**Title**

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**Publication Date**

2024

Peer reviewed|Thesis/dissertation

# *Listeria monocytogenes* as a vector for cancer immunotherapies

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A thesis submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

In

Microbiology

in the

Graduate Division

of the

University of California, Berkeley

Committee in Charge:

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Spring 2024

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Abstract

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*Listeria monocytogenes* engineered to express tumor antigens as a cancer vaccine has yielded mixed results in the clinic. Here, we utilized an attenuated strain of *Listeria monocytogenes* (*ΔactA, Lm*) that does not express tumor antigen to explore the immunological response to *Listeria* itself in the context of intravenous (IV), intratumoral (IT), or a combination of IV+IT administration into tumor-bearing mice. Unexpectedly, we found that *Lm* persisted in tumors of immune competent mice, regardless of the administration route. While IT *Lm* alone led to the recruitment of immunosuppressive immune cells that promoted tumor growth, IV *Lm* followed by IT *Lm* controlled tumor growth. IV *Lm* vaccination generated a pool of anti-*Lm* cytotoxic CD8 T cells that killed *Lm*-infected non-tumor cells to control tumor growth. Our findings reveal a differential impact of IT *Lm* administration on tumor progression that depends on the presence of anti-*Lm* CD8 T cells, rather than antitumor CD8 T cells, for antitumor therapeutic efficacy.

# Acknowledgements

I would like to thank my advisor Michel DuPage for taking a chance on me. When I first came into the lab I had no previous experience on mouse work and limited knowledge of immunology. He gave me the opportunity to grow as a scientist and supported me through my development during my grad school experience. He has been a huge supporter of me as a scientist and as a mentor, with his help I have been able to grow as a scientist and I am proud of what we have accomplished.

Also I would like to thank my undergraduate students Laura, Elina, Julia and Nicole. They helped me develop as a mentor. I am very grateful and proud of them, I have no doubt that they will go far in any field they decide to pursue.

Finally I would like to thank the DuPage lab as a whole, Maru, Janneke, Chenyu, Alissa, Jessica, Stephanie, Jesse, Tim, Ola and Brenna. I could not have gotten through experiments without the support of everyone, especially without the help of Tim and Jesse.

## I. Cancer immunotherapy introduction

Cancer immunotherapy is now recognized as the fourth pillar of cancer treatment, the rest being surgery, chemotherapy and radiotherapy. Cancer treatments that harness the power of the immune system are not a completely recent idea, in fact during the turn of the century in the late 1800s, William Coley, a bone surgeon, noticed reported cases of miraculous recovery, in which some patients had a strong fever induced by erysipelas prior to carcinoma surgery underwent remission (REF?). This seemingly miraculous cure led him to believe that patients, specifically patients with superficial tumors, such as sarcomas, could be treated directly by inducing a strong fever derived from *Streptococcus pyogenes*. He would then go on to treat more than a thousand patients with "Coley's Toxins" , a mixture of live and dead streptococcal bacteria[1]. This treatment led to some responses in patients, and was in use, although notably with limited success, until radiotherapy became widely adopted. Remarkably, and to a degree unknowingly, Coley was able to harness the power of the immune system against bacterial infections to control tumors, before much was truly known about the immune system, its interactions with bacteria during infection and the role of the immune response against cancer. Almost a century later, a vaccine against tuberculosis, widely used throughout the world, the Bacille Calmette-Guerin vaccine (BCG), a live attenuated form of Mycobacterium bovis became the standard of care for early stage bladder cancer, although the specific mechanism behind the methods of action are not completely understood, and are still an active area of research[2] The BCG vaccine has been the most successful bacterial-derived immunotherapy to date[3].

In the mid 2010s checkpoint blockade was approved by the FDA and ushered the newest pillar in the treatment against cancer. Checkpoint blockade works by using monoclonal antibodies against checkpoint inhibitors expressed in a variety of cells, often T cells, and has been described as a way to "release the breaks of the immune system against cancer". The first FDA approved monoclonal antibody Ipilimumab works by blocking interactions between CTLA-4 in regulatory T cells (Tregs) and B6/B7 in antigen presenting cells like dendritic cells[4]. CTLA-4 works as a T Cell inhibitor by outcompeting CD28 expressed on CD4s and CD8s effector T cells by having more affinity and avidity with B6/B7 expressed in dendritic cells. This aforementioned outcompeting by CTLA-4 leads to anergy of effector T cells in tumors, since they are unable to get the "signal 2" from dendritic cells required for T cell activation, leading to less effector T cells in the tumor microenvironment (TME), which is ultimately associated with poor prognosis in patients. The next checkpoint inhibitor approved by the FDA was Nivolunab, a PD-1 monoclonal antibody inhibitor. PD-1 is expressed early on by activated T cells, and once PD-1 interacts with its ligand PD-L1 leads to T cell

anergy and loss of effector function, which is important during normal immune response, to minimize damage to healthy tissues[5]. However in the response against cancer, cancer cells have taken advantage of this immunosuppressive phenotype and express PD-L1 to suppress activated effector T cells that would otherwise help clear the tumor by killing cancer-antigen-specific T cells. PD-L1 is also expressed in immunosuppressive cells such as Tregs and Myeloid-derived suppressor cells (MDSCs) during the normal course of infection, to limit damage to healthy tissue after the infection is cleared. Checkpoint inhibitors have been used against metastatic melanoma, renal cell carcinoma, head and neck cancers and non-small lung cancer [6]. Other monoclonal antibodies have been developed against PD-L1 and are used in combination with αPD-1 and αCTLA-4 which has been shown to have a synergistic effect and better outcomes than a single therapy alone. However, not all patients benefit from checkpoint blockade, the numbers being around 20-30% of patients actually benefiting from checkpoint blockade treatments [7]. The rest are divided into two categories, (1) those who did not respond to initial treatment and (2) those who relapsed[8]. Studies and clinical trials have been conducted to mechanistically understand why patients do not respond to checkpoint blockade therapies. Some patients do not respond due to defects in T cell behavior in the tumor microenvironment, thus, better strategies for long term T cell activation in the tumor microenvironment are necessary. Tumor intrinsic resistance has also been shown in patients that are poor responders to checkpoint blockade, tumors downregulate janus kinases JAK1 and JAK2, in the context of cancer these kinases, when exposed to INF-γ from T cells, downregulate PD-L1 and and secrete chemokines that attract T cells[9]. Melanoma patients that expressed β-catenin, a suppressor of CCL4 secretion which in turn, is important in T cell and DC recruitment to tumors saw resistance to checkpoint blockade due to poor tumor infiltration of CD103 DCs and T cells, the same was observed in mouse preclinical models[10]. Gain of function mutations such as BRAF can inhibit melanoma antigen expression in tumors, other cancers can downregulate expression of MHC I, leading to less recognition by T cells and ultimately escaping tumor immune surveillance[11]. Tumor extrinsic factors that affect the effectiveness of checkpoint blockade have also been identified, cancer cells and MDSCs have been shown to express indoleamine-2,3-dioxygenase (IDO) which recruits further suppressive MDSCs, Tregs and directly induces T cell dysfunction. The presence of adenosine in tumors is also associated with M2 macrophage induction, recruitment of MDSCs, NK and T cell effector inhibition. Neutrophils recruited to tumors can also be detrimental to checkpoint blockade treatment when present at higher ratios to lymphocytes, possibly because they can eventually become suppressive MDSCs in the tumor microenvironment. The extracellular matrix contains latent TGF-ß, which has been shown to have a negative effect on atezolizumab therapy in preclinical models, directly

inhibiting T cell tumor infiltration and function[12]. T cells themselves can be a detrimental factor on checkpoint blockade treatment. For example, the expression of TIM-3 in CD8 T cells is associated with a lack of response in PD-1 treated non-small cell lung cancer [13] . Finally, large numbers of Tregs in the tumor microenvironment are associated with poorer prognoses in patients undergoing checkpoint blockade treatment, however this can be mitigated with α-CD25 antibodies to deplete Tregs, although this therapy comes with its own adverse reactions[14]. Taken together, while checkpoint blockade is a huge step in the right direction in the development of medicine against cancer, additional barriers are present before the vast majority of patients can benefit from treatment. Thus, further research into checkpoint inhibitors, biomarkers for treatment effectiveness, suppressive mechanisms in the tumor microenvironment, and T cell effector enhancement is required for broader effectiveness of checkpoint blockade treatment .

Cytokines were once thought to be a miracle in cancer treatment, hence, for example, the aptly named tumor necrosis factor (TNF), which unfortunately had extreme side effects when administered in patients systemically due to its broad effects in tissues, TNF can also lead to a cytokine storm, and unfortunate tumor growth due to increased angiogenesis with very limited anti-cancer results via inducing tumor cell death [15]. TNF offers a special case on paradoxical treatment, since localized administration has been shown to be beneficial for organ-localized solid tumors, especially so when used in combination with chemotherapy, allowing the uptake of the drug into cancer cells and ultimately the destruction of the vasculature that is crucial for tumor control [16]. However when used systematically TNF can lead to negative organism-wide effects that can ultimately lead to death [17] (REF?). In contrast with TNF treatment, high-dose IL-2 (HDIL-2) has been approved by the FDA against metastatic renal cell carcinoma and metastatic melanoma almost universally in combination with adoptive T cell therapy (ACT) due to, mechanistically, IL-2 acting as a survival signal in T cells which are responsible for clearing cancer in an antigen specific manner[18]. IL-2, as is the case with many cytokines, has different effects on the different types of cells and at different dosages, leading to a modest use of HDIL-2 in cancer treatments[19]. Drawbacks arise due to the binding of IL-2 to epithelial cells expressing IL-2Rα which induces vasodilation and vascular leak syndrome, in addition to expansion of Tregs which have a high expression of IL-2Rα and can suppress the T cell response by acting as IL-2 “sinks” [20]. Furthermore due to the short half life of interleukins in the bloodstream, they are administered in large doses which can further expand risks of vascular leak syndrome, hypertension, acute renal insufficiency and in rare cases myocarditis[21]. To date IL-2 therapies are mostly used in conjunction with CAR-T cell therapies, ACT or to stimulate and expand T cells or NK cells.

Type 1 interferons (IFN) also have many targets depending on tissue and context, but they are mainly a response and activator of the innate immune response via upregulation of MHC-I, furthermore type 1 IFN can promote apoptosis in cancers in a caspase dependent manner, can skew the immune response to a type 1 T cells response and promote DC maturation[22]. In combination with the vacular-targeting antibody bevacizumab (anti-VEGF), type 1 IFN treatment has been approved as a first line of treatment against metastatic renal cell carcinoma, as an adjuvant for completely resected stage III or IV high-risk melanoma, AIDS-related Kaposi's sarcoma, hairy cell leukemia, and cervical intraperitoneal neoplasms[23]. On the other hand type 1 IFN treatments can have some strong side effects such as, fever, fatigue, intestinal problems, myalgias, and increase in pancreatic enzymes, especially in high level IV doses, meaning proper dosage is of high importance.

Toll-like receptor (TLR) agonists are also being studied as treatments for a variety of cancers. To date no TLR treatments have been approved by the FDA, however, TLR agonists are used in a variety of cancer treatments to function as adjuvants for the immune response, e.g. BCG vaccine treatment for bladder cancer and TLR7/8 agonists (Imiquimod) are used as topical applications to treat superficial basal cell carcinoma, since systemic application can be toxic[24]. Simply put, TLRs recognize pathogen associated molecular patterns (PAMPs), damage associated molecular patterns (DAMPs), and work by acting on a wide range of cells, especially innate immune cells, ultimately bridging, recalling and activating innate and adaptive immune cells to the site of infection. The biggest advantage of TLR agonist therapies is the potential to turn “cold” into “hot” tumors, leading to better prognosis and better synergy with checkpoint blockade and other immunotherapies[25]. Furthermore, TLR agonists can induce IFNγ and TNFα leading to MHC-I upregulation, resistance to PD-1, and DC maturation. A variety of cancers are known to overexpress TLRs, these include, esophageal, lung, ovarian, colorectal, and squamous cell carcinomas of the head and neck (SCCHN), however, it's important to note that in the case of SCCHN, meta analysis shows that overexpression of TLRs are correlated with poor prognosis in patients[25], thus expression of TLRs and prognosis is highly varied from different types of cancer. LPS, when used in treatment against hepatoblastomas overexpressing TLR4, has been shown to promote an anti-tumor response, while TLR4 expression is associated with tumor progression in hepatocellular carcinoma and cervical cancer[26]. In some cases TLR agonist treatment can also inadvertently recruit Tregs to tumors, which is associated with detrimental prognoses due to enhanced immunosuppression in the TME [27]. These findings support the importance of identifying biomarkers when selecting appropriate treatments. In a preclinical cancer model[28] a TLR7 agonist (Resiquimod) was applied systemically leading to reduced tumor volume, increased CD8/Treg ratio in mice, and

was further enhanced when used in combination with anti PD-L1 checkpoint blockade, while results were not observed in monotherapy alone[28]. Taken together, further studies where biomarkers are identified and a better understanding of the role of TLR agonists' impact in tumor acceleration or control in different types of cancer are needed. All this, plus a better understanding of the efficacies and limitations of combination therapies with checkpoint blockade are required for better cancer treatments when using TLR agonists.

