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Listeria monocytogenes Cancer Vaccines: Bridging Innate and Adaptive Immunity

Zachary T. Morrow[1] · Zachary M. Powers[1] · John-Demian Sauer[1]

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Abstract

Purpose of Review Immunotherapy has emerged as a promising cancer treatment; however, success in only select clinical indications underscores the need for novel approaches. Recently *Listeria monocytogenes*–based vaccines have been developed to drive tumor-specific T cell responses. Here, we discuss recent preclinical studies using *L. monocytogenes* vaccines, innate immune pathways that influence T cell priming, and new vaccine strategies in clinical trials.

Recent Findings Recent studies indicate that in addition to inducing antigen-specific T cell responses, *L. monocytogenes* vaccines remodel the TME. In addition, several innate immune pathways influence adaptive immune responses to *L. monocytogenes* and modulating these pathways holds promise to enhance antitumor T cell responses.

Summary The interplay between innate and adaptive immune responses to *L. monocytogenes* is poorly understood. Understanding these interactions will facilitate the design of better anti-cancer vaccines and improved use of combination therapies.

Keywords *Listeria monocytogenes* · Immunotherapy · Cancer vaccines · Innate immunity · Adaptive immunity · Tumor microenvironment

Introduction

To achieve a robust and durable antitumor response, an immunotherapy approach must achieve two goals: (1) the generation of antigen-specific T cell responses [1, 2] and (2) modulation of the immunosuppressive tumor microenvironment (TME) [3]. Checkpoint inhibitors have revolutionized the treatment landscape for many tumor types by reinvigorating a preexisting pool of T cells. Yet, only a small fraction of patients respond [4], with a large proportion eventually becoming treatment resistant [5]. Likewise, many tumors are poorly immunogenic, and methods to elicit antigen-specific T cell responses have failed for most tumor types [6–8], demanding novel approaches.

Bacteria, a long-forgotten treatment for cancer, have the potential to overcome the immunosuppressive TME and drive antigen-specific T cell responses and as such are poised to make a resurgence as part of a therapeutic regimen. Indeed, the ability of bacteria to stimulate antitumor immune responses was first appreciated in the 1890s when William Coley observed tumor regressions in sarcoma patients purposefully infected with *Streptococci* [9]. Despite remarkable responses, the use of bacteria to treat cancer fell out of practice in favor of more consistent treatments such as radiotherapy and cytotoxic agents. However, evidence accumulated over the twentieth century indicating that the immune system has an active role against cancer. In the 1950s, Paul Ehrlich proposed the cancer immunosurveillance hypothesis and the 1970s saw a revival of the use of bacteria to treat cancer when Alvaro Morales demonstrated that intravesical administration of attenuated *Mycobacterium tuberculosis* (the Bacillus Calmette-Guerin [BCG] vaccine) could prevent recurrence of non-muscle-invasive bladder cancer [10].

There are currently eighteen active or recruiting clinical trials utilizing bacteria to treat cancer with countless others

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✉ John-Demian Sauer
Sauer3@wisc.edu

[1] Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, School of Medicine and Public Health, Madison, WI 53706, USA

completed in the last two decades. Of the active/recruiting trials, three will employ *Salmonella*, one will use *Clostridium novyi*, two will use *Enterococcus gallinarum*, one will use *Bifidobacterium longum*, and eleven will see *Listeria monocytogenes* used as a therapeutic platform [11]. *L. monocytogenes* has unique and advantageous properties compared with other bacteria or oncolytic viruses used to treat cancer. In this review, we will highlight aspects of *L. monocytogenes* biology that make it a particularly attractive immunotherapy agent as well as describe our current understanding of the mechanisms of *L. monocytogenes*–induced antitumor activity while highlighting recent and current clinical trials.

L. monocytogenes Life Cycle and Induction of Antigen-Specific T Cell Responses

L. monocytogenes is a gram-positive bacterium best known as a food-borne pathogen and the causative agent of listeriosis [12]. *L. monocytogenes* infection occurs when the bacterium is ingested and disseminates to the liver, spleen, and central nervous system. Cellular access is gained through phagocytosis or by receptor-mediated endocytosis. Two *L. monocytogenes* proteins, internalin A and internalin B, facilitate receptor-mediated endocytosis through interactions with host E-cadherin or C-met, respectively [13, 14]. Once internalized, *L. monocytogenes* is encapsulated in a phagosome and secretes phospholipases and a pore-forming toxin, listeriolysin O (LLO), to escape the maturing phagolysosome [15, 16]. In the host cytosol, *L. monocytogenes* replicates and expresses ActA, a secreted protein that polymerizes host actin to propel itself into neighboring cells facilitating an almost exclusively intracellular lifecycle [17].

