Cancer Immunotherapy Targeting the High Molecular Weight Melanoma-Associated Antigen Protein Results in a Broad Antitumor Response and Reduction of Pericytes in the Tumor Vasculature

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Abstract

The high molecular weight melanoma-associated antigen (HMW-MAA), also known as melanoma chondroitin sulfate proteoglycan, has been used as a target for the immunotherapy of melanoma. This antigen is expressed on the cell surface and has a restricted distribution in normal tissues. Besides its expression in a broad range of transformed cells, this antigen is also found in pericytes, which are important for tumor angiogenesis. We generated a recombinant Listeria monocytogenes (Lm-LLO-HMW-MAA) that expresses and secretes a fragment of HMW-MAA (residues 2,160–2,258) fused to the first 441 residues of the listeriolysin O (LLO) protein. Immunization with Lm-LLO-HMW-MAA-C was able to impede the tumor growth of early established B16F10-HMW-MAA tumors in mice and both CD4+ and CD8+ T cells were required for therapeutic efficacy. Immune responses to a known HLA-A2 epitope present in the HMW-MAA2160-2258 fragment were detected in the HLA-A2/Kb transgenic mice immunized with Lm-LLO-HMW-MAA-C. Surprisingly, this vaccine also significantly impaired the in vivo growth of other tumorigenic cell lines, such as melanoma, renal carcinoma, and breast tumors, which were not engineered to express HMW-MAA. One hypothesis is that the vaccine could be targeting pericytes, which are important for tumor angiogenesis. In a breast tumor model, immunization with Lm-LLO-HMW-MAA-C caused CD8+ T-cell infiltration in the tumor stroma and a significant decrease in the number of pericytes in the tumor blood vessels. In conclusion, a Lm-based vaccine against HMW-MAA can trigger cell-mediated immune responses to this antigen that can target not only tumor cells but also pericytes in the tumor vasculature. [Cancer Res 2008;68(19):8066–75]

Introduction

The human high molecular weight melanoma-associated antigen (HMW-MAA) is a membrane-bound protein of 2,322 residues, also known as melanoma chondroitin sulfate proteoglycan, which is overexpressed on >90% of the surgically removed benign nevi and melanoma lesions (1). Besides tumors of melanocytic origin, HMW-MAA has also been found to be expressed by basal cell carcinoma, tumors of neural crest origin (astrocytomas, gliomas, neuroblastomas, and sarcomas), some forms of childhood leukemias, and lobular breast carcinoma lesions. In vitro experimental data show that HMW-MAA is involved in the adhesion, spreading, and migration of melanoma cells and may have a role in cell invasion and metastasis (1, 2). Nevertheless, HMW-MAA has a restricted expression in normal tissues, although it is expressed at high levels on both activated pericytes and pericytes in tumor angiogenic vasculature, which are associated with neovascularization in vivo (3, 4). A murine homologue of HMW-MAA, known as AN2, has 80% homology to HMW-MAA, besides a similar expression pattern and function (5).

Because of its cell surface localization, immunotherapies for this antigen have focused on inducing a humoral response against HMW-MAA. In a phase I/II clinical trial, the development of anti-HMW-MAA humoral response elicited by active immunization was associated with prolonged survival of patients with melanoma (6). In contrast, despite the well-established role of cell-mediated immune responses in tumor immunotherapy, limited information is available about the generation of T-cell immunity against HMW-MAA. Recently, Peng and colleagues (7) showed that antigen-specific CTLs against HMW-MAA can be generated in mice in a CD4-dependent manner. Nevertheless, the role of cell-mediated immune responses against HMW-MAA in affecting the in vivo growth of tumorigenic cells has not yet been investigated.

Listeria monocytogenes (Lm) is a Gram-positive facultative intracellular bacterium that has been extensively used as a vaccine vector for several diseases. Mouse models have shown that recombinant Lm expressing tumor-associated antigens (TAA) is a potential therapeutic approach able to eradicate established malignant tumors (8–11). Lm is able to infect phagocytic and antigen-presenting cells (APC), such as macrophages and dendritic cells, where it can access the cytosolic compartment after lysing the phagolysosome membrane (12). In the cytosol, the bacterium replicates and spreads to neighboring cells (12). This unusual intracellular life cycle allows antigens secreted by Lm to be processed and presented in the context of both MHC class I and II molecules, resulting in strong CD8+ and CD4+ T-cell–mediated immune responses (13). The listeriolysin O (LLO) protein is the major virulence factor of Lm responsible for the lysis of the phagolysosome. LLO, which is highly immunogenic (14), induces maturation of antigen-specific T cells into Th1 cells (15) and IFN-γ secretion by T cells (16, 17). Our group has previously shown that genetically fusing an antigen to a nonhemolytic form of LLO enhances the immunogenicity of TAAAs compared with the antigen expressed alone in the same system, resulting in a better therapeutic efficacy against established tumors (9, 10, 18, 19).
Here, we describe the therapeutic and immunologic properties of a recombinant Lm-based vaccine (Lm-LLO-HMW-MAA-C) that secretes a fragment of HMW-HMAA fused to LLO. We show that this vaccine is able to eradicate established B16F10 tumors engineered to express HMW-MAA. This therapeutic effect is dependent on both CD4+ and CD8+ T cells. Further, using the HLA-A2/Kb transgenic mouse, we detected HMW-MAA–specific responses induced by the vaccine against an epitope previously shown to be restricted to the HLA-A2 molecule (7). Interestingly, we show that this vaccine can also significantly delay the growth of tumors that do not express the HMW-MAA antigen or its homologue AN2. This effect on tumor growth is associated with infiltration of the tumor stroma by CD8+ T cells and a significant reduction in the pericyte coverage in the tumor vasculature.