Dr William Coley used therapeutic bacteria as a treatment for cancer and while his positive results were limited, one can say he was ahead of his time. With recent advances in the field of immunology, molecular biology and precision therapies, we can now truly think of bacterial administrations as cancer immunotherapy. Bacterial treatments when attenuated can be well tolerated by immunocompetent patients. Bacteria have the capacity to elicit the innate and adaptive response, where other treatments can only induce activation in a limited manner. Bacterial therapies also have the capacity to be genetically manipulated in ways that can serve as delivery systems for toxins, chemotherapies, and other forms of targeted treatments. Bacteria can find immunosuppressive niches such as the anerobic areas of tumors and can work in combination with existing therapies. Therapeutic bacteria such as *E.coli, Salmonella, Listeria, Clostridium*, among many others, will be discussed in the next sections of the thesis to recapitulate the pros, cons, and questions that remain. Some of the critical outstanding questions for improving bacteria derived cancer treatments are: the **dosage of bacteria** in patients due to the less than ideal treatment modality of having to inject patients multiple times with live bacteria; the **colonization of tumors** derived from bacterial treatments - are some species more prone to tumor colonization than others?; can the **inflammatory response against bacteria be harnessed to control tumors?;** and whether **antigen specific** expressing bacteria are necessary for treatment to have an effect on tumor control. My thesis work aims to answer the aforementioned questions and further the viability of cancer bateria therapies by understanding the mechanism of action of the immune response against bacteria in a way that can harness the inflammatory response against cancer.

## II. BACTERIA AS CANCER IMMUNOTHERAPY REVIEW

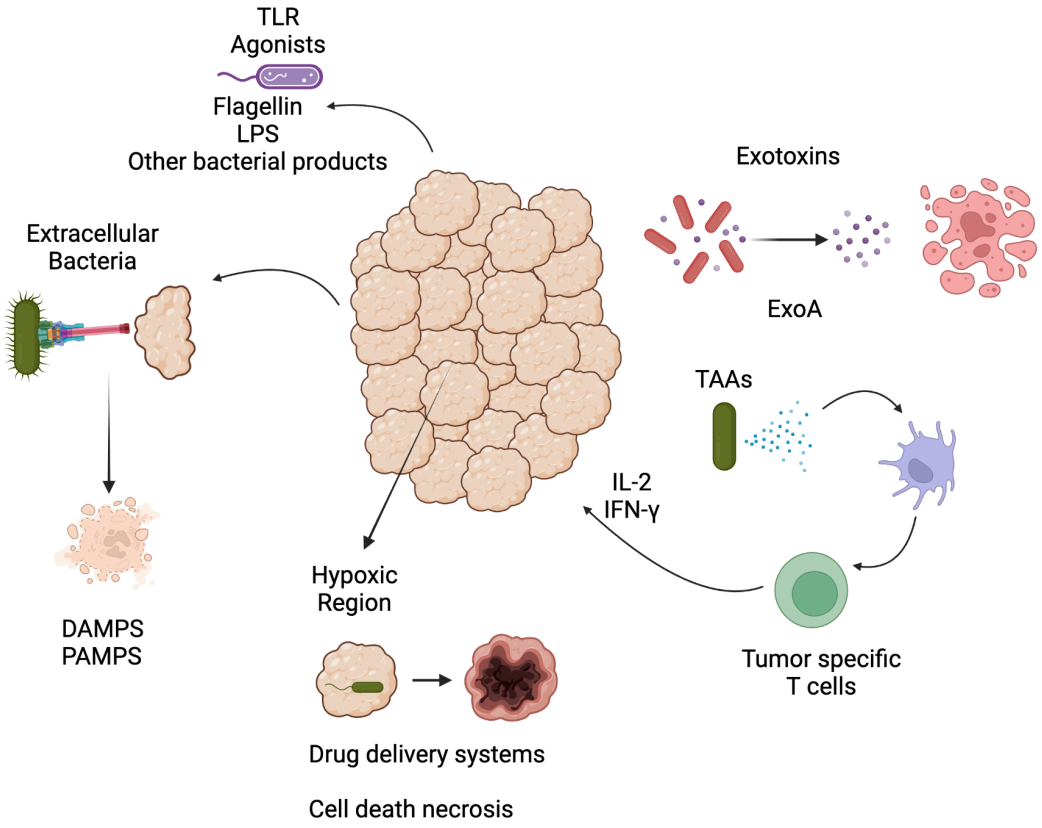


Fig 1. Modes of action of bacterial cancer immunotherapies

BCG vaccine

Bacteria as treatment against cancer takes many shapes and forms, depending on what species/strains are used and what types of cancers are being treated. To date there have been many clinical trials using bacterial mediated cancer immunotherapies, however bacteria derived treatments are yet to receive wide approval by the FDA. Furthermore, the mechanism of action of the Bacillus Calmette–Guérin (BCG) vaccine, a live attenuated *Mycobacterium bovis* vaccine that is used as treatment against non-muscle invasive bladder cancer (NMIBC) is yet to be completely mechanistically understood all while being the most successful form of bacteria-derived cancer immunotherapy. In this section, I will review the state of the field regarding bacteria derived cancer treatments, starting with the BCG vaccine, the questions yet to be answered, and future perspectives about the field of bacteria-derived cancer immunotherapies.

The BCG vaccine as a treatment for high-risk non-muscle invasive bladder cancer (NMIBC) to avoid recurrence and progression of disease has been used for the better part of the past four decades. The vaccine is a live attenuated form of *Mycobacterium bovis,* a vaccine against tuberculosis, still widely used in large parts of the world to prevent severe tuberculosis in children[29]. The BCG vaccine is thought to work through the activation of both the innate and the adaptive immune response, invasion of tumors, ultimately leading to tumor destruction. While the precise mechanism of action of the BCG vaccine against NMBC is not completely understood, much work has been done to understand the precise mechanism of action and it is proposed that a combination of the possible mechanisms of action are likely to be correct. These are, as mentioned before, the activation of the innate immune system via TLR activation. BCG has been shown to activate TLR2, TLR4, and TLR9 [30]. The induction of NOD- like receptors have also been shown to be activated by the BCG vaccine in mouse models[31]. Furthermore, colonization of *Mycobacterium bovis* into urethral and bladder cancer cells has been shown to be important for cancer control,[32] where mycobacterial fibronectin is thought to attach to the urothelium. Experiments where attachment to urothelium was disrupted led to loss of anticancer effects of the BCG vaccine in mouse models, however, it is important to note that these studies are yet to replicated[33].

The precise modes of colonization have shown inconclusive results whether experiments have been performed *in vitro* or *in vivo,* thus, precise mode of colonization and the importance of such cancer cell colonization is still being investigated. Some *in vitro* studies have suggested that patients receiving intravesical BCG show uptake into urothelial cells, following *in vitro* experiments showing bladder cancer cell lines are able to uptake BCG in a non-specific way, involving macropinocytosis, thus the answer

might be a mixture of urothelial uptake to stimulate innate TLR activation and bladder cancer uptake to further express BCG antigen in MHC-I[34]. Bladder cancer cells that have internalized BCG are known to secrete pro-inflammatory cytokines (IL-6, IL-8, GM-CSF and TNFα), inducing what is thought to be an inflammatory TME, which can shift the balance of immunosuppression and lead to better infiltration of innate and adaptive immune cells[35]. One group showed indirect evidence involving mannose-binding protein FimH expressing BCG had better internalization capabilities in urothelial cells and better bladder cancer control than wild type BCG[36]. All this considered, there is no conclusive evidence *in vivo* that internalized BCG into bladder cancer cells is necessary for tumor control[36].

T cells play an interesting role in mycobacterial infections, especially given the fact that chronic infection brought about from *Mycobacterium tuberculosis* persists even after an antigen-specific response is mounted against it. This is in part due to T cell responses to *Mycobacterium tuberculosis* being marked by a delayed response brought about by *Mycobacterium tuberculosis* inhibiting host apoptotic machinery, delayed migration of DCs into adjacent lymph nodes, and a critical early induction of suppression by regulatory T cells [37, 38, 39]. However once activated, the T cell response is responsible for maintaining control of Tuberculosis infection in the chronic stage, this is an area of active research and many questions remain unanswered. Mainly, how are antigen specific effector T cells maintained for long periods of time? And how do these antigen-specific T cells not succumb to exhaustion and anergy, as seen in chronic viral infections or what is observed in the TME [40] ? Because of this critical role of T cells in the course of *Mycobacterium tuberculosis*, it's crucial to understand the role of the BGC vaccine in the context of NMIBC.

The activation of tumor specific T cells, which can reach the tumor has been found to be necessary for BGC treatment efficacy, where mouse models lacking T cells showed a lack of efficacy of the BCG vaccine against cancer[41]. Importantly, CD4s from the Th1 lineage seemed to play a bigger role in the efficacy of BCG, this correlated with a normal chronic infection of Tuberculosis [42]. Mechanistically the BCG vaccine has been shown to shift from a Th2 to a Th1 inflammatory response in mice bladder tumor models with an increase of IL-2 and IFN-γ secretion, this was supported in experiments with IFN-γ knock out mice where mice did not respond to BCG treatment [43]. Further mouse models have also suggested T cells specific for bladder cancer are responsible for tumor control. Experiments showed that mice bearing bladder cancer that received adoptively transferred T cells from mice that had no exposure to bladder cancer and had been vaccinated with the BCG vaccine were not able to control tumors as well as mice that had been exposed to bladder cancer previously [42]. Of note, one clinical trial in humans with intermediate- and high-risk NMIBC treated with BCG, where urine samples were taken before and after treatment for cytokine analysis, concluded that

post-BCG Th1 associated cytokines ( IL-2, IL-6, IL-8, IL-18, IL-1ra, TRAIL, IFN-γ, IL-12[p70]) in urine were an indicator for recurrence and effectiveness of treatment[44].

An important area of research is whether the T cell response mounted by the BGC vaccine in the context of NMIBC has to be directed against *Mycobacteria* antigen or against cancer antigen? Some animal model experiments showed that a prior vaccination with BCG prior to intravesical treatment with BCG led to increased T cell infiltration and better tumor control compared to no prior exposure. Of note, researchers in a phase 1 clinical trial were also able to correlate a positive PPD test with better performance of BGC treatment in humans, meaning there may be correlation between memory T cells from early life vaccination against Tuberculosis to better tumor control by the BCG vaccine later in life. This assumption means that BCG specific T cells play a large role in tumor control, albeit it is not certain whether this is via direct killing of NMIBC or if colonization of BCG-specific T cells shifts the TME to a proinflammatory environment [45]. However, it is important to note that other groups have noted no benefit from adoptive transfer of BCG-specific T cells into tumor-bearing mice[42]. The role of bladder cancer specific CD4 T cells has also been explored. Adoptive transfer of CD4 T cells into mice with tumors indicated that mice rejected bladder cancer when adoptively transferred CD4 T cells were from mice previously cured of bladder cancer, however, mice that received CD4 T cells from mice that had been inoculated with the BCG vaccine, but were NOT tumor-bearing, saw no protection against bladder cancer[42]. This opens up the question of whether a combination of both bladder cancer and BCG-specific CD4 T cells working in tandem are required for tumor control in NMIBC in a manner that shifts the TME for a suppressive to a more proinflammatory one.

Finally, another proposed mechanism of action is what has been seen in *in vitro* experiments where a large ratio of BCG to bladder cancer cells led to inhibited bladder cancer cell growth and even killing of the bladder cancer cells, although this phenomenon has yet to be replicated *in vivo* [46]. Taken together, a great deal of evidence shows that the BCG vaccine is a viable treatment for NMIBC and a complete mechanism of action would not only benefit patients with NMIBC but also the field of immunotherapy in general.

*E. coli*

*E. coli* is a gram negative, facultative anaerobic, commensal bacteria, widely studied due in part to its ease of genetic manipulation. *E. coli* is recognized as a model organism and has its own advantages as cancer bacterial immunotherapy due to the aforementioned facultative anaerobic lifecycle, lending itself to have better survivability

and colonization capabilities in the anerobic areas of the tumor microenvironment compared to other bacteria. This makes *E. coli* an attractive vector for drug delivery systems, and it could play an important role in combination with existing therapies for better targeted deliveries. The next section will expand on *E. coli* biology, summarizing recent studies, and the open questions regarding *E. coli* as a vector for cancer immunotherapies .

