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***Listeria* and *Salmonella* Bacterial Vectors of Tumor-associated antigens for Cancer Immunotherapy**

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Abstract

This review covers the use of the facultative intracellular bacteria, *Listeria monocytogenes* and *Salmonella enterica* serovar *typhimurium* as delivery systems for tumor-associated antigens in tumor immunotherapy. Because of their ability to infect and survive in antigen presenting cells, these bacteria have been harnessed to deliver tumor antigens to the immune system both as bacterially expressed proteins and encoded on eukaryotic plasmids. They do this in the context of strong innate immunity, which provides the required stimulus to the immune response to break tolerance against those tumor-associated antigens that bear homology to self. Here we describe differences in the properties of these bacteria as vaccine vectors, a summary of the major therapies they have been applied to and their advancement towards the clinic.

Keywords

Listeria monocytogenes; *Salmonella enterica*; tumor immunotherapy; tumor associated antigens; tumor vasculature

1. Using bacteria to overcome challenges in tumor immunotherapy

Tumor immunotherapy is predicated on the existence of tumor-associated antigens (TAAs) to which the immune system can respond. Since the pioneering work of Thierry Boon that began two decades ago [1] many such antigens have been discovered. However, these TAAs are often endogenous antigens, which are either over-expressed, have unregulated expression or have accumulated mutations that differentiate them from the wild type protein. Most TAAs are intracellularly located, so it is now believed that cell mediated immunity characterized by a strong cytotoxic T lymphocyte (CTL) response against a TAA is the most effective anti-tumor strategy. However, since the majority of tumor antigens bear strong homology to self proteins [2], and they are initially presented to the immune system by tumor cells in the absence of co-stimulatory signals, they are likely to have induced immune tolerance rather than active T cell responses [2]. The challenge of tumor immunotherapy is to overcome these tolerogenic events. Here, we will describe the advantages that bacterial based immunotherapeutics may have in enhancing the immune response to tolerogenic tumor antigens.

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Bacterial pathogens were one of the earliest, non-surgical, cancer interventions. William Coley, a New York City surgeon in the late nineteenth century was the first investigator to recognize the potential of bacteria for cancer immunotherapy. Coley noticed that tumors often shrank in cancer patients who contracted acute bacterial infections [3]. In collaboration with Robert Koch, he developed a mixture of bacterial toxins for use in patients with inoperable cancer [3]. However, safety concerns plus the fact that the toxins were only effective in eliminating sarcomas led to a decline in the use of Coley's toxins for cancer therapy as radiation- and chemo-therapy treatments were developed [3]. Nevertheless, BCG is still in clinical use for the treatment of bladder cancer after resection of primary bladder surface tumors. Intravesical administration of BCG to the bladder on a weekly basis can prevent tumor recurrence in almost 60% of patients [4].

The success of Coley's toxins is almost certainly due to the fact that pathogens in general, and bacteria in particular, induce a strong pro-inflammatory innate immune response early in infection through the action of Pathogen Associated Molecular Patterns (PAMPs). It has long been recognized that infection with pathogens can exacerbate auto-immunity or cause the relapse of patients in remission from auto-immune disease [5]. The ability of pathogens to overcome tolerance associated with tumors, has led to the development of a variety of pathogen-based immunotherapies, which includes viral vectors, such as adenovirus, AAV, vaccinia, avipox, VEEV and MVA, and the bacterial vectors *BCG*, *Salmonella*, *L. monocytogenes*, and *Streptococcus*. The use of bacteria, rather than viruses has some advantages. Bacterial strains can be readily and irreversibly attenuated, their infection can be easily curtailed with antibiotics and they can be cheaply produced in simple media free from animal products and cells. Of the bacteria that have been explored as cancer immunotherapeutics the most advanced are the facultative intra-cellular pathogens, *Listeria* and *Salmonella*, which we will focus on in this review.

2. The Biology of *Listeria monocytogenes* and *Salmonella enterica* serovar typhimurium

Facultative intracellular bacteria are organisms that are free-living but have evolved virulence factors that allow them to infect host animal cells and enable them to survive the microbicidal environment of phagocytic cells. Key to understanding the potential of intracellular bacteria as carriers of passenger antigens to the immune system is knowledge of their cellular localization and mechanisms for inducing immunity. *Listeria* and *Salmonella* are oral pathogens that naturally invade the host at the gut mucosa. Thus, their first encounter with the immune system is most likely to be with phagocytic cells in the Peyer's patches. *Salmonella enterica* serovar typhimurium is a facultative anaerobic Gram-negative bacterium. *Listeria monocytogenes* is a facultative, intracellular, gram-positive rod that is resistant to adverse environmental conditions. Once inside the host both *Salmonella* and *Listeria* invade the intestinal mucosa and are ultimately captured by phagocytes. However the mechanism by which these organisms evade the immune response during the intracellular stages of their infectious cycle differ remarkably.

Bacterial products can signal through toll-like receptors and induce inflammatory cytokine cascades that drive potent cellular immune responses against pathogens as well as tumors.

The burst of innate immunity that precedes the adaptive mucosal immune response must be overcome for a successful infection to take place. In order to survive the hostile environment of the phagosome, intra-cellular bacteria secrete a variety of virulence factors that modify phagolysosomal microbicidal mechanisms such as defensins, reactive oxygen and nitrogen intermediates, and lysosomal enzymes that are active at acid pH. Some mechanisms are common to many intracellular bacteria but others are unique to each species. *S. enterica* [6] can prevent acidification of the phagosome by inhibition of phagosome-lysosome fusion, thus constructing an innocuous vacuole within which the bacteria can live and replicate. *Salmonella* virulence factors encoded in the *phoP* locus induce the formation of spacious vacuoles from phagosomes that allow bacterial persistence and growth in these organelles [7]. However others, such as *Listeria monocytogenes*, are less efficient at modifying the phagolysosome. Indeed, until quite recently [8], it was believed that *listeriae* were destined to die if they remained in the phagosomal compartment. It is now known that *Listeria* can modify phagosomes, to large compartments, termed spacious *Listeria* associated phagosomes or SLAPS that are LAMP-1 positive, using a mechanism that involves the virulence factor listeriolysin O (LLO) [8]. Nevertheless, *Listeria* has a rather unique strategy for avoiding destruction in the phagolysosome, displayed by only a small group of intracellular bacteria, which use virulence factors that allow them to escape from the phagosome and live in the less hostile environment of the cell cytoplasm [9, 10].