Materials and Methods

Mice. Female C57BL/6, BALB/c, and FVB/N mice were purchased from Charles River Laboratories. A breeder pair of HLA-A2/Kb was generously provided by Dr. L. Sherman (The Scripps Research Institute, La Jolla, CA). These mice were maintained and bred at the Veterans’ Administration Hospital affiliated with the University of Pennsylvania. The FVB/N HER-2/neu transgenic mice (20) were housed and bred at the Veteran’s Administration Hospital affiliated with the University of Pennsylvania. Mice were 6 to 8 wk old when used at the start of the experiments, which were done in accordance with regulations by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Antibodies. The anti-HMW-MAA monoclonal antibody (mAb) VT80.12 has been previously described (21). Goat anti-mouse IgG-PE, anti-CD8b.2-FITC, anti-IFN-γ-PE, anti-mouse CD31-FITC, anti-mouse CD8c-PE, rat IgG2a, and rat IgG2b isotype controls were purchased from BD Biosciences. Anti-α-smooth muscle actin (aSMA)-Cy3 and anti-Flag M2 mAbs were purchased from Sigma. Secondary anti-rabbit Alexa Fluor 488 was purchased from Invitrogen and anti-rat NG2 from Chemicon. The anti-CD4 mAb GK1.5, anti-CD8 mAb 2.43, and anti-CD25 mAb PC61 were purchased from Invitrogen and anti-rat NG2 from Chemicon. The anti-CD4 mAb VT80.12, anti-CD8 mAb 2.43, and anti-CD25 mAb PC61 were purified using protein G-Sepharose columns (Amersham Biosciences).

Flow cytometry. Cells were harvested and washed in fluorescence-activated cell sorting (FACS) buffer [PBS-2% fetal bovine serum (FBS)] and Fc receptors were blocked with 2.4G2 hybridoma supernatant. After washing, cells were resuspended in 50 μL of FACS buffer containing the appropriate antibodies and incubated at 4°C in the dark for 30 min. Cells were washed twice and, when necessary, incubated with a secondary antibody. Otherwise, cells were fixed in 2% formaldehyde and analyzed using a FACS caliber cytometer and CellQuest pro software (BD Biosciences).

Cell lines. Cell culture medium and supplements were purchased from Life Technologies (Invitrogen). B16F10 cells were maintained in DME; REpCA and J774 cells in RPMI 1640. Medium was supplemented with 10% FBS, 10 mmol/L HEPES buffer, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, and 50 μg/mL gentamicin. The NT-2 cell line was maintained as previously described (10). All cell cultures were kept at 37°C and 5% CO2. B16F10 cells were transfected with pcDNA3.1+ plasmid containing the full-length HMW-MAA cDNA sequence (7), using Lipofectamine 2000 (Invitrogen), as recommended by the manufacturer. Stable transfected cells were maintained in medium supplemented with 1 mg/mL G418, and single clones were isolated using limiting dilution. Individual clones were screened for HMW-MAA expression by flow cytometry using the mAb VT80.12.

Construction of Lm-LLO-HMW-MAA-C vaccine. A fragment corresponding to HMW-MAA residues 2,160 to 2,258 was amplified by PCR using the primers 5′-TGGCTCGAGGCCACCTGGGCTTTAATGCTGGC-3′ (forward primer; XhoI site underlined) and 5′-CCCGGTTTACCTTGCCATCATTCTCGGATATCCGAGGAGTCATGCCCGG-3′ (reverse primer; XmaI site underlined, stop codon in bold, and Flag sequence in italics). The PCR product was ligated into pCR2.1-TOPO plasmid (Invitrogen), confirmed by sequencing, and subsequently excised by double digestion with XhoI and XmaI (New England Biolabs). The fragment was ligated into a pGGS5-based plasmid downstream and fused to a gene encoding for the first 441 residues of the LLO protein, whose expression is driven by the hly promoter. The construction of the pGGS5 has been described in detail elsewhere (9). The resultant plasmid was electroporated into the PrfA-defective Lm strain XFL-7 (kindly provided by Hao Shen, University of Pennsylvania, Philadelphia, PA), which is derived from the Lm strain 1040S. Positive clones were selected on brain heart infusion (BHI; Difco) plates supplemented with 34 μg/mL chloramphenicol and 250 μg/mL streptomycin. The resultant strain was named Lm-LLO-HMW-MAA-C, which was subsequently passaged twice in vivo as previously described (22).

