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Harnessing *Listeria monocytogenes* to target tumors

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Abbreviations: ActA, actin assembly; ALT, alanine transferase; APC, antigen presenting cells; AST, aspartate amino acid transferase; CFU, colony forming units; CTL, cytotoxic T lymphocytes; DC, dendritic cells; DPI, diphenylene iodonium; ECM, extracellular matrix; ECOG, eastern cooperative oncology group; ER, endoplasmic reticulum; FCUI, fusion protein consisting of yeast cytosine deaminase and uracil phosphoribosyl transferase; FLK-1, fetal liver kinase-1; GI, gastrointestine; GILT, γ-interferon-inducible lysosomal thiolreductase; HGF, hepatocyte growth factor; HMW-MAA, high molecular weight-melanoma associated antigen; HPV, human papillomavirus; HSPG, heparan sulphate proteoglycans; IFN, interferon; LLO, listeriolysin O; LM, *Listeria monocytogenes*; MCSP, melanoma chondroitin sulfateproteoglycan; MVD, tumor microvascular density; NADPH, nicotinamide adenine dinucleotide phosphate; PNP, purine-deoxynucleoside phosphorylase; PSA, prostate specific antigen; ROS, reactive oxygen species; TAA, tumor associated antigen; TLR, toll-like receptors; TNF, tumor necrosis factor; TRP, tyrosinase related protein; VEGFR, vascular endothelial growth factor receptor

Because of its cytosolic localization, *Listeria monocytogenes* (LM) has long been considered an attractive tool for delivering tumor-associated antigens (TAA) antigens in vivo to combat cancer. LM directly infects antigen-presenting cells (APC) such as monocytes, macrophages and dendritic cells (DC), thereby delivering the TAA into their cytoplasm, resulting in processing and presentation of the antigen to the immune system. This activates adaptive and innate immune responses to the TAA, mediating tumor cell cytolysis. Recently we discovered additional pathways by which Listeria can be harnessed to induce tumor cell death, which suggest new directions in the development of vaccines or therapies against cancer. In one approach, we have used Listeria to induce immune responses that destroy tumor vasculature. Another new pathway involves selective infection of cancer cells with Listeria, followed by tumor cell death through the production of high levels of reactive oxygen species (ROS) and through Listeria-specific cytotoxic T lymphocytes (CTL). This review will focus on the most recent studies on the multiple pathways of LM and how they can be harnessed in the battle against cancer.

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Delivery of Tumor Associated-Antigens (TAA) into Antigen Presenting Cells (APC)

One of the main problems in cancer vaccination is the poor delivery of the TAA into APC. LM is an excellent candidate to improve delivery of TAA in vivo since LM is an intracellular pathogen that can deliver the TAA, either as cDNA or as an expressed and secreted protein, directly into APC with high efficiency through active phagocytosis followed by lysis of the phagosome.[1-3] APC infected by Listeria include monocytes, macrophages and dendritic cells (DC). However, LM can also induce its own internalization in various cell types that are normally non-phagocytic.[4] These include epithelial cells, fibroblasts, hepatocytes, endothelial cells, as well as neurons.[5-14]

Prior to phagocytosis, LM binds to the surface of APC through specific receptors. For instance, on phagocytic cells LM binds to C3bi and C1q complement receptors,[15-18] or to the macrophage scavenger receptor.[19,20] However, LM can also bind to receptors on tumor cells such as the Met receptor for hepatocyte growth factor (HGF)[21] expressed in many breast cancers[22] or to components of the extracellular matrix (ECM) such as heparan sulphate proteoglycans (HSPG)[23] and fibronectin,[24] both expressed in many cancers including breast cancer, glioma, prostate cancer and melanoma,[25-28] or to E-cadherin,[5] which is expressed by breast tumors and metastases.

The bacterial ligands of these entry receptors identified to date are surface proteins, such as the internalins, InlA and InlB, the actin-polymerizing protein ActA, and p60. Also, putative adhesions have been identified such as surface protein Ami, which is similar to InlB,[29-31] and lap, which is involved in attachment to Caco-2 cells.[32] In addition to surface proteins, lectins may be involved in LM adhesion to eukaryotic cells

through lectin-like ligands such as L-fucosylalanine, p-aminophenyl α-D mannopyranoside moieties.[33,34] Finally, it has been described that LM adhesins containing α-D-galactose are involved in the uptake by mouse CB1 dendritic cells,[2] and by human HepG22 hepatocarcinoma cells,[35] upon recognition of a carbohydrate receptor at the eukaryotic cell surface.