Listeria monocytogenes invades cells either through direct phagocytosis or by binding to host cells via virulence factors called internalins [11]. Once inside the phagosome, *Listeria* secretes the membrane-active virulence factors LLO and phospholipase C, which degrade the phagolysosomal membrane. This results in the release of *Listeria* into the comfortable environment of the cytoplasm where they undergo cell division. *Listeria* can become motile through the expression of ActA, an actin polymerase, at the cell surface in an apolar manner. Motile bacteria that reach the cell membrane can protrude out of the host cell in pseudopodia and are subsequently phagocytosed by neighboring cells, which they then infect [9,10].

The changes in the microbicidal properties of the phago-lysosomal compartment induced by the uptake of bacteria is accompanied by an enhancement of the antigen presenting function of the phagocytic cell. Bacterial phagocytosis stimulates macrophages to secrete a variety of chemokines that recruit new cells to the site of infection, inflammatory cytokines that increase vascular permeability and lymphokines that promote the expression of MHC molecules, co-stimulatory molecules and molecules associated with antigen processing. All of these act to promote an early Th1 response and cell mediated immunity. IL-12, produced by macrophages and dendritic cells in response to bacterial PAMPS, is the key lymphokine in this process. IL-12 acts on NK cells to release IFN- γ that further activates macrophages and promotes the destruction of the intra-cellular bacteria in the phagosome. The production of IFN- γ by NK cells, promoted by IL-12, has been shown to be a crucial factor in early host defense mechanisms against intracellular bacteria such as *Salmonella*, *Mycobacteria* and *Listeria* [12]. In addition to its role in propagating innate immunity, IFN- γ also drives the early antigen-specific CD4⁺ T cell response to the Th1 phenotype required to generate CD8⁺ T cells and clear bacterial infection via adaptive cell-mediated immunity.

The activation of the microbicidal properties of macrophages early in infection will fail to kill *Listeria* that have succeeded in escaping into the cytoplasm of the cell. Following colonization of the macrophage cytosol *Listeria* is eliminated by CD8⁺ CTL that recognize and lyse infected cells. Indeed, the presence of live bacteria in the cytosol facilitates the generation of these cells by displaying antigenic peptides bound to MHC class I molecules [13]. Proteins secreted by *Listeria* into the cytosol, such as the virulence factors, LLO and ActA, can be processed by the classical endogenous pathway of antigen processing and loaded onto MHC class I molecules after transport to the ER by specialized chaperone molecules. Thus secreted bacterial virulence factors are a potent source of peptides for the generation of CTL. CD8⁺ cells are essential for the clearance of listerial infections and CTL have been found that are specific for many of the secreted listerial virulence factors that are required for phagosomal lysis and cell to cell spread [13]. *Listeria* has long been recognized as a well-studied model organism with which to study CD8⁺ T cell responses to bacteria [14]. Over a decade ago, the property of *Listeria* to target secreted proteins to the MHC class I pathway of antigen processing prompted us to examine the ability of these bacteria to act as vaccine vectors [15, 16].

3. *Listeria* and *Salmonella* as TAA protein vectors

Listeria has been explored as a vaccine vector for both infectious [17] and neoplastic [18] disease since it was first demonstrated in 1992 that it could deliver passenger antigens to the immune system with the induction of CTL responses [16]. Initially, efforts were focused on using *Listeria* to deliver protein antigens. A number of recent reviews have described earlier efforts to harness the unusual ability of *Listeria* to target protein antigens to both the MHC class I and II pathways of antigen processing [17–20]. Here we will review more recent studies focused on overcoming obstacles encountered in preclinical studies developing *Listeria* for the efficacious immunotherapeutic treatment of cancer. An early finding is that maximum anti-tumor efficacy was only achieved if the protein antigen was expressed as a fusion protein with the listerial virulence factors LLO [21] or ActA [22]. These bacterial products appear to have adjuvant properties that increase the antigenicity of otherwise poorly immunogenic tumor antigens. In addition both proteins contain PEST domains, regions rich in proline, glutamic acid, serine and threonine, which may direct the fused proteins to the ER for degradation and MHC I loading [23]. Elimination of the PEST domain in LLO conferred lost adjuvant activity for fused protein antigens [24]. In our laboratory, we routinely engineer *Listeria* to express the TAA genes of interest under the LLO (*hly*) promoter, which is the strongest listerial promoter [25]. Using these expression strategies, our lab and others have shown that *Listeria* is a particularly good vector for TAA immunization. A complete listing of all the pre-clinical studies using *Listeria* to deliver tumor antigens as proteins in mouse models of cancer can be found in recent reviews [18–20]. They include both natural tumor antigens such as PSA, Mage-b, HER-2/neu, tyrosinase and HPV-16 E7 as well as the use of viral proteins, such as influenza and LCMV nucleoprotein as model tumor antigen targets. These recombinant, protein expressing strains of *Listeria* have been tested in mouse models of melanoma, prostate, breast, cervical, renal and colon cancer. In addition, the ability of *Listeria* to successfully break tolerance in transgenic mice in which an oncogenic tumor antigen is expressed under the control of a

tissue specific promoter has also been demonstrated in a mouse model for HPV-16 E7 induced thyroid cancer [26] and in an autochthonous HER-2/neu expressing breast cancer model [27, 28].

