High Efficacy of a Listeria-Based Vaccine against Metastatic Breast Cancer Reveals a Dual Mode of Action

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Abstract
Most cancer vaccines induce CTL responses to tumor-associated antigens (TAA). Killing of tumor cells occurs through TAA-specific CTL-mediated cytolysis. Here, we show that one preventive followed by two therapeutic immunizations with an attenuated Listeria monocytogenes (LM)-based vaccine eradicates all metastases and almost the entire primary tumor in the syngeneic, aggressive mouse breast tumor model 4T1. We provide strong evidence that this is due to the combined result of direct kill by Listeria infecting the tumor cells and by CTL responses against Listeria antigens. We showed by electron microscopy that LM expressing truncated listeriolysin O (LLO) and amino acid fragments 311 to 660 of TAA Mage-b (LM-LLO-Mage-b311-660) and the control strain LM-LLO infect tumor cells in vitro and in vivo. In vitro data indicate that tumor cell death occurs through activation of NADPH oxidase and increased intracellular Ca2+ levels, both resulting in the production of high ROS levels. Because both LM-LLO and LM-LLO-Mage-b311-660 showed equally strong efficacies in vitro, we concluded that LM-LLO was crucial and Mage-b was of less importance. We found strong CTL responses to LM-LLO in the spleen, and depletion of CD8 T cells in vivo resulted in significant tumor regrowth (52%) in LM-LLO–vaccinated mice, indicating that LM-LLO–specific CTL indeed partially contributed to tumor cell kill in vivo. This dual mode of action of a Listeria-based vaccine has not been described before and may provide new directions in the development of more effective vaccines against metastatic breast cancer. [Cancer Res 2009;69(14):5860–6]

Introduction
Breast cancer is the most common cancer among women around the world (1), and 30% to 40% of the women diagnosed with breast cancer will progress to metastatic disease (2). Current treatment options for metastatic cancer include surgery followed by chemotherapy or radiation and/or adjuvant therapy (3). Although first-line endocrine therapy with tamoxifen or the newer third generation aromatases is promising (4), the cure rate of metastatic breast cancer is low (5). In previous studies, we found evidence that vaccination with Mage-b DNA was effective against metastases in various metastatic mouse breast tumor models (6, 7). Mage is an attractive tumor-associated antigen (TAA) because it is expressed in >90% of all breast cancers but not in normal cells (8). To further improve the vaccine efficacy of Mage-b, we used an attenuated Listeria monocytogenes (LM) as DNA delivery system. LM is an intracellular pathogen that delivers the vaccine antigen directly into APCs such as macrophages with high efficiency (9). Cell entrance of macrophages by LM occurs through active phagocytosis, and the LM escape into the host cytosol by perforating the phagosomal membrane through the action of a cytolysin, listeriolysin O (LLO; refs. 10, 11). Once in the cytosol, the vaccine antigen produced by the LM is processed and presented as short peptides via the MHC class I and class II pathways, generating both CD4 and CD8 T-cell responses (12). Killing of tumor cells occurs through CD8 T cells. Previous studies have shown that TAA Her2/neu, expressed by an attenuated LM as fusion protein with LLO, is effective against primary tumors in a syngeneic mouse breast tumor model NT-2 (13). LLO, required for the establishment of intracellular infections (14, 15), also improves immunogenicity of poor immunogenic antigens (13).

In the study presented here, we show that one preventive followed by two therapeutic immunizations with LM-LLO-Mage-b311-660 completely eradicates the metastases and reduces the primary tumors by 90% in a poorly immunogenic metastatic mouse breast tumor model 4T1. Our in vitro and in vivo data strongly suggest that this is due to infection of the tumor cells with Listeria bacteria, resulting in tumor cell kill by high ROS levels and by Listeria–specific CTL. These results point the way toward novel approaches in the use of Listeria vaccines as antitumor agents.

Materials and Methods

Mice. Normal female BALB/c mice (3 mo old) were obtained from The Jackson Laboratory and maintained in the animal husbandry facility of the Pacific Medical Center Research Institute according to the Association and Accreditation of Laboratory Animal Care guidelines.

