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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-b$_{311-660}$) 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 NADP$^+$ oxidase and increased intracellular Ca^{2+} levels, both resulting in the production of high ROS levels. Because both LM-LLO and LM-LLO-Mage-b$_{311-660}$ showed equally strong efficacies *in vivo*, 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 includes 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-b$_{311-660}$ 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-b$_{311-660}$ was developed in our laboratory (16). The *Listerial* pGG-34 plasmid, expressing the positive regulatory factor A (prfA) 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, glutamic acid, serine, and threonine (PEST) sequence is still present. Mutations have been introduced into the prfA gene (expressed by the pGG34 vector), which reduced the pathogenicity of the LM (13). pcDNA3.1-Mage-b/V5 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, Biodesign 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

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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amino acids, 2 mmol/L L-glutamine, insulin (0.5 HSP units/mL), penicillin (100 units/mL), and streptomycin (100 μg/mL).

Immunization and tumor challenge. Three different immunization protocols have been tested in this study: (*a*) three preventive vaccinations given 1 wk apart (days 1, 8, and 15) with tumor challenge (10^5 cells injected into a mammary fat pad) at 10 d after the last immunization (day 25); (*b*) one preventive vaccination, followed by tumor challenge at day 5, with two therapeutic vaccinations at days 8 and 15; or (*c*) three therapeutic vaccinations given 3 d after tumor challenge. Injections of 0.1 × LD_{50} (10^7 bacteria) of LM-LLO-Mage-$b_{311\text{-}660}$, the control vector LM-LLO, or saline was given i.p. Fourteen to 18 d after tumor challenge, the mice were euthanized and analyzed for tumor weight, frequency, and location of metastases, as well as for immunologic responses in the spleen. Primary tumors extend to the chest cavity lining and metastases (visible by the naked eye as nodules) predominantly to the mesenteric lymph nodes and less frequently to the diaphragm, portal liver, spleen, and kidneys (16).

CD8 depletion *in vivo*. CD8 T cells were depleted in 4T1 tumor-bearing mice with 0.5 mg of anti-CD8 antibodies 2.43 (18) on days 3, 7, 8, 9, 14, 15, and 16, whereas one preventive and two therapeutic immunizations with LM-LLO were given at days 1, 8, and 15 and tumor challenge at day 5. Mice were euthanized and analyzed for tumor weight and frequency of metastases 14 d after tumor challenge. The antibodies were purchased from BioXCell. This antibody has been shown by many investigators to specifically deplete only CD8 T cells by >95% (13). As control, isotype-matched rat antibodies against HRPN were used (13, 17).

***In vitro* analysis of immune responses against Mage-b and *Listeria*.** Spleen cells were isolated from vaccinated and control mice with 4T1 tumors and metastases. To detect Mage-b–specific immune responses, 2 × 10^5 cells from spleens were restimulated with 5 × 10^4 autologous bone marrow (BM) cells (transfected with pcDNA3.1-Mage-b plasmid DNA and pCMV-GM-CSF plasmid DNA; 1 μg of each plasmid DNA per 5 × 10^6 BM cells), using the Nucleofector kit of AMAXA. To detect *Listeria*-induced immune responses, 5 × 10^4 autologous BM cells were infected with 10^3 LM-LLO for 1 h and subsequently treated with gentamicin. Two days later, the frequency of IFNγ-producing cells was determined by ELISPOT for both restimulation assays according to standard protocols (Pharmingen), using an ELISPOT reader (CTL Immunospot S4 analyzer, Cellular Technology Ltd.). Spleen cells were depleted for CD8 T cells using magnetic bead depletion techniques according to the manufacturer's instructions (Miltenyi Biotec, Inc.). Fluorescence-activated cell sorting analysis showed that ≥90% of all CD8 T cells were depleted.