In constructing and testing *Listeria*-based immunotherapeutics targeting HER-2/neu, some interesting advantages and obstacles were observed. Because *Listeria* has difficulty secreting large and hydrophobic molecules Her-2/neu was expressed by constructing five different *Listeria*-based constructs each expressing one of three extracellular domain fragments or two intracellular domain fragments [29]. Each of these constructs was able to impact tumor growth in a subcutaneous mouse model for breast cancer [29] and slow growth in an autochthonous model for breast cancer [27]. This was initially surprising since other vaccine approaches had only identified a single epitope in HER-2/neu for the FVB mouse [30]. However, delivering the proteins fused to LLO and with *Listeria* as a shuttle vector revealed several immuno-subdominant epitopes not previously identified [29, 31]. However, when extensive vaccination with each of the five constructs was attempted to prevent the occurrence of autochthonous tumors in the FVB/HER-2/neu transgenic mouse, it was found that eventually tumors grew out that had undergone immunoediting with the loss of CTL epitopes [27]. In an attempt to shift the immune pressure away from individual regions of the molecule, a *Listeria* fusion vaccine strategy was designed that expresses a chimeric product composed of three immunodominant regions of Her-2/neu, two in the extracellular domain and one in the intracellular domain [28]. Vaccination with the Her-2/neu chimeric *Listeria* vaccine induced regression of established tumors, prevented lung metastasis in mouse models of breast cancer and appeared to relieve immunoediting in the autochthonous model of the disease [28].

Salmonella enterica serovar typhimurium has been used to deliver tumor antigens to the immune system both as DNA plasmids and as proteins. Since *Salmonella* is gram-negative highly attenuated strains have been constructed to reduce the toxicity associated with LPS [32]. Given the ability of *Salmonella* to systemically infect animals through the gut, attention has been focused on using *Salmonella* as an oral delivery agent for tumor antigens. There are more examples of *Salmonella*'s use as a DNA delivery system (described below) rather than to express proteins designed to stimulate anti-tumor immune responses. Nevertheless *Salmonella* has been used to deliver both model and natural tumor antigens as proteins.

Early studies focused on using beta-galactosidase as a model tumor antigen carried by the *Salmonella typhimurium aroA* vaccine carrier strain. Two promoters were tested one that was activated upon infection and one that resulted in constitutive expression. Although both vaccines resulted in antigen-specific CTL, responses the expression of antigen *in vivo* was more effective at controlling the growth of a fibrosarcoma transfected with beta-galactosidase [33]. Later it was shown that expression of the antigen on infection resulted in a classical Th1 type of response compared to a mixed Th1/Th2 response induced by *Salmonella* that constitutively expressed beta-galactosidase. This was associated with better control of fibrosarcoma growth in a lung seeding model of metastases [34]. Natural tumor antigens targeted by *Salmonella* include the HPV-16 L1 protein. Expression of L1 by *Salmonella* resulted in intracellular viral like particles, which appeared to be effective in

inhibiting the growth of an HPV immortalized mouse cell line, which had retained expression of this antigen [35]. However, in all of these studies no attempt was made to promote antigen secretion by the bacterium and, unlike the case with *Listeria* that lives in the cytosol, it remains unclear how such antigens are released by *Salmonella* and target the MHC class I antigen processing pathway. Indeed, the superior ability of antigen secreted by *Listeria* to access the endogenous pathway of antigen processing, may be the reason why a *Listeria* construct expressing chicken ovalbumin was a much more effective anti-tumor immunotherapeutic against a melanoma tumor transfected with ovalbumin than *Salmonella* expressing this antigen [36].

A creative approach to this problem takes advantage of *Salmonella*'s type III secretion system, which can directly "inject" antigens through membranes and into the cytosol of infected cells [37, 38]. Nishikawa and colleagues applied this approach to the cancer testis antigen NY-ESO-1 and showed that the *S. typhimurium*-NY-ESO-1 construct could deliver antigen to the cytosol of human cells and activate NY-ESO-1-specific T cells from peripheral blood lymphocytes of cancer patients *in vitro*. Oral administration of *S. typhimurium*-NY-ESO-1 to mice eradicated established NY-ESO-1-expressing tumors [37]. The anti-tumor immunity induced by this vaccine was associated with a profound resistance to suppression by CD4⁺CD25⁺ regulatory T cells [38]. Another stratagem to ensure secretion of the tumor antigen by *Salmonella* employed the hemolysin (HlyA) secretion system of *Escherichia coli*, the prototype of type I secretion systems. In addition, the antigen, prostate specific antigen (PSA), was fused to cholera toxin subunit B, a potent mucosal adjuvant. Fusing PSA to CtxB is believed to enhance antigen secretion and uptake by antigen presenting cells (APCs) and was included to assist in delivering PSA to induce a cytotoxic CD8⁺ T-cell response after oral delivery. This novel *Salmonella* secretory system readily induced cell mediated immunity and prevented the growth of a PSA expressing tumor in mice [39].

4. *Listeria* and *Salmonella* as Vectors for cDNA and mRNA Delivery

The use of a bacterial vector to deliver plasmids to the immune system that contain genes that are under the control of a eukaryotic promoter has been termed "bactofection" by Goebel and colleagues [40]. There are several advantages associated with using facultative intracellular bacteria such as *Salmonella* or *Listeria* as carriers for DNA vaccines. One is their propensity to be taken up by phagocytic cells, which includes professional APCs. After phagocytosis many bacteria die in the phagolysosome and deliver the plasmid to this compartment or, in the case of *Listeria* may escape to the cytosol. If the bacterium is engineered to die in this compartment, through expression of a suicide gene, then it will deliver its tumor antigen-encoding cDNA to the cytosol. The delivered cDNA is then transcribed and the antigen expressed and presented by the APC. Compared to conventional DNA vaccine delivery approaches, which largely target the plasmid to myocytes, such as i.m. injection, gene gun or electroporation [41, 42], delivery by intracellular bacteria could be both simpler and more effective in stimulating an adaptive immune response against a tumor antigen. In addition purified cDNA plasmids encoding for tumor antigens are poorly immunogenic unless supplemented with costimulatory molecules such as MIP-1 α and GM-CSF [43, 44]. Using bacteria as the delivery agent provides these adjuvants in abundance.

Thus, using *Listeria* or *Salmonella* to deliver cDNA for antitumor immune therapy, allows for the selective delivery of cDNA to APCs for efficient processing of tumor antigens and effective costimulatory molecule production in response to the bacterial PAMPS.