Plasmids and LM. The LM-LLO-Mage-b311-660 was developed in our laboratory (16). The Listerial pGG-34 plasmid, expressing the positive regulatory factor A (pfrA) and LLO, was developed in the laboratory of Yvonne Paterson, University of Pennsylvania (17, 18). The LM-LLO used in this study is attenuated, i.e., the coding region for the COOH terminal part of the LLO (cytolytic domain that binds cholesterol in the membranes) protein has been deleted, but the proline, glutaminic acid, serine, and threonine (PEST) sequence is still present. Mutations have been introduced into the pfrA gene (expressed by the pGG34 vector), which reduced the pathogenicity of the LM (13). pcDNAJ.1-Mage-b/N5 was developed in our laboratory (6). Mouse granulocyte macrophage colony-stimulating factor plasmid (pCMV-GM-CSF) was provided by Dr. Stephen Johnston (Center for Innovations in Medicine, Bodesign Institute at Arizona State University; ref. 19).

Cells and cell culture. The 4T1 cell line, derived from a spontaneous mammary carcinoma in a BALB/c mouse (20), was cultured in DMEM supplemented with 10% fetal bovine serum, 1 mmol/L mixed nonessential
Immune responses, 5

Cell lines was assessed

pCMV-GM-CSF plasmid DNA; 1

cells), using the Nucleofector kit of AMAXA. To detect

restimulation assays according to standard protocols (Pharmingen), using

LLO for 1 h and subsequently treated with gentamicin. Two days later, the

Mage-b311-660. On the next day, mice were euthanized and tumors and

grown on glass-bottom dishes with or without 10^8 bacteria of LM-LLO or

was given i.p. Fourteen to 18 d after tumor challenge, the mice were

extends to the chest cavity lining and metastases (visible by the

primary tumors extend to the diaphragm, portal liver, spleen, and kidneys (16).

Euthanized and analyzed for tumor weight, frequency, and location of

was given i.p. Fourteen to 18 d after tumor challenge, the mice were

and subsequently treated with gentamicin. Two days later, the

Infection of tumor cells

In vivo

Infection of tumor cells

in vivo

Infection of tumor cells

in vitro

Detection of Listeria and Mage-b proteins in infected tumor cells.

In vitro analysis of immune responses against Mage-b and Listeria.

CD8 depletion in vivo

LM-LLO-Mage-b311-660 for 2 h. Subcellular localization of the p47 phox

PEST antibodies (dilution, 1:1,000).

Detection of bacteria. The infectivity rate of the tumor

Evaluation of cell death.

Evaluation of cell death. Death of tumor or normal cells induced by

LM-LLO or LM-LLO-Mage-b311-660 was determined in vitro as follows. 4T1

Microscopy (EM) using a Philips Tecnai 10 Electron Microscope (21, 22).

Infection of tumor cells in vivo. BALB/c mice were injected with

10^5 4T1 tumor cells in a mammary fat pad and, 10 d later, were injected i.p.

with the usual immunization dose, i.e., 0.1 × LD50 (10^3 CFU) of LM-LLO-

Mage-b311-660. On the next day, mice were euthanized and tumors and

metastases were analyzed by EM for the presence of Listeria bacteria.

Antibody binding was visualized with a 1:400 dilution of Alexa Fluor 555–

conjugated antimouse IgG (Molecular Probes; Invitrogen). Fluorescence

microscopy was performed on a TE2000 Nikon inverted microscope with a

Photometrics and MetaMorph software (Molecular Devices).

Results

Combined preventive and therapeutic immunization completely eradicates metastases. In a previous study, we developed a LM-LLO-Mage-b311-660 vaccine (16). After the combination of two preventive and one therapeutic immunization, a significant effect was observed on the metastases but not on the primary tumors (16). In the current study, we initially aimed to further improve our vaccination protocol. We reasoned that because the vaccine effect on the tumor was likely due to immune responses, preventive immunizations only (tumor challenge at 10 days after the last immunization) should increase the vaccine efficacy, but this was not the case. A moderate but significant effect was observed on the metastases by the LM-LLO-Mage-b311-660 vaccine, but again, no effect was observed on the primary tumors (Fig. 1A). A similarly modest effect was also observed after three therapeutic immunizations (Supplementary Fig. S1). However, one preventive immunization, followed by two therapeutic immunizations, completely eradicated metastases, and the growth of primary tumors was reduced by almost 90% (Fig. 1B). Moreover, inexplicably, the control strain LM-LLO was almost as effective as LM-LLO-Mage-b311-660.