Following adherence, LM enters the APC through phagocytosis, and some bacteria will escape into the host cytosol by perforating the phagosomal membrane through the action of a cytolysin, listeriolysin O (LLO).[36,37] Once in the cytosol, the endogenously produced TAA are processed, transported into the endoplasmic reticulum (ER) for loading onto major histocompatibility complex (MHC) class I molecules and presented as short peptides via the MHC class I pathway, resulting in the activation of CD8 T-cells. However, most LM are actually destroyed and processed in the phagolysosome. Any TAA proteins expressed by LM in this compartment will be processed by lysosomal degradation and loaded onto MHC class II molecules, resulting in the activation of CD4 T-cells. Other studies provide evidence that the TAA may also activate the immune system through cross-presentation of the exogeneous TAA by DC though the pathways involved are not yet well characterized.[38]

Activation of Adaptive and Innate Immune Responses by TAA-Based LM Vaccines

TAA peptides associated with MHC class I and II molecules, activate CD8 and CD4 T-cells, respectively.[39,40] Boosting with the TAA-based LM vaccine results in activation of memory CD4 and CD8 T-cells to the TAA. Activation of TAA-specific CD8 memory CTL results in the killing of tumor cells through effector CTL-mediated cytolysis and may be enhanced by the activation of memory CD4 T helper cells.[40] Also innate immune responses will be activated by Listeria through the activation of toll-like receptors (TLR) on macrophages and the subsequent production of interleukin (IL)-12, tumor necrosis factor (TNF)α and IL-1.[39,40] These lymphokines attract natural killer (NK) cells and neutrophils to the infection site. IL-12 activates NK cells and T-cells to produce interferon (IFN)γ, which in turn activates bactericidal properties of macrophages, stimulates DC to take up bacterial antigens at the site of infection, and regulates the development of CTL persistence.[39,40] DC expressing TNFα and inducible nitric oxide synthase (iNOS) are essential for bacterial control.[41] It is interesting that LLO-mutants are able to induce CD8 T-cell responses, but fail to provide protective immunity.[40,42] Also, only live Listeria-based vaccines provide protection while heat-killed Listeria, despite the induction of strong memory CD8 T-cell responses, does not.[43] One reason could be that CD8 T-cells activated by live LM are cytolytic in vitro, while CD8 T-cells activated by heat-killed LM are not.[44] However, another report describes that cytolytic activity is not required for protection.[45] These results suggest that other factors may play a role in the protection provided by live Listeria.

Preclinical Studies of Listeria-Based Vaccines in Animal Models with Cancer

Various Listeria-based vaccines expressing TAA have been tested in animal models with cancer (**Table 1**). The first demonstration used an LM, secreting the influenza nucleoprotein (NP) as a fusion protein with LLO, and showed that this induced regression of established colorectal cancer, renal cancer or melanoma, expressing the same antigen as the vaccine, in mice, which correlated with NP-specific immune responses.[46,47] Also, vaccination of mice with LM expressing human papilloma virus (HPV) E7 as fusion protein with LLO induced regression of established TC-1 tumors, immortalized by HPV-16 in mice.[48] Interestingly, vaccination with LM-E7 or LM-LLO-E7 missing the PEST (a sequence rich in proline, glutamic acid, serine and threonine) in the amino terminus of LLO, only induced slower growth of TC-1 tumors, despite strong immune responses to E7.[48,49] The Lm-LLO-E7 vaccine was also tested for its ability to break tolerance to a self-tumor antigen using a mouse that was transgenic for HPV-16, E6 and E7.[50] These mice develop autochthonous thyroid tumors, as the transgene is under the control of the thyroglobulin promoter. Lm-LLO-E7, and a similar recombinant vaccine that expresses E7 fused to the listerial virulence factor ActA were found to delay the appearance of thyroid tumors and slow their growth in the E6/E7 transgenic mouse.[51] Further evidence that Listeria based tumor antigen expression systems can break tolerance was provided by Bruhn et al. who showed that vaccination with Listeria, expressing TAA tyrosinase-related protein (TRP-2) induced prophylactic and therapeutic protection of mice from challenge by B16 melanoma tumors expressing TRP-2.[52]

Immunotherapy of breast tumors with Listeria-based vaccines expressing TAA has focused on two tumor antigens, Mage and HER-2/neu. Our laboratory has shown that vaccination with LM-LLO, expressing a melanoma-associated antigen (Mage), Mage-$b_{311-660}$, completely eradicated the metastases and almost completely eradicated the primary tumors (90%) in a metastatic mouse breast tumor 4T1, expressing Mage-b.[53] However, this dramatic effect was not solely due to Mage-b-induced immune responses but also due to infection and subsequent kill of the tumor cells by LM-LLO bacteria, as we discuss in detail below. Also, vaccination with Listeria vaccines expressing fragments of Her2/neu, fused to LLO, were effective against primary tumors in a syngeneic mouse breast tumor model, NT-2, expressing Her2/neu, which correlated with strong immune responses to Her2/neu.[54] These vaccines were also effective against NT-2 when implanted into a syngeneic HER-2/neu transgenic mouse,[55] which displays profound tolerance to HER-2/neu. Female HER-2/neu transgenic mice develop mammary tumors by about 5–9 mo of age but the HER-2/neu expressing Listeria vaccines delayed the appearance of mammary tumors in these mice.[56] In order to move these findings in breast cancer towards clinical applications, Seavey et al. improved the design of the Listeria-LLO-HER-2/neu vaccines, by creating a single Listeria construct that expressed a chimeric gene incorporating most of the known HLA epitopes of HER-2/neu fused to LLO.[57]