The ease of oral immunization by *Salmonella* based vaccines has been harnessed to assist other vaccine approaches, such as DNA vaccines, that are not so easily administered [45]. Many of these target pro-angiogenic molecules in mouse colon, breast, and lung carcinoma and will be reviewed below, but others target tumor associated antigens such as alpha-fetoprotein for the treatment of hepatocellular carcinoma [46], gp 100 for melanoma therapy [47, 48] CEA for colon cancer [49, 50] and murine transcription factor Fos-related antigen 1, which is overexpressed in the aggressive murine breast carcinoma, D2F2 [51]. An unusual target antigen that was delivered as a DNA vaccine by *Salmonella* is the acquired multidrug resistance-1 (MDR-1) gene that emerges on tumor membranes with chemotherapy [52]. Reisfeld and colleagues orally administered *Salmonella* carrying a DNA vaccine encoding MDR-1 three times at 2-week intervals and showed partial protection against challenge two weeks later with either MDR-1 expressing CT-26 colon carcinoma cells or MDR-1 expressing Lewis lung carcinoma cells. The vaccine appeared to overcome tolerance to MDR-1 and induce antigen-specific CD8⁺ T cells [52].

A clear advantage in using *Listeria* rather than *Salmonella* to deliver plasmids to APCs is that *Listeria* can colonize the cytosol of cells. Goebel and colleagues took advantage of this property by constructing an attenuated *Listeria* strain that expressed a suicide cassette [40, 53, 54]. This cassette consisted of a phage lysin under the transcriptional control of the *actA* promoter responsible for the expression of the ActA virulence factor that allows *Listeria* to move through the cell. This promoter has maximum activity when *Listeria* is in the cytosolic phase of its infectious cycle thus expression of the phage lysin under the control of the *actA* promoter leads to the death of the bacterium and release of the cDNA plasmid to the cytosol after *Listeria* has exited the phagolysosome. The cytosolic cDNA plasmid is then in the appropriate compartment to translocate to the nucleus. The development of cytosol-specific suicide cassettes has resulted in improved gene expression compared to earlier bactofection vectors that did not include them. Using this delivery system for the reporter gene, eGFP, Goebel and colleagues demonstrated that *Listeria* bactoected cells were able to efficiently express and present this protein to eGFP-specific CD8⁺ T cells [40, 53].

While the pioneering studies of Goebel and colleagues demonstrated that the delivery of plasmid cDNA by *Listeria monocytogenes* could stimulate CD8⁺ T cells *in vitro*, we have found this to be a less effective use of the vector compare to protein delivery *in vivo* [55]. We directly compared *Listeria monocytogenes* strains that endogenously expressed and secreted the HPV-16 E7 tumor antigen as a protein [21] with a newly constructed strain [55] based on Goebel's DNA delivery vectors [40]. Although the latter was more effective than i.m. delivery of the plasmid alone, it was a great deal less effective in eliminating subcutaneous transplanted tumors that expressed E7 and in stimulating anti-E7 immune responses [55]. It seems likely that the decreased effectiveness of *Listeria* delivered plasmid DNA vaccines compared to protein based vaccines is due to the delayed expression of the tumor antigen, because of the need for transcription and translation. In order to expedite the expression of the tumor antigen after *Listeria* infection, strains have been developed to

deliver translation-competent mRNA encoding for model tumor antigens [56]. Similar to the plasmid DNA delivery system, the mRNA strains contain a suicide cassette that kills the *Listeria* vector upon entry of the bacterium into the cytosol after which it releases T7 polymerase transcribed mRNA that contains an IRES element rendering it translation-competent in the eukaryotic host cell. This approach resulted in earlier expression of the tumor antigen and augmented *in vivo* antitumor immune responses when compared to a plasmid DNA delivery strain. Nevertheless, although the mRNA delivery system was more effective than the plasmid DNA delivery strain, it was still not as effective as the *Listeria* strains that endogenously expressed and secreted the tumor antigen [56]. Clearly, despite a decade of research developing *Listeria monocytogenes* as a vector for the delivery of eukaryotic expression systems, further improvements are required for these strategies to exceed or match the effectiveness of *Listeria* as a protein delivery system (55, 56). Nevertheless such efforts are important since they could overcome some of the difficulties associated with expression and secretion of large, hydrophobic proteins by *Listeria*, thus broadening the targets for *Listeria* based tumor immunotherapy.

5. *Listeria* and *Salmonella* as vectors for anti-angiogenic molecules

Tumor cells are inherently unstable and can evolve under immune pressure to avoid the immune system by down regulating tumor antigens, MHC class I molecules and molecules required for efficient antigen processing and immunoediting CTL epitopes in antigens. In an attempt to overcome these difficulties immunotherapy has been directed against cell types that are required for tumor vasculature, which may be more stable or have less opportunity to escape immune responses. Folkman and colleagues first suggested that antiangiogenesis therapy, which interrupts a tumor's vascular network may be effective in controlling tumor growth [57]. Today many anti-angiogenesis therapies for cancer are available some of which are in clinical use [58].

Blood vessels are constructed from endothelial cells and are lined 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 [59 – 61]. When tumors cells begin to grow and divide they can obtain sufficient oxygen and nutrients by passive diffusion from the interstitial fluid. However, once the tumor has reached a critical mass, 2–3mm in diameter, an 'angiogenic switch' must occur that requires the formation of new blood vessels. 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 or Flk-1 plays an important role in tumor growth, invasion and metastasis [62, 63] and is thus strongly indicated as a therapeutic target [64, 65].

Immunotherapy targeting tumor vasculature was first demonstrated by Reisfeld and colleagues who used VEGFR2 DNA vaccines delivered orally by *Salmonella typhimurium* [64]. In addition to VEGFR2 these workers have used *Salmonella* to deliver a variety of

DNA vaccines encoding pro-angiogenic molecules [66]. These include endoglin (CD105), a co-receptor in the TGF-beta receptor complex, which is over-expressed on proliferating endothelial cells in the breast tumor neovasculature [67] and murine platelet derived growth factor receptor-beta (mPDGFRbeta) [68] which is required for pericyte recruitment to blood vessels [60]. These vaccines are all capable of inducing potent cell-mediated protective immunity against these self-antigens, resulting in efficient suppression of angiogenesis in the tumor vasculature, activation of cytotoxic T cells and marked suppression of tumor growth.