To find an explanation for this difference in vaccine efficacy, we compared Mage-b–specific CTL responses after the three preventive immunizations with the more successful combination of one preventive and two therapeutic immunizations. Restimulation of spleen cells of vaccinated and control mice, bearing 4T1 tumors and metastases, with autologous BM cells expressing Mage-b, showed strong Mage-b–specific CD8 T-cell responses after both vaccination strategies (Fig. 1C, i and ii), suggesting that CTL-mediated tumor cell kill was not the only cause of this strong in vivo effect in the combination strategy (one preventive followed by two therapeutic immunizations). As expected, three therapeutic immunizations showed reduced Mage-b–specific immune responses in the spleen (Supplementary Fig. S1), likely induced by the primary tumors. However, the similar in vivo efficacies of the
control and vaccine strain in the combined immunization suggested that the *Listeria* bacteria itself may have an effect on the tumor cells, a hypothesis that we further pursued as described below.

**Listeria infects and kills tumor cells.** To analyze whether the *Listeria* bacteria could have a direct effect on tumor cells, 4T1 tumor cells were cultured with the LM-LLO-Mage-b311-660 or with the control strain LM-LLO in vitro. LM-LLO-Mage-b311-660 and LM-LLO not only infects but also kills 4T1 tumor cells with high efficiency in the complete absence of immune cells (Fig. 2A). A similar result was observed with a human breast tumor cell line MCF7 (Fig. 2A). Although the infection rate of MCF7 was five times higher than of 4T1 tumor cells, i.e., after 1 hour of incubation, 20% of the 4T1 and 100% of the MCF7 tumor cells were infected (Fig. 2A) and after 2 to 3 hours of incubation, the infection rate of both tumor cell lines was 100% (data not shown). Interestingly, LM-LLO and LM-LLO-Mage-b311-660 killed the tumor cells (4T1 and MCF7) with the same efficacy. Therefore, we concluded that LM-LLO and not Mage-b311-660 mediated the direct tumor cell kill in vitro.

Using EM, we showed the presence of *Listeria* bacteria in the tumor cells both in vitro (cultured 4T1 and MCF7 tumor cell lines) and in vivo (4T1 metastases and primary tumor; Fig. 2B).

**Mechanism(s) of tumor cell kill by *Listeria*.** We then asked which pathways could be triggered by LM-LLO and LM-LLO-Mage-b311-660 resulting in tumor cell death. Bacteria can trigger apoptosis through a large variety of mechanisms that include the secretion of protein synthesis inhibitors, pore-forming proteins, or molecules responsible for the activation of the endogenous death machinery in infected cells (23). It is known that LM activates NADPH oxidase in macrophages and neutrophils (24–26). Here, we show that LM-LLO and LM-LLO-Mage-b311-660 induce the death of 4T1 and MCF7 tumor cells through the activation of NADPH oxidase and subsequent production of ROS (Fig. 3A, i). Trolox, a scavenger of OH· radicals, and apocynin or DPI, both selective inhibitors of NADPH oxidase, significantly decreased LM-LLO-induced and LM-LLO-Mage-b311-660–induced cell death of 4T1 or MCF7 tumor cells (Fig. 3A, ii), which shows the involvement of NADPH oxidase–mediated ROS in tumor cell death. Accordingly, we examined whether ROS was produced in tumor cells upon *Listeria* infection. Live cell microscopy with H2DFFDA or CM-H2XRos revealed that cytosolic ROS were produced through activated NADPH oxidase and that mitochondrial ROS were produced as well (Fig. 3B). Our findings collectively imply that NADPH oxidase–mediated ROS production and subsequent mitochondrial dysfunction contribute to LM-LLO or LM-LLO-Mage-b311-660–induced tumor cell death.