Infection of tumor cells *in vitro*. The infectivity rate of the tumor cell lines was assessed *in vitro*. Cells (5 × 10^5/mL) were infected with 10^8 (per well) of LM-LLO or LM-LLO-Mage-$b_{311\text{-}660}$ for 1 h at 37°C in culture medium, as described above. After incubation with gentamicin (50 μg/mL) for 1 h (killing all extracellular *Listeria* bacteria), cells were washed with PBS and lysed in sterile water and serial dilutions were plated onto LB agar to determine the infection rate the next day or analyzed by electron 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 × LD_{50} (10^7 CFU) of LM-LLO-Mage-$b_{311\text{-}660}$. On the next day, mice were euthanized and tumors and metastases were analyzed by EM for the presence of *Listeria* bacteria.

Evaluation of cell death. Death of tumor or normal cells induced by LM-LLO or LM-LLO-Mage-$b_{311\text{-}660}$ was determined *in vitro* as follows. 4T1 or MCF7 cells (3 × 10^3) plated on 96-well plates were infected with 10^6 (per well) of LM-LLO or LM-LLO-Mage-$b_{311\text{-}660}$ for 3 h at 37°C. Gentamycin (50 μg/mL) was added until live and dead cells were counted using trypan blue staining on the next day. Recombinant LLO or LLOΔPEST (LLO without PEST sequence; 100 ng/mL) were added in 4T1 cell cultures. Cell death was evaluated as described above. Trolox (100 μmol/L), apocynin (500 μmol/L), diphenylene iodonium (DPI; 50 nmol/L), or membrane permeable Ca^{2+}-chelator BAPTA (2 μmol/L) were concomitantly cultured with the *Listeria* bacteria.

Immunostaining. Immunostaining was performed in 4T1 cultures grown on glass-bottomed dishes with or without 10^8 bacteria of LM-LLO or LM-LLO-Mage-$b_{311\text{-}660}$ for 2 h. Subcellular localization of the $p47^{phox}$ subunit of NADPH oxidase was evaluated in cultures fixed with 4% paraformaldehyde for 20 min on ice. After preincubation in blocking buffer (0.01% PBS, 2% goat serum, 0.2% Triton-X, 0.1% bovine serum albumin) for 30 min, the cultures were incubated with a 1:500 dilution of mouse anti-$p47^{phox}$ antibody (BD Biosciences, Transduction Laboratories). Primary 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 (MolecularDevices).

Detection of *Listeria* and Mage-b proteins in infected tumor cells. 4T1 tumor cells (5 × 10^5) were infected with LM-LLO or LM-LLO-Mage-$b_{311\text{-}660}$ (10^8 bacteria), then treated with gentamicin (50 μg/mL) for 2 h, washed thrice with PBS, and analyzed by Western blotting. LLO (containing PEST motif) and LLO fused with Mage-$b_{311\text{-}660}$ were detected with anti-PEST antibodies (dilution, 1:1,000).

Imaging of ROS and Ca^{2+}. 4T1 or MCF7 tumor cells (2 × 10^5/mL) grown on glass-bottomed dishes were infected with 10^6 of LM. Two hours later, cells were loaded with 5 μmol/L 5- (and -6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate for intracellular ROS, 100 nmol/L Mitotracker Red CM-H_2XROS for mitochondrial ROS, or 10 μmol/L calcium green-1 (Molecular Probes; Invitrogen) for intracellular free Ca^{2+} in HBSS for 25 min at 37°C and washed thrice with HBSS. The signals of ROS or Ca^{2+} were observed at room temperature on the stage of a TE2000 Nikon inverted microscope with a Photometrics and Coolsnap HQ CCD camera controlled by MetaMorph software (Molecular Devices).

Results

Combined preventive and therapeutic immunization completely eradicates metastases. In a previous study, we developed a LM-LLO-Mage-$b_{311\text{-}660}$ 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-$b_{311\text{-}660}$ vaccine, but again, no effect was observed on the primary tumors (Fig. 1*A*). 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. 1*B*). Moreover, inexplicably, the control strain LM-LLO was almost as effective as LM-LLO-Mage-$b_{311\text{-}660}$.