*E. coli* has been used as a vector to deliver tumoricidal agents, such as the strain of *E. coli* K-12 which can colonize necrotic and hypoxic regions of a variety of tumor models[47]. Furthermore, *E. coli* K-12 has been engineered to secrete the bacteria-derived toxin Cytolysin A (ClyA), a pore causing toxin, is secreted in a vesicle-mediated pathway. Mouse studies showed a single intravenous injection of *E. coli* K-12 led to colonization and some tumor control in mouse models, however, eventually mice lost tumor control and disease progressed faster thereafter [47]. Another study showed that a genetically modified *E. coli* could secrete α-hemolysin specifically in tumors due to poor survivability of *E. coli* in the periphery. However, α-hemolysin is toxic to mammalian cells and secretion must be tightly regulated for treatment to be effective, thus further studies are needed to scale into the clinic[48]. Furthermore, *E.coli* has also been used for the deliberate targeting of anti-cancer drugs into the tumor microenvironment. The strain *E.coli* Nissle 1917 is known to penetrate tumors and colonizes necrotic sections of said tumors via production of minicells when minCD is deleted, this led to a way to deliver anticancer drugs directly to cancer cells[49]. Taken together *E. coli* is an attractive vector for drug deliveries, but further research is needed to scale up treatments into humans. [50]

*E. coli* strain Nissle 1917 has also been studied in combination with TGF-β blockade (Galunisertib), a group found that mouse models treated with Galunisertib in combination with *E. coli* Nissle 1917 responded better to checkpoint blockade treatment, potentially limiting toxicity of treatment. Response to treatment was mediated by more tumor specific T cells infiltrating the TME of 4T1 tumor bearing mice, and better DC activation[51]. This study suggested that gut microbiota can have a direct effect on checkpoint blockade treatment effectiveness, something that has been appreciated in human clinical trials as well, where gut microbiota can be a biomarker for checkpoint blockade effectiveness and is something that will be expanded later in this chapter[52]. It is also well documented that checkpoint blockade fails to help patients with "cold" tumors[53], to address this, a uropathogenic strain of *E.coli* CP-1 derived from a patient with chronic prostatitis has been shown to colonize the mouse prostate and induce a proinflammatory response in a tissue-specific manner via intra-urethral administration in a manner not to dissimilar from how the BCG vaccine is thought to infiltrate tumors. Furthermore, orthotopic prostate cancer models when treated with *E.coli* CP-1 in combination with anti-PD-1 checkpoint blockade resulted in increased

survival rate, decreased tumor burden, increasing T cell tumor infiltration, CD8 cytotoxicity, M1 macrophage increase, DC maturation and NK infiltration [51,54]. An *E.coli* strain that tightly regulates its function of drug delivery was engineered to respond to thermal stimuli and secrete TNF-α directly into tumors when stimulated[55]. Using the non-pathogenic backbone of *E. coli* Nissle1917, researchers were able to stimulate expression TNF-α which led to localized delivery of pro-inflammatory cytokine and tumor control [56]. Meanwhile another group used ultrasound to activate exogenous IFN-γ genes when an *E.coli* MG1655 strain reached tumors[57]. Ultrasound was used to heat the bacteria allowing it to express desired genes[58]. This also led to M1 polarization of macrophages, increased infiltration of CD4s and CD8s into the TME, and improved tumor control[58].

Taken together, due to its ease of genetic manipulation, its lifecycle and effective colonization of the TME using *E. coli* as a vector for cancer immunotherapies is an attractive avenue of study. Whether it be colonizing necrotic/anerobic areas of the TME or engineered to secrete tumoricidal treatments directly into tumors *E. coli* is a versatile model organism that has seen a number of applications, especially in the drug delivery aspect of treatments. However, questions remain to be answered and further research is needed to fully understand the potential of *E. coli*. Questions such as, whether the lack of immunogenicity of *E. coli* can be detrimental once it has colonized the TME?, whether *E. coli* can act to recruit immunosuppressive cell populations such as Tregs or MDCS?, and how this can affect combination therapies or drug delivery systems? It is important to note that other bacteria can induce a strong immune response once the TME has been infiltrated and strains reviewed here lack the immunogenicity to induce an immune response but rather rely on the ease of genetic manipulation to deliver therapies directly into the TME. Furthermore, in the context of *E. coli*, what is the significance of varying the dosing regimen of the aforementioned strains?, and what is the importance of bacterial long-term survivability of *E. coli* in the tumor microenvironment? How do these studies pair with existing checkpoint blockade? And what is the role of the immune system once these deliverables have been secreted into the tumor? These questions are yet to be answered. Taken together, much of the work that has been done with *E. coli* has focused on the delivery of therapeutics to the TME with success in mouse models. However, further research is needed to understand the immune response once *E. coli* has reached the TME, the dosage of *E. coli* - especially how tumors might react with multiple bacterial treatments, and whether or not *E. coli* is feasible in the clinic.

*Clostridium* genus

The *Clostridium* genus, a gram-positive, spore forming, anaerobic genus of bacteria, ubiquitously found in nature has also shown promise as a vector for cancer immunotherapy. *Clostridium* provides an effective mechanism of colonization of tumors derived from its strictly anaerobic lifecycle. While in aerobic environments, most *Clostridium* species are able to form spores that lie dormant until more suitable conditions are encountered, such as what is found in the anerobic regions of solid tumors. This provides a mechanism for systemic administration of *Clostridium* spores that can activate once solid tumors are reached, where an anerobic and necrotic environment is better suited for the *Clostridium* lifecycle, providing potential for targeted cancer therapies. During the 1970s *Clostridium* was used in clinical trials due to it being found present in solid tumors, however at the time, human trials ended due to not providing significant benefit to patients compared to standard treatments[59] . Further clinical trials were performed with non-pathogenic *C. butyricum* M55 spores (later reclassified as *C. sporogenes* ATCC13732) which was first observed to colonize tumors in mice, have been found to induce oncolysis, and later tolerated in humans with up to $1x10^9$ spores intravenously administered into patients with Glioblastoma, an extremely aggressive form of brain cancer with poor prognosis[60]. Ultimately, several patients developed abscesses that had to be surgically removed, and the rate of malignancy recurrence remained unaffected, even after multiple treatments with *C. butyricum* M55 spores[61]. Due to this unfortunate development, *C. butyricum* M55 spores have not moved forward as a viable bacteria cancer immunotherapy.

In the early 2000's, a promising strain of attenuated *Clostridium*, *Clostridium novyi*-NT (*C. novyi*-NT) was developed by eliminating α-toxin responsible for its toxicity[62]. The α-toxin present in *Clostridium* is responsible for gas gangrene, tissue necrosis and possesses hemolytic activity[63]. *C. novyi*-NT, is non-pathogenic when administered in animal models, and well tolerated when injected locally into tumors via intratumoral (IT) or systemically via intravenous injection (IV) [60,62]. *C. novyi*-NT spores are able to colonize hypoxic areas of tumors and locally germinate, leading to tumor necrosis, tumor cell lysis, and tumor regression in animal models [64]. Several groups have found when using combination therapy *C. novyi*-NT with existing chemotherapy, tumor control was achieved in preclinical animal models, ultimately leading to better tumor control than with either therapy alone. Interestingly, 20-30

percent of animals treated with a sole IV injection of *C. novyi*-NT spores saw complete remission in CT26 colorectal carcinoma tumor models. The mechanism of rejection was immune mediated, because tumors were rejected after follow-up tumor rechallenge, although this group did not go into detail when characterizing immune mechanisms of tumor control, this is yet to be explored [65]. One group found that injecting *C. novyi*-NT spores intratumorally led to better tumor control compared to IV only in mice, and furthermore, and when scaled to larger animals they found direct IT injections of *C. novyi*-NT into canine tumors led to 37.5 percent response with 3 complete and 3 partial responses in spontaneously developed tumors[66]. It is important to note that canine treatments closely resemble what is observed in the clinical setting, since these animals come into trials already having exhausted other forms of treatment.[67]

Although *C. novyi*-NT spore treatment has shown great improvement in the recent decade, and shows great promise for targeted treatment directed into solid tumors independent of antigen expression, the precise immune mechanism of tumor control is yet to be completely understood, with several questions regarding *C. novyi*-NT persisting[68]. As mentioned before, the mechanism and role of the immune system in contributing to long-term tumor control, further research into how to avoid tumor lysis syndrome and abscess development in patients will reduce toxicity. How *C. novyi*-NT will fare in combination with recently developed checkpoint inhibitors is yet to be answered. A recent phase 1 clinical trial which used single dose IT injections of *C. novyi*-NT resulted in 41 percent of patients (9 out of 22) decreasing in tumor size and 86 percent of patients having stable disease as best overall response[69]. The study concluded that IT injections of *C. novyi*-NT in humans are tolerated and feasible, while toxicities are significant but manageable[69]. Thus if immunological mechanisms are clarified, *C. novyi*-NT could become an attractive form of treatment in the near future.

### *Salmonella*

*Salmonella* is gram-negative, non-spore forming, rod-shaped, pathogenic bacteria. *Salmonella* plays a significant role in human foodborne illness, being the most common form of food pathology. However, when attenuated, but still invasive, *Salmonella* is a viable option for bacteria-derived cancer therapies due to it being a facultative anaerobe that is able to selectively colonize tumors, combined with its ability to infect cancer cells directly, and it being a potent inducer of innate and adaptive immune responses[70].

The *Salmonella* genus consists primarily of *Salmonella enterica* and *Salmonella bongori, Salmonella* life cycle primarily affects the human and animal digestive tracts, where its virulence factors include multiple flagella, capsule, plasmids, adhesion systems and type 3 secretion systems (T3SS) which are encoded in Salmonella pathogenicity islands (SPI 1-2,and others)[71]. Once inside the host's digestive track, *Salmonella* is able to colonize its host by actively making contact with the host cells using type 3 secretion factors to facilitate bacterial uptake into the host cell via macropinocytosis. Inside the vacuole of enterocytes, *Salmonella* further secretes effector proteins which protect *Salmonella* from host lysosomes further evading host defenses[72]. However, *Salmonella* cannot evade host defenses permanently, once outside the vacuole, *Salmonella* infection ultimately leads to pyroptosis in the host cell, releasing *Salmonella* to infect further enterocytes alongside DAMPs. Different SPIs play different roles in the course of a *Salmonella* infection, SPI-1 are required for host cell uptake and and macrophage apoptosis, SPI-2 is more general for the systemic infection, replication inside macrophages and interfere with MHC presentation machinery, dampening the immune response against *Salmonella* [73]. SPI-3 is necessary for survival within macrophages and growth in less than ideal low magnesium conditions[74]. SPI-4 has been found to be important in for toxin secretion and intracellular macrophage survival, importantly SPI-5 consists of T3SS proteins and SPI-6 has been found to be important in response to extracellular stimuli and and transport into intracellular space of host cells[75].

Due to the aforementioned intracellular life cycle of *Salmonella*, its potent virulence, specifically T3SS which facilitates uptake of *Salmonella* into non-phagocytic cells and the ultimately recognition from the innate and adaptive immune system, *Salmonella* has become a promising organism for bacteria derived cancer immunotherapies[76]. More specifically, five key properties of *Salmonella* have been described as advantageous. (1) Tumor targeting capabilities, (2) broad number of cancer targets, (3) oncolytic activity, (4) proinflammatory immunomodulatory effects, and (5) ease of gene modification [77,78].

*Salmonella* is known to reach and persist in tumors when administered systemically via IV injections in mice, once in the TME, the innate response begins by recruiting neutrophils, macrophages, natural killer cells to the TME, and activating DCs. Then the DCs will migrate to tumor-draining lymph nodes to activate T and B cells against *Salmonella*, ultimately leading to long lasting adaptive T cell memory response[79] . One study using “cold " B16F10 tumors showed that when treated with attenuated *Salmonella* BRD509E via I.P tumors grew slower than the non-treatment group[80]. Furthermore, *Salmonella* has been shown to inhibit metastases in a variety of tumor models [81]. Mechanistically, *Salmonella* induced tumor control appears to be mediated by the innate and adaptive responses due in part to *Salmonella* strains with a mutated Lipid

A or purine systems being unable to elicit tumor control. *Salmonella* BRD509E treatment also had an effect on CD11b+Gr-1+ myeloid cells, which are known to be suppressive myeloid cells in the TME, however in the case of *Salmonella* BRD509E treatment they exhibit a more proinflammatory phenotype. These CD11b+Gr-1+ myeloid cells upregulated activation markers like MHC-II, costimulatory markers CD80/86, and Sca-1/Ly6A proteins, however for this study this group did not focus on the T cells response against tumors or *Salmonella* BRD509E [82].

A separate group showed that *Salmonella* is able to aggregate in tumors due to chemotaxis to necrotic and quiescent cells and a preferred low oxygen environment, when it is present outside of its normal place of infection, i.e. outside the intestinal tract [70]. Another study showed that when mice are vaccinated with *Salmonella* prior to tumor inoculation, then followed by a localized injection of DCs preloaded with *Salmonella* with an intact T3SS led to tumor colonization and killing of B16F10 melanoma by CD8 T cells specific to *Salmonella* antigen, which was presented on MHC-I in cancer cells, when compared with infection with a Salmonella T3SS knock out mutant, which ultimately showed no tumor control[83]. Similar studies have shown that *Salmonella*, when administered systemically, can reduce programed death ligand-1 (PD-L1) expression in tumors [77].