Effect of Lm-LLO-HMW-MAA-C on tumor growth. Mice were given s.c. 2 × 106 of B16F10-HMW-MAA, B16F10, or RENCA tumor cells on the flank in 0.2 mL PBS. On day 3 after tumor inoculation, mice were immunized i.p. with 2.5 × 107 colony-forming units of Lm-LLO-HMW-MAA-C. This dose was determined as one tenth of the minimum dose observed to have adverse effects on mice and it was used in all experiments. In the NT-2 model, mice were given 1 × 106 tumor cells and immunized 7 d later with Lm-LLO-HMW-MAA-C. Immunizations were repeated weekly, totaling three doses of the vaccine in all experiments. In the control groups, mice received either PBS or an equivalent dose of an irrelevant Lm vaccine (Lm-LLO-E7 or Lm-LLO-NT-ESO-1) i.o. (20). Tumors were measured every 2 d with calipers, and the shortest and longest surface diameters were recorded for each individual tumor. Mice were sacrificed when they developed open wounds or tumors reached 20 mm in diameter. Tumor-free surviving mice challenged with B16F10-HMW-MAA were rechallenged in the opposite flank with the same cell line 7 wk after the first inoculation. The FVB/N HER-2/neu transgenic mice were immunized every 3 wk with either an irrelevant Lm vaccine or Lm-LLO-HMW-MAA-C, totaling six doses, starting vaccination when mice were 6 wk old with the last dose given at age 21 wk. These mice were observed weekly for the appearance of mammary tumors.

In vivo cell depletions. For CD4 and CD8 in vivo depletions, 500 μg of GK1.5 and 2.43 antibodies, respectively, were given i.p. on days 1, 2, 6, and 9. The control groups either received an anti-β2-galactosidase antibody or were left untreated. For CD25 depletion, 500 μg PC61 was given i.p. on days 0 and 2. These antibodies were tested and confirmed to induce depletion of the target cells by flow cytometry (data not shown).

Transfer of antitumor immunity (Winn assay). C57BL/6 mice were injected twice at a 1-wk interval with Lm-LLO-HMW-MAA-C. Control mice were left untreated. One week after the last immunization, mice were sacrificed and the spleens were harvested. CD8+ T cells were enriched from the splenocyte suspension by negative magnetic separation (Dynal Mouse CD8 Cell Negative Isolation Kit, Invitrogen) and comprised 85% of the total cells as assessed by flow cytometry. CD8+ T cells either naive or Lm-LLO-HMW-MAA-C–immunized mice were mixed in PBS with B16F10-HMW-MAA at a ratio of 10:1 and 0.2 mL of the cell suspension, containing 2 × 106 tumor cells and 2 × 105 CD8+ T cells, was inoculated s.c. on the flank of naive mice. Tumors were measured every 2 d with a caliper and the size was recorded as the mean tumor diameter.

Synthetic peptide. The HLA-A2–binding synthetic peptide LILPLFLYI, which corresponds to HMW-MAA residues 2,238 to 2,246, was purchased from EZBiolog.

Murine IFN-γ assays to detect antigen-specific CD8+ T cells. Spleens from immunized mice were harvested 1 wk after the last immunization. After lysing BSCs, splenocytes were stimulated with 1 μmol/L of the HMW-MAA2238-2246 peptide and IFN-γ production was detected by either enzyme-linked immunospot (ELISpot) or intracellular cytokine staining. ELISpots were performed according to the manufacturer's instructions (Mabtech) and spot-forming cells (SFC) were counted using a dissecting microscope. Intracellular cytokine staining for IFN-γ was done as previously described (22). Data were collected using a FACS caliber cytometer and analyzed using CellQuest Pro software. Cells were gated on CD8+ and analyzed for intracellular IFN-γ.

Immunofluorescence. On day 84 after tumor inoculation, mice were sacrificed and the NT-2 tumors were surgically excised, cryopreserved in OCT freezing medium, and cryosectioned for 8- to 10-μm-thick sections. For immunofluorescence, samples were thawed and fixed using 4% formalin. After blocking (2.4G2 conditioned medium/10% FBS/5% rat serum and...
mouse serum), sections were stained with primary antibodies in blocking solution in a humidified chamber at 37 °C for 1 h. Samples were stained with secondary antibody following the same procedure as for primary staining. 4,6-Diamidino-2-phenylindole (DAPI; Invitrogen) staining was performed according to the manufacturer's instructions. Intracellular stains (uSMA) were done in PBS/0.1% Tween/1% bovine serum albumin solution. Slides were coveredslipped using Biomedia mounting solution with antifading agents, set for 24 h, and kept at 4 °C until imaging using Spot Image Software (2006) and Olympus BX51 series fluorescent microscope. Images were merged using Spot Image Software and quantification was performed after a region of interest was gated using Image Pro Software (2006). All images are a merged series of three different channels captured for the same exposure time.

Evaluation of possible toxicity associated with inhibition of angiogenesis. Six- to 8-wk-old FVB/N female mice were immunized three consecutive times weekly with either a control Lm vaccine or Lm-LLO-HMW-MAA-C. On the fourth week, safety studies were conducted. For pregnancy and fertility, five mice per group were allowed to mate with individual housed males. Coitus was monitored and confirmed by the presence of a vaginal plug. Time to gestation, pup weight at birth, and total litter size were measured. The wound-healing assay used in this study was done according to previously described methods used in antiangiogenesis studies (23). Briefly, mice were anesthetized, hair was removed, and skin was cleaned with an aseptic wipe. Two circular wounds, 3 mm in diameter, were punched from the skin using a sterile skin biopsy tool (Acuderm). Wounds were not treated and no infection was observed. Average time to wound closure was monitored and considered complete when a scar was formed without any visible scar left.