As protein based delivery systems appear to be more efficacious than DNA delivery by *Listeria* [55] we attempted to construct vectors that express VEGFR2 (Flk-1). However Flk-1 is a very large molecule of 1345 residues, which is too large to be expressed by *Listeria*. We thus selected three regions of the molecule, of about 200 to 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. These were each expressed as fusion proteins with the microbial adjuvant LLO [69]. 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 [69]. 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.

Pericytes can also act as targets for anti-angiogenesis immunotherapy. [60, 61]. Pericytes act as support cells for the normal function and integrity of vascular capillaries. Pericyte loss disrupts vessel integrity leading to vessel collapse, tissue starvation and hypoxia. These cells, which arise from vascular smooth muscles cells, express a glycoprotein, high molecular weight melanoma associated antigen (HMW-MAA) and expression increases during angiogenesis [70]. 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, neuroblastomas and sarcomas and by over 90% of benign nevi and melanoma lesions [71]. 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 cloned into *Listeria* fused to LLO. 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 [72]. 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 [72] indicating that it had few adverse effects.

6. Discussion and concluding remarks

The efficacy of bacterial based immunotherapy depends upon our knowledge of the inherent capability of bacterial vaccine strains to stimulate anti-tumor immune responses. Our understanding of the immune responses elicited in response to bacterial vectors has revealed qualitative differences in terms of the benefits as well as the limitations of employing one bacterium over another including *Salmonella* and *Listeria* [36]. Each bacterium may occupy a unique niche within the ever-expanding spectrum of vaccine options.

Bacterial based cancer immunotherapeutics have demonstrated great promise in pre-clinical settings and are beginning to make their way from the bench to the bedside. The use of live pathogen based vectors raises concerns about safety in potentially immunocompromised hosts. Attenuation of the virulence factors associated with each of the bacterial vectors under current investigation for clinical use has enabled significant development of the most promising bacterial species. The application of facultative intracellular bacteria as cancer vaccine carriers for human disease has not been rapid but both *Salmonella* and *Listeria* have entered or completed phase I cancer clinical trials. The NY-ESO-1 type III secreting *Salmonella* that expresses NY-ESO-1 is currently being tested by the Ludwig Cancer Institute for safety, immunogenicity and clinical responses in patients with NY-ESO-1 expressing melanoma, although no results have yet been published [73]. A first-in-human clinical Phase I safety study of Lm-LLO-E7, a *Listeria* vaccine that secretes an LLO-E7 fusion product [21] showed that vaccinations were safe for use in patients with late stage invasive carcinoma of the cervix [74]. 15 patients were enrolled and experienced flu-like symptoms, which were alleviated by non-prescription symptomatic treatment. At the end of the study more than half of the patients treated with the vaccine had stable disease and one patient was considered a partial responder. This study showed for the first time that a live-attenuated *Listeria* immunotherapeutic is safe to be administered to late stage invasive cancer patients [74]. Follow up on these patients, which is performed at three month intervals, shows that three are long-term survivors and are still alive three years post-treatment. This survival rate is substantially greater than the median survival rate established by the NCI's Gynecologic Oncology Group, which is between 3.8 and 6.2 months for cervical cancer patients at the same stage of advanced disease.

However, despite these promising results, the development of bacterial vaccines for cancer treatment faces a number of technical and biological challenges in addition to the challenges that face all immune based therapies associated with host tolerance to tumor-associated self-antigens, which are poorly immunogenic and genetically unstable, and the immune suppressive microenvironment that prevails in tumors. Bacteria are unable to carry out a variety of post-translational protein modifications observed in mammalian cells and do not readily fold proteins greater than about 60kD. In addition the necessity to secrete protein antigens expressed by bacteria limits the number of proteins that can be targeted by this approach. Furthermore, the expression of foreign antigens in bacteria, which it does not require or use, places a metabolic burden on the organism that can affect growth rate and

select for plasmid loss in the case of plasmid transformed bacteria or bacteria that have undergone mutations to silence antigen expression [75, 76].

As one considers the potential of bacterial vectors for the treatment of cancer the question arises as to how previous exposure to the wild type versions of attenuated vaccine strains may affect the ability of the vaccine to mount an immune response. Recent studies indicate that this may not be a limitation in using these vectors [77]. Leong and colleagues found that although mice with pre-existing cellular immunity to *L. monocytogenes* displayed attenuated CD8⁺ T cell responses to recombinant strains of *Listeria* expressing a target antigen such as OVA, which were dependent on dose and time of exposure to wild-type *Listeria*, these could be overcome by repeated immunizations. Nevertheless it may be important to monitor future patients for pre-existing immunity so that potential vaccinations may be effectively administered to treat separate incidences of cancer.