A phase I clinical trial using *Salmonella typhimurium* (VNP20009) resulted in well tolerated treatment of up to $3\times10^8$ cfu/$m^2$, while higher doses led to more negative side effects, including, thrombocytopenia, anemia, persistent bacteremia, hyperbilirubinemia, diarrhea, vomiting, nausea, elevated alkaline phosphatase, and hypophosphatemia. Patients who received VNP20009 expressed more pro-inflammatory cytokines in serum but, in contrast, few patients saw tumor colonization, all while none of the patients treated observed tumor regression[84]. VNP20009 attenuation - done by deletion of the purI and msbB genes, is quickly cleared from the murine periphery, because these deletions result in less systemic production of TNF-a due to deletion of msbB gene, thus lacking N-terminal of lipid A and leading to no induction of septic shock *in vivo*, while retaining the ability to colonize B16F10 tumors in mouse models [85]. Conclusions from the aforementioned clinical trial were that further research is required to understand dosage and tumor localization to understand if there are benefits to patients when using VNP20009 *Salmonella* against melanoma[84]. Due to lack of successful results in patients when administering a single dose of *Salmonella VNP20009* research has shifted to combination therapies with chemotherapies and radiotherapy, while combination with checkpoint blockade remains to be tried in humans[78]. One study involving MC38 tumor bearing mice treated with attenuated *Salmonella* BRD509E in combination with αPD-L1 found inhibition of tumor growth mediated by a decrease in tumor associated granulocytes, upregulation in MHC-II expression by monocytes in tumors and greater infiltration of effector T cells

into the TME, ultimately leading to a more immunogenic tumor microenvironment[77]. T cells were found to have higher expression of IFN-γ, GranzymeB, CXCL9 and CXL10 contributing to the proinflammatory environment of the TME [77]. Another study, using attenuated *Salmonella* LVR01 which has a deletion on the aroC gene, and has been used as a vector to vaccinate deer against prion disease (U.S. Patent No. 8685718). This study used *Salmonella* LVR01 in combination with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) therapy treating B-cell non-Hodgkin lymphoma (B-NHL) models in mice. Results showed when administering *Salmonella* LVR01 three times via IT while mice receiving CHOPS had greatly increased the tumor control effectiveness when compared to *Salmonella* LVR01 or CHOPS treatment alone. Furthermore, immunologically, there was an increase of tumor infiltrating T cells in all treated groups being single or combination therapy. Interestingly, combination therapy recipients showed a further increase of CD8 T cells into the TME, a decrease of Tregs, while also reducing chemotherapy toxicity and expressing more A20 antigen[86]. *Salmonella* LVR01 does not express A20 antigen itself, but the combination CHOPS + LVR01 in A20 expressing tumors led to tumor antigen being presented to CD8 T cells which are then recruited into the TME[87]. Another study using attenuated systemic *Salmonella* in combination with low dose doxorubicin in mouse breast cancer models saw no toxicity and decreased tumor burden, but the greatest tumor control was observed with the highest dose of doxorubicin at the expense of higher toxicity. This study also suggests higher infiltration of CD8s into the TME with combination therapy, and inadvertently also increased Tregs recruited into the TME [88]. Furthermore, a number of studies have suggested that accumulation and persistence of *Salmonella* in tumors is enhanced when treating with chemotherapy, this could be in part due to combination chemotherapy and *Salmonella* treatment reducing tumor microvasculature, furthering the hypoxic state of tumors, leading to a positive feedback loop of colonization [89,90]. Mechanistically combination chemotherapy and *Salmonella* treatment is thought to have synergistic effects due to *Salmonella* upregulating expression of connexin 43 (Cx43) enhancing gap junction communication, which is decreased in a variety of cancers, leading to enhanced antigen presentation by DCs, and further facilitating tumor apoptotic signals and delivery of chemotherapy drugs[91,92]. Another idea put forward is the ability of *Salmonella* to increase chemosensitivity of cancers by shifting cells from G0/G1 phase to the S/G2/M phase in the cell cycle[93]. This shift is advantageous due to chemotherapies being more effective on proliferating cells than quiescent cancer cells. One study pushed this further by adding methioninase to trap cancer cells in S/G2 phase, having better tumor control than combination chemotherapy and *Salmonella* treatment alone[94].

*Salmonella* has also been employed in combination with radiotherapy to enhance and sensitize tumors to respond better to radiotherapy with preclinical success in

mouse models. One study suggested that combination radiation with *Salmonella* led to better tumor control and survival rate in the B16F10 model than monotherapy by inducing more apoptosis in combination therapy treated mice [95]. Studies performed in CT26 tumors models also showed efficacy of combination radiotherapy therapy and attenuated *Salmonella* strain ΔppGpp/pBAD-ClyA administered via IV, also showing increased tumor colonization after radiotherapy treatment due to radiotherapy making tumors more anerobic, helping *Salmonella* colonization[96]. Previous studies from the same group observed *E. Coli* colonization after radiotherapy treatment [96 47]. Mechanistically results from multiple studies conclude that when administering attenuated *Salmonella* after radiotherapy treatment induces higher levels of apoptosis in tumor models, increased inflammatory cytokines (GM-CSF, IL-2 and IL-12), radiosensitization induced activation and recruitment of DCs in the TME, inhibition of T cell exhaustion, plus all the added effects mentioned above regarding a proinflammatory TME [97].

As mentioned above, *Salmonella* has also been used in combination with checkpoint blockade in mouse models, here we will go into more detail. Attenuated *Salmonella* treatment has been shown to decrease PD-L1 in mouse and human cell lines (B16F10 and LL2) via inhibition of AKT/mTOR/p70s6K signaling pathway while enhancing effector T cell infiltration into the TME, ultimately leading to better tumor control [98]. Other studies have shown that systemic IV injections of attenuated *Salmonella* actually increased PD-L1 in dendritic cells and macrophages, however, these studies did not correlate PD-L1 expression with tumor growth inhibition [99, 100]. Further studies combining attenuated ovalbumin-producing A1-R *Salmonella* and anti PD-L1 showed antigen-specific OVA tumor-specific CD8 T cells were rescued with combination therapy and ultimately led to tumor rejection when compared to either single therapy alone [101]. This study further suggested antigen specific *Salmonella* in combination with α-PD-L1 checkpoint blockade can lead to T cell infiltration of long-established B16F10 tumors and rejection of 32% of tumors[78] . Interestingly, α-CTLA in combination with *Salmonella* did not have the same effect as α-PD-L1 treatment [101]. Another group used *Salmonella* itself as a vector for checkpoint blockade carrying siRNA targeting PD-1. Here researchers used a combination *Salmonella* and Nifuroxazide which is known to improve anti tumor immunity and impair colorectal cancer metastasis in a STAT3 dependent manner[102]. Results showed that combination Nifuroxazide and *Salmonella* expressing siRNA for PD-1 led to better tumor control of a colorectal cancer model when compared to monotherapy alone, although Nifuroxazide is not associated with increased proinflammatory stimulation, it is with tumor killing, which in turn can release tumor antigen and confer a proinflammatory state the to the TME[103]. Another study from the same group used the same siRNA expressing *Salmonella* strain in combination with pimozide, which is known to inhibit melanomas by creating ROS. Here, researchers found that combination therapy yielded the best results, where tumor

burden was decreased, mice had prolonged survival, and greater infiltration of T cells into the TME, ultimately leading to apoptosis in B16F10 models via caspase 3[104]. However, these studies did not focus on the proinflammatory profile of tumor infiltrating T cells[105]. Another group used Salmonella-siRNA targeting Indoleamine 2, 3-Dioxygenase 1 (IDO) in combination with α-PD-1 to treat CT26 or MC38. Results showed that *Salmonella*-siRNA and α-PD-1 had positive effects on tumor control, while combination therapy had no additive effects [106]. Taken together *Salmonella* has shown promise when combined with checkpoint blockade and in many cases has a synergistic effect. There are remaining questions regarding the complete mechanism of action before *Salmonella* can be scaled to human trials, especially since the dose of attenuated *Salmonella* can impact the toxicity of checkpoint blockade.

Because *Salmonella* has been extensively studied, it is a great candidate for scaling treatment to human clinical trials. Here, I will summarize clinical trial data and future perspectives of using *Salmonella* as a vector for cancer immunotherapy. As mentioned before, the strain VNP20009 was used to treat melanoma in a phase I clinical trial, but patients failed to see positive results in tumor control, regardless of VNP20009 colonization into tumors[84]. An update with four more patients to the aforementioned study with updated results, indicates that patients are able to tolerate VNP20009 better for a longer period of time than in the previous study, going from a 30 minute infusion to a 4 hour period which was well tolerated[107]. This addendum to the study again showed similar results to the previous study in that no patient showed VNP20009 colonization in tumors and VNP20009 offered no effect on tumor control[107]. In a pilot clinical trial using a modified strain of VNP20009 injected via IT engineered to express *E. coli* cytosine deaminase gene, producing CD enzyme, patients saw no major adverse effects and 2 out of the 3 saw tumor *Salmonella* colonization, which persisted after 15 days of treatment[108]. It is known that the CD enzyme, when combined with 5-Fluorocytosine, inhibits thymidylate synthase and DNA synthesis, leading to cell death in cancer cells. This pilot study showed that tumor colonization is feasible and well-tolerated, especially when administered via IT, but it did not explore the therapeutic anti-tumor effects[109]. More recent trials have involved the oral route of *Salmonella* as cancer immunotherapy, albeit with limited results. A phase I clinical trial using VXM01 an oral tumor vaccine of attenuated *Salmonella* expressing VEGFR2, an antigen expressed in tumor vasculature, was used to quantify safety, dose immunogenicity, and T cell activation in patients with advanced pancreatic cancer[110]. Patients received $10^6$ VXM01 CFUs monthly for up to 6 months, results from this trial showed that 8 out of 16 patients who received VXM01 had at least a 3 fold increase in VEGFR2-specific CD4 and CD8 T cells compared to placebo, which peaked at 21 days and decreased over the course of 3 months, and a correlated decrease of tumor perfusion[111]. Ultimately patients saw mild side effects, and the goal was to understand

the dosing regimen of VXM01 effects on long term VEGFR2-specific T cells [111,110]. Another study used oral administration of attenuated *Salmonella* expressing human IL-2 (SalpIL2) in patients with metastatic gastrointestinal cancer to test dose escalation tolerance ($10^5$ to $10^{10}$). Results showed no adverse toxicity when dose was escalated to $10^{10}$, ultimately there was no survival advantage[112]. However, there was an increase in circulating NK cells and NKT cells while there was no difference observed in CD4 or CD8 T cells, this increase in NK immunogenicity prompted researchers to conclude that multiple doses and possibly combination with other immunotherapies should be undertaken[113]. Taken together, combination therapies with *Salmonella* show promise due to low toxicity, observed tolerance and tumor colonization. However major roadblocks must still be overcome. It's important to note that differences in efficacy in preclinical to clinical studies have major differences in efficacy, which can be attributed to a number of differences between animal preclinical and human clinical trials. (a) Over-attenuation of bacteria derived therapies when scaled into humans. Over-attenuation can have detrimental effects on immunogenicity of therapy, which is ultimately the purpose of bacteria derived treatments, leading to a less than ideal immune response against the bacteria, which in turn leads to less immunogenicity towards tumors. (b) The importance of the route of bacterial administration.t Some engineered strains are better at colonizing the TME than others, thus, intratumoral injections have been recognized as a potential candidate for better tumor infiltration. (c) Animal tumor models often rely on tumor cells lines that in many cases fail to replicate the heterogeneity of tumors in patients, better models, such as GEM cancer models, are needed to recapitulate what is observed in cancer patients. (d) Fundamental changes to clinical trials could be introduced, since bacterial therapies often rely on a strong immune response that many patients lack when they are eligible for clinical trials. All things considered *Salmonella* derived cancer bacterial treatments show great promise while suffering from many of the same roadblocks as other bacteria-derived cancer immunotherapies. If these roadblocks can be addressed, *Salmonella* can become a strong vector for cancer immunotherapies.

*Pseudomonas*

*Pseudomonas aeruginosa* is a facultative anaerobic, biofilm producing, capsule gram-negative, abundantly found in nature specially in fresh water and soil.

*Pseudomonas aeruginosa* mainly affects immunocompromised patients, especially those with cystic fibrosis and those with chronic respiratory infections[114]. Due to *P. aeruginosa's* ability to produce biofilm, it is a major cause of persistent infections because biofilms are a natural barrier against the immune system[115]. Ultimately leading to massive collateral damage from prolonged inflammation at the site of infection. Of note, an estimated 60-70 percent of hospital acquired infections are from microbial biofilms[116]. *P. aeruginosa* pathogenesis comes from expressing type IV polar pili, a single polar flagellum, and chaperone pili, which are necessary for motility and adhesion. However, in patients with cystic fibrosis *P. aeruginosa* has been found to lack type IV pili. *P. aeruginosa* has also been shown to downregulate flagella expression when exposed to purulent sputum, this downregulation is thought to reduce TLR5 recognition[117]. During infection *P. aeruginosa* has also been shown to express an altered form of LPS, a more highly acylated Lipid A variant, which is a more potent agonist of TLR4[118]. *P. aeruginosa* also expresses Type 2 and Type 3 secretion systems which are important for secreting effector proteins into target cells and can be "hijacked" to deliver anti tumor molecules as will be expanded later in this section[119]. Furthermore *P. aeruginosa* T2SS mediates secretion of exotoxin A, which is responsible for inhibition of target cell protein synthesis, while Type 3 secretion systems are responsible for ExoU an inhibitor of inflammasome activation leading to necrotic cell death of host, usually innate immune phagocytic cells[119]. Mice that were infected with $T2SS^{KO}/T3SS^{WT}$ *P. aeruginosa* succumbed rapidly compared to $T2SS^{WT}/T3SS^{KO}$ infections in acute pneumonia models[119]. This delay in killing in the presence of T2SS is thought to be responsible for the chronic inflammation during a prolonged infection of *P. aeruginosa*. It is important to note that high variability in strains has been recognized, as mentioned previously patients with cystic fibrosis infections show a clear phenotype that is acquired through mutations and selection of the environment, however strains that have been isolated from patients without cystic fibrosis show a very different phenotype of *P. aeruginosa* virulence factors[120]. For example up to 50 percent of recovered strains are T3SS negative from cystic fibrosis negative patients, this can make it difficult for accurate assessment of laboratory experimental strain outcomes, ultimately leading to making it more difficult to generalize results.