Internalized *L. monocytogenes* undergo one of two fates inside the host cell. Inside antigen-presenting cells (APCs), some bacteria are degraded in the phagolysosome [18], facilitating antigen processing and presentation on major histocompatibility class II (MHC-II) complexes and induction of CD4+ T cell responses [19], while some bacteria escape the phagosome and enter the cytosol. Here, secreted antigens are degraded by the proteasome and loaded onto major histocompatibility class I (MHC-I) complexes facilitating CD8+ T cell responses [20–22]. Access to the host cytosol is required for a strong CD8+ T cell response and *L. monocytogenes* lacking LLO do not induce protective CD8+ T cell responses [22]. In fact, CD8+ T cell responses are suppressed by infection with *L. monocytogenes* that fail to escape host phagosomes [23]. However, LLO-deficient *L. monocytogenes* elicit CD4+ T cell responses indicating that cytosolic access is not necessary for MHC-II antigen presentation [22]. Similarly, antigen secretion is required to generate robust antigen-specific CD8+ T cell populations. *L. monocytogenes* expressing non-secreted antigens induce poor memory T cell responses compared with *L. monocytogenes* expressing secreted versions of the same antigens [24].

Thus, in comparison with other intracellular bacteria which have been proposed as therapeutic platforms (like *Salmonella* which tend to reside within host phagosomes [25]), *L. monocytogenes* is uniquely adept at generating strong antigen-specific T cell responses due to its ability to escape the phagosome and thrive in the host cytosol. Survival in the APC cytosol supports CD8+ T cell priming and the generation of a pool of memory T cells capable of providing protective immunity upon secondary challenge. Discussed in more detail below, aspects of *L. monocytogenes* biology such as cell-to-cell spread and host cell tropism can be manipulated to engineer safe and effective cancer vaccines.

Therapeutic *L. monocytogenes* Platforms

L. monocytogenes vaccines are primarily administered intravenously (IV) and are phagocytosed by host APCs [26]. To utilize a pathogen as a cancer vaccine, two essential steps must be taken: (1) the pathogen's ability to cause disease must be attenuated while retaining immunogenicity and (2) the bacterium must secrete a target antigen(s) to drive antitumor CD8+ T cell responses. Two main companies, Advaxis and Aduro Biotech, as well as a variety of academic labs, have pioneered this technology. A comprehensive review of possible attenuation strategies can be found elsewhere [27]; therefore, we will focus on strategies that are currently in clinical trials. Importantly, as discussed below, the Aduro Biotech and Advaxis platforms differ in both mechanisms of attenuation and mechanisms of tumor-associated antigen (TAA) expression; however, these platforms have not been directly compared.

Aduro Biotech's therapeutic platform, termed Live Attenuated Double Deleted (LADD), utilizes a strain of *L. monocytogenes* lacking *actA* and *inlB*. Loss of ActA prevents cell-to-cell spread and dissemination of bacteria, while deletion of InlB curbs liver toxicity by preventing receptor-mediated endocytosis into hepatocytes [28]. ActA-deficient *L. monocytogenes* are 1000-fold attenuated compared with wild-type bacteria [29], and importantly, the LADD strain induces similar antigen-specific T cell responses to wild-type *L. monocytogenes* [28]. Advaxis takes a different approach to attenuate *L. monocytogenes*. The Advaxis vaccine platform, termed LmddA, is deleted at *actA*, and the *dal* and *dat* loci which are required for the synthesis of D-alanine, effectively making the bacteria auxotrophic and replication deficient in vivo [30]. By comparison, the LADD strain is replication competent, which may enhance the absolute abundance of antigens produced within an infected cell.

In addition to differences in mechanisms of attenuation, the Aduro Biotech and Advaxis platforms utilize different approaches for tumor antigen expression. Aduro Biotech's vaccine platform utilizes expression of TAA's fused to the *actA* N-terminal 100 amino acids under the control of the ActA promoter, stably integrated into the *L. monocytogenes* genome [31]. Advaxis on the other hand episomally expresses TAAs fused to LLO under the control of the *hly* promoter [30]. Creating in-frame fusions with endogenous *L. monocytogenes* proteins enhances vaccine immunogenicity [32], although the mechanisms underlying the enhanced immunogenicity of the fusion-based vaccines are not completely understood. Fusion of TAAs to ActA enhances vaccine immunogenicity and antitumor activity [33], as does fusion of TAAs to LLO [32]. However, the enhanced immunogenicity of ActA-based fusions may not rely entirely on enhanced bacterial secretion. Injected protein-based vaccines consisting of TAAs either fused to or administered in conjunction with ActA are more effective than TAAs alone, suggesting that in addition to enhancing antigen presentation, *L. monocytogenes* ActA may also act as an adjuvant to enhance immune responses [33]. Similar adjuvant-like effects have been observed for LLO [34].

