while vaccination with Lm-LLO-CD105B demonstrated a trend toward a significant reduction ($P = 0.07$) (Fig. 6c). Similarly, in the 4T1-Luc tumor model, each CD105 vaccine significantly reduced tumor-specific hemoglobin concentration in comparison with control vaccination (Fig. 6d). The significant reduction in expression of CD31 expression and reduced concentration of tumor-specific hemoglobin concentration suggest that each CD105 vaccine effectively targets tumor neovascularization, likely resulting in their significant efficacy against primary and metastatic tumors.

Discussion

Numerous studies confirm that high expression of CD105 is associated with tumor neovascularization [29–31, 45, 46].

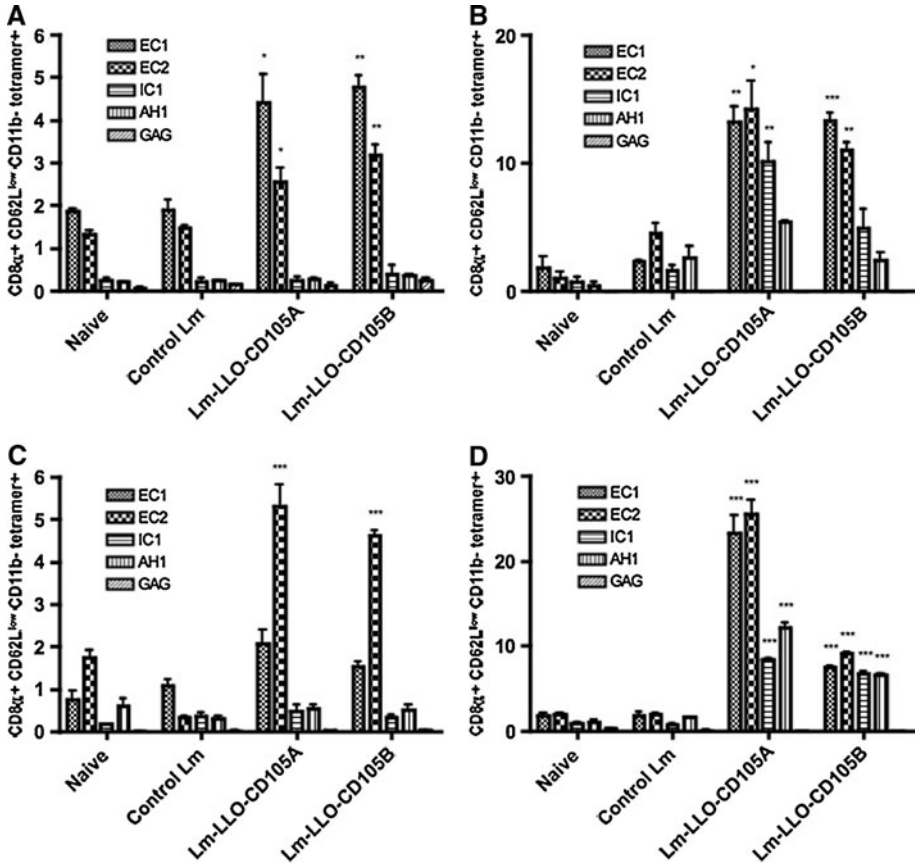

Fig. 5 Induction of $CD8^+$ T cell responses to endogenous tumor antigens. After vaccination with control or CD105 vaccines, splenocytes and tumor-infiltrating lymphocytes were isolated and assayed for $CD8^+CD62L^-CD11b^-$ lymphocytes specific for epitopes of Her-2/neu (EC1, EC2, and IC1), gp70 (AH1), or HIV (GAG) as a control by tetramer analysis. Results are depicted as percent of lymphocytes. **a** Her-2/neu-specific $CD8^+$ lymphocytes in the spleens of CD105-vaccinated NT-2 tumor-bearing FVB/N mice. **b** Her-2/neu-specific $CD8^+$ tumor-infiltrating lymphocytes in CD105-vaccinated NT-2 tumor-bearing FVB/N mice. **c** Her-2/neu-specific $CD8^+$ lymphocytes in the spleens of CD105-vaccinated 4T1-Luc tumor-bearing Balb/c mice. **d** Her-2/neu-specific and gp70-specific $CD8^+$ tumor-infiltrating lymphocytes in CD105-vaccinated 4T1-Luc tumor-bearing Balb/c mice