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. 1*C, 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

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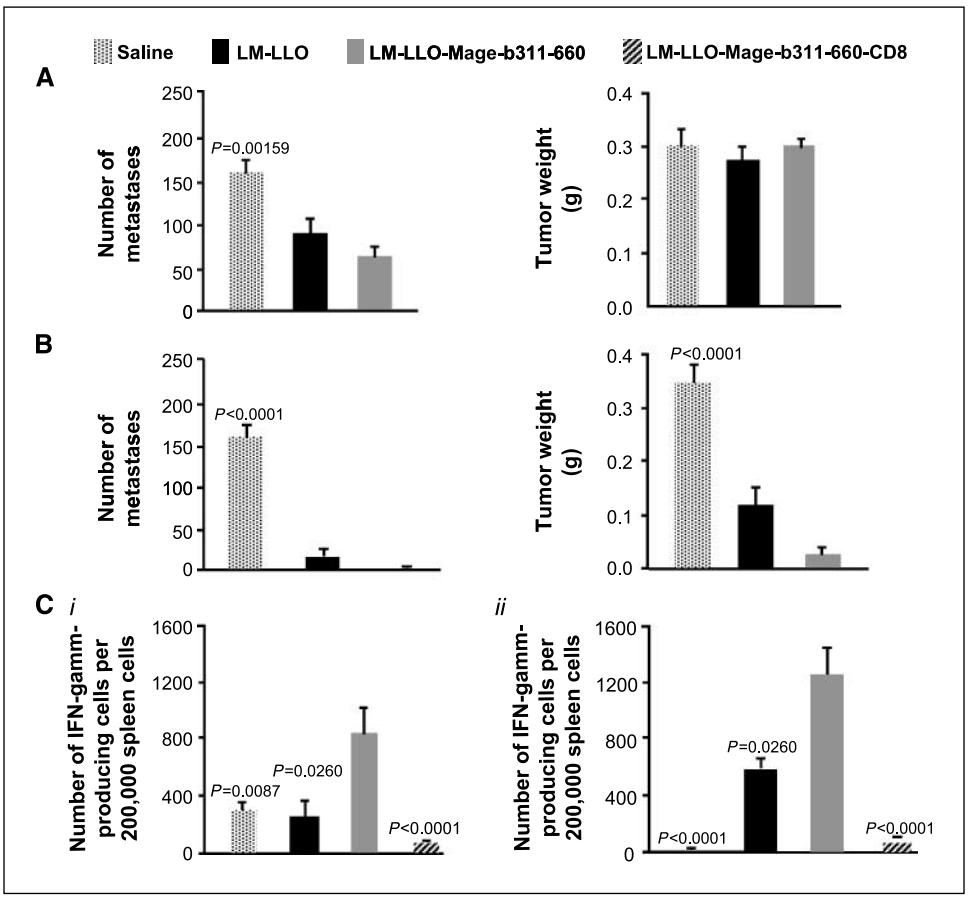

Figure 1. Effect of LM-LLO and LM-LLO-Mage-$b_{311-660}$ *in vivo* and *in vitro*. The frequency of metastases and tumor weight was determined after three preventive immunizations (*A*) and after a combination of one preventive and two therapeutic immunizations (*B*). Also Mage-b–specific immune responses (*C*) were analyzed after three preventive immunizations (*i*) and after the combination of one preventive and two therapeutic immunizations (*ii*) by ELISPOT. For this purpose, spleen cells were pooled per group, restimulated with BM cells (transfected with pcDNA3.1-Mage-b and pCMV1-GM-CSF), and analyzed for the production of IFNγ 72 h later. The involvement of CD8 T cells was determined by negative depletion, using magnetic beads with anti-CD8 antibodies. Controls such as BM cells transfected with pcDNA3.1-Mage-b or pCMV1-GM-CSF or nontransfected BM cells did not produce IFNγ (data not shown). *Columns,* average of two to three independent experiments, $n = 5$ mice per group; *bars,* SE. Significant differences between Mage-b and other groups were analyzed using Mann-Whitney test. $P < 0.05$ is significant.