The efficacy of ActA- and LLO-TAA fusion vaccines at generating CD8+ T cell responses has been directly compared. In mice with human papillomavirus (HPV)-16 E7-expressing tumors, vaccination with *L. monocytogenes* expressing LLO fused with E7 resulted in a greater number of E7-specific CD8+ T cells compared with mice immunized an ActA-E7 fusion vaccine. Interestingly, both vaccine strategies conferred similar tumor control [35]. In an HPV-16-driven model of autochthonous thyroid cancer, the frequency of E7-specific CD8+ T cells was no different in the spleens of immunized mice, while in tumors, the LLO vaccine produced a threefold increase in E7-specific T cells [36]. Tumor control was similar for both vaccines. Given that both ActA and LLO have ostensible adjuvant-like properties, why differences in T cell number did not correlate with differences in tumor control is unclear. Perhaps LLO and ActA differ in their adjuvant-like properties resulting in different changes in the tumor microenvironment. Alternatively, TAA expression or secretion levels may have differed between the two vaccines, a consideration that was not directly addressed.

While fusion of TAAs to secreted *L. monocytogenes* virulence factors increases immunogenicity, it remains unclear if there is an optimal antigen fusion partner. Although LLO-TAA vaccines appear to induce greater numbers of antigen-specific T cells than ActA-TAA vaccines in some experimental settings, it is difficult to argue in favor of either as both seem to control tumors to the same extent. Future studies are needed to understand why LLO fusions induce more antigen-specific T cells, and why this does not translate to enhanced tumor control over ActA-based vaccines. Although LLO- and ActA-TAA fusions are the most extensively studied in the context of cancer immunotherapy, *L. monocytogenes* secretes a plethora of proteins inside host cells [37], any of which may be even better fusion partners for TAAs in *L. monocytogenes* vaccines. Finally, it is unclear if the replication-competent LADD strain or replication-deficient LmddA strain is superior at generating antitumor immune responses. A direct comparison would prove valuable to inform future vaccine design strategies.

L. monocytogenes Effects on Immune Cells in the Tumor Microenvironment

L. monocytogenes–based vaccines generate robust antigen-specific CD8+ T cell responses; however, tumor protection likely involves additional mechanisms. Mounting evidence suggests *L. monocytogenes* immunization dramatically alters the tumor microenvironment (TME). This may include alterations in the frequency and/or function of both pro- or antitumor immune cells. Suppressive cell types found in the TME include regulatory T cells (T-regs) on the adaptive side [38], and myeloid-derived suppressor cells (MDSCs) on the innate side [39], which both dampen immune responses through various mechanisms.

Deng et al. demonstrated that *L. monocytogenes* vaccines control tumor growth in two different tumor models in different mouse backgrounds. Importantly, this study attributed the effectiveness of *L. monocytogenes* vaccination to modulation of the TME, most notably decreases in tumor-infiltrating FOXP3+ T-reg frequency, increases in pro-inflammatory cytokine production, and a shift in tumor-associated macrophage (TAM) phenotype from M2 to M1. Similarly, PD-1 expression was decreased on tumor-infiltrating CD8+ T cells relative to unvaccinated animals. Notably, some of these results (decreased PD-1 expression and FOXP3+ T-reg frequency) were also observed in mice vaccinated with empty *L. monocytogenes* suggesting that the bacterium alone affects the TME immune milieu in addition to contributing to T cell priming [40••]. Additional groups have also noted decreases in T-reg frequency [41, 42] and PD-1 expression associated with *L. monocytogenes* vaccination [43]. Consistent with observations from Deng et al., in a 4T1 breast cancer model, LADD treatment repolarized TAMs from M2 to M1. Interestingly however, in this model, macrophage polarization was dependent on LADD accumulation in the TME suggesting that in addition to priming T cells in the periphery, *L. monocytogenes* vaccines can act locally at the tumor site if access is granted [44]. Others have demonstrated that *L. monocytogenes* vaccines are particularly effective due to alterations in the frequency of MDSCs. *L. monocytogenes* vaccination reduces the number of MDSC-like cells both in the peripheral blood and in the TME of tumor-bearing

animals. Notably, in the remaining MDSC-like cells, expression of the pro-inflammatory cytokine IL-12 was increased, reflecting an overall phenotypic shift from immunosuppressive to pro-inflammatory [45, 46].