Statistical analysis. Data were analyzed using either the nonparametric Mann-Whitney test or the parametric t test when appropriate. The log-rank test was used for survival data. All statistical analyses were done with the Statistical Package for the Social Sciences 15.0 software. Statistical significance was based on a value of P ≤ 0.05.

Results

Construction and design of HMW-MAA expressing Lm vaccines. We designed three Lm-based vaccines expressing distinct HMW-MAA fragments based on the position of previously mapped (7) and predicted HLA-A2 epitopes. The fragment A comprised HMW-MAA residues 360 to 554, fragment B residues 701 to 1130, and fragment C residues 2,160 to 2,258 (Supplementary Fig. S1A). These vaccines were constructed similarly, and although fragments A and B were expressed and secreted by Lm (Supplementary Fig. S1B), they had a much lower therapeutic efficacy in mouse models than the Lm-LLO-HMW-MAA-C vaccine. Because the fragments were chosen for the presence of HLA-A2 epitopes, it is possible that the reduced potency of these vaccines is because fragments A and B lack H-2b epitopes. For this reason, only the results obtained with Lm-LLO-HMW-MAA-C are shown and discussed here.

The Lm-LLO-HMW-MAA-C vaccine vector is based on the avirulent Lm XFI-7 strain, which lacks the transcriptional factor PrfA and is derived from the 10403S strain. The Lm XFI-7 was transformed with a plasmid constructed for the expression and secretion of the HMW-MAA2160-2258 fragment fused to the first 441 residues of LLO. This plasmid also contains a copy of the prfA gene that partially restores the virulence of this fragment, which is necessary for plasmid retention in vivo. To test whether the LLO-HMW-MAA2160-2258 fusion protein was produced and secreted by Lm-LLO-HMW-MAA-C, we analyzed proteins from culture supernatants by Western blot. A ~62-kDa band corresponding to the LLO-HMW-MAA2160-2258 fusion protein was detected using a polyclonal anti-LLO antibody (Supplementary Fig. S1B). The secretion of LLO-HMW-MAA2160-2258 was relatively weak likely due to the high hydrophobicity of this fragment, which corresponds to the HMW-MAA transmembrane domain. Despite the expression of the LLO-HMW-MAA2160-2258 fusion protein, Lm-LLO-HMW-MAA-C retained its growth capacity in BHI broth (data not shown) and also intracellularly on infection of J774 cells, suggesting that this vector retains the ability to escape the vacuole and invade the cytosol of host cells (Supplementary Fig. S1C).

Therapeutic efficacy of Lm-LLO-HMW-MAA-C in a tumor model expressing HMW-MAA. The B16F10 melanoma was chosen as a mouse tumor model to test the therapeutic efficacy of the Lm-LLO-HMW-MAA-C vaccine because HMW-MAA is expressed in a high proportion of melanoma lesions in humans. The B16F10 cell line, which does not express the mouse HMW-MAA homologue AN2, was transfected with the full-length HMW-MAA gene. A stable clone expressing HMW-MAA was selected (Supplementary Fig. S2), whose in vivo growth characteristics were similar to the parental B16F10 cell line (Supplementary Fig. S3). To verify the effect of Lm-LLO-HMW-MAA-C vaccination on tumor growth, mice were inoculated with B16F10-HMW-MAA cells and immunized 3 days later with the Lm-LLO-HMW-MAA-C vaccine. In the control groups, mice were either immunized with an irrelevant Lm or left untreated and no significant difference in the growth of B16F10-HMW-MAA was observed between these groups (Fig. 1A and B). On the other hand, in the group immunized with the Lm-LLO-HMW-MAA-C vaccine, five of eight mice (62.5%) were tumor-free on day 56 after tumor challenge (Fig. 1C). This difference was highly significant when compared with the Lm control group (P < 0.05). This finding was reproducible and early treatment with Lm-LLO-HMW-MAA-C led to tumor regression in 37.5% to 62.5% of mice in three independent experiments. Importantly, no mice given the parental B16F10 cell line remained tumor-free on immunization with Lm-LLO-HMW-MAA-C, suggesting that the vaccine is specifically targeting the HMW-MAA protein. However, as discussed below, Lm-LLO-HMW-MAA-C vaccination did slow down the growth of the parental B16F10 tumor (Fig. 4A).

We also tested the long-term immunity to HMW-MAA by giving a second lethal dose of the B16F10-HMW-MAA tumor cell line to those mice that remained tumor-free 7 to 8 weeks after the first challenge. Whereas all the naive mice developed palpable tumors within the first 10 days after injection, >90% of the mice in the group treated with Lm-LLO-HMW-MAA-C rejected this tumor cell line after the second challenge (P < 0.001; Fig. 1D).