References

1. Van den Eynde B, Hainaut P, Hérin M, et al. Presence on a human melanoma of multiple antigens recognized by autologous CTL. *Int J Cancer*. 1989; 44:634–640. [PubMed: 2529220]
2. Pardoll D. Does the immune system see tumors as foreign or self? *Annu Rev Immunol*. 2003; 21:807–39. [PubMed: 12615893]
3. McCarthy EF. The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. *Iowa Orthop J*. 2006; 26:154–8. [PubMed: 16789469]
4. Shelly MD, Kynaston H, Court J, et al. A systemic review of intravesical bacillus Calmette-Guérin plus transurethral resection vs transurethral resection alone in Ta and T1 bladder cancer. *BJU Int*. 2001; 88:209–16. [PubMed: 11488731]
5. Rapp NS, Gilroy J, Lerner AM. Role of bacterial infection in exacerbation of multiple sclerosis. *Am J Phys Med Rehabil*. 1995; 74:415–8. [PubMed: 8534384]
6. Buchmeier NA, Heffron F. Inhibition of macrophage phagosome-lysosome fusion by *Salmonella typhimurium*. *Infect Immun*. 1991; 59:2232–8. [PubMed: 2050395]
7. Alpuche-Aranda CM, Racoosin EL, Swanson JA, et al. *Salmonella* stimulate macrophage macropinocytosis and persist within spacious phagosomes. *J Exp Med*. 1994; 179:601–8. [PubMed: 8294870]
8. Birmingham CL, Canadien V, Kaniuk NA, et al. Listeriolysin O allows *Listeria monocytogenes* replication in macrophage vacuoles. *Nature*. 2008; 451:350–4. [PubMed: 18202661]
9. Portnoy DA, Chakraborty T, Goebel W, et al. Molecular determinants of *Listeria monocytogenes* pathogenesis. *Infect Immun*. 1992; 60:1263–7. [PubMed: 1312514]
10. Southwick FS, Purich DL. *Listeria* and *Shigella* actin-based motility in host cells. *Trans Am Clin Climatol Assoc*. 1998; 109:160–73. [PubMed: 9601135]
11. Cossart P, Pizarro-Cerdá J, Lecuit M. Invasion of mammalian cells by *Listeria monocytogenes*: functional mimicry to subvert cellular functions. *Trends Cell Biol*. 2003; 13:23–31. [PubMed: 12480337]
12. Biron CA, Gazzinelli RT. Effects of IL-12 on immune responses to microbial infections: a key mediator in regulating disease outcome. *Curr Opin Immunol*. 1995; 7:485–96. [PubMed: 7495512]
13. Pamer EG, Sijts AJ, Villanueva MS, et al. MHC class I antigen processing of *Listeria monocytogenes* proteins: implications for dominant and subdominant CTL responses. *Immunol Rev*. 1997; 158:129–36. [PubMed: 9314081]
14. Mackaness GB. The immunological basis of acquired cellular resistance. *J Exp Med*. 1964; 120:105–20. [PubMed: 14194388]
15. Weiskirch LM, Paterson Y. *Listeria monocytogenes*: a potent vaccine vector for neoplastic and infectious disease. *Immunol Rev*. 1997; 158:159–69. [PubMed: 9314084]

16. Schafer R, Portnoy DA, Brassell SA, et al. Induction of a cellular immune response to a foreign antigen by a recombinant *Listeria monocytogenes* vaccine. *J Immunol.* 1992; 149:53–9. [PubMed: 1607662]
17. Paterson Y, Johnson RS. Progress towards the use of *Listeria monocytogenes* as a live bacterial vaccine vector for the delivery of HIV antigens. *Expert Review of Vaccines.* 2004; 3:S119–34. [PubMed: 15285711]
18. Seavey MM, Verch T, Paterson Y. Anticancer Vaccine Strategies. In: Liu, D., editor. *Handbook of Listeria monocytogenes.* CRC Press; 2008. p. 481–511.
19. Wood LM, Guirnalda PG, Seavey MM, et al. Cancer Immunotherapy using *Listeria monocytogenes* and Listerial virulence factors. *Immunological Research.* 2008; 42:233–45.
20. Wallecha A, Carroll KD, Maciag PC, et al. Multiple effector mechanisms induced by recombinant *Listeria monocytogenes* anti-cancer immunotherapeutics. *Advances in Applied Microbiology.* 2009; 66:1–27. [PubMed: 19203646]
21. Gunn GR, Zubair A, Peters C, et al. Two *Listeria monocytogenes* vaccine vectors that express different molecular forms of human papilloma virus-16 (HPV-16) E7 induce qualitatively different T cell immunity that correlates with their ability to induce regression of established tumors immortalized by HPV-16. *J Immunol.* 2001; 167:6471–9. [PubMed: 11714814]
22. Sewell DA, Douven D, Pan ZK, et al. Regression of HPV-positive tumors treated with a new *Listeria monocytogenes* vaccine. *Arch Otolaryngol Head Neck Surg.* 2004; 130:92–7. [PubMed: 14732776]
23. Rogers S, Wells R, Rechsteiner M. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science.* 1986; 234:364–8. [PubMed: 2876518]
24. Sewell DA, Shahabi V, Gunn GR 3rd, et al. Recombinant *Listeria* vaccines containing PEST sequences are potent immune adjuvants for the tumor-associated antigen human papillomavirus-16 E7. *Cancer Res.* 2004; 64:8821–5. [PubMed: 15604239]
25. Mengaud J, Vicente MF, Cossart P. Transcriptional mapping and nucleotide sequence of the *Listeria monocytogenes* *hlyA* region reveal structural features that may be involved in regulation. *Infect Immun.* 1989; 57:3695–701. [PubMed: 2509367]
26. Sewell DA, Pan Z-K, Paterson Y. *Listeria*-based HPV-16 E7 vaccines limit autochthonous tumor growth in a transgenic mouse model for HPV-16 transformed tumors. *Vaccine.* 2008; 26:5322–7.
27. Singh R, Paterson Y. Immunoediting sculpts tumor epitopes during immunotherapy. *Cancer Research.* 2007; 67:1887–92. [PubMed: 17332314]
28. Seavey MM, Pan Z-K, Maciag PC, et al. A novel human Her-2/neu chimeric molecule expressed by *Listeria monocytogenes* can elicit potent HLA-A2 restricted CD8⁺ T cell responses and impact the growth and spread of Her-2/neu⁺ breast tumors. *Clinical Cancer Research.* 2009; 15:924–32. [PubMed: 19188163]
29. Singh R, Dominiecki ME, Jaffee EM, et al. Fusion to Listeriolysin O and delivery by *Listeria monocytogenes* enhances the immunogenicity of HER-2/neu and reveals subdominant epitopes in the FVB/N mouse. *J Immunol.* 2005; 175:3663–73. [PubMed: 16148111]
30. Ercolini AM, Machiels JP, Chen YC, et al. Identification and characterization of the immunodominant rat HER-2/neu MHC class I epitope presented by spontaneous mammary tumors from HER-2/neu-transgenic mice. *J Immunol.* 2003; 170:4273–80. [PubMed: 12682262]
31. Singh R, Paterson Y. Vaccination strategy determines the emergence and dominance of CD8⁺ T-cell epitopes in a FVB/N rat HER-2/neu mouse model of breast cancer. *Cancer Res.* 2006; 66:7748–57. [PubMed: 16885378]
32. Schödel F, Curtiss R 3rd. *Salmonellae* as oral vaccine carriers. *Dev Biol Stand.* 1995; 84:245–53. [PubMed: 7796961]
33. Medina E, Guzmán CA, Staendner LH, et al. *Salmonella* vaccine carrier strains: effective delivery system to trigger anti-tumor immunity by oral route. *Eur J Immunol.* 1999; 29:693–9. [PubMed: 10064087]
34. Medina E, Paglia P, Rohde M, et al. Modulation of host immune response stimulated by *Salmonella* vaccine carrier strains by using different promoters to drive the expression of the recombinant antigen. *Eur J Immunol.* 2000; 30:768–77. [PubMed: 10741391]