Taken together, the data indicate that *L. monocytogenes* vaccination is particularly effective due in part to its effects in the TME in addition to contributing to T cell priming. While the importance of *L. monocytogenes*' ability to generate antigen-specific T cells cannot be understated, *L. monocytogenes* vaccination also induces a profound reduction in the frequency of immunosuppressive cell types including FOXP3+ T-regs and MDSCs. Additionally, tumor-infiltrating immune cells display a markedly more immunogenic phenotype in response to *L. monocytogenes* vaccination. CD8+ T cells have reduced PD-1 expression, while TAMs display increased iNOS and reduced expression of M2 markers. Given the effects of *L. monocytogenes* vaccination, tumors heavily infiltrated with immunosuppressive cell types or where PD-1 blocking antibody has failed despite high PD-1 expression would make rational targets. Additionally, *L. monocytogenes* vaccination might be particularly well suited to tumors with high CD8+ T cell infiltration but low effector function due to its ability to induce a pro-inflammatory state.

Combination Approaches for Enhanced *L. monocytogenes* Vaccines

Although in many cases *L. monocytogenes* is administered in combination with standard of care chemotherapies, significant interest exists in exploring *L. monocytogenes* in combination with other immunotherapies and radiation therapy. Combining immune checkpoint inhibitors with therapeutic *L. monocytogenes* vaccination is a logical next step in enhancing vaccine efficacy. TAA-expressing *L. monocytogenes* vaccination in combination with α-PD-1 antibody was shown to induce complete tumor regression in 20% of mice bearing HPV-positive TC-1 tumors, whereas no mice were cured by either monotherapy [42], while a similar strategy eradicated all tumors in a model of breast cancer [40••]. Another recent study explored combining TAA-expressing *L. monocytogenes* vaccination with α-PD-1 antibody in a cancer with low mutational burden, pancreatic ductal adenocarcinoma (PDAC) [47]. The vaccine targeted Annexin-A2, which is frequently overexpressed in metastatic lesions of pancreatic ductal adenocarcinoma but is rarely mutated. Astonishingly, despite targeting a self-antigen, the vaccine resulted in tumor control which was enhanced by α-PD-1 antibody and induction of Annexin-A2-specific T cells [48]. Others have similarly demonstrated the capacity of *L. monocytogenes*–based vaccines to break central tolerance against several proteins [35, 49, 50]. Critical questions include how *L. monocytogenes* breaks central tolerance, and which self-antigens are suitable targets for *L. monocytogenes*–based vaccines. While a discussion of epitope discovery is beyond the scope of this review, it is important to mention that many preclinical studies are using epitopes specific to mice which may not apply to human disease. Future studies will need to employ humanized mouse models to test immunogenic epitopes in relevant human cancer antigens. Indeed, a recent study utilizing human prostate cancer antigens in HLA-A2- and HLA-DRI-expressing mice demonstrated the increased efficacy of combining DNA tumor vaccines with *L. monocytogenes* tumor vaccines in specific prime-boost regimens [51].

Finally, studies on the use of combination therapy with *L. monocytogenes* are not limited to checkpoint inhibitors and DNA vaccines as two recent studies investigated the combined effect of TAA-expressing LADD and radiation therapy. Treatment with TAA-expressing LADD alone resulted in tumor control, and this was enhanced by combination with radiation treatment. This correlated with a massive influx of TAA-specific T cells with enhanced effector activity [52, 53]. Ultimately, to rationally combine *L. monocytogenes*–based immunotherapies with other treatment modalities, we need a better mechanistic understanding of why some tumors respond better to combined *L. monocytogenes* vaccination and immune checkpoint blockade as well as how the kinetics of dosing influence efficacy. Likewise, although there have been no preclinical studies combining α-CTLA-4 antibody with *L. monocytogenes* vaccination, *L. monocytogenes* vaccination was shown to induce CTLA-4 expression on CD4+ T cells in the spleens and livers of mice bearing metastatic colon cancer. While slowed tumor growth was observed in vaccinated mice, no cures were achieved [43] suggesting that CTLA-4 blockade might be a rational choice in this model. Other immune checkpoints such as VISTA, Lag-3, Tim-3, and TIGIT (as well as others) have largely been unexplored as monotherapies or in combination with therapeutic anti-cancer vaccines [54]. Therefore, future studies are needed to address if these checkpoints are relevant during therapeutic *L. monocytogenes* vaccine schemes and if so, which tumor types are most likely to respond to which combination therapies.