Depletion of either CD8+ or CD4+ cells abrogates the effect of Lm-LLO-HMW-MAA-C on tumor growth. To evaluate the role of different cell types in the efficacy of Lm-LLO-HMW-MAA-C, we depleted in vivo CD8+, CD4+, and CD25+ cells by antibody administration before and during the treatment course. In the nonimmunized control group, all mice developed tumors (Fig. 2A), whereas 50% of the mice immunized with Lm-LLO-HMW-MAA-C and given a control antibody remained tumor-free for at least 56 days after tumor challenge (Fig. 2B). On the other hand, none of the mice receiving either the anti-CD8 or anti-CD4 antibodies could control the tumor growth, showing that both cell types play an important role in the antitumor immunity generated by the Lm-LLO-HMW-MAA-C vaccine (Fig. 2C and D). However, administration of the anti-CD25 antibody did not improve the efficacy of the Lm-LLO-HMW-MAA-C vaccine, suggesting that the subset of CD4+CD25+ T regulatory cells does not have a significant influence in this model (Fig. 2D).

Transfer of CD8+ T cells from Lm-LLO-HMW-MAA-C–immunized mice confers antitumor immunity against B16F10-HMW-MAA tumor cells. Lm vectors are recognized for
their ability to generate strong CD8+ T-cell responses, which are important for tumor rejection. To verify if the CD8+ T cells generated on immunization with Lm-LLO-HMW-MAA-C had antitumor activity against the B16F10-HMW-MAA cell line, we inoculated tumor cells mixed with CD8+ T cells from either immunized or nonimmunized mice and followed the tumor growth for 4 weeks. When mixed with CD8+ T cells from mice immunized with Lm-LLO-HMW-MAA-C, 50% of the naive animals did not develop tumors in 4 weeks compared with none in the control group (P < 0.01; Fig. 3A). This result indicates that vaccination with Lm-LLO-HMW-MAA-C induces CD8+ T cells able to inhibit the in vivo growth of the B16F10-HMW-MAA cell line.

Immunization with Lm-LLO-HMW-MAA-C induces CD8+ T cells specific for HMW-MAA in HLA-A2/Kb mice. The HMW-MAA fragment expressed and secreted by the Lm-LLO-HMW-MAA-C vaccine contains the HLA-A2–restricted epitope 2238LILPLLFYL2246 (7). To test whether immunization with Lm-LLO-HMW-MAA-C was able to induce immune responses against this epitope, we vaccinated either HLA-A2/Kb transgenic mice or C57BL/6 mice with Lm-LLO-HMW-MAA-C and analyzed the production of IFN-γ by ELISpot after stimulation with HMW-MAA2238-2246 peptide. Following two immunizations, a significantly higher number of SFC were detected in splenocytes from the HLA-A2/Kb transgenic mice but not in the C57BL/6 mice (Fig. 3B). Similar results were obtained using intracellular staining for IFN-γ. After one immunization with Lm-LLO-HMW-MAA-C, IFN-γ production was detected in 0.51% of the CD8+ T cells from the HLA-A2/Kb transgenic mice stimulated with the HMW-MAA2238-2246 peptide compared with 0.06% in the absence of the peptide (Fig. 3C). No responses could be detected in nontransgenic C57BL/6 mice treated with Lm-LLO-HMW-MAA-C that rejected the B16F10-HMW-MAA were challenged a second time with the same cell line 7 wk after the first challenge. Data were pooled from independent experiments and the proportion of mice that remained tumor-free after the second challenge is plotted against time expressed in days of follow-up.
mice (data not shown). These results show that immunization with the \( Lm \)-LLO-HMW-MAA-C vaccine can induce CD8\(^+\) T-cell responses against a HMW-MAA epitope restricted to the HLA-A2 molecule.

Effect of \( Lm \)-LLO-HMW-MAA-C immunization on the growth of tumors not engineered to express HMW-MAA.

Interestingly, we also observed that immunization of mice with \( Lm \)-LLO-HMW-MAA-C could affect the growth of several different tumors that were not engineered to express HMW-MAA, such as the parental B16F10, RENCA, and NT-2 tumors, which were derived from distinct mouse strains. In the RENCA, which is a spontaneous renal carcinoma cell line derived from the BALB/c mouse, and B16F10 models, mice were immunized weekly with \( Lm \)-LLO-HMW-MAA-C thrice, starting 3 days after tumor challenge. Immunization with \( Lm \)-LLO-HMW-MAA-C significantly delayed the growth of these tumors (Fig. 4A and B). In the NT-2 tumor model, which is a mammary carcinoma cell line expressing the rat HER-2/neu protein and is derived from the FVB/N HER-2/neu transgenic mouse (24), immunization with \( Lm \)-LLO-HMW-MAA-C 7 days after tumor inoculation not only impaired tumor growth but also induced regression of the tumor in one of five mice (Fig. 4C). Furthermore, these results could not be attributed to a nonspecific \( Lm \) effect because a control \( Lm \) vaccine strain did not affect the growth of B16F10 (Supplementary Fig. S4), RENCA (Fig. 4B), or NT-2 tumors (Fig. 4C). We also evaluated the effect of \( Lm \)-LLO-HMW-MAA-C immunization in a spontaneous tumor model using the FVB/N HER-2/neu transgenic mouse. These mice express the rat HER-2/neu proto-oncogene under the control of the mouse mammary tumor virus promoter (20). In this transgenic mouse strain, \( >90\% \) of the females develop focal mammary tumors after a latency of about 4 to 6 months. Immunization with \( Lm \)-LLO-HMW-MAA-C significantly delayed the median time for the onset of mammary tumors in these mice (39 weeks) compared with a control \( Lm \) vaccine (25 weeks; Fig. 4D).