Table 1. Re-clinical studies with listeria-based vaccines that target tumor antigens in mice with cancer

Cancer model	Listeria-antigen	Origin of antigen[1]	Tumor/mouse model	Antitumor responses	References
Colon, kidney	LM-LLO-NP	Influenza virus	CT26, RENCA	Protect against tumor challenge. Regression of established tumors	46
Melanoma	LM-LLO-NP	Influenza virus	B16	Regression of established tumors and lung metastases	47
Cervical, H & N	LM-LLO-E7	HPV-16	TC-1[2]	Regression of	48
	LM-PEST$^+$E7	HPV-16	TC-1	Established tumors	49
	LM-LLO (PEST$^-$)-E7	HPV-16	TC-1	Slowing of tumor growth	49
	LM-E7[3]	HPV-16	TC-1	Slowing of tumor growth	48, 49
	Lm-LLO-E7	HPV-16	TC-1/E6 & E7 Tg mouse[4]	Regression of established tumors	50
	Lm-LLO-E7	HPV-16	Autochthonous tumors in the E6 & E7 Tg mouse	Slowing of tumor growth	51
	Lm-ActA-E7[5]	HPV-16			
Melanoma	LM-TRP-2[3]	Mouse	B16	Protect against tumor challenge.	52
Breast	LM-LLO-Mage-b	Mouse	4T1	Eradication of metastases and 90% of primary tumors	53
Breast	LM-LLO-Neu[6]	Rat	NT2	Regression of established tumors	54
	LM-LLO-Neu[6]	Rat	NT2/rat HER-2/neu Tg mouse[7]	Stopped growth of established tumors	55
	LM-LLO-Neu[6]	Rat	Autochthonous tumors in the rat HER-2/neu Tg mouse	Slowing of tumor growth	56
	Lm-LLO-Neu-chimera[8]	Human	NT2 & autochthonous tumors in the rat HER-2/neu Tg mouse	Regression of primary established NT-2 and lung metastases. Slows autochthonous tumor growth	57
Prostate	LM-LLOdal$^-$dat$^-$ actA^{-9} PSA	Human	TPSA23	Regression of established tumors	58

[1]Non-mouse antigens will be recognized as foreign unless the mouse is transgenic for that antigen. [2]Lung epithelial cells transformed/immortalized by v-Ha-ras, E6 and E7, epitopes of HPV. [3]Antigen is not fused to LLO and is integrated into the chromosome. [4]C57BL/6 mouse transgenic for HPV-16 E6 and E7 under the thyroglobulin promoter develops autochthonous thyroid tumors. [5]Antigen is fused to the listerial actin polymerase, ActA. [6]Expressed as five separate fragments. [7]FVB mouse transgenic for rat HER-2/neu under the MMTV promoter develops autochthonous breast tumors. [8]Complete Neu is represented as a fusion of HLA restricted epitopes. [9]Host strain lacks ActA and alanine racemases. Plasmid complements racemase deficiency. LM, *Listeria monocytogenes*; LLO, ListeriolysinO; PEST, a sequence rich in proline, glutamic acid, serine and threonine in the amino terminus of LLO; NP, influenza nucleoprotein; TRP, tyrosine-related protein; HPV, human papilloma virus; H & N, head and neck cancer.

Recently, a LM-LLO-PSA construct, expressing prostate-specific antigen (PSA), but lacking the *dal* and *dat* genes (which are responsible for D-alanine synthesis), and ActA (involved in actin polymerization required for cell to cell spread) has been developed by Wallecha et al.[58] to further improve its safety. Vaccination with this Listeria vaccine induced regression of established prostate adenocarcinoma and improved survival of mice with prostate cancer tumors. For an overview of vaccine studies with Listeria-based vaccines expressing tumor-associated antigens in preclinical animal models see **Table 1.**

Harnessing Listeria to Kill Tumor Vasculature

Tumor cells can evade antigen-specific immunotherapy by downregulating tumor antigens, MHC class I molecules or molecules required for efficient antigen processing. We have also shown extensive immunoediting of CTL epitopes in response to active immunotherapy against a tumor antigen know to contribute to the tumor phenotype.[56] One possible solution to this problem could be to direct immunotherapy against tumor vascular cells, which are required for tumor growth, but which may be more genetically stable than tumor cells.