Clinical Trials

Current Clinical Trials

A comprehensive review of the state of *L. monocytogenes* vaccine trials was recently published [27] and as such we will focus our discussion here on new avenues of clinical application, namely combination therapy and precision medicine approaches. Aduro Biotech's CRS-207 (LADD platform expressing the TAA mesothelin) is currently in clinical trials for patients with pancreatic cancer and mesothelioma in

various combinations with pembrolizumab, ipilimumab, nivolumab, chemotherapeutics, and IDO inhibitors (NCT02243371, NCT03190265, NCT03006302, and NCT01675765). Aduro Biotech's other platform currently in clinical trials, pLADD, is based on an all-together different approach to engineering anti-cancer vaccines and is one mirrored in the ADXS-NEO platform described below. In the era of personized medicine, treatment options are increasingly focusing on driving T cell responses toward patient-specific mutations termed neoantigens. Indeed, neoantigens generate an enhanced repertoire of strongly immunogenic epitopes which are often presented in the context of MHC-I and recognized as foreign antigens [55]. By sequencing colorectal tumors and formulating LADD-based vaccines using predicted neoantigen epitopes, the pLADD approach is taking personalized medicine to the next level (NCT03189030). While the active trials will be completed, Aduro Biotech announced that it will not be initiating any further clinical trials based on its LADD platform leaving open the question of the future of pLADD and the LADD platform more generally.

Advaxis has also developed a patient-specific vaccine termed ADXS-NEO that similarly uses patient-specific neoantigens expressed by *L. monocytogenes* to personalize tumor immunotherapy (NCT03265080). Here patients with multiple tumor types are included and combination with pembrolizumab is being explored. In addition to ADXS-NEO, Advaxis has three other *L. monocytogenes* vaccines in active clinical trials. ADXS11-001 expressing HPV E7 is in active trials for patients with HPV-positive oropharyngeal cancer, cervical cancer, and anorectal cancer as a monotherapy (NCT02002182, NCT01266460, NCT02853604, and NCT02399813). ADXS31-142 targets the prostate cancer antigen PSA and is being tested in combination with pembrolizumab in prostate cancer (NCT02325557). Finally, ADXS-503 is a vaccine formulated to express epitopes from ten frequently mutated genes in a variety of tumor types and is being used in combination with pembrolizumab (NCT03847519). This is intended to be an *off the shelf* treatment for multiple tumor types.

Safety of *L. monocytogenes*–Based Clinical Trials

Overall, the published clinical data suggest *L. monocytogenes* anti-cancer vaccines are well tolerated. Adverse clinical events associated with vaccination, most often pyrexia and chills, are frequently reported but are well managed [56–59]. However, one patient that received an Advaxis *L. monocytogenes* vaccine in 2013 died in 2015, with trace amounts of the bacteria detected in her blood. The FDA put a hold on *L. monocytogenes* vaccine trials, which was lifted shortly after. Then again, briefly, in 2016, the FDA put a halt on an Aduro Biotech trial due to the detection of disseminated bacteria in the blood of a cervical cancer patient treated with CRS-207 [60]. The trials were reinstated after Aduro Biotech and Advaxis reevaluated their patient monitoring and management practices and will exclude patients that receive immunosuppressive drugs or have certain prosthetics including indwelling ports.

New platforms are being developed for use in immunocompromised patients. Recently, a strain of *L. monocytogenes* that dies upon entry into host cells was shown to be cleared rapidly in immunocompromised mice [61]. Importantly, this strain retains comparable immunogenicity to LADD-based vaccines. Therefore, therapeutic *L. monocytogenes* vaccines are incredibly safe and well tolerated, especially in comparison with chemotherapy and other cytotoxic agents.

Engineering More Efficacious Anti-cancer Vaccines: Modulating Innate Immune Pathways Activated by *L. monocytogenes*

L. monocytogenes infection activates many innate immune signaling pathways [62]. Access to the cytosol is essential for *L. monocytogenes* activation of protective CD8+ T cell responses [22] leading to the hypothesis that innate immune pathways triggered specifically by cytosolic bacteria are essential for driving robust CD8+ T cell priming. Consistent with this hypothesis, Toll-like receptor (TLR) signaling, which is triggered at the cell surface or in endosomes, is robustly triggered by *L. monocytogenes* infection but is dispensable for T cell priming [63–66, 67•]. Surprisingly however, many cytosolic innate immune signaling pathways are not only dispensable for optimal T cell priming; in some cases, they appear to be actively detrimental.

STING/cGAS

One of the earliest known innate responses specific to cytosolic *L. monocytogenes* was activation of type I interferons [68]. Considerable effort was focused on elucidating the bacterial PAMP(s) and host signaling pathway(s) leading to IFNβ production with the expectation that it would be a key signal for T cell priming due to the canonical role of IFNβ in T cell expansion and activation [69]. These efforts ultimately demonstrated that *L. monocytogenes* activates the Stimulator of interferon genes (STING) pathway directly through the secretion of cyclic-di-AMP [70, 71] as well as through cytosolic DNA recognition by the cyclic GMP-AMP synthase (cGAS) [72]. cGAS binds cytosolic DNA and catalyzes the formation of cyclic-di-nucleotides (CDNs) which are recognized by STING, initiating a signaling cascade through interferon regulatory factor 3 (IRF3) and resulting in the production of the type I interferon (IFN) IFNβ. Surprisingly however, STING-deficient mice develop enhanced T cell responses and better

protective immunity relative to wild-type mice [67•]. This strongly suggests that during *L. monocytogenes* immunization, limiting systemic STING-induced inflammation produces an ideal environment for T cell priming. Deficits in cellular immunity are mediated by type I interferons, as IFNαβR-deficient mice generate comparable T cell responses to STING-deficient mice [67•].