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-$b_{311-660}$ or with the control strain LM-LLO *in vitro*. LM-LLO-Mage-$b_{311-660}$ and LM-LLO not only infects but also kills 4T1 tumor cells with high efficiency in the complete absence of immune cells (Fig. 2*A*). A similar result was observed with a human breast tumor cell line MCF7 (Fig. 2*A*). 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. 2*A*) 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-$b_{311-660}$ killed the tumor cells (4T1 and MCF7) with the same efficacy. Therefore, we concluded that LM-LLO and not Mage-$b_{311-660}$ 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-$b_{311-660}$ 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-$b_{311-660}$ induce the death of 4T1 and MCF7 tumor cells through the activation of NADPH oxidase and subsequent production of ROS (Fig. 3*A, 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-$b_{311-660}$–induced cell death of 4T1 or MCF7 tumor cells (Fig. 3*A, 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 H_2DFFDA or CM-H_2XRos revealed that cytosolic ROS were produced through activated NADPH oxidase and that mitochondrial ROS were produced as well (Fig. 3*B*). Our findings collectively imply that NADPH oxidase-mediated ROS production and subsequent mitochondrial dysfunction contribute to LM-LLO or LM-LLO-Mage-$b_{311-660}$–induced tumor cell death.

It was obvious that trolox and/or apocynin could only prevent 50% of the LM-LLO–induced cell death (Fig. 3*A, 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 Ca^{2+} levels in a macrophage cell line, J774 (27). Therefore, we analyzed intracellular Ca^{2+} levels in 4T1 cells after the addition of LM-LLO. Indeed, LM-LLO increased intracellular Ca^{2+} levels, as shown with Ca^{2+} green-1 (Fig. 3*C, i*). BAPTA, a membrane permeable Ca^{2+} chelator, reduced mitochondrial ROS detected with CM-H_2Ros (Fig. 3*C, ii*) and LM-LLO–induced tumor cell death by 50% (Fig. 3*C, iii*). Moreover, BAPTA combined with apocynin very effectively

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reduced mitochondrial ROS detected with CM-H_2Ros (Fig. 3*C, ii*) and prevented the LM-LLO–induced cell death by 80% (Fig. 3*C, 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-$b_{311-660}$, 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 LLOΔpest did not induce tumor cell death (Fig. 3*D*). 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. 3*D*), suggesting that PEST is involved in the activation of NADPH oxidase. LLO protein did not induce an increase in intracellular Ca^{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-$b_{311-660}$ 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-$b_{311-660}$ bacteria resulted in overexpression of *Listerial* proteins and Mage-$b_{311-660}$ (in addition to relatively low levels of natural Mage-b; ref. 16; Fig. 4*A*). In addition, macrophages will be infected, as well, by LM-LLO or LM-LLO-Mage-$b_{311-660}$, resulting in strong CTL responses against both Mage-$b_{311-660}$ (Fig. 4*B*) and the highly immunogenic *Listerial* proteins, as shown here *in vitro* (Fig. 4*C*). Because the combined (preventive/therapeutic) vaccination with LM-LLO and LM-LLO-Mage-$b_{311-660}$ eradicated metastases and primary tumors