Immunization of mice with the \( Lm \)-LLO-HMW-MAA-C vaccine induces infiltration of the tumor by CD8\(^+\) T lymphocytes and decreases the number of pericyte-positive tumor vessels. Although NT-2 cells do not express the HMW-MAA homologue AN2, immunization of FVB/N mice with \( Lm \)-LLO-HMW-MAA-C significantly impaired the growth of NT-2 tumors and eventually led to tumor regression (Fig. 4C). One hypothesis is that activated pericytes present in tumor blood vessels, which express the AN2/HMW-MAA marker, could be a potential target for HMW-MAA vaccines. Because of the more pronounced effect of \( Lm \)-LLO-HMW-MAA-C vaccination in NT-2 tumors, we used this tumor model to evaluate CD8\(^+\) T cells and pericytes in the tumor site by immunofluorescence. Staining of NT-2 tumor sections for CD8 showed infiltration of CD8\(^+\) T cells into the tumors and around blood vessels in mice immunized with the \( Lm \)-LLO-HMW-MAA-C vaccine but not in mice immunized with the control vaccine (Fig. 5A). We also analyzed pericytes in NT-2 tumors by double staining with αSMA and NG2 antibodies. The NG2 protein is the rat

**Figure 2.** In vivo depletion of either CD4\(^+\) or CD8\(^+\) cells abrogates the efficacy of \( Lm \)-LLO-HMW-MAA-C vaccine. In this experiment, 40 C57BL/6 mice were inoculated s.c. with B16F10-HMW-MAA cells on the left flank and either left untreated (\( n = 8 \); A) or immunized i.p. with \( Lm \)-LLO-HMW-MAA-C (\( n = 32 \)) on days 3, 10, and 17 (B–D). For CD8, CD4, and CD25 in vivo cell depletions, mice were treated with 2.43 (anti-CD8; C), GK1.5 (anti-CD4; D, left), or PC61 (anti-CD25; D, right) antibodies. B, an anti-α-galactosidase antibody was given to mice in the control group. Tumor sizes were measured for each individual tumor and the values were expressed as the mean diameter in millimeters. Each line in A, B, C, and D represents an individual mouse.
homologue of HMW-MAA and the NG2 antibody used in this study has been shown to cross-react with the mouse homologue AN2. Data analysis from three independent NT-2 tumors showed a significant decrease in the number of pericytes in mice immunized with Lm-LLO-HMW-MAA-C compared with control (P < 0.05; Fig. 5B). Similar results were obtained when the analysis was restricted to cells stained for aSMA, which is not targeted by the vaccine (data not shown). This finding agrees with the hypothesis that Lm-LLO-HMW-MAA-C vaccination might potentially affect blood vessel formation in the tumor site by targeting pericytes.

Immunization with HMW-MAA-C has no effect on wound healing, pregnancy, and fertility in mice. To evaluate whether Lm-LLO-HMW-MAA-C causes toxicity that is associated with angiogenesis inhibition, we studied wound healing, pregnancy, and fertility in immunized mice. No significant difference was observed in the time required for wound closure in mice immunized with Lm-LLO-HMW-MAA-C compared with control (P > 0.05; Fig. 6A). Similarly, Lm-LLO-HMW-MAA-C immunization had no effect on fertility, gestation length, and pup mass at birth (Fig. 6B).

Discussion

HMW-MAA is a potential antigen for cancer immunotherapy that is expressed in a wide range of tumors and also in pericytes found in tumor angiogenic vasculature. Additionally, HMW-MAA may play a role in cell invasion and metastasis (1, 2). In this study, we generated a recombinant Lm that expresses and secretes a fragment of the HMW-MAA protein fused to a nonhemolytic form of the LLO protein, despite the high hydrophobicity of the LLO-HMW-MAA2238-2246 fragment. Additionally, Lm-LLO-HMW-MAA-C retains its ability to replicate in the cytosol of host cells. It is believed that secretion of the LLO-antigen fusion protein and cytosol invasion are important features of a Lm-based vaccine because antigens secreted at this stage would potentially be targeted to the MHC class I processing pathway, consequently leading to activation of antigen-specific CD8+ T cells (11). In fact, most of the Lm epitopes presented to CD8+ T cells are derived from proteins secreted by the bacterium in the cytosol (13, 25, 26). Besides, invasion of the cytosol by Lm has been shown to enhance the maturation of murine dendritic cells and generate higher number of functional CD8+ T cells (27) and protective immunity (28, 29).
Recombinant live-attenuated \textit{Lm} has been extensively investigated as a potential vector to deliver and elicit cellular immunity against TAAs. As a bacterial vector, \textit{Lm} induces a potent innate immune response with activation of immune cells and production of several chemokines and cytokines, such as interleukin (IL)-6, IL-12, IL-18, IFN-\(\gamma\), and tumor necrosis factor-\(\alpha\), which are important to generate effector T cells (13). As a vaccine vector, \textit{Lm} has the ability to activate and deliver antigens into the intracellular environment of professional APCs, such as macrophages and dendritic cells, resulting in strong cellular immune responses against these antigens while raising minimum humoral responses against the bacterium itself (30). This is an advantage over live viral vaccine vectors, which induce strong humoral responses against the vector on primary immunization, limiting their use in subsequent immunizations. Additionally, \textit{Lm} does not integrate its genetic material into the host genome, and unlike viral vectors, it can be eliminated quickly by a wide range of antibiotics without compromising its efficacy (31).