In contrast, evidence from human tumors suggests that STING activation at the tumor site can be beneficial to the generation of T cell responses. Analysis of gene signatures from human cancer metastases demonstrated that CD8+ T cell infiltration correlates with IFNβ expression [73] and STING expression in tumors can predict prognosis [74]. Work from multiple groups utilizing various tumor models has demonstrated that STING activation at the tumor site has potent antitumor effects [45, 75, 76]. Why systemic STING activation during *L. monocytogenes* infection is detrimental to the generation of adaptive immunity, but beneficial in the TME in some models, is just beginning to be unraveled.

In a 4T1 mouse breast cancer model, TAA-expressing *L. monocytogenes* vaccination and intratumoral CDN injection result in enhanced tumor control compared with the vaccine or CDNs alone [45]. However, antigen-specific CD8+ T cells were decreased by CDN treatment compared with the *L. monocytogenes* vaccine alone. Rather, tumor control in CDN-treated mice was attributed to Caspase-3-dependent apoptosis in the tumor cells. Finally, reducing the amount of CDNs 10,000-fold led to an expansion of antigen-specific T cells in mice that received the combination treatment compared with vaccine alone [45] hinting that modest STING activation may be beneficial to generating T cell responses. Excessive IFNβ may inhibit the induction of robust cellular immunity through numerous mechanisms [77, 78]. Consistent with this observation, Sivick et al. found that high doses of STING agonist delivered to directly to tumor beds kill tumor cells through Caspase-3-dependent apoptosis, but limits systemic T cell immunity, whereas low doses at the tumor site activate innate immune cells to prime CD8+ T cell responses resulting in greater systemic immunity [79•]. Therefore, it appears that the magnitude of STING signaling is critical for shaping the adaptive immune response.

Collectively, although systemic activation of the STING/cGAS pathway appears to inhibit cellular immunity during *L. monocytogenes* infection, the extent of STING activation in acute infection models may exceed a threshold for enhancing CD8+ T cell responses. Experiments utilizing *L. monocytogenes* strains that limit secretion of CDNs or are less susceptible to cytosolic bacteriolysis (limiting *L. monocytogenes* genomic DNA in the cytosol) may lead to the generation of vaccine platforms that induce increased T cell responses. Furthermore, the combined approach utilizing low-dose intratumoral CDN injection to optimize T cell priming at the tumor bed and IV-delivered *L. monocytogenes* to optimize priming in the periphery should synergize to yield a profoundly more effective immunotherapeutic approach.

Inflammasomes

In addition to STING/cGAS activation, activation of the inflammasome is an innate response specific to *L. monocytogenes* in the cytosol [80–85]. Canonical inflammasomes are cytosolic multi-protein complexes comprised of a receptor, the adaptor apoptosis-associated Spec-like protein containing a Caspase recruitment domain (ASC), and Caspase-1 [86]. Inflammasome activation leads to the secretion of the inflammatory cytokines IL-1β and IL-18 [87], secretion of inflammatory lipid signaling molecules known as eicosanoids [88], and an inflammatory type of cell death known as pyroptosis [89]. The AIM2 inflammasome is the predominant receptor activated during *L. monocytogenes* infection due to bacteriolysis in the host cytosol [84].

Surprisingly, similar to STING activation, inflammasome activation limits the generation of adaptive immunity during *L. monocytogenes* infection [90, 91••, 92]. Although the obvious hypothesis is that inflammasome-mediated APC death via pyroptosis could impair adaptive immunity by destroying the cells responsible for priming CD8+ T cells, Theisen et al. demonstrated instead that the inflammation associated with inflammasome activation is responsible for inhibiting optimal T cell priming [91••]. Although the mechanisms underlying inflammasome-mediated inhibition of T cell priming remain unknown (inhibition is independent of IL-1R/IL18R (data not shown)), limiting activation of this pathway may produce a more efficacious vaccine due to its effects on both immune cells and tumor cells.

Multiple studies have documented a role for inflammasome activation in cancer development and progression in both humans and mice [93–96]. Patient data demonstrate a positive correlation between IL-1β and IL-18 and pro-tumor cytokine expression [97], whereas lower IL-1β and IL-18 expression confers better prognosis [97–99]. Inflammasome activation also inhibits NK cell responses and enhances tumor growth in a model of melanoma. Depleting inflammasome activity specifically in MDSC-like cells completely abrogated these defects, strongly suggesting that inflammasome activation in immune cells is detrimental to antitumor immunity [100]. Inflammasome activation also promotes an M2 phenotype in TAMs [101] and blocking IL-1R signaling reduces the accumulation of MDSCs in the TME [102]. As with many innate immune responses, however, magnitude and location are likely important as there is also conflicting data that suggests inflammasome activation can be protective against cancer in some contexts [103–107].