*P. aeruginosa* mutants have been recognized and used in bacterial cancer immunotherapies in animal models and human clinical trials. The mutant *Pseudomonas aeruginosa*-mannose sensitive hemagglutinin (PA-MSHA) is characterized by high expression of mannose-sensitive hemagglutination fimbriae, which attenuates *P. aeruginosa* by lowering the exposure of LPS and flagella to the immune system while MSHA being a TLR4 agonist[121]. Mechanistically, PA-MSHA is thought to work by inducing tumor apoptosis via caspase 3, 8 and 9 activation, inhibition of cancer proliferative signaling machinery, and by repolarization of TME macrophages towards a

proinflammatory M1 phenotype [122, 123, 124, 125]. In human bladder cancer cell lines PA-MSHA suppressed tumor proliferation by inducing apoptosis, meanwhile in nude mice xenograft transplanted mice also saw an increase of tumor control, mediated by PA-MSHA induced apoptosis[126]. Lewis lung carcinoma tumor-bearing mice with intact immune systems treated with PA-MSHA resulted in slower tumor progression and longer survival mediated by a more proinflammatory TME showcased with higher numbers of T cells and activated DCs[127]. Mechanistically this outcome is thought to be induced by the TLR4 agonists effects of PA-MSHA which induced higher co-stimulatory signaling, cytokine secretion and stronger T cell activation[128]. In comparison, heat inactivated PA-MSHA has been used in human clinical trials in combination with chemotherapy for breast, lymphoma and NSCLC cancer. The NSCLC phase III clinical trial using heat killed PA-MSHA was used to test whether the addition of the bacteria could enhance the effects of chemotherapy, resulting in borderline statistical significance. Remarkably, the addition of PA-MSHA did not increase toxicity or have a negative effect on quality of life in patients[129]. Although the results were marginally significant, this study represents a positive impact of bacteria derived cancer treatments independent of cancer antigen specificity. Due to preclinical results showing that PA-MSHA had an antiproliferative effect on breast cancer cell lines, a phase II clinical trial using PA-MSHA in the context of HER2 negative metastatic breast cancer interestingly showed that patients with moderate immune-related adverse events (fever, skin induration) caused by PA-MSHA had higher survival (25.4 vs 16.4 months) and longer progression free survival (8.2 vs 3.1 months) compared to patients that had mild or no immune related reaction to the injection of PA-MSHA [130].

*P. aeruginosa* has also been used as a vaccine vector for tumor antigen delivery. *P. aeruginosa* has been engineered to deliver tumor-specific antigens via T3SS directly into DCs to prime the CD8 T cell response against tumors, producing in B16 tumor models long lasting antitumor immune responses[131]. In another study using an engineered strain of *P. aeruginosa* (CHA-OST S54-Ova) which had deletions on the toxin delivery system of the T3SS (ExoS and ExoT) instead replacing it with the C terminus of the Ovalbumin protein for immunogenicity[132]. Once this strain was administered in mice inoculated with B16-OVA tumors, mice were able to clear tumors in a CD8 T cell-dependent manner and were resistant to further tumor challenges with B16-OVA[133]. This result showed that the T3SS machinery in *P. aeruginosa* can be used safely for antigen delivery in tumors, however it's important to note that tumor antigen specificity in tumors can lead to antigen escape and many tumors are heterogeneous in nature, thus, the development of non specific antigen treatments should also be of importance when developing strains.

Exotoxins produced by *P. aeruginosa* have also been widely studied in the context of cancer therapies, which target specific tumor cells to influence proliferation, differentiation and ultimately leading to apoptosis, killing the target cancer cell. These bacteria-mediated toxin cancer therapies (immunotoxins) are cytolytic fusion proteins which work by generating the catalytic component of the protein responsible for the killing and a receptor binding such as a surface ligand specific for tumor are combined to form the immunotoxin[134]. Variable fragment parts of monoclonal antibodies that recognize specific tumor surface proteins or overexpressed protein in the surface of cancer cells are commonly used in the binding domain of immunotoxins[135]. Once bound to the surface of tumor cells, immunotoxins are endocytosed, leading to the catalytic effects of the immunotoxins to take effect inside of the cell leading to tumor cell death[136]. One challenge noted from these immunotoxins is because of the immunogenicity of peptides such as ExoA, generated immunotoxins are truncated to reduce immunogenicity leading to less recognition by B and T cells[137]. This can be a double-edged sword, since by making the immunotoxins less recognizable by the immune system, treatment can progress without the immune system recognizing and neutralizing immunotoxins before they can have the desired effect, usually after multiple doses of treatment. While also by merit of being less immunogenic, immunotoxin treatment could have a less pro-inflammatory effect in the TME compared to treatments that are able to elicit a more pro-inflammatory immune derived response within the TME. Exotoxin A (ExoA) produced by *P. aeruginosa* disrupts protein synthesis, is cytotoxic against cancers and is the most widely used toxic virulence factor applied in building immunotoxins for targeted cancer therapies. Interestingly, compared to a number of bacteria-derived cancer therapies, there is a fair amount of mechanistic knowledge regarding Exo A derived immunotoxic mechanism of action. *P. aeruginosa* Exo A acts as an adenosine diphosphate ribosyltransferase which can irreversible ribosylate eukaryotic protein prolongation elongation factor 2 (EF-2), leading to inhibition of protein synthesis which in turn lead to cell death via apoptosis[138]. In the TME, treatment with *P. aeruginosa* Exo A immunotoxins has been observed to have interesting effects on degrading oncogenic signaling proteins and growth factors, such as vascular growth factor used in tumor angiogenesis[139]. However these off-target effects are still an area of active research.

To date there have been a number of phase I and II clinical trials involving *P. aeruginosa* immunotoxins in a variety of cancers (B cell lymphoma, ovarian, mesothelioma, breast, esophageal, among other cancers)[140]. Toxicity and limited efficacy were the major setbacks of these studies, and while in murine models, combination immunotoxins with existing chemotherapy showed a synergistic effect, this effect has not yet been deemed safe to further proceed into humans, because in patients with adenocarcinomas, combination *P. aeruginosa* immunotoxins with chemotherapy resulted

in clinical activity, but was not well-tolerated in patients[141]. Taken together, there is a great deal of mechanistic evidence on how *P. aeruginosa* can be beneficial in bacterial cancer immunotherapies in a variety of tumor models. However, there are still roadblocks to overcome before widespread adoption of this treatment is feasible. The fine line between over attenuation and high adverse side-effects is the main obstacle for *P. aeruginosa* in becoming a more successful avenue of treatment.

*Listeria monocytogenes*

*Listeria monocytogenes* is a facultative gram-positive bacteria, taxonomically placed in the Firmicute phylum, most closely related to bacterial members of the Bacilli, Lactobacilli and Enterococci, used for decades as a model organism for infection and activation of the innate and adaptive immune response [142]. Although natural infection is via the oral route, most basic research has been conducted in mice using either intravenous (IV) or intraperitoneal (IP) routes of administration. Indeed, beginning with the classic work of George Mackaness in the 1960s, *L. monocytogenes* emerged as a highly quantitative and reproducible murine model system to study basic aspects of innate and adaptive immunity[143]. In a normal infection *L. monocytogenes* is a food contaminant usually not presenting a serious threat to immunocompetent individuals, however, *L. monocytogenes* does present a danger to pregnant individuals, newborns, the elderly, and other immunocompromised patients. If infection progresses, *L. monocytogenes* can infect the central nervous system leading to meningitis and death[144].

In murine models, intravenous infection of *Listeria* consists of a quick uptake of *Lm* by macrophages (MØs) and dendritic cells (DCs) in the spleen and liver, where the vast majority of bacteria are killed by the innate response of MØs and Neutrophils[145]. Mice with an intact immune system can clear the infection in 7-10 days and develop long lasting immunity which is mostly dependent on $CD8^+$ T cells[146]. In more detail, the primary determinant of virulence in *L. monocytogenes* is Listeriolysin O (LLO) a cholesterol dependent cytolysin, necessary for phagosome and vacuole escape when spreading from cell to cell[147]. LLO is secreted inside of the vacuole, forming pores in the vacuole allowing *L. monocytogenes* access to the cytosol of the host[145]. Once *L. monocytogenes* has accessed the cytosol, a cell surface transmembrane protein (ActA) is highly upregulated, which recruits and activates host Arp2/3 complex inducing the polymerization of host actin filaments allowing motility and intracellular and extracellular *L. monocytogenes* spread[148].

ActA mutants of *L. monocytogenes* are able to break free from the host phagosome and have access to the cytosol, ultimately leading to a robust innate and adaptive response towards *L. monocytogenes* while reducing virulence 1000-fold compared to ActA WT strains due to ActA mutants not being capable of spreading cell to cell.[149] On the other hand, LLO mutant strains are unable to access host cell's cytosol, therefore unable to spread cell-to-cell because they do not escape the vacuole, inducing a strong innate response clearing infection while not inducing a strong adaptive response[150].

After an IV injection of *L. monocytogenes,* the bacteria is found inside macrophages in a matter of minutes located in the marginal zone of the spleen, followed by DCs present in the white pulp, similarly to other intracellular bacterial infections, *L. monocytogenes* stimulates TLRs extracellularly and intracellularly[151]. Of note, MyD88 and IFN-γ play an important role on the innate immune response against *L. monocytogenes* where as few as 10 bacteria can be lethal against MyD88 and IFN-γ deficient mice in the context of a WT *L. monocytogenes* infection[152]. However when the same MyD88 and IFN-γ deficient mice are infected with ActA-deficient *L. monocytogenes*, the same phenotype was not observed due to killing of infected cells from recruited neutrophils[153].

Induction of type I interferon also plays a major role in the clearance of *L. monocytogenes.* As discussed above, MyD88 mediated TLR signaling plays an important role in recognition, moreover, type I interferon intact mice are more susceptible to *L. monocytogenes* infection than MyD88 deficient mice[154] due an inability to recruit $Ly6G^+$ monocytes to the site of infection, this shows a level of redundancy in the infection course of *L. monocytogenes*, where MyD88-deficient plus IFNAR-deficient mice, which lack Interferon-alpha/beta receptor can still recruit monocytes, but when the whole type I IFN pathway is disrupted mechanisms of defense fall apart.

Like many other intracellular pathogens, *L. monocytogenes* has evolved ways to inhibit host inflammasome machinery to increase virulence and reduce host cell death. LLO the master virulence factor of *L. monocytogenes*, has the capacity of inducing necrosis if unregulated, however, under normal infection conditions LLO contains a series of residues that allow LLO to perform well under low pH conditions such as of the vacuole, but limits its activity at neutral pH, like what is found in the cytosol. Importantly, *L. monocytogenes* mutants which highly express LLO, ultimately lead to necrosis and have their virulence highly attenuated, quickly being cleared by the innate immune response, specifically neutrophils and a decrease of the adaptive response by having less *L. monocytogenes* specific CD8 T cells when mice were re-challenged with a functional LLO strain [155,156].

On the other hand *L. monocytogenes* suppresses expression of flagellin *in vivo*, thus mitigating the role of pyroptosis due to the lack of recognition via NLRC4 inflammasome, as seen with the expression of LLO when *L. monocytogenes* flagellin is

highly expressed in the cytoplasm of host cells, they quickly undergo pyroptosis, leading to release of DAMPs, IL-1β and IL-18, ultimately leading to quick clearance by the the innate immune response[157]. This clearance of *L. monocytogenes* when inflammasome machinery is activated also leads to decreased adaptive immune cell activation[157]. While the mechanism behind this phenomenon is not completely understood, it has been hypothesized that quick cytokine inflammation can be detrimental to T cell priming and activation[156]. Taken together, due to *L. monocytogenes* being a "professional" intracellular pathogen it has evolved to evade intracellular recognition, dampening innate immunity and thus, ultimately being cleared by the adaptive response, due to ultimately activating both innate and adaptive respite makes *L. monocytogenes* an attractive vector for cancer immunotherapies when properly attenuated .

The role of apoptosis during a *L. monocytogenes* infection is better understood in the context of hepatocyte infection, which contrast to dogma that apoptosis is not immunogenic. During *L. monocytogenes* infection of hepatocytes, apoptosis is shown to recruit neutrophils to the site of infection, likely in a TNFα dependent manner[158]. Furthermore, an infection of *L. monocytogenes* can trigger apoptosis in lymphocytes in a non-invasive manner, but rather, it's due to the close proximity of infected phagocytic cells to lymphocytes. This phenomenon has been observed in two ways. (1) Extracellular released LLO when phagocytic cells are killed, making extracellular LLO act similarly to perforin, killing T cells in a granzyme dependent manner via caspase 3,6 and 9[159]. (2) Abundant levels of type I IFN produced during the course of *L. monocytogenes* infection[160]. This is thought to happen by virtue of type I IFN being an antigen-independent activator of T cells, possibly rendering T cells more susceptible to apoptosis[161]. Interestingly, apoptotic bodies are shown to induce immunosuppression, this could be of evolutionary advantage to *L. monocytogenes* during the course of infection. Mechanistically, in the final stages of apoptosis cells are seen to generate membrane-bound vesicles known as apoptotic extracellular vesicles, a subset of these apoptotic bodies are recently described to have importance in cell signaling leading to apoptotic debris clearance and intracellular communication. Of note, phagocytic cells that uptake apoptotic bodies are shown to produce IL-10, an anti-inflammatory cytokine [162]. IL-10 antagonizes production of IFN-γ, a crucial cytokine in the clearance of *L. monocytogenes* from the impaired generation of the T cell response in an IL-10 abundant environment. Mice deficient in IL-10 were as much as 50 fold less susceptible to *L. monocytogenes* compared to WT mice, leading to the assertion that IL-10 plays an important role in the course of infection aiding *L. monocytogenes*, the flipside being that mice which lack T cell are also less susceptible to *L. monocytogenes* infection, emphasizing the interplay and balance of IL-10, apoptotic T cells and virulence in the course of *L. monocytogenes* pathogenesis [162,163]. Taken together, it's important to

understand the role of cell death in the course of *L. monocytogenes* infection, due to the careful balance that is present between the bacteria and the immune system, which can lead to better selection of strains and applications in cancer immunotherapies.