It was obvious that trolox and/or apocynin could only prevent 50% of the LM-LLO–induced cell death (Fig. 3A, ii). This suggests that, in addition to NADPH oxidase-mediated ROS, other pathway(s) are involved in tumor cell death. It has been shown by others that LLO is involved in the rapid increase in intracellular Ca2+ levels in a macrophage cell line, J774 (27). Therefore, we analyzed intracellular Ca2+ levels in 4T1 cells after the addition of LM-LLO. Indeed, LM-LLO increased intracellular Ca2+ levels, as shown with Ca2+ green-1 (Fig. 3C, i). BAPTA, a membrane permeable Ca2+ chelator, reduced mitochondrial ROS detected with CM-H2Ros (Fig. 3C, ii) and LM-LLO–induced tumor cell death by 50% (Fig. 3C, iii). Moreover, BAPTA combined with apocynin very effectively...
reduced mitochondrial ROS detected with CM-H2Ros (Fig. 3C, ii) and prevented the LM-LLO-induced cell death by 80% (Fig. 3C, iii). These results imply that NADPH oxidase and excessive intracellular calcium contribute to tumor cell death upon LM-LLO infection causing mitochondrial failure.

As concluded earlier, LM-LLO, but not Mage-b\textsubscript{311-660}, is involved in direct tumor cell kill. Thus, we analyzed the involvement of LM-LLO in tumor cell kill in more detail. Because LLO is required to establish intracellular infections (14, 15), we were wondering whether LLO could be involved in tumor cell death. A sequence rich in PEST at the amino terminus of LLO is thought to control the production of LLO (28). Therefore, we analyzed the effect of LLO protein with and without PEST on 4T1 tumor cells in vitro. Incubation of 4T1 tumor cells with LLO protein killed 80% to 90% of the 4T1 tumor cells, whereas LLO\textsubscript{ΔPEST} did not induce tumor cell death (Fig. 3D). These results indicate that the PEST sequence is involved in tumor cell death. Moreover, apocynin was able to prevent 50% of the 4T1 tumor cell death induced by LLO protein (Fig. 3D), suggesting that PEST is involved in the activation of NADPH oxidase. LLO protein did not induce an increase in intracellular Ca\textsuperscript{2+} levels (data not shown).

Expression of Mage-b and Listerial proteins by tumor cells and immunologic consequences. As shown above, LM-LLO and LM-LLO-Mage-P\textsubscript{311-660} are able to infect and kill tumor cells in the complete absence of immune cells in vitro. However, infection of the tumor cells with Listeria bacteria does have consequences for the immune system in vivo. We show here that infection of tumor cells in vitro with LM-LLO or LM-LLO-Mage-P\textsubscript{311-660} bacteria resulted in overexpression of Listerial proteins and Mage-b\textsubscript{311-660} (in addition to relatively low levels of natural Mage-b; ref. 16; Fig. 4A). In addition, macrophages will be infected, as well, by LM-LLO or LM-LLO-Mage-P\textsubscript{311-660} resulting in strong CTL responses against both Mage-b\textsubscript{311-660} (Fig. 4B) and the highly immunogenic Listerial proteins, as shown here in vitro (Fig. 4C). Because the combined (preventive/therapeutic) vaccination with LM-LLO and LM-LLO-Mage-P\textsubscript{311-660} eradicated metastases and primary tumors

![Figure 2](image_url). Listeria infects and kills tumor cells. A, both LM-LLO and LM-LLO-Mage-b\textsubscript{311-660} infected 4T1 and MCF7 with high efficiency after 1 h of infection. Experiments were performed in triplicates and repeated thrice. Columns, average of three infection experiments; bars, SE. Both LM-LLO and LM-LLO-Mage-b\textsubscript{311-660} killed 4T1 and MCF7 with the same efficiency (unpaired $t$ test, $P > 0.05$). In this experiment, 4T1 tumor cells were incubated with the Listeria bacteria for 3 h and then treated with gentamicin. On the next day, dead and alive tumor cells were determined with trypan blue. From each sample, nine fields were blindly analyzed by two different investigators. Experiments were performed in triplicates and repeated thrice. Columns, average of three experiments; bars, SE. B, EM analysis shows the Listeria bacteria (LM-LLO-Mage-P\textsubscript{311-660}) inside 4T1 and MCF7 tumor cells after 1-h infection in vitro. Bar, 200 μm. The Listeria bacteria also infected 4T1 primary tumor and metastases in vivo with high efficiency, as shown here after one immunization with LM-LLO-mage-b\textsubscript{311-660}. Bar, 400 μm (top) and 50 μm (bottom). In this experiment, tumors and metastases of three different mice that received LM-LLO-Mage-b\textsubscript{311-660} were analyzed. Representative for the three different mice analyzed. White arrows, Listeria bacteria.
almost with the same efficiency, we concluded that most of the in vivo effect here was derived from LM-LLO. Depletion of CD8 T cells in mice that received one preventive and two therapeutic immunizations with LM-LLO showed an increase in tumor growth by 52% compared with LM-LLO alone (Supplementary Fig. S2), suggesting that Listeria-specific CD8 T cells, at least partially, contribute to tumor reduction in vivo. CD8 depletions did not alter the frequency of metastases, and it is possible that direct kill may play a more important role here.