Although it is unclear why inflammasome activation has both pro- and antitumorigenic functions during cancer development, in the context of *L. monocytogenes* vaccination, it

appears that limiting inflammasome activation should result in enhanced vaccine efficacy. Several inflammasome inhibitors are being tested in preclinical models showing antitumor benefits [108] and could be paired with *L. monocytogenes* vaccines for a combination approach. Additionally, strains of *L. monocytogenes* that are less susceptible to cytosolic bacteriolysis may prove to be better at priming T cells by limiting AIM2 activation.

Eicosanoids

Finally, eicosanoid production is also associated with *L. monocytogenes* access to the cytosol [109, 110]. Eicosanoids are produced following the liberation of arachidonic acid from the plasma membrane by the cytosolic phospholipase A2 at which point arachidonic acid can be further metabolized by cyclooxygenases or lipoxygenases [111]. Among the best studied eicosanoids, prostaglandin E2 (PGE_2) is produced from arachidonic acid first through the activity of cyclooxygenase-1 or cyclooxygenase-2 (COX-1 and COX-2) to produce PGH_2, and then subsequently into PGE_2 by a prostaglandin E synthase [112]. Inhibition of both COX enzymes by indomethacin (an ibuprofen analog) impairs adaptive immunity to *L. monocytogenes* infection. However, specific knockout of COX-1 enhances adaptive immunity, while COX-2 inhibition by celecoxib impairs adaptive immunity, suggesting contrasting roles for the two enzymes [113•]. PGE_2 is the key eicosanoid downstream of COX-2 necessary for optimal T cell priming as add back of PGE_2 alone to celecoxib-treated mice restored both T cell priming and protective immunity. Together, these data suggest that eicosanoid signaling plays a key role in *L. monocytogenes*–stimulated immunity and that care should be taken in the choosing of analgesics following administration of *L. monocytogenes* vaccines.

In contrast to its essential role in *L. monocytogenes* T cell priming, COX-2 inhibition enhances the therapeutic efficacy of non–*L. monocytogenes* anti-cancer vaccines. In 4T1 tumor-bearing mice, expression of COX-2 by tumor cells impairs T cell ingress into tumors and inhibition by celecoxib in the context of dendritic cell vaccines improves tumor control [114]. Similarly celecoxib treatment in the context of adenovirus vaccination improves tumor control due to increased T cell influx into tumors [115]. Indeed, tumor-derived PGE_2 has been shown to inhibit immune cell infiltration [116], indicating that local, tumor-derived PGE_2 can impair vaccine efficacy by inhibiting immune cell infiltration in a tumor cell–autonomous manner. Given the contrasting roles of COX-2 systemically and in the TME, COX enzyme inhibitors could