35. Revaz V, Benyacoub J, Kast WM, et al. Mucosal vaccination with a recombinant *Salmonella typhimurium* expressing human papillomavirus type 16 (HPV16) L1 virus-like particles (VLPs) or HPV16 VLPs purified from insect cells inhibits the growth of HPV16-expressing tumor cells in mice. *Virology*. 2001; 279:354–60. [PubMed: 11145916]
36. Stark FC, Sad S, Krishnan L. Intracellular bacterial vectors that induce CD8(+) T cells with similar cytolytic abilities but disparate memory phenotypes provide contrasting tumor protection. *Cancer Res*. 2009; 69:4327–34. [PubMed: 19435919]
37. Nishikawa H, Sato E, Briones G, et al. *In vivo* antigen delivery by a *Salmonella typhimurium* type III secretion system for therapeutic cancer vaccines. *J Clin Invest*. 2006; 116:1946–54. [PubMed: 16794737]
38. Nishikawa H, Tsuji T, Jäger E, et al. Induction of regulatory T cell-resistant helper CD4⁺ T cells by a bacterial vector. *Blood*. 2008; 111:1404–12. [PubMed: 17986662]
39. Fensterle J, Bergmann B, Yone CL, et al. Cancer immunotherapy based on recombinant *Salmonella enterica* serovar *Typhimurium aroA* strains secreting prostate-specific antigen and cholera toxin subunit B. *Cancer Gene Ther*. 2008; 15:85–93. [PubMed: 18084243]
40. Pilgrim S, Stritzker J, Schoen C, et al. Bactofection of mammalian cells by *Listeria monocytogenes*: improvement and mechanism of DNA delivery. *Gene Ther*. 2003; 10:2036–45. [PubMed: 14566363]
41. Donnelly JJ, Ulmer JB, Shiver JW, et al. DNA vaccines. *Annu Rev Immunol*. 1997; 15:617–48. [PubMed: 9143702]
42. Ulmer JB, Donnelly JJ, Parker SE, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science*. 1993; 259:1745–9. [PubMed: 8456302]
43. McKay PF, Barouch DH, Santra S, et al. Recruitment of different subsets of antigen-presenting cells selectively modulates DNA vaccine-elicited CD4⁺ and CD8⁺ T lymphocyte responses. *Eur J Immunol*. 2004; 34:1011–20. [PubMed: 15048711]
44. Peng X, Tremblé J, Paterson Y. Plasmid Listeriolysin O (LLO) acts as an adjuvant to enhance the potency of an HPV-16 E7 DNA vaccine by improving both CD4⁺ and CD8⁺ T cell responses. *Cancer Immunol Immunother*. 2007; 56:797–806. [PubMed: 17102978]
45. Reisfeld RA, Niethammer AG, Luo Y, et al. DNA vaccines suppress tumor growth and metastases by the induction of anti-angiogenesis. *Immunol Rev*. 2004; 199:181–90. [PubMed: 15233734]
46. Chou CK, Hung JY, Liu JC, et al. An attenuated *Salmonella* oral DNA vaccine prevents the growth of hepatocellular carcinoma and colon cancer that express alpha-fetoprotein. *Cancer Gene Ther*. 2006; 13:746–52. [PubMed: 16410824]
47. Xiang R, Lode HN, Chao TH, et al. An autologous oral DNA vaccine protects against murine melanoma. *Proc Natl Acad Sci U S A*. 2000; 97:5492–7. [PubMed: 10779556]
48. Weth R, Christ O, Stevanovic S, et al. Gene delivery by attenuated *Salmonella typhimurium*: comparing the efficacy of helper versus cytotoxic T cell priming in tumor vaccination. *Cancer Gene Ther*. 2001; 8:599–611. [PubMed: 11571538]
49. Xiang R, Silletti S, Lode HN, et al. Protective immunity against human carcinoembryonic antigen (CEA) induced by an oral DNA vaccine in CEA-transgenic mice. *Clin Cancer Res*. 2001; 3 (Suppl):856s–864s. [PubMed: 11300483]
50. Xiang R, Primus FJ, Ruehlmann JM, et al. Dual-function DNA vaccine encoding carcinoembryonic antigen and CD40 ligand trimer induces T cell-mediated protective immunity against colon cancer in carcinoembryonic antigen-transgenic mice. *J Immunol*. 2001; 167:4560–5. [PubMed: 11591784]
51. Luo Y, Zhou H, Mizutani M, et al. Transcription factor Fos-related antigen 1 is an effective target for a breast cancer vaccine. *Proc Natl Acad Sci U S A*. 2003; 100:8850–5. [PubMed: 12857959]
52. Niethammer AG, Wodrich H, Loeffler M, et al. Multidrug resistance-1 (MDR-1): a new target for T cell-based immunotherapy. *FASEB J*. 2005; 19:158–9. [PubMed: 15498893]
53. Dietrich G, Bubert A, Gentschev I, et al. Delivery of antigen-encoding plasmid DNA into the cytosol of macrophages by attenuated suicide *Listeria monocytogenes*. *Nat Biotechnol*. 1998; 16:181–5. [PubMed: 9487527]
54. Spreng S, Dietrich G, Niewiesk S, et al. Novel bacterial systems for the delivery of recombinant protein or DNA. *FEMS Immunol Med Microbiol*. 2000; 27:299–304. [PubMed: 10727885]