Folkman and colleagues[59] first suggested targeting tumor vasculature with anti-angiogenic therapy to control tumor growth. Blood vessels are assembled from vascular endothelial cells and supported by mural cells called pericytes, which stabilize the vessels and promote angiogenesis. Both cell types are crucial to vascular function but tumors in general have poor vasculature with sparse pericyte coverage.[60,61] Tumor cells cannot grow past a critical mass of 2–3 mm in the absence of a blood supply. Initially tumors co-opt existing blood vessels in tissue beds but as the tumors grow they must lay down their own vasculature. A key molecule in this process is vascular endothelial growth factor receptor 2 (VEGFR2), which is also called fetal liver kinase 1 (Flk-1) in the mouse. Expression of VEGFR2 on endothelial cells and binding of VEGF-A leads to the rapid differentiation, proliferation and migration of these cells into tube-like structures. Because of this, VEGFR2 plays an important role in tumor growth, invasion and metastasis,[62,63] making it an attractive therapeutic target[64,65] for Listeria based delivery.

However Flk-1 is a very large molecule of 1345 residues, which is too large to be expressed as a single molecule by Listeria. We thus selected three regions of the molecule, of about 200–300 residues each that appeared to contain the majority of known and putative CTL epitopes for the breast tumor models with which we wished to test them[66] and expressed these as fusion proteins with the microbial adjuvant, LLO. Two of these vaccines, which expressed the fragments 68–277 and 792–1081, were effective tumor immunotherapeutics in a transplantable breast tumor model and also reduced the appearance of experimental micrometastases in the lung.[66] In addition, they promoted epitope spreading to an endogenous tumor antigen, HER-2/neu; reduced tumor microvascular density (MVD); and prevented the long-term growth of spontaneous tumors all without significantly affecting normal tissue angiogenesis. Thus targeting endothelial cells through Flk-1 could induce epitope spreading to an endogenous tumor protein and lead to tumor death.[66]

Pericytes can also act as targets for anti-angiogenesis immunotherapy.[60,61] Pericytes are required for the normal function and integrity of vascular capillaries. Their loss disrupts vessel integrity leading to vessel collapse and hypoxia. Pericytes arise from vascular smooth muscles cells and express a glycoprotein, called high molecular weight melanoma associated antigen (HMW-MAA) in humans. HMW-MAA expression by pericytes increases during angiogenesis.[67] HMW-MAA is a cell surface, highly glycosylated, proteoglycan that interacts with the extracellular matrix and binds VEGF-A, matrix metalloproteinases and bFGF. It has been found in the CNS and is expressed by basal cell carcinoma, tumors of neural crest origin (astrocytomas, gliomas and neuroblastomas) and sarcomas.[68] It was originally identified as a melanoma specific marker and is found on over 90% of benign nevi and melanoma lesions.[68] HMW-MAA is also known as melanoma chondroitin sulfate proteoglycan (MCSP) and as NG2 in the rat and AN2 in the mouse. We chose to explore HMW-MAA as an anti-angiogenesis target because of its high expression on pericytes. Three different regions of the HMW-MAA molecule were fused to LLO and cloned into Listeria. Only one out of the three cloned regions, residues 2160–2258, showed any efficacy. This Listeria-LLO-HMW-MAA vaccine was able to slow the growth of a number of transplanted subcutaneous tumor cells, reduce the appearance of micrometastases in a lung seeding model and slow the appearance of breast tumors in a transgenic mouse model for breast cancer.[69] In addition, the vaccine induced a significant reduction in tumor volume, MVD and pericyte coverage, which correlated with an increase in CD8 T-cell infiltration into the tumor microenvironment. However targeting HMW-MAA did not appear to influence the ability of mice to lay down normal tissue vasculature during pregnancy or wound healing[69] indicating that it had few adverse effects. Taken together these studies suggest that Listeria can break tolerance to ubiquitous self-molecules and destroy tumor vasculature with little consequence to normal vasculature indicating that this may be a promising approach for tumor immunotherapy.

Listeria can Infect and Kill Mouse and Human Breast Tumor Cells

Recently, we made the interesting finding that Listeria can infect and kill mouse and human breast tumor cells in vitro.[70] After 1 h of co-culture, 20% of 4T1 and 100% of MCF7 tumor cells were infected with Listeria. However, after 2–3 h of incubation the infection rate of both tumor cell lines was 100%. Also in vivo, Listeria efficiently infects primary breast tumors and metastases. At this point, we do not know which receptors on the breast tumor cells are involved in invasion by Listeria. Possible candidates are HSPG or HGF, since both receptors are highly expressed in many breast cancers.[21-23,25,26] Another candidate is E-cadherin, which is highly expressed in many cancers, including breast cancer.[71] The higher infection level of the human MCF-7 than of the mouse 4T1 may be the result of higher expression of the receptors on MCF7 than on 4T1.