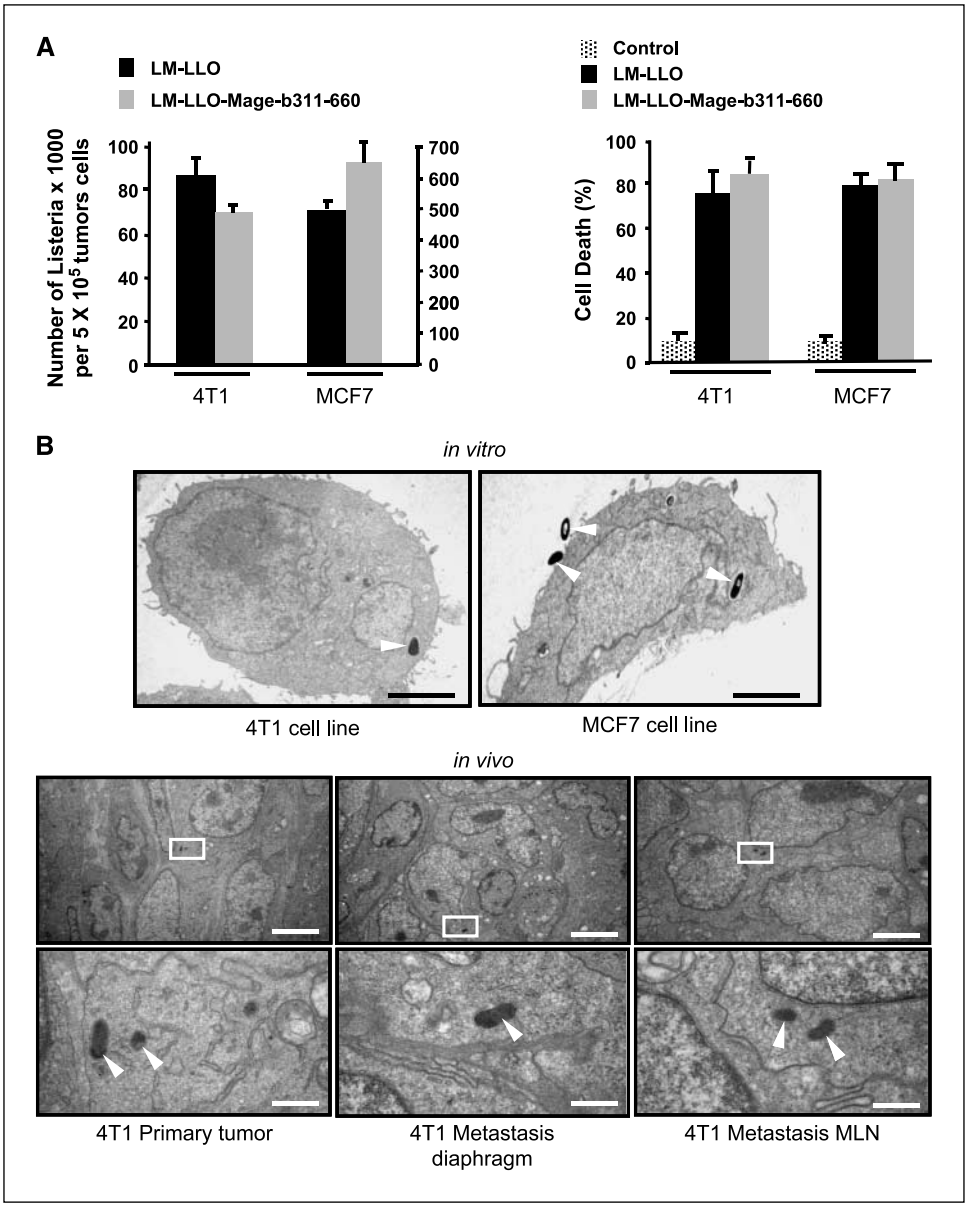

Figure 2. *Listeria* infects and kills tumor cells. *A,* both LM-LLO and LM-LLO-Mage-$b_{311-660}$ infected 4T1 and MCF7 with high efficiency after 1 h of infection. Experiments were performed in triplicates and repeated thrice. *Columns,* average of three infection experiments; *bars,* SE. Both LM-LLO and LM-LLO-Mage-$b_{311-660}$ 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-$b_{311-660}$) 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-$b_{311-660}$. *Bar,* 400 μm (*top*) and 50 μm (*bottom*). In this experiment, tumors and metastases of three different mice that received LM-LLO-Mage-$b_{311-660}$ were analyzed. Representative for the three different mice analyzed. *White arrows, Listeria* bacteria.