Because of the strong innate and adaptive cell response induced, intravenous injections of recombinant *L. monocytogenes* strains have been developed as vaccine vectors and are currently in clinical trials for a variety of cancers[164]. These recombinant *L. monocytogenes* strains have been engineered to express personalized tumor antigens[165]. Mechanistically, this treatment may work by the following ordered process leading to immunity: (**I**) *Lm* uptake by antigen presenting cells (APCs), (**II**) *Lm* expression of personalized tumor antigens in these APCs, (**III**) processing and presentation of the tumor antigens on MHC class I to $CD8^+$ cytotoxic T cells, and (**IV**) recognition and killing of tumor cells by the activated $CD8^+$ T cells. However, the efficacy of *L. monocytogenes* treatment for cancer patients is still under investigation. While this strategy may potently enhance the anti-tumor $CD8^+$ T cell response, it does not address the obstacle of the immunosuppressive tumor microenvironment. Clinical trials in the mid 2000's used a live attenuated double deleted (LADD) strain of *L. monocytogenes* [166]. This LADD strains contains deletions in the ActA promoter rendering it unable polymerize host Actin to spread from cell to cell, which ultimately leads to a significant (1000-fold) reduction in virulence compared to WT *L. monocytogenes* [145]. This, coupled with the deletion of internalin B, which renders *L. monocytogenes* unable to infect hepatocytes, ultimately renders *L. monocytogenes* safer for patient treatment[167].

These genetic modifications, however, still let the double-deleted *L. monocytogenes* harbor the ability of inducing a potent innate and adaptive immune response. This, in combination with the further ease of genetic manipulation of *Listeria*, which can be engineered to express tumor specific antigen, makes this LADD strain particularly attractive for clinical trials. The aforementioned LADD (JNJ-757) has been used in clinical trials, where the strain expresses mesothelin a human glycoprotein which is expressed in the surface of healthy pleura, peritoneum and pericardium cells while also accounts for 30%-70% the Non-small cell lung cancer (NSCLC) neoantigen expression, associated with poor prognosis in patients with NSCLC[168]. And while in animal models LADD JNJ-757 in combination with anti-PD1 treatment led to decreased tumor size and prolonged survival rates in human clinical trials, overall results were mixed, with some patients developing a T cell response against tumor expressing mesothelin while others did not, leading researchers to question whether more tumor expressing antigens are required for a stronger immune response, or if an immune response can be elicited independent of tumor specific antigens, or whether the lack of efficacy was due to other external factors [168].

Pancreatic ductal cell carcinoma (PDAC) has not yet benefited from advances in checkpoint blockade and recent developments in immunotherapy, PDAC is known to express tumor associated antigens such as mesothelin and it has been identified as a "cold" tumor by poor infiltration of T cells, resulting in one of the most challenging cancers to treat. Because *L. monocytogenes* has been recognized to elicit a more pro-inflammatory tumor microenvironment, it is of great interest to use *L. monocytogenes* as a vector for cancer immunotherapies in so-called "cold" tumors[169]. Clinical trials used the LADD strain expressing mesothelin tumor antigen have been performed to treat PDAC(Lm-Mesothelin, CRS-207), and phase I results indicated that LADD strain expressing tumor antigen was well tolerated, and patients that had longer survivals were the the ones that mounted a stronger T cell response to the cancer vaccine[170]. Because of the promising results from phase I clinical trials, several phase II clinical trials commenced. These included CRS-207 in combination with cyclophosphamide(Cy) a chemotherapy drug and GVAX, a tumor cancer vaccine that consists of irradiated PDAC cells secreting GM-CSF. Of note, when GVAX and Cy were used in combination with CRS-207 patients had better overall survival and 12 month survival than patients treated with GVAX and Cy (6.1 vs. 3.9 months and 24% vs. 12% respectively). There was also immune-synergy when adding CRS-207 to the treatment[171]. It was shown that the CD8 T cell response started earlier when treated with CRS-207 than without it [170,172]. Another phase II study compared CRS-207 alone, CRS-207+Cy/GVAX, and standard of care chemotherapy. This study yielded no statistical significance between groups, but anecdotally, patients that received CRS-207 alone had an effect of overall survival, but not significant enough to warrant phase III studies, indicating that there is merit to the use of *L. monocytogenes* as a vector for immunotherapy, but further research is necessary[171]. Due to preclinical models showing that *L. monocytogenes* in combination with checkpoint blockade yielded positive results in mice, a third clinical trial, employed the aforementioned CRS-207+Cy/GVAX with or without the addition of nivolumab, an anti-PD1 antibody. Results indicated similar overall survival between treatments, while other metrics such as disease control and 12/18 month survival were improved with the addition of checkpoint blockade. Of interest, long term survival in patients receiving nivolumab in top of CRS-207+Cy/GVAX, had correlated CD8 T cell tumor infiltration, and reduction of myeloid suppressive cells[171]. Unfortunately, when combined with nivolumab, patients also suffered with adverse events associated with checkpoint blockade therapies [171]. Due to these reasons there have not been any subsequent phase III clinical trials involving CRS-207 in treatment of PDAC, reinforcing the notion that there are positive effects with CRS-207 cancer immunotherapies but further research is required to improve these *L. monocytogenes* against cold tumors.

Malignant Pleural Mesothelioma, although rare, is a form of cancer that arises from prolonged exposure to asbestos, which has a high mortality rate. However due to advances on checkpoint blockade, the overall survival has increased in recent phase III clinical trials[173]. This development leads to the possibility of synergistic effect of CRS-207 *L. monocytogenes* in combination with checkpoint blockade, furthermore Malignant Pleural Mesothelioma, highly expresses mesothelin, because of this using CRS-207 is an attractive from cancer immunotherapy. Remarkably, phase I clinical trials determined that CRS-207 in combination with chemotherapy was safe with up to $1x10^9$ CFU CRS-207 and in contrast with the aforementioned PDAC, 89% of patients (31/35) saw tumor reduction when treated with CRS-207 in combination with pemetrexed and cisplatin[174]. Mechanistically, in this phase I study, patients that responded favorably to therapy saw no adverse results with CRS-207 infusions, and furthermore, showed higher infiltration of DCs, NK cells, an increased CD8/Treg ratio, and a shift to a more M1 phenotype in tumors following CRS-207 treatment [174]. Alternatively, a recent phase II clinical trial was terminated due to low enrollment and lack of response using Pembrolizumab in combination with CRS-207 in previously treated malignant pleural mesothelioma. It was difficult to draw conclusions from 10 patients, and there was insufficient data to evaluate clinical activity of the treatment[175]. Taken together, these encouraging results warrant further research and clinical trials using combination CRS-207 plus chemotherapy.

Osteocarcinomas are highly aggressive, mostly affecting children, usually manifesting in longer bones before metastasizing into organs. Because of the aggressiveness of osteo carcinomas current treatments are lacking and consist of amputations and chemotherapy. However, due to the aggressiveness of the disease, the high propensity for micrometastases before treatment, and the high expression of HER2/neu, makes osteocarcinomas extremely difficult to treat[176]. Interestingly, canines suffer from osteosarcomas at rates higher than humans, while also having a high expression HER2/neu, poor prognosis and limited treatment options[177]. Because of these similarities, canines can serve as a model for pre clinical trials, especially because canine and human HER2 share >90% homology. ADXS31-164 is a live attenuated form of *L. monocytogenes* engineered to express the intracellular domain 1 and extracellular domains 1 and 2 of chimeric human epidermal growth factor receptor 2 (cHER2), the treatment in canines mirrors human treatment from amputation to chemotherapy, followed by treatment with ADXS31-164[178]. Strinkinly, dogs had improved 1, 2 and 3 year survival rates (77.8%, 67%, and 56%), compared to what was seen before with no added ADXS31-164 (55%, 28%, and 22%). Furthermore, when treated with ADXS31-164, 83% of canines developed Her2-specific T cells, leading to the synergistic effect of ADXS31-164 in combination with standard treatments[179]. Given these encouraging early

results, and given the homology between HER2 in humans, clinical trials in humans for osteocarcinomas should be highly considered, especially due to the fact that treatment options that increase survival rate have not kept up with other types of cancer.

The discovery and implementation of checkpoint blockade has been helpful for some cancers as mentioned before, while many others remain challenging to treat, thus, new ways of improving checkpoint blockade in combination with different treatment modalities are an area of great interest. As mentioned before *L. monocytogenes* vaccines induce a strong innate and adaptive immune response, especially inducing a strong $CD8^+$ T cell response. Because of this, there has been an interest in combining checkpoint blockade with *L. monocytogenes* vaccine treatments in cancer immunotherapies. Early preclinical studies used *L. monocytogenes* (Lm-LLO-E7) expressing antigen HPV-16 E7 fused to a truncated fragment of LLO, rendering *L. monocytogenes* attenuated but still able to induce a strong adaptive immune response. Lm-LLO-E7 when used in combination with anti-PD-1 checkpoint blockade improved the treatment efficacy of TC1 mouse tumor model compared with Lm-LLO-E7 treatment alone[180]. When treating mice with Lm-LLO-E7, researchers also found a reduction of Tregs and suppressor myeloid cells commonly found in tumors which are ultimately associated with poor prognosis in patients[180]. As mentioned in a previous section antigen specific *L. monocytogenes* (Lm-ANXA2) in combination with anti-PD-1 was used in pre-clinical PDAC tumor models to achieve a synergistic therapeutic effect than either therapy alone, leading to an increase of pro inflammatory cytokines (IFN-γ) in the tumor microenvironment [181]. Other groups have studied more challenging tumor models, such as hepatocellular carcinoma (HCC). A recent mouse study showed *L. monocytogenes* expressing multivalent HCC antigen (Lmdd-MPFG) in combination with anti-PD-1 led to better tumor control, in contrast to what has been reported in human clinical trials of anti-PD-1 against HCC[182]. It is interesting to note that Lmdd-MPFG induces PD-L1 expression, implying that there is natural synergy between Lmdd-MPFG and anti-PD-1 checkpoint blockade[183]. Results are promising but it is important to note that patients with HCC are known to undergo hyperprogressive disease with anti-PD-1 treatment, because of this, further research should be conducted to assess the safety and efficacy of combination Lmdd-MPFG/anti-PD-1 therapies [183].

Patients that benefit from checkpoint blockade treatment are a relatively small part of the overall population suffering from disease, in order to mitigate this problem more personalized forms of treatment are being investigated. Recently, adoptive cell transfers (ACT) have garnered great interest where cell-based vaccines, engineered T cells (CAR-T cells) or the patient's own cancer T cells are transferred back to patients and used to fight cancer in an antigen specific manner[184]. However there are several hurdles needed to be overcome for this highly personalized form of treatment to be

more broadly effective. Since these therapies have historically been monolithic in nature, tumors are able to escape antigen specific treatment and are known to relapse in a more aggressive manner, and due to the immunosuppressive state of many solid tumors, new approaches are needed for a more reliable form of treatment. *L. monocytogenes* has been used by some groups to expand the antigen repertoire being recognized by adoptive cell transfer treatments and infiltrate the TME by infecting immunosuppressive cell populations to produce a better form of adoptive cell transfer treatment. Their therapeutic model, deemed Reenergized ACT (ReACT) where $CD8^+$ T cells are engineered to express both tumor antigen (gp100) and *Lm* derived OVA in two distinct TCRs. Experimentally, they found that after tumor inoculation and growth, followed by injecting both engineered $CD8^+$ T cells and *Lm*-OVA drastically increased tumor tumor control (69%) compared to (10%) when treated with only $CD8^+$ T cells[185]. Of interest, they found mechanistically that this therapeutic effect relied on cDC1 $Batf3^+$ dendritic cells responsible for cross priming of T cells. Consistently with similar intratumoral *L. monocytogenes* treatments, they found a decrease of MDSCs and Tregs in tumors[186]. This new treatment modality of ACT treatment in combination with bacteria derived immunotherapy is encouraging, however further research is needed to fully understand the mechanism of action against a variety of tumors, especially models that are known to undergo antigen escape, and the autoimmune side effects that have been observed with ACT, specifically CAR T cells. These side effects are thought to arise due to cancer associated antigens sharing similarities to proteins expressed in healthy cells. [187].