Although the presence of bacteria in tumor cells has been previously recognized,[72-74] the direct kill and immunological consequences, as described below, are novel observations. For instance, Yu et al. have shown that attenuated pathogens such as *Vibrio cholera*, and *Salmonella typhimurium*, enter tumors and metastases then replicate.[73] In this same study, they demonstrated that an attenuated LM carrying a green fluorescence protein infects PC-3 human prostate tumor cells in a xenograft model in vivo. Very recently, also Stritzker et al. have shown that an attenuated LM infects tumor cells.[74] However, in contrast to our study, they did not report Listeria induced killing of the infected tumors.

ROS-Mediated Tumor Cell Kill by Listeria

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.[75] It is known that Listeria activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in macrophages and neutrophils.[76-78] We have shown that Listeria induces death of 4T1 and MCF7 tumor cells through the activation of NADPH oxidase and subsequent production of ROS.[70] We demonstrated the involvement of NADPH oxidase-mediated ROS in tumor cell death, using Trolox, a scavenger of OH• radicals, and apocynin or diphenylene iodonium (DPI). Both selective inhibitors of NADPH oxidase prevented 50% of the Listeria-induced tumor cell death. Using live cell microscopy and H_2DFFDA or CM-H_2XRos, we also demonstrated that cytosolic ROS were produced through activated NADPH oxidase, and that mitochondrial ROS were produced as well. 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.[79] We found that Listeria increased intracellular Ca^{2+} levels resulting in the production of high levels of mROS.[70] These results imply that NADPH oxidase and excessive intracellular calcium contribute to tumor cell death upon LM-LLO infection causing mitochondrial failure. An example of Listeria-induced mitochondrial failure in vitro and in vivo is shown in **Figure 1.**

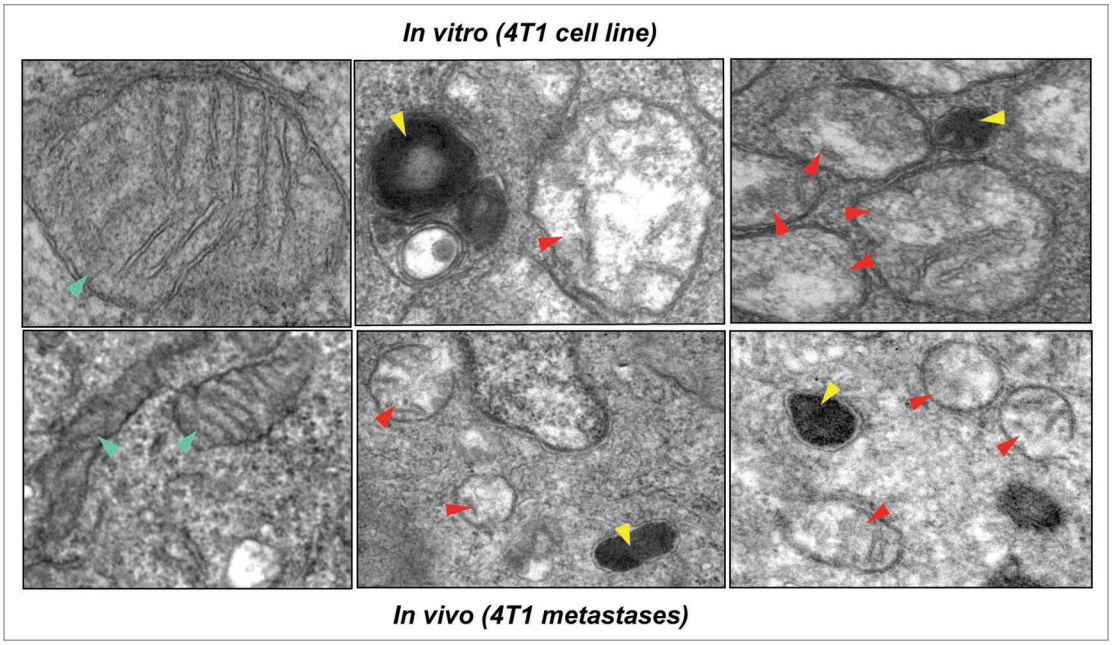

Figure 1. Listeria induce mitochondrial failure. EM analysis shows mitochondrial damage in tumor cells after infection with Listeria bacteria (LM-LLO-Mage-$b_{311-660}$) in vitro (top) and in vivo (bottom). The 4T1 tumor cell line was co-cultured with LM-LLO-Mage-$b_{311-660}$ for 1 h.[70] (top/left) A non-damaged mitochondrion (green arrow) of a non-infected 4T1 cell (negative control) (top/middle and right) damaged mitochondria (red arrows) in a 4T1 tumor cell infected with Listeria (yellow arrow). In vivo, Listeria induced mitochondrial damage in 4T1 tumor cells after one immunization with LM-LLO-Mage-$b_{311-660}$.[70] (bottom/left) A 4T1 tumor cell with non-damaged mitochondria (green arrows) of a metastasis (in diaphragm) of a non-immunized mouse (negative control) (bottom/middle) A 4T1 tumor cell with a Listeria bacterium (yellow arrow) and damaged mitochondria (red arrows) of a metastasis (in diaphragm) of mice immunized with LM-LLO-Mage-$b_{311-660}$; (Bottom/right) A 4T1 tumor cell with a Listeria bacterium (yellow arrow) and damaged mitochondria (red arrows) of a metastasis in the mesenteric lymph nodes (MLN) of mice immunized with LM-LLO-Mage-$b_{311-660}$. Magnifications (top) x44,000, 0.5 μ sections; (bottom) x20,500, 1 μ sections.