In previous studies, we showed that vaccination of mice with \textit{Lm} recombinant vectors induced regression of established tumors expressing target antigens such as human papillomavirus 16 E7 and HER-2/\textit{neu} (9, 10). These studies also show that fusion of the antigen to a nonfunctional LLO increases the immunogenicity of the target antigen and the efficacy of the cancer immunotherapy (9, 10, 32). In agreement with these studies, \textit{Lm}-LLO-HMW-MAA-C was able to specifically impede the growth of recently established B16F10 tumors expressing HMW-MAA, besides conferring a long-term immunity against this tumor.

\begin{figure}
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Vaccination of mice with \textit{Lm}-LLO-HMW-MAA-C impairs the growth of HMW-MAA/AN2–negative tumors. \textbf{A}, C57BL/6 mice were inoculated s.c. with B16F10 cells and either immunized i.p. on days 3, 10, and 17 with \textit{Lm}-LLO-HMW-MAA-C \(n=8\) or left untreated \(n=7\). \textbf{B}, BALB/c mice were inoculated s.c. with RENCA cells and immunized i.p. on days 3, 10, and 17 with either \textit{Lm}-LLO-HMW-MAA-C \(n=8\) or an equivalent dose of a control \textit{Lm} vaccine. \textbf{C}, FVB/N mice were inoculated s.c. with NT-2 tumor cells and immunized i.p. on days 7, 14, and 21 with either \textit{Lm}-LLO-HMW-MAA-C \(n=8\) or an equivalent dose of a control \textit{Lm} vaccine \(n=8\). Tumor sizes were measured for each individual tumor. Points, mean diameter in millimeters; bars, SE. *, \(P \leq 0.05\), Mann-Whitney test. \textbf{D}, immunization with \textit{Lm}-LLO-HMW-MAA-C significantly delays the onset of mammary tumors in the FVB/N HER-2/\textit{neu} transgenic mouse. Mice were immunized i.p. every 3 wk with either \textit{Lm}-LLO-HMW-MAA-C \(n=20\) or an equivalent dose of a control \textit{Lm} vaccine \(n=14\), starting when mice were 6 wk old and totaling six doses of vaccine.}
\end{figure}
immunotherapy has been previously shown and it is consistent with our findings (9, 10). As expected, CD8+ T cells from mice immunized with Lm-LLO-HMW-MAA-C could inhibit the growth of these tumors in vivo, in contrast to CD8+ T cells from nonimmunized mice. In addition, in the HLA-A2/Kb transgenic mouse, vaccination with Lm-LLO-HMW-MAA-C induced CD8+ T-cell immunity against a HMW-MAA epitope restricted to the HLA-A2 molecule, which had been previously mapped (7). Together, these results suggest that vaccination with Lm-LLO-HMW-MAA-C induces CD8+ T-cell responses against HMW-MAA that are important to the therapeutic effect of this vaccine.

In contrast to CD8+ T-cell immunity, the role of CD4+ T cells in antitumoral immunity is less well characterized and may differ depending on the tumor model and vaccine strategy used. In the B16F10-HMW-MAA model and Lm-LLO-HMW-MAA-C vaccine, CD4+ cells were essential to control tumor growth. Although Lm infection is capable of activating CD8+ T cells even in the absence of CD4 help (33), several studies have reported that CD4+ T cells play an important role in antitumor immunity in B16F0 and B16F10 melanomas and other tumors (34–37). Interestingly, depletion of CD25+ cells did not improve the efficacy of the Lm-LLO-HMW-MAA-C vaccine against the B16F10-HMW-MAA tumor. CD25 is constitutively expressed by regulatory T cells and anti-CD25 antibody is commonly used to deplete these cells in vivo. However, CD25 is also expressed by recently activated effector CD4+ T cells, and the elimination of CD25+ T cells, although may predominantly reduce regulatory CD25+CD4+ T cells, may also impair the responses by reducing effector T cells (38). On the other hand, we and others have shown that fusion of the target antigen to LLO might be a major factor in modulating regulatory T cells (9, 39, 40) and also broadening the regions of an antigen recognizable as targets by CTLs (10). These results support the strategy of fusing tumor antigens to LLO to enhance the immunogenicity of such antigens and improve the therapeutic efficacy of Lm-based cancer vaccines (9, 10, 18, 32).