55. Souders NC, Verch T, Paterson Y. *In vivo* bactofection: *Listeria* can function as a DNA-cancer vaccine. *DNA Cell Biol.* 2006; 25:142–51. [PubMed: 16569193]
56. Loeffler DI, Schoen CU, Goebel W, et al. Comparison of different live vaccine strategies *in vivo* for delivery of protein antigen or antigen-encoding DNA and mRNA by virulence-attenuated *Listeria monocytogenes*. *Infect Immun.* 2006; 74:3946–57. [PubMed: 16790768]
57. Folkman J. Tumor angiogenesis: therapeutic implications. *NJEM.* 1971; 285:1182–6.
58. Esteva FJ. Monoclonal antibodies, small molecules, and vaccines in the treatment of breast cancer. *The Oncologist.* 2004; 9 (Suppl 3):4–9. [PubMed: 15163841]
59. Zwaans BM, Bielenberg DR. Potential therapeutic strategies for lymphatic metastasis. *Microvasc Res.* 2007; 74:145–58. [PubMed: 17950368]
60. Betsholtz C, Lindblom P, Gerhardt H. The role of pericytes in vascular morphogenesis. *EXS.* 2005; 94:115–25. [PubMed: 15617474]
61. O’Keeffe MB, Devlin AH, Burns AJ, et al. Investigation of pericytes, hypoxia, and vascularity in bladder tumors: association with clinical outcomes. *Oncol Res.* 2008; 17:93–101. [PubMed: 18669161]
62. Folkman J. Tumor angiogenesis and tissue factor. *Nat Med.* 1996; 2:167–8. [PubMed: 8574960]
63. Shibuya M. Vascular endothelial growth factor (VEGF)-Receptor2: its biological functions, major signaling pathway, and specific ligand VEGF-E. *Endothelium.* 2006; 13:63–9. [PubMed: 16728325]
64. Niethammer AG, Xiang R, Becker JC, et al. A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth. *Nat Med.* 2002; 8:1369–75. [PubMed: 12415261]
65. Luo Y, Markowitz D, Xiang R, et al. FLK-1-based minigene vaccines induce T cell-mediated suppression of angiogenesis and tumor protective immunity in syngeneic BALB/c mice. *Vaccine.* 2007; 25:1409–15. [PubMed: 17113202]
66. Xiang R, Luo Y, Niethammer AG, et al. Oral DNA vaccines target the tumor vasculature and microenvironment and suppress tumor growth and metastasis. *Immunol Rev.* 2008; 222:117–28. [PubMed: 18363997]
67. Lee SH, Mizutani N, Mizutani M, et al. Endoglin (CD105) is a target for an oral DNA vaccine against breast cancer. *Cancer Immunol Immunother.* 2006; 55:1565–74. [PubMed: 16565828]
68. Kaplan CD, Krüger JA, Zhou H, et al. A novel DNA vaccine encoding PDGFRbeta suppresses growth and dissemination of murine colon, lung and breast carcinoma. *Vaccine.* 2006; 24:6994–7002. [PubMed: 17050049]
69. Seavey MM, Maciag PC, Al-Rawi N, et al. An anti-vascular endothelial growth factor receptor 2/fetal liver kinase-1 *Listeria monocytogenes* anti-angiogenesis cancer vaccine for the treatment of primary and metastatic Her-2/neu⁺ breast tumors in a mouse model. *J Immunol.* 2009; 182:5537–46. [PubMed: 19380802]
70. Schlingemann RO, Rietveld FJ, de Waal RM, et al. Expression of the high molecular weight melanoma-associated antigen by pericytes during angiogenesis in tumors and in healing wounds. *The Am J Path.* 1990; 136:1393–1405.
71. Campoli MR, Chang CC, Kageshita T, et al. Human high molecular weight-melanoma-associated antigen (HMW-MAA): a melanoma cell surface chondroitin sulfate proteoglycan (MSCP) with biological and clinical significance. *Crit Rev Immunol.* 2004; 24:267–96. [PubMed: 15588226]
72. Maciag PC, Seavey MM, Pan ZK, et al. Cancer immunotherapy targeting the high molecular weight melanoma-associated antigen protein results in a broad antitumor response and reduction of pericytes in the tumor vasculature. *Cancer Res.* 2008; 68:8066–75. [PubMed: 18829565]
73. Old LJ. Cancer vaccines: an overview. *Cancer Immun.* 2008; 8 (Suppl 1):1. [PubMed: 18333603]
74. Maciag PC, Radulovic S, Rothman J. The first clinical use of a live-attenuated *Listeria monocytogenes* vaccine: a Phase I safety study of Lm-LLO-E7 in patients with advanced carcinoma of the cervix. *Vaccine.* 2009; 27:3975–83. [PubMed: 19389451]
75. Galen, JE.; Pasetti, MF.; Sztein, MB., et al. Attenuated *Salmonella* and *Shigella* as live vectors carrying either prokaryotic or eukaryotic expression systems. In: Levine, MM.; Kaper, JB.; Rappuoli, R.; Liu, MA.; Good, MF., editors. *New Generation Vaccines.* 3. Marcel Dekker; 2004. p. 354–66.

76. Peters C, Paterson Y. Enhancing the immunogenicity of bioengineered *Listeria monocytogenes* by passaging through live animal hosts. *Vaccine*. 2003; 21:1187–94. [PubMed: 12559797]
77. Leong ML, Hampl J, Liu W, et al. Impact of preexisting vector-specific immunity on vaccine potency: characterization of *Listeria monocytogenes*-specific humoral and cellular immunity in humans and modeling studies using recombinant vaccines in mice. *Infect Immun*. 2009; 77:3958–68. [PubMed: 19528221]