Tumor Cell Death Mediated by Listeria-Specific CTL

Infection of tumor cells with Listeria bacteria results in overexpression of listerial proteins. In addition, macrophages will be infected with Listeria resulting in strong CTL responses against the highly immunogenic listerial proteins.[39,40] Therefore, it is expected that Listeria-specific CTL will kill the infected tumor cells in vivo. Indeed, 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 to LM-LLO alone, suggesting that Listeria-specific CD8 T-cells, at least partially, contribute to tumor reduction in vivo.[70] A schematic view of the dual mechanism of LM-based vaccine resulting in tumor cell kill is presented in **Figures 2A and B.**

The Role of LLO in the Battle Against Cancer

LLO is required by Listeria to establish intracellular infections.[6,80] As discussed earlier, LM enters the APC through phagocytosis, and escapes into the host cytosol by perforating the phagosomal membrane through the action of a cytolysin, listeriolysin O (LLO).[36,37] A sequence rich in proline, glutamic acid, serine and threonine (PEST) at the amino terminus of LLO is thought to control the production of LLO.[81] Others found that the γ-interferon-inducible lysosomal thiolreductase (GILT) is responsible for the activation of LLO in vivo.[82] We found that LLO protein killed 80–90% of the 4T1 tumor cells in vitro, while LLOΔpest did not induce tumor cell death, and that this could be prevented with apocynin.[70] These results suggest that the PEST sequence is involved in the activation of NADPH oxidase. Also, while LM-LLO killed 80–90% of the tumor cells, LM-OVA, lacking LLO, was not able to infect and kill tumor cells (unpublished results).

LLO also plays a central role in vivo. Vaccination with LM-LLO-E7 strongly eradicated E7-expressing tumors, while LM-E7 or LM-LLO-E7 (lacking PEST) did not, despite the presence of strong immune responses to E7 in all three vaccine studies.[48,49] Our vaccine studies showed that immunization with LM-LLO was as effective as LM-LLO-Mage-b against 4T1 metastases, despite the presence of strong Mage-b responses in the LM-LLO-Mage-b vaccinated mice.[70] Also, while LM-LLO was highly effective against 4T1 metastases in vivo, LM-OVA was not (unpublished results). These in vivo studies indicate that LLO plays a further role in protection from tumor challenge than just the TAA-induced immune responses. Again, heat-killed Listeria are not protective. A favored hypothesis is that killed bacteria do not enter the cytosol of macrophages following phagocytosis, thereby resulting in insufficient antigen presentation.[40] However, as we discussed earlier, heat-killed Listeria induce CTL responses[43] but CTL activated by heat-killed Listeria are not cytolytic, in contrast to CTL activated by live Listeria.[44] Another report describes that cytolytic activity is not required