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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-$b_{311-660}$) 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-$b_{311-660}$ 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-$b_{311-660}$ 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

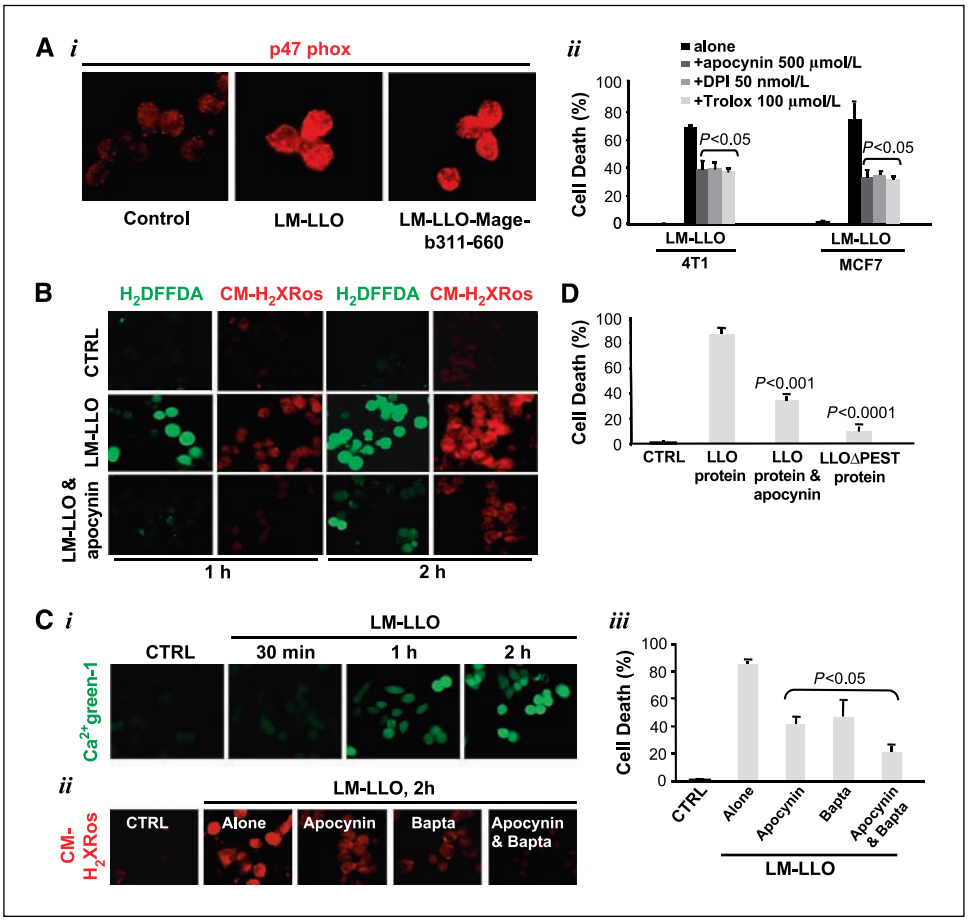

Figure 3. Mechanism(s) of tumor cell kill by LM-LLO-Mage-$b_{311-660}$ and LM-LLO. *A, Listeria*-induced tumor cell death by activation of NADPH oxidase and production of ROS. 4T1 tumor cells were incubated with LM-LLO or LM-LLO-Mage-$b_{311-660}$ for 2 h and analyzed for activation of NADPH oxidase 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 H_2DFFA (cROS and mROS) or CM-H_2XRos (mROS). *C,* LM-LLO also induced increase in intracellular Ca^{2+} levels in 4T1 tumor cells, as shown with Ca^{2+}-green-1 (*i*). LM-LLO–induced mitochondrial disruption (*ii*) in 4T1 tumor cells and subsequent tumor cell death (*iii*) could be prevented with apocynin an/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 (LLOΔPEST) 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 (*Aii, Ciii, D*) or a representative (*Ai, B, Ci, Cii*) of two experiments performed. The error bars represent SE.