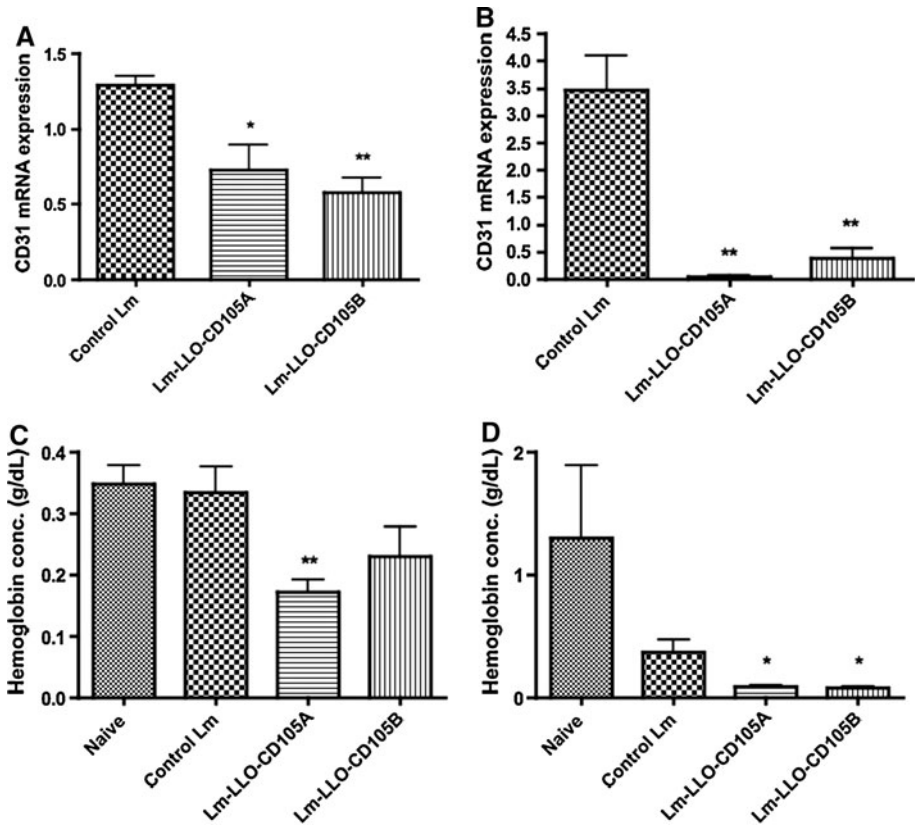

Fig. 6 Reduced tumor vascularity after CD105 vaccination. Vascularity of primary tumors after *Listeria*-based CD105 vaccines was determined by tumor-specific expression of the vascular endothelium marker, CD31, and total hemoglobin concentration of tumors. Significant reduction in CD31 mRNA expression after Lm-LLO-CD105A and Lm-LLO-CD105B vaccination in comparison with control vaccination in **a** NT-2 primary tumors and **b** 4T1-Luc primary tumors. **c** Significant reduction in tumor-specific hemoglobin concentration after Lm-LLO-CD105A vaccination and reduced hemoglobin concentration after Lm-LLO-CD105B vaccination ($P = 0.07$) in NT-2 primary tumors. **d** Significant reduction in tumor-specific hemoglobin concentration after Lm-LLO-CD105A and Lm-LLO-CD105B vaccination in 4T1-Luc primary tumors as compared with control vaccination

While there have been attempts to target the elevated expression of CD105 found in tumor vasculature, a CTL-mediated approach using *Listeria*-based vaccines could be one of the most effective strategies [10, 32, 33]. After optimizing the vaccines for proficient expression and secretion of recombinant CD105 fusion proteins, each of the vaccines significantly inhibited growth of implanted primary tumors and significantly limited metastatic spread, while Lm-LLO-CD105A significantly delayed time to occurrence of autochthonous mammary tumors (Figs. 1, 2). The relatively higher efficacy of Lm-LLO-CD105A compared with Lm-LLO-CD105B in several assays (Figs. 2b, 4b, c, 6c) may be explained by the more proficient secretion of antigen by Lm-LLO-CD105A (Fig. 1b). Additionally, with regard to reducing primary tumor growth, *Listeria*-based vaccines targeting CD105 expression on tumor vasculature are either equal or superior to vaccines targeting other tumor vasculature antigens (Fig. 3). The results presented in this manuscript suggest that targeting elevated expression of CD105 on tumor vasculature with *Listeria*-based vectors results in significant anti-tumor immune responses and significant reductions in established primary and metastatic tumor growth.