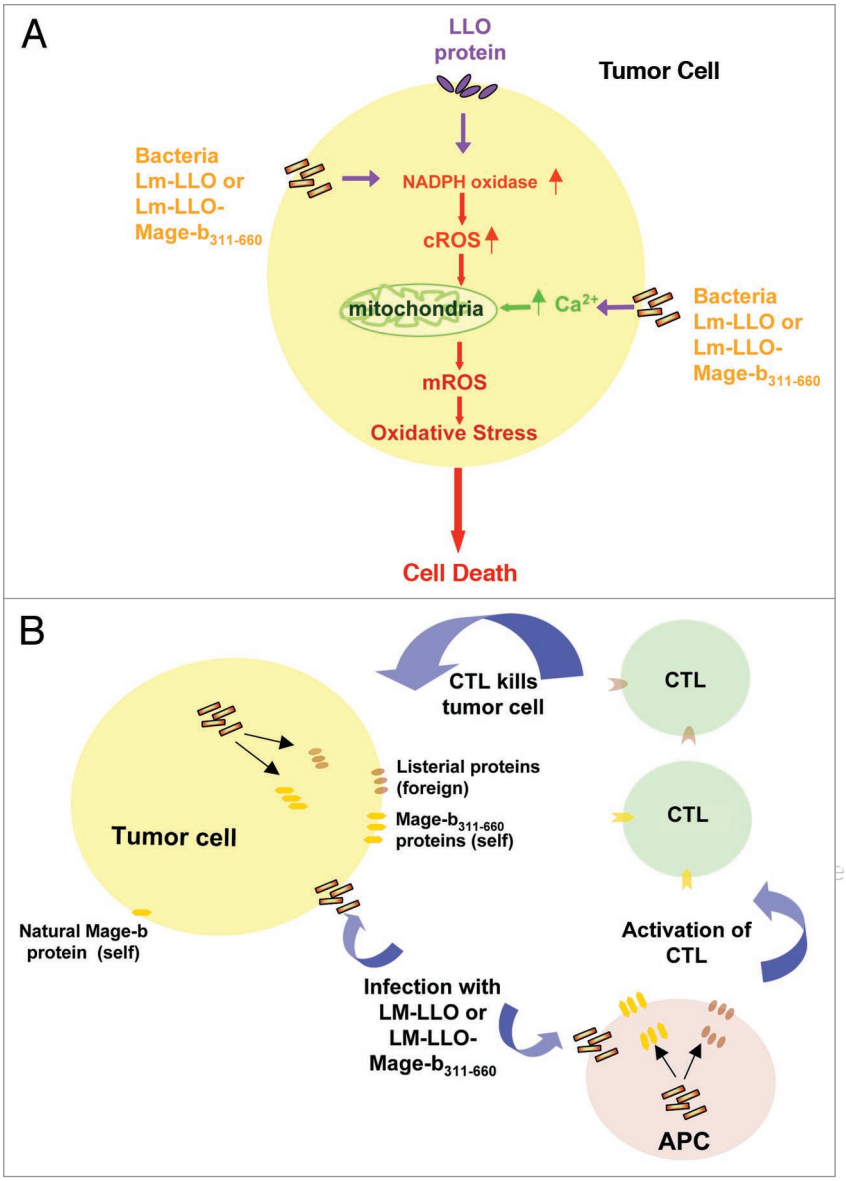

Figure 2. (A) ROS-induced tumor cell kill. Infection of tumor cells with LM-LLO or LM-LLO-Mage-$b_{311-660}$ results in the activation of NADPH oxidase and subsequent production of cytosolic reactive oxygen species (cROS), followed by mitochondrial disruption and production of mitochondrial (m)ROS, and results in increased intracellular Ca^{2+} levels, followed by mitochondrial disruption and subsequent production of mROS.[70] The high ROS levels, usually produced by cells to kill the intracellular bacteria, were more detrimental to the tumor cells than to the Listeria bacteria. The high ROS levels induced by both pathways resulted in oxidative stress and subsequent cell death. Also extracellular LLO protein but not LLOΔPEST was able to induce activation of NADPH oxidase and subsequent cell death. (B) Tumor cell kill mediated by Listeria-specific CTL. Immunization of tumor-bearing mice with Listeria bacteria (LM-LLO-Mage-$b_{311-660}$ or LM-LLO) leads to infection of antigen-presenting cells (APC) such as macrophages, resulting in expression of listerial and Mage-$b_{311-660}$ antigens, which subsequently stimulate cytotoxic T lymphocytes (CTL) specific for these antigens. As shown earlier, we found evidence that not only APC were infected with Listeria bacteria but also 4T1 tumor cells in vitro and in vivo.[70] As a result, infected tumor cells express Mage-$b_{311-660}$, in addition to naturally expressed Mage-b, as well as Listeria proteins, thereby changing poorly immunogenic tumor cells into a highly immunogenic target for Listeria-specific CTL that were activated by Listeria-infected APC.

for protection.[45] Based on comparative studies in several tumor models that have established the requirement for LLO in effective tumor immunotherapy mediated by Listeria, we hypothesize that dead Listeria with non-functional LLO cannot infect and cannot kill tumor cells. It is clear that LLO plays a crucial role in protection from tumor challenge and in the direct kill of tumor cells by Listeria. However, more research is needed to further unravel the LLO-mediated pathways in the battle against cancer.

Listeria are Protected from Immune Clearance in the Tumor Environment

Our in vivo studies suggest that Listeria selectively infects tumor cells. Mice were not sick after three immunizations with Listeria. No pathological damage in tissues [liver, spleen, gastro intestines (GI)] was observed after three immunizations with LM-LLO-Mage-$b_{311-660}$ and liver functions such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were unaffected.[70] Only some inflammatory spots (concentration of lymphocytes) were observed in the liver. One reason is that our Listeria (LM-LLO) is highly attenuated by mutations in the virulence gene prfA, which strongly downregulates its pathogenicity and virulence in vivo compared to wild-type Listeria.[48] This does not explain why the Listeria selectively infects and kills tumor cells. To answer this question, we co-incubated primary cultures of normal human and mouse fibroblasts with the Listeria bacteria. To our surprise, Listeria infected and killed the normal cells almost as efficiently as the tumor cells in vitro.[70] However, in vivo this was not the case. We found evidence that in vivo the Listeria bacteria are cleared very efficiently by the immune system in normal tissues.[70] Three d after immunization, the Listeria bacteria could no longer be cultured from normal tissues such as spleen, liver, kidneys, heart and lungs and gastro-intestines, while the Listeria multiplied in the metastases and primary tumors. We had shown earlier that Mage-b-specific immune responses are completely suppressed in the tumor microenvironment but not in normal tissues.[53,70] Also Listeria-specific immune responses are partly reduced in the tumor environment but not in normal tissues. Therefore, we concluded that Listeria bacteria in the tumor microenvironment are protected from clearance by the immune system compared to normal tissue.