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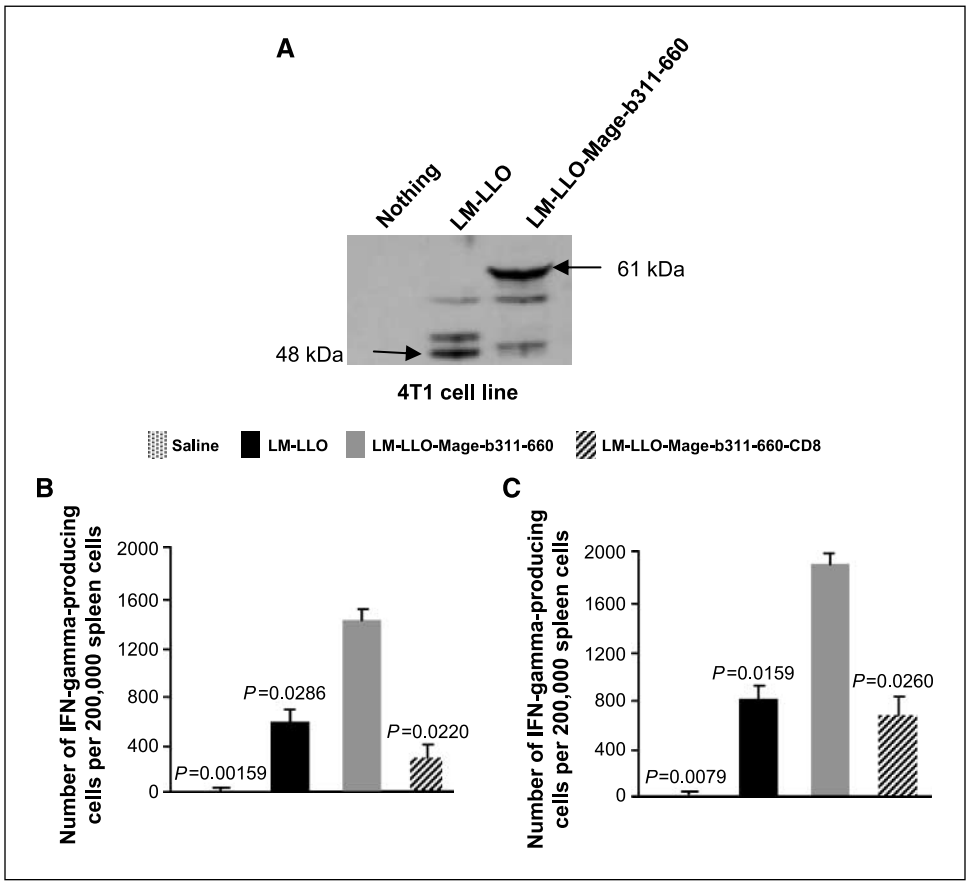

Figure 4. Expression of Mage-$b_{311-660}$ and *Listerial* proteins by 4T1 tumor cells. 4T1 tumor cells were incubated with LM-LLO or LM-LLO-Mage-$b_{311-660}$ for 2 h and subsequently analyzed for the expression of Mage-$b_{311-660}$ (fused with LLO; 61 kDa) and *Listeria* (LLO; 48 kDa) proteins by 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-$b_{311-660}$ 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-$b_{311-660}$ 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-$b_{311-660}$ 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.

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