Previous efforts to target elevated CD105 expression on tumor vasculature have resulted in favorable outcomes [10, 32–34]. Several studies employed a passive immunotherapy approach by conjugating anti-CD105 mAbs to either a toxin, deglycosylated ricin A-chain (dgRA), or a radioactive isotope, ^{125}I [32, 33]. Each of these methods resulted in significant growth suppression of tumors, however, if translated to the clinic, would likely require indefinite injections due to the absence of adaptive immune responses. Alternatively, a DNA-based vaccine strategy was employed by Lee et al. [10] to target tumor vasculature for killing by CTLs. In this strategy, a plasmid encoding for CD105 is delivered to lymphoid organs by an attenuated Salmonella typhimurium vector. Prophylactic vaccination with this DNA-based CD105 vaccine led to $CD8^+$ T cell-dependent responses that resulted in reduced tumor growth and reduced metastases after tumor challenge. However, our approach has several advantages over these prior studies. While a similar approach was taken by Lee et al. to stimulate the induction of CD105-specific CTL responses, we have taken the more clinically relevant and challenging approach of treating already established tumors in a therapeutic setting. While established tumors (>125 mm^3) are known to reside in a highly immunosuppressive microenvironment, complete tumor regression was observed in 37.5% of Lm-LLO-CD105A and 25% of Lm-LLO-CD105B-vaccinated mice at experiment end (Fig. 3a, c) [47, 48]. A great deal of this effectiveness is likely due to the ability of our vector, attenuated *Listeria*, to break tolerance and elicit a robust CTL response to *Listerial*-secreted recombinant proteins [49].

As with other anti-angiogenic therapies, there is an inherent safety concern regarding off-target complications in healthy tissues [50, 51]. However, this is of less concern when targeting endoglin compared with other vascular markers since whole animal imaging studies using radiolabeled anti-CD105 mAbs have demonstrated that expression of CD105 is specific for vasculature associated with tumors [46]. This tumor-specific expression of CD105 may be because vascular endothelial cells weakly express CD105, while vascular endothelial cells undergoing proliferation highly express CD105 [29–31, 45, 46]. In addition to tumor-specific expression of CD105, anti-angiogenesis therapies have a greater impact on tumor vasculature because of the disorganized structure of rapid neovascularization [52]. In support of this argument, multiple intravenous injections of non-human primates with monoclonal antibody targeting CD105 had no detrimental impact on animal health [53]. In fact, previous anti-angiogenesis *Listeria*-based vaccines have demonstrated no significant impact on pregnancy or wound healing, which are two normal processes that are highly dependent on neovascularization [6, 7]. While safety issues are always a concern before and during clinical testing, we believe the evidence suggests it is reasonable to expect few complications from immunotherapy targeting CD105.

As demonstrated in this manuscript, *Listeria*-based vaccines targeting CD105 limit the growth of established and metastatic breast tumors. Additionally, vaccination against CD105 with our *Listeria*-based vaccines resulted in the stimulation of tumor-specific CTLs that infiltrated tumors and likely facilitated the observed anti-tumor responses and reduction in tumor vascularization. Translation of these results into the clinic may require some modification to the route of vaccine administration, as intravenous injection may be more acceptable than intraperitoneal injection. Previous studies, however, demonstrate that *Listeria*-based vaccines are still highly effective when administered intravenously in both preclinical and clinical settings of cancer [54, 55]. Therefore, vaccination with *Listeria*-based CD105 vaccines may be a promising novel strategy to target neovascularization in tumor immunotherapy.

Conflict of interest Yvonne Paterson wishes to disclose that she has a financial interest in Advaxis, a vaccine and therapeutic company that has licensed or has an option to license all patents from the University of Pennsylvania that concern the use of *Listeria monocytogenes* or Listerial products as vaccines.

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