Systemic injection of Lm can nonspecifically impair the growth of some tumors, an effect that is dependent on natural killer (NK) cells (41). Besides tumor killing properties, NK cells are also a major primary source of IFN-γ during infection with Lm, probably contributing to the antitumor and antiangiogenic effects of Lm vaccination (42, 43). However, Lm-LLO-HMW-MAA-C vaccine can affect the growth of different transplantable tumors, which were not engineered to express HMW-MAA, more efficiently than other Lm vectors expressing irrelevant antigens. This effect was observed with RENCA, NT-2, and B16F10 tumors. Additionally, the Lm-LLO-HMW-MAA-C vaccine also significantly delayed the onset of mammary tumors in FVB/N HER-2/neu transgenic mice. Although these tumor cells could express the AN2 protein at very low levels, an attractive hypothesis to explain this property of Lm-LLO-HMW-MAA-C is that vaccination against HMW-MAA targets pericytes in the tumor angiogenic vasculature, thus impairing the blood vessel formation in the tumor site. The AN2/HMW-MAA molecule is

Figure 5. Immunization with Lm-LLO-HMW-MAA-C promotes tumor infiltration by CD8+ cells and decreases the number of pericytes in blood vessels. A, NT-2 tumors were removed and sectioned for immunofluorescence. Staining groups are numbered (1–3) and each stain is indicated on the right. Sequential tissues were either stained with the pan-vessel marker anti-CD31 or the anti-NG2 antibody for the HMW-MAA mouse homologue AN2, in conjunction with anti-CD8a for possible TILs. Group 3 shows isotype controls for the above antibodies and DAPI staining was used as a nuclear marker. A total of five tumors were analyzed and a single representative image from each group is shown. Arrows, CD8+ cells around blood vessels. B, sequential sections were stained for pericytes by using the anti-NG2 and anti-α-SMA antibodies. Top, double staining/colocalization of these two antibodies (yellow in merge image) is indicative of pericyte staining. Pericyte colocalization was quantitated using Image Pro Software and the number of colocalized objects is shown in the graph (bottom). A total of three tumors were analyzed and a single representative image from each group is shown. *, P ≤ 0.05, Mann-Whitney test. Columns, mean; bars, SE.
expressed at high levels in activated pericytes such as in wound healings and pericytes in the tumor stroma (44), which are important cells in vascular development, stabilization, maturation, and remodeling (3, 4). Immunofluorescence staining showed a significant reduction in the number of pericytes expressing both αSMA and AN2 markers in NT-2 tumors from mice immunized with Lm-LLO-HMW-MAA-C compared with tumors from mice immunized with a control Lm vaccine. This finding suggests that vaccination with Lm-LLO-HMW-MAA-C can indeed target pericytes in the tumor vasculature, which might contribute to the improved efficacy of this vaccine in slowing down the tumor growth. Additionally, we observed an infiltration of CD8+ T cells around blood vessels and in the stroma of tumors from mice immunized with Lm-LLO-HMW-MAA-C but not in tumors from the control group. This is an essential characteristic in a vaccine because the presence of tumor-infiltrating lymphocytes (TIL) has been correlated with clinical responses in cancer immunotherapy (45). Noteworthy, we did not observe possible toxicity associated with blood vessel damage in mice immunized with Lm-LLO-HMW-MAA-C, such as wound healing, pregnancy, or fertility problems.

An interesting possibility is to target HMW-MAA in combination with metronomic therapies to reduce tumor angiogenesis. It has been previously reported in a preclinical mouse model that inhibition of platelet-derived growth factor receptor signaling, which reduces pericytes counts, enhances the therapeutic efficacy of metronomic chemotherapy or vascular endothelial growth factor receptor inhibition (46). Additionally, targeting pericytes in the tumor stroma might cause a certain degree of vasculitis that could promote the infiltration of the tumor by T cells specific to TAs and improve the efficacy of cancer immunotherapies. In this study, we did not investigate a possible role of antibodies against HMW-MAA that could be generated during the immunotherapy. Antibodies are not thought to play a role in tumor immunotherapy using recombinant Lm as a vaccine vector (8, 47), as we have previously shown using a HER-2/neu tumor model (48). Thus, enhanced efficacy might be achieved by combining Lm-LLO-HMW-MAA-C with active antibody-mediated therapies also targeting HMW-MAA. In a previous study, melanoma patients had prolonged survival on immunization with an anti-idiotypic mAb that mimics the determinant defined by an anti-HMW-MAA antibody (6).

In summary, these results indicate that immunization with Lm-LLO-HMW-MAA-C can induce cell-mediated immune responses against HMW-MAA that can affect the in vivo growth of a melanoma tumor expressing this antigen. This vaccine has shown potential to target not only tumors expressing HMW-MAA but also other tumors, likely due to its effects in reducing the number of pericytes in the tumor vasculature. This characteristic makes this antigen suitable to be used as a universal antigen in cancer immunotherapy. In addition, even if the vaccine is not powerful enough to cause complete regression of all tumor lesions, it could be combined with other
antigens and therapies to improve the efficacy of the therapeutic regimen.

Disclosure of Potential Conflicts of Interest

Y. Paterson and P.C. Maciag wish to disclose that they have a financial interest in Advaxis, Inc., 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 Lm or listerial products as vaccines. The other authors disclosed no potential conflicts of interest.

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References


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