Discussion

Whereas LM-based cancer vaccines are supposed to exert their effect through killing of tumors cells by CTL responses to TAAs, our present work shows that this is not necessarily the only and most efficient mechanism. Here, we show a dramatic effect of a Listeria-based vaccine on metastases and primary tumor after one preventive followed by two therapeutic immunizations in a highly aggressive and poorly immunogenic mouse model 4T1. We provide strong evidence that efficient tumor cell kill can be achieved through a dual mode of action by LM involving both direct kill and CTL responses to Listeria antigens. EM studies showed that both the vaccine (LM-LLO-Mage-b311-660) and control strain (LM-LLO) infect tumor cells in vitro and in vivo and that both kill tumor cells in vitro through the generation of high levels of ROS resulting in oxidative stress. Evidence for tumor cell kill by CTL responses to LM-LLO is based on in vitro and in vivo data. We showed in vitro that infection of the tumor cells with LM-LLO and LM-LLO-Mage-b311-660 resulted in high expression of Listeria and Mage-b proteins and strong Listeria-specific and Mage-b-specific CTL responses in the spleen of tumor-bearing vaccinated mice. However, we showed that the combined vaccination with LM-LLO and LM-LLO-Mage-b311-660 eradicated metastases and primary tumors almost with the same efficiency in vivo, indicating that most of the in vivo effect was derived from LM-LLO. Depletion of CD8 T cells in LM-LLO-vaccinated mice resulted in tumor regrowth (~50%), suggesting

Figure 3. Mechanism(s) of tumor cell kill by LM-LLO-Mage-b311-660 and LM-LLO. A, Listeria-induced tumor cell death by activation of NADPH oxidase and production of ROS. 4T1 tumor cells were incubated with LM-LLO or LM-LLO-Mage-b311-660 for 2 h and analyzed for activation of NADPH oxidase using antibodies to p47phox (i). NADPH oxidase–induced cell death was prevented by apocynin, whereas ROS-induced cell death was prevented by trolox and DPI (ii). Significant differences were found between 4T1 tumor cells treated with inhibitors compared with untreated 4T1. Unpaired t test, P < 0.05 is significant. A similar result was observed with MCF7. B, prevention of Listeria-induced ROS with apocynin. 4T1 tumor cells were incubated with LM-LLO, in the presence or absence of apocynin, and showed decreased production of ROS with H2DFFA (cROS and mROS) or CM-H2XRos (mROS). C, LM-LLO also induced increase in intracellular Ca2+ levels in 4T1 tumor cells, as shown with Ca2+-green-1 (i). LM-LLO-induced mitochondrial disruption (ii) in 4T1 tumor cells and subsequent tumor cell death (iii) could be prevented with apocynin or BAPTA. Whereas both apocynin and BAPTA were able to prevent tumor cell death by 50% when added separately, 80% to 90% of tumor cell death could be prevented when added combined with infected tumor cell cultures (iii). Significant differences were found between 4T1 tumor cells treated with inhibitors compared with untreated 4T1. Unpaired t test, P < 0.05 is significant. D, the PEST motif in LLO of the Listeria bacteria activates NADPH oxidase and is involved in tumor cell death. This could be prevented with apocynin. LLO protein (LLO) with or without the PEST motif (LLO-PEST) was added to 4T1 cultured, and cell death was determined the next day. Significant differences between LLO protein and other groups were analyzed. Unpaired t test, P < 0.05 is significant. Experiments were performed in triplicates and repeated twice. The results presented here are the average (Ai, Cii, D) or a representative (Ai, B, Ci, Cii) of two experiments performed. The error bars represent SE.
Figure 4. Expression of Mage-b311-660 and Listerial proteins by 4T1 tumor cells. 4T1 tumor cells were incubated with LM-LLO or LM-LLO-Mage-b311-660 for 2 h and subsequently analyzed for the expression of Mage-b311-660 (fused with LLO; 61 kDa) and Listeria (LLO; 48 kDa) proteins by Western blotting using anti-PEST antibodies (A). Spleen cells (pooled) of vaccinated (preventive/therapeutic) and control mice bearing 4T1 metastases and primary tumors were analyzed for Mage-b-specific and Listeria-specific immune responses by ELISPOT using BM cells (transfected with pcDNA3.1-Mage-b and pCMV1-GM-CSF, B) and BM cells infected with LM-LLO (C), respectively. The involvement of CD8 T cells was determined by negative depletion, using magnetic beads with anti-CD8 antibodies. Columns, average of two independent experiments, n = 5 mice per group; bars, SE. Significant differences between Mage-b and other groups were determined. Mann-Whitney test, P < 0.05 is significant.