Due to the easy of genetic manipulation of *L. monocytogenes* has also been employed in heterologous vaccination treatments, *L. monocytogenes* is used to express tumor specific antigens was shown to induce a more potent anti tumor response compared to DNA vaccine alone in a preclinical study. In this study researchers used a prime/ boost approach that was most effective when priming with a DNA vaccine followed by attenuated *Lm* expressing PAP antigen (similar to the LADD strain), but not when primed with attenuated *Lm* expressing PAP (LADD-PAP) followed by DNA vaccination[188] . Dosage regimen in this study was performed by doing weekly doses of LADD-PAP, ultimately leading to better tumor control than the DNA vaccine or LADD-PAP alone. One group used a heterologous vaccination regimen where mice are first vaccinated with peptide vaccine expressing EGFRvIII antigen which is known to be expressed by a number of cancers, followed by an attenuated strain of *L. monocytogenes* that also expressed EGFRvIII. Researchers found there was a more robust CD8+ T cell response against EGFR when heterologous prime boost dosing regimen with vaccines followed by *L. monocytogenes* treatments [189]. One of the drawbacks of homologous prime/boost vaccination courses is the loss of immunogenicity to the vector backbone

once the adaptive response is able to recognize and neutralize said backbone. To address this challenge, heterologous vaccination using a separate but still potent inducer of the innate and adaptive immunity is key to success. One group used the overexpression of p53 observed in cancers to test the heterologous vaccine treatment. They first vaccinated mice with p53 expressing modified vaccinia Ankara (MVA) in combination with attenuated *L. monocytogenes* expressing p53 (LmddA-LLO-p53) or a second dose of MVA. results showed that mice that received heterologous vaccine treatment with MVA followed by LmddA-LLO-p53 controlled 4T1p53 tumors better than homologous MVA vaccine treatment[190].

Issues arise when eliciting an immune response against specific tumor antigens due to the phenomena called "tumor antigen escape" [191], where, due to antigen-specific therapy, the immune system "selects" for cancer cells that do not express immune-targeted tumor antigens and ultimately gives rise to tumors that are resistant to therapy, often times leading to a more aggressive and difficult to treat cancer[191]. To this day, clinical trials that employ LADD strains of *Listeria* express a single tumor antigen for a variety of clinical trials against a multitude of different types of cancer. Consensus is that the field is still relatively new and different approaches are needed. While patients do seem to tolerate systemic injections of attenuated *Listeria*, some side effects do persist, such as fever and hypotension, which as mentioned in previous sections might be crucial for a good anti tumor response, but more research is required to understand dosage and if patients benefit from multiple treatments[150]. This is coupled with the fact that many patients enrolled in these clinical trials show late stage progression of disease with already weakened immune systems from chemotherapy and radiotherapy, and ultimately natural aging.

Because of the ease of genetic manipulation and some of the adverse effects noted in patients when treating with attenuated *L. monocytogenes*, several groups have worked on different types of attenuation in regards to safer cancer immunotherapy. There is a fine line of attenuation when constructing mutants due to some virulence is necessary for a robust innate and adaptive immune response, especially since many of the currently employed strains of *L. monocytogenes* express tumor specific antigens. One of these attenuation methods employ *L. monocytogenes* that once inside of the host cell "self-destructs" delivering antigenic cargo that can then be presented to T cells. Mechanistically, these "self-destructing" stains work by expressing phage lysin from the ActA promoter, attenuating *L. monocytogenes,* while still inducing a strong immune response and reducing APC death from infection [192193]. The LADD strain has also received a boost in attenuation, while keeping its robust T cell activation, working in a similar fashion to the self-destruct strains. *Listeria monocytogenes* recombinase-induced intracellular death (Lm-RIID) is built on the backbone of the LADD strain, in addition to

Cre recombinase expression from the ActA promoter, while essential genes for bacterial viability being flanked by LoxP elements[194]. Infections in immunocompromised mice lead to self limited infection while immunocompetent mice still induce a strong $CD8^+$ T cell response. In mice lung CT26 metastasis tumor models, *Lm*-RIID in combination with α-PD-1 led to similar tumor control as what has been observed with LADD strain in combination with α-PD-1[194]. Another attenuated strain of *L. monocytogenes*, rsΔ2, can be administered intramuscularly or orally, which has been a challenging route of delivery. rsΔ2 is engineered to lyse upon entering the host cell, by expressing *Listeria*-specific phage and delivering protein antigen and eukaryotic expression vector to the infected cell[195]. In mouse models rsΔ2 was able to induce a potent T cell response against ovalbumin protein, showing further promise to express cancer antigen[164]. While attenuation techniques have improved and show great promise, these strains have yet to be scaled to human clinical trials.

Several questions arise when looking at the data, such as, whether having expression of multiple antigens might be beneficial? Can *Listeria* treatments be paired with checkpoint blockade while at the same time this combination have a synergistic effect? Would abandoning TAAs might be a more effective way to induce the immune response in the tumor microenvironment? Other *Listeria* derived vaccines express antigen from the Listeriolysin O (LLO) promoter, however this genetically modified version of LLO is truncated (tLLO)(ADXS11-001) leads to a lack of pore formation once *Listeria* has been internalized by infected cells. While this approach makes *Listeria* less virulent than a LLO-WT, it also is important to note that it makes *Listeria* a potent inducer of the CD8 T cell response against induced TAA[196]. These vaccines target HPV oncolytic proteins expressed in HPV derived cancers and preclinical studies have shown tumor control with HPV-transformed tumors. In a phase II clinical trial employing the aforementioned *Listeria* vaccine in patients with cervical cancer [197], where results showed an encouraging 12 month 34.9% combined overall survival rate, there was no added benefit when patients were treated with ADXS11-001 and cisplatin[197]. Again, the question still remains about the efficacy of ADXS11-001 when paired with checkpoint inhibitors, as the synergistic effect of eliciting a strong immune response against TAA paired with checkpoint inhibitors could be beneficial. A recent phase III double-blind clinical trial (NCT02853604) is currently the only phase III *Listeria* derived clinical trial, however, the study ended early for business reasons and the complete results are not yet publicly available [198]. Consensus among phase II trials is that Listeria derived treatments are well tolerated and the modality requires follow up. Taken together, *L. monocytogenes* shows great promise in the field of bacteria cancer immunotherapies. A great deal of research has been done with *L. monocytogenes* as a vector for cancer immunotherapies, specially to induce a TAA specific T cell response, albeit with limited success in the clinic. Future research should focus on recognizing

biomarkers induced from these therapies, as in better characterization of CD8 and CD4 T cells and the suppressive environment of the TME when treated with *L. monocytogenes*, since it is better understood now that bacteria can persist in tumors (REF?). This paired with better understanding in dosing regimens and a better understanding of the fine line between attenuation and virulence could help the field to make bacteria mediated cancer immunotherapies more viable.

*H. pylori* and gut microbiota in the promotion and treatment of cancer

Not all bacteria can be employed against cancer, the other side of the bacteria derived cancer immunotherapies are cancer promoting bacteria, such as *Helicobacter pylori*, a gram-negative, flagellum producing bacteria, present in about half of the world's population, where it colonizes the gastric mucosa. *H. pylori* is responsible for gastric cancer and a variety of gastric illnesses[199]. *H. pylori* is thought to be responsible for tumorigenesis in the gastric mucosa during infection where after inducing chronic inflammation few patients progress to gastric and duodenal ulcers where less than 1 percent progress to gastric cancer[200].

Furthermore, *H. pylori* infections have been shown to have a detrimental effect on cancer immunotherapies. Where mice inoculated with MC38 or B16-OVA tumor models then treated with checkpoint inhibitors followed by infection of *H. pylori* saw a reduction of activated anti-tumor T cells, dampening the innate response mechanistically by obstructing the cross presentation activities of DCs ultimately leading larger tumors compared to α-CTLA 4/PD-L1 treated uninfected controls. Previous studies have shown that infections of H pylori does indeed affect the DC activation process in the mesenteric lymph nodes [201].

Further analysis into human data correlating the presence of *H pylori* in serum with PD-1 checkpoint blockade treatment in patients with NSCLC showed decreased survival in patients positive with *H. pylori.* Tumor samples from the aforementioned experiments yielded less monocyte-derived cells in the TME, less type 1 IFN, IFN-γ and IL-6 compared with patients negative for *H pylori*[202]. While these results are in need of corroboration, it is important to note that trends and correlations were shared between preclinical mouse models and retrospective studies. *H. pylori* can be used as an indicator of effectiveness in checkpoint blockage treatments, and further studies into how to mechanistically reverse the effects of *H. pylori* on checkpoint blockade is crucial for advancements of cancer immunotherapies.

In complete contrast to the detrimental effects of *H. pylori*, commensal bacteria *Bifidobacterium*, which is found in breast feeding neonates, and associated with longevity in humans, has been found to have positive synergistic correlation with

checkpoint blockade effectiveness [203]. In fact, the interplay between cancer immunotherapies and microbiota composition has gained great appreciation over the past few years due to discoveries that suggest different gut microbiota can have effects on checkpoint blockade treatment effectiveness[204]. Mice that were treated with antibiotics failed to respond to anti-CTLA4 therapy in the context of melanoma models compared with intact microbiota counterparts, and specifically mice that were given *B. fragilis* via oral gavage[205]. In short, gut microbiota affect response and toxicity to cancer therapies, ultimately affecting their mechanism of action. For example mice with or without gut microbiota when treated with cyclophosphamide had different outcomes, mice with intact microbiota had better translocation of gram-positive bacteria to lymphoid organs leading to a skewed IL-17 response from T cells compared to gut microbiota free mice[206]. Furthermore, in the case of immunotherapies, mice models showed that animals with intact gut microbiota had better response to CpG-oligonucleotide immunotherapy than antibiotic treated mice, via TNF-α production from T cells and myeloid cells, ultimately leading to better overall tumors response[207]. Differences in abundance of *Bifidobacterium* present in microbiota also have differences of response to checkpoint blockade, JAX mice have increase presence of *Bifidobacterium* compared to TAC mice and have been shown to response better to anti-PD-L1 treatment against melanoma tumor models[208]. In human studies with patients with metastatic melanoma, the presence of *Bifidobacterium* correlated with better response to anti-PD-L1 treatment[209]. Further studies where germ-free mice received *Bifidobacterium* fecal transplants showed resensitization to anti-PD-L1 immunotherapy by inducing CD8 memory T cells, ultimately helping immunotherapies [209,210]. Interestingly, systemic treatment of *Bifidobacterium* that was genetically engineered to express the anti-angiogenesis protein endostatin via IV injection led to bacterial tumor infiltration and tumor control compared with no *Bifidobacterium* injections[211]. On the contrary, research has shown the negative effects of *Bifidobacterium* and other lactic acid bacteria in cancer therapies. Correlation exists between lactic acid bacteria in the gut microbiota and increased angiogenesis in tumors[212]. Furthermore, *Bifidobacterium* is known to be associated with an anti-inflammatory role, leading to induction of Tregs and expression of IL-10 and gut microbiota has been associated with changes in Tregs metabolism shifting to be more reliant on short chain fatty acids which are produced by *Bifidobacterium* [213]. However, more studies are required for the direct correlation between *Bifidobacterium* presence in the gut microbiota and cancer therapy effectiveness [214]. Human clinical trials and preclinical studies using *Bifidobacterium* have overall been inconclusive, but as mentioned previously, the presence of *Bifidobacterium* is associated with protection against colorectal cancer in mice and human patients [215]. Other cancers have not shown a benefit with the presence of *Bifidobacterium*, such as head and neck cancer, where a clinical study showed no improvement in patients who received a

cocktail of *Bifidobacterium* spp. and *Lactobacillus* spp[216]. Encouragingly, multiple clinical trials are ongoing or have just finished focusing on the role of *Bifidobacterium* in patient gut microbiota with immunotherapies, immunochemotherapy, or the role of *Bifidobacterium* in patients during and after cancer treatments [217] [218] [219]. It goes without saying that further research is necessary to completely understand the role of *Bifidobacterium* in gut microbiota and the implications of the interaction between gut microbiota and immunotherapies aforementioned clinical trials have not yet produced publicly available results.

III. Attenuated *Listeria* colonize tumors and either promote or inhibit tumor growth dependent on the presence of *Listeria*-specific cytotoxic CD8 T cells

Including material from published work:

Jesse Garcia Castillo[1,3], **Sebastian Fernandez**[2,3], Timothy Campbell[1], Jacob Williams[1], Diego Gonzalez-Ventura[1], Julia Ybarra[1], Nicole Flores Hernandez[1], Elina Wells[1], Daniel A. Portnoy[1,2], and Michel DuPage[1]

Chapter preface:

Cancer immunotherapies have become a pillar in the treatment of cancer in recent decades, however many hurdles have to be overcomed for broader patient applications. *Listeria monocytogenes* is a known potent inducer of the innate and adaptive response and has been used in clinical trials in an antigen dependent manner against a variety of cancers. Here we describe a novel approach to tumor control using systemic and localized injections of *Listeria monocytogenes* that has a positive effect on tumor control in a variety of cancers independent of antigen expression.

**Title**:

Cellular mechanisms underlying beneficial versus detrimental effects of bacterial antitumor immunotherapy

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**Abstract**

*Listeria monocytogenes* engineered to express tumor antigens as a cancer vaccine has yielded mixed results. Here, we utilized an attenuated strain of *Listeria* (*ΔactA, Lm*) that does not express tumor antigen to explore the immunological response to *Listeria* itself in the context of intravenous (IV), intratumoral (IT), or a combination of IV+IT administration into tumor-bearing mice. Unexpectedly, we found that *Lm* persisted in

tumors of immune competent mice, regardless of the administration route. While IT *Lm* alone led to the recruitment of immunosuppressive immune cells that promoted tumor growth, IV *Lm* followed by IT *Lm* controlled tumor growth. IV *Lm* vaccination generated a pool of anti-*Lm* cytotoxic CD8 T cells that killed *Lm*-infected non-tumor cells to control tumor growth. Our findings reveal a differential impact of IT *Lm* administration on tumor progression that depends on the presence of anti-*Lm* CD8 T cells, rather than antitumor CD8 T cells, for antitumor therapeutic efficacy.