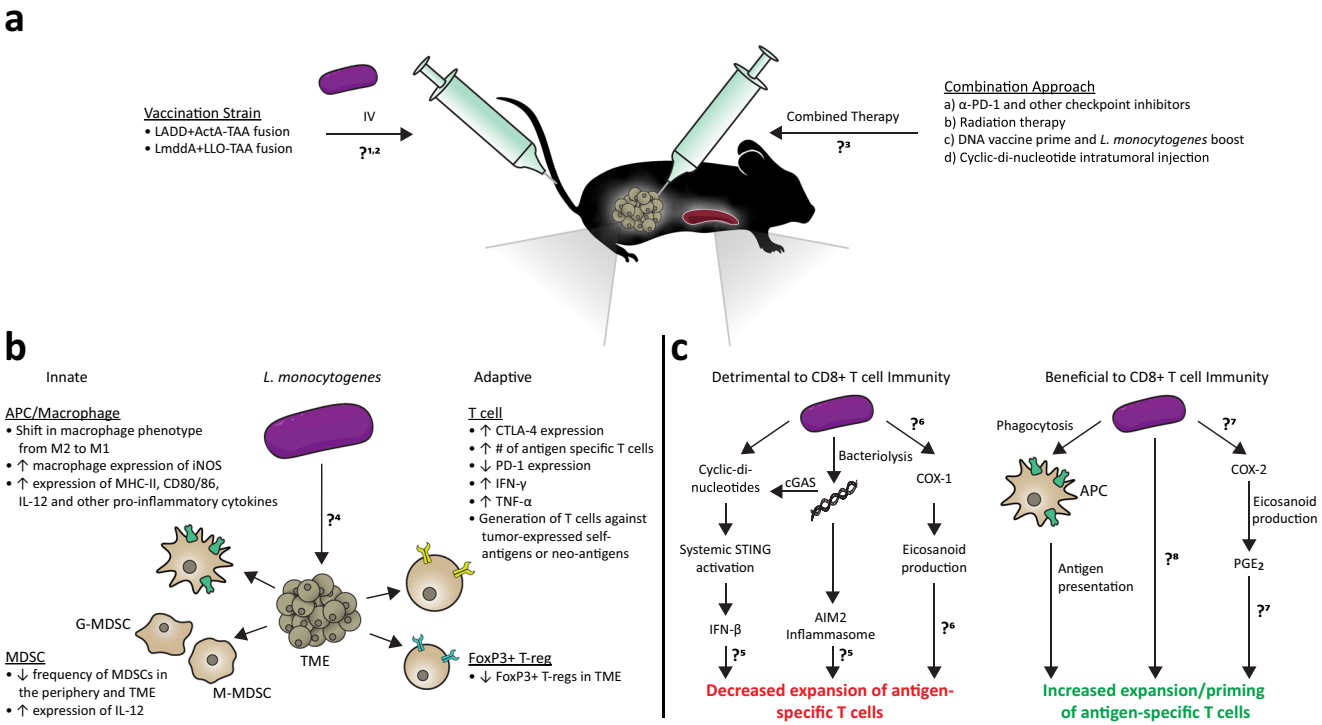

Fig. 1 (A) Schematic representation of *L. monocytogenes* anti-cancer vaccine administration, and (B) the effects on innate immune cells (left) and adaptive immune cells (right) in the TME, and (C) innate immune pathways triggered by *L. monocytogenes* that are detrimental (left) and beneficial to adaptive immunity (right). Numbered question marks reference the future directions discussed in the "Conclusion" section

be withheld immediately after vaccination allowing for development of optimal T cell responses and administered later to enhance immune cell infiltration into tumors.

The function of PGE_2 during vaccination is poorly understood as PGE_2 has both pro- and anti-T cell functions in various vaccine contexts and has historically been associated with impaired immune function [117–122]. More specifically, there are four PGE_2 receptors, EP1, EP2, EP3, and EP4 [123], each ascribed varying functions [119, 121, 124–126]. Which PGE_2 receptors drive pro–T cell priming functions vs those that shut down inflammation and T cell function remains to be fully elucidated. How *L. monocytogenes* infection drives PGE_2 production and whether or not strains that modulate PGE_2 production may be ideal vaccine platforms need to be addressed. Furthermore, pharmacologic inhibitors and/or agonists of specific PGE_2 receptors could be combined with therapeutic *L. monocytogenes* vaccines to enhance the development of adaptive immunity. Ultimately the role of PGE_2, the COX enzymes, and eicosanoids more broadly in anticancer vaccines and specifically *L. monocytogenes* vaccines remains to be fully elucidated.

Conclusion

L. monocytogenes is poised to become a major player in the therapeutic armament against cancer. The infectious cycle that results in secretion of antigens directly into the cytosol while driving robust and specific innate immune responses uniquely positions *L. monocytogenes* as a powerful platform to generate adaptive immune responses toward ectopically expressed antigens. Nevertheless, many questions remain (Fig. 1):

1. What is the optimal attenuated vaccine platform (LADD, LmddA, other?) for driving both antigen-specific T cell responses and beneficial modulation of the TME?
2. What is the ideal TAA fusion partner (LLO, ActA, other?) and how do these partners augment immunogenicity?
3. What combination therapies (checkpoint inhibitors, radiation therapy, and others) will be most effective when paired with a *L. monocytogenes* vaccine and which indications will require such therapeutic augmentations?
4. How does *L. monocytogenes* vaccination modulate the TME and can this be optimized?
5. Why are systemic STING and inflammasome activation detrimental and can these pathways be modulated with either pharmacological agents or with modified *L. monocytogenes* strains to enhance vaccine efficacy?
6. How does *L. monocytogenes* activate COX-1 and what are the downstream products of COX-1 that inhibit T cell immunity?
7. Which cell types make and respond to PGE_2, what are the relevant PGE_2 receptors and can PGE_2 signaling be modulated in a rational way to enhance vaccine efficacy?
8. And finally, what other innate immune pathways are activated by *L. monocytogenes* that can be capitalized upon to enhance vaccine efficacy?

To fully harness the potential of *L. monocytogenes* vaccines, these and many other questions must be answered. Nevertheless, as evidenced by the approval of a *L. monocytogenes*–based osteosarcoma immunotherapy [127] for use in dogs last year which more than doubled median survival, future of *L. monocytogenes* cancer vaccines is now.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

Disclaimer Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

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