Clinical Trials with Listeria-Based Vaccines and Safety Issues

Thus far, two clinical trials with attenuated Listeria-based vaccines have been reported. One clinical trial involves a single dose escalation study with an attenuated live Listeria vaccine, lacking virulence factors ActA (responsible for actin polymerization and resultant movement within eukaryotic cells and intercellular spread), and plcB (encoding a phospholipase or lecithinase,[83] involved in secondary vacuole escape). This vaccine has been tested orally (doses 10^6–10^9 CFU) in twenty healthy adult volunteers for its safety and toxicity. There were no positive blood cultures, three volunteers had temporarily abnormal liver functions, and humoral and cellular responses were strongly induced to the Listeria. They concluded that the attenuated LM is safe in adult volunteers without serious long-term health sequelae.

The other clinical trial used an attenuated live Listeria strain with mutations in the prfA (a virulence gene required for survival of the Listeria) which expressed human papilloma virus (HPV) E7 protein fused to LLO.[49,84] This vaccine designated as LM-LLO-E7, has been tested in fifteen cancer patients (doses 10^9 to 10^{10} CFU) with advanced carcinoma of the cervix in a Phase I/II clinical trial. All patients had a history of failed surgery, chemotherapy and/or radiation. Vaccinations with LM-LLO-E7 were at least 30 d after the last treatment with chemotherapy or radiation in order to retain immune competence. Immune competence in the patients with remaining tumor was confirmed by a delayed hypersensitive (DTH) screening panel, and Eastern Cooperative Oncology Group (ECOG) performance status ≤2 (Karnofsky index >60%). All patients received two immunizations intravenously with a 3-w time interval and Ampicillin 5 d after each vaccination. Vaccination resulted in flu-like symptoms in all patients. Six out of 15 patients showed grade 3 side effects. Liver functions such as AST and ALT increased after the first immunization, but went back to normal levels at the end of the study. The study concluded that the LM-LLO-E7 vaccine was safe in cancer patients with advanced adenocarcinoma of the cervix paving the way for a Phase II clinical trial.

Concluding Remarks and Future Prospects

One conclusion that can be made from the preclinical studies is that Listeria has multiple functions that are particularly useful to combat cancer. Listeria-TAA-based vaccines are able to activate TAA-specific CTL, mediating tumor cell cytolysis and NK cells that may kill tumor cells as well. Targeting tumor vasculature using Listeria that expresses pro-angiogenic molecules is an effective immunotherapeutic strategy that can circumvent the need for a known tumor antigen, since it induces epitope spreading to endogenous tumor antigens. We have also shown that Listeria can infect and kill tumor cells directly through high levels of ROS and through Listeria-specific CTL. In vitro and in vivo studies strongly suggest that LLO plays a central role in the multiple pathways of Listeria against cancer.

With the knowledge that Listeria selectively infects tumor cells, the opportunities to develop constructs that further improve tumor cell death are unlimited. Any gene that can induce apoptosis, necrosis, autophagy or other type of cell death could be cloned into Listeria. While CD8 T-cell responses to the TAA Mage-b were completely inhibited in the tumor environment, Listeria-specific CD8 T-cell responses were still detectable. It seems that immune responses to a self molecule such as Mage-b are much more strongly inhibited than immune responses to foreign antigens (Listeria) in the tumor environment. Therefore, any gene that is foreign and highly immunogenic could be a suitable candidate for cloning into the Listeria. Listeria can also be used to deliver drugs into tumor cells. Stritzker et al. delivered prodrug converting enzyme genes successfully into tumor cells in vitro using an attenuated Listeria.[74] This Listeria expressed LLO and the prodrug converting enzyme genes purine-deoxynucleoside phosphorylase (PNP) or a fusion protein consisting of yeast cytosine deaminase and uracil phosphoribosyl transferase (FCUI).

The clinical trials conducted with attenuated Listeria show that it is tolerated much better with less severe side effects than chemotherapy or radiation. Important criteria that may further improve the success rate of Listeria-based therapies against cancer in clinical trials could be to target tumors in tissues that are natural niches for Listeria infection. Thus pancreatic, liver and colon cancer, as well as brain tumors may be most suitable for Listeria-based therapies.

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