that Listeria-specific CTL responses were indeed, at least partially, effective against the primary tumor. CD8 depletions did not alter the frequency of metastases. It is possible that the metastases are directly killed by the Listeria bacteria. Because we only did CD8 depletion studies, we cannot rule out that natural killer cell responses and CD4 T-cell responses are involved, as well. Although the presence of bacteria in tumors has been recognized earlier (29–31), the direct kill and immunologic consequences, as shown with the attenuated LM in the current study, has not been reported thus far.

If Listeria is mediating direct tumor cell kill in vivo, why are normal cells not affected? Indeed, primary cultures of normal mouse or human fibroblasts were infected and killed by LM-LLO as efficiently as the tumor cells in vitro (Supplementary Fig. S3). However, mice that were immunized with LM-LLO or LM-LLO-Mage-b311-660 seemed completely healthy. Analysis of the liver, spleen, and gastrointestinal tissues (known targets for LM infection) for pathologic damage after three immunizations with LM-LLO or LM-LLO-Mage-b311-660 showed a few inflammatory spots (concentration of lymphocytes) in the liver only (data not shown). Also, liver functions such as AST and ALT were unaffected by the Listeria bacteria (Supplementary Table S1). To explain this apparent lack of effect on normal tissues, we hypothesize that in vivo the Listeria are cleared very efficiently by the immune system from normal tissues, as we have shown for the spleen in this study (3 days after infection, Listeria could not be cultured anymore from the spleen). At the same time, vaccine-induced immune responses are, at least, partially suppressed in the tumor environment, as we found in the draining lymph nodes of the current (data not shown) and a previous study (16) and have also been shown by others (32). Therefore, Listeria bacteria in the tumor microenvironment may be protected from clearance by the immune system, but not in the normal tissues. This is in keeping with the results obtained by others reporting only flu-like symptoms of LM-LLO–based vaccines in cancer patients in phase I/phase II clinical trials (33).

In summary, our results show that different immunization strategies with LM-LLO-Mage-b311-660 provide different effects on metastatic breast cancer in the highly aggressive mouse model 4T1. The combined preventive/therapeutic immunization was superior over three preventive or three therapeutic immunizations. Our results strongly suggest that this is due to the combination of direct kill and Listeria-specific CTL-mediated tumor cell cytolysis. Reduced efficacies after exclusive preventive or therapeutic immunizations may be due to vaccine-induced immune responses or direct kill as separate actions, respectively. This dual mode of action through tumor cell infection has not been described before and opens up new strategies for eradicating breast cancer effectively through the combination of direct kill and immune responses against highly immunogenic antigens rather than against weak TAA.

Acknowledgments

Received 12/20/08; revised 5/8/09; accepted 5/11/09, published OnlineFirst 7/7/09.

Grant support: NIH grant 1RO1 AG025096-01 and American Federation for Aging Research grant A000106.

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We thank Ivy Hsie and Dr. ZhenHang Meng for their technical assistance with EM and pathology, respectively, and Wilber Quispe, Denise Asafu-Adjei, and Ilyssa Ramos for finishing the experiments of this article.
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