Introduction:

At the turn of the 20th century, William Coley was the first to demonstrate the efficacy and application of bacterial cancer immunotherapies through combinations of intratumoral and systemic injections of cultured bacteria (gram-positive *Streptococci* and gram-negative *Serratia*) into patients with bone and soft-tissue sarcomas [1,220,221]. Today, direct intravesical administration of Bacillus Calmette-Guerin (BCG), a live attenuated strain of the gram-positive intracellular pathogen *Mycobacterium bovis*, is a standard of care for non-muscle-invading bladder cancer and the only FDA approved remnant of bacterial cancer therapy[222]. Recently, the discovery that bacteria naturally colonize many solid tumors has uncovered the potential of bacteria to serve as a drug delivery system or even as a cancer detection probe [223–232] . Improved methods to genetically manipulate bacterial genomes has also made it feasible to engineer bacteria that express tumor antigens, cytokines or cytotoxic proteins to control cancer [233–236]. Hence, multiple breakthroughs in manipulating bacteria and their activity in the tumor microenvironment has reignited interest in the potential for effective bacterial-based cancer immunotherapies.

*Listeria monocytogenes* engineered to express tumor-associated antigens and injected intravenously has effectively controlled multiple cancer models in mice [237–240]. To date, *Listeria* engineered to express tumor antigens and injected intravenously is the most tested bacterial-based immunotherapy in clinical trials for cancer[241]. Despite strong evidence of the vaccine's capacity to safely generate tumor antigen-specific CD8 T cells, the anti-cancer efficacy in patients has been mixed[172,174,197,242–244]. While select trials have shown improved outcomes in patients with otherwise untreatable cancers, such as malignant pleural mesothelioma or cervical cancer [174,242], many *Listeria*-based vaccine trials have not shown significant improvement in tumor control compared to standards of care. Therefore, it is critically important to discern why these vaccines fail despite generating antitumor CD8 T cells. In contrast to other bacterial cancer therapies, whether *Listeria* directly colonizes tumors has not been adequately investigated, likely because colonization was never a goal for its use against cancer in patients. A better

understanding of the dynamics of *Listeria* interaction with host immune cells, its migration, and its persistence in tissues may reveal vulnerabilities of the current *Listeria*-based strategies as well as lead to improved uses of *Listeria* as an immune adjuvant directly within tumors.

Effective antitumor immunity requires a robust pool of antitumor immune cells and a permissive tumor microenvironment (TME) that allows for antitumor immune cells to function [233,245–247]. However, the TME is often highly immunosuppressive, allowing cancer cells to evade the immune system despite the presence of tumor-specific cytotoxic CD8 T cells [248,249]. Thus, the failure of *Listeria* vaccines might be due to *Listeria's* singular use as a systemic therapy to generate antitumor CD8 T cells, without harnessing its capacity to stimulate the innate arm of the immune system and reverse immunosuppression directly within tumors. *Listeria* is rich in mechanisms to stimulate innate immunity directly in tumors[250]. Direct intratumoral injection of bacteria or bacterial products may have underlied the success of William Coley's early therapies[251]. Recent preclinical studies as well as clinical trials have demonstrated the feasibility and efficacy of direct intratumoral injection of immune adjuvants naturally generated by *Listeria* and other bacterial species that can activate host cell pattern recognition receptors (PRRs) and stimulate the innate immune response against tumors [252–254]. Direct IT injection of TLR1/2 agonists or cyclic dinucleotides (CDNs) was shown to increase CD8 T cell infiltration, polarize tumor-associated macrophages (TAMs) toward an inflammatory M1 anti-tumor phenotype, and synergize with checkpoint blockade therapy to control multiple tumor types in mice [254–258]. Thus, PRR agonists injected directly into tumors can drive potent antitumor immunity without necessitating tumor antigen vaccination. However, a better understanding of *Listeria*'s impact on the TME and the landscape of immune cells is needed before mechanisms to synergize the innate and adaptive immune-activating capacities of *Listeria* can be translated into a more effective treatment of cancer.

Here, we show that either intratumoral (IT) or intravenous (IV) injection of *ΔactA*-attenuated *Listeria* (*Lm*) that is not engineered to express tumor antigens did not control tumor growth. Instead, IT *Lm* promoted tumor progression and *Lm* persisted predominantly in polymorphonuclear cells (PMNs) within the TME. *Lm* persistence in tumors was the result of IT *Lm* recruiting PMNs into the TME where they were polarized into a myeloid-derived suppressor cell phenotype (PMN-MDSC) that could suppress antitumor CD8 T cells [256,257]. In contrast, prior vaccination of mice against *Lm* by IV injection, either early in tumor development or prior to tumor initiation, led to strong tumor control upon IT *Lm* administration, even against highly aggressive sarcomas from genetically engineered mice. Tumor control required perforin-mediated killing by CD8 T cells specific to *Lm* antigen, which could eliminate PMN-MDSCs harboring *Lm* within the TME. Killing of *Lm*-infected host cells mediated significant

tumor control without the direct killing of tumor cells by tumor-specific or *Listeria*-specific CD8 T cells. Altogether, these results show that generating a strong, localized anti-bacterial CD8 T cell response in tumors is sufficient to overcome the suppressive TME and inhibit tumor growth. Importantly, this work redefines the goals for effective anti-cancer bacterial therapies by revealing a critical role for direct T cell targeting of conserved bacterial antigens, which is more broadly applicable and rapidly translatable compared to engineering bacteria to express patient-tailored tumor-specific neoantigens. These findings are fundamental to developing better *Listeria*-based cancer therapies.

## Results

Figure 1. Intratumoral injection of *L. monocytogenes* leads to persistent colonization and increased tumor growth.

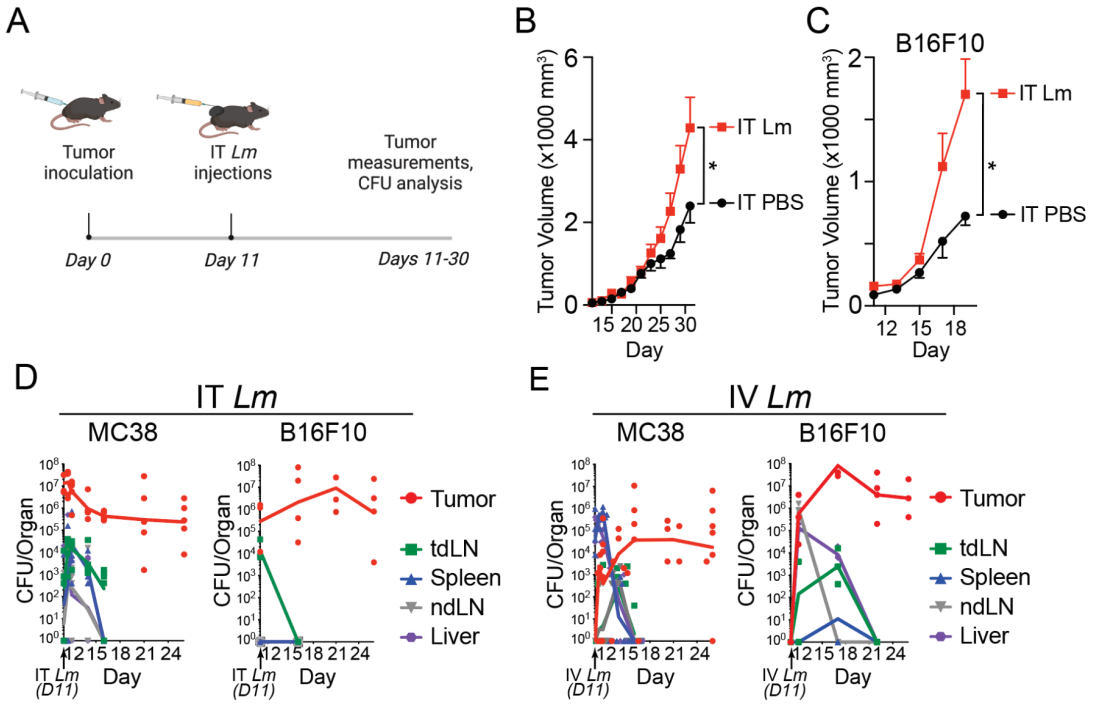


**Figure 1 | Intratumoral injection of *Lm* leads to increased tumor growth and persistent colonization of *Lm* within tumors.**

(A) Experimental design.

(B-C) C57BL/6J WT mice were injected with $5x10^5$ MC38 (B, data shown from n= 4-5 mice per group from one of three experiments) or $2x10^5$ B16F10 (C, data shown from n=4-5 mice per group from one of two experiments) tumor cells and then $5x10^7$ *Lm* CFUs IT 11 days later and tumors measured.

(D-E) CFU analysis for *Lm* from indicated tissues after injection IT (D, $5x10^7$ CFU, n=4-8 mice pooled from two experiments) or IV (E, $1x10^6$ CFU, n=4-5 mice pooled from two

experiments) of *Lm* 11 days after MC38 or B16F10 tumor inoculation. tdLN = tumor-draining lymph node, ndLN = non-tumor-draining LN.
For all plots, *P<0.05, **P<0.01 by two-way ANOVA, mean ± s.e.m.

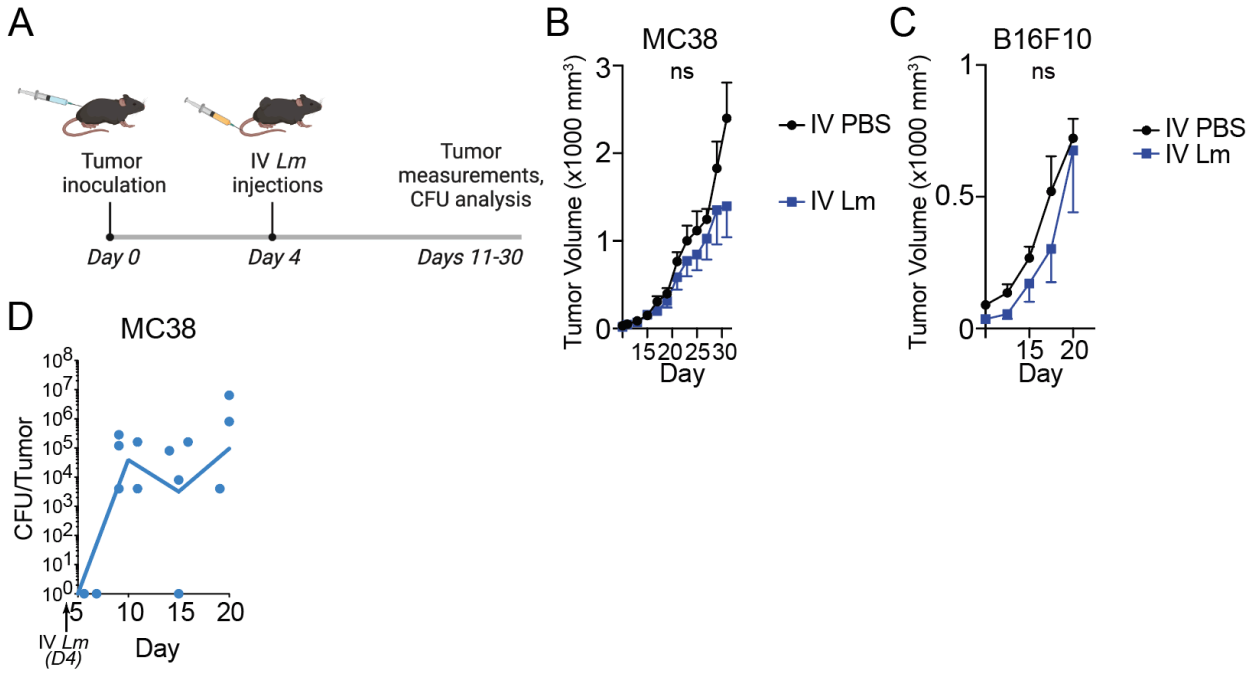


**Figure S1 | Intravenous injection of *Lm* alone does not slow tumor growth.**
(A) Experimental design.
(B-C) C57BL/6J WT mice were injected with $5x10^5$ MC38 (B, data shown from n= 5 mice per group from one of three experiments) or $2x10^5$ B16F10 (C, data shown from n= 5 mice per group from one of two experiments) tumor cells and then $1x10^6$ *Lm* CFUs IV 4 days later and tumors measured.
(D) CFU analysis for *Lm* in MC38 tumors from mice injected IV with $1x10^6$ CFU 4 days after tumor inoculation showed seeding and persistence of *Lm* in tumors as in (B) (n=4-5 mice per time point).
For all plots, statistical analysis was done by two-way ANOVA, mean ± s.e.m.

Intratumoral injection of attenuated *Listeria* increases tumor growth.

Immune adjuvants (i.e. PRR agonists) alone are effective against cancers when delivered directly into tumors without the addition of engineered tumor antigens[252,258] . To test whether antitumor responses could be generated by direct intratumoral (IT) injection of an attenuated strain of *L. monocytogenes*, which is not engineered to express tumor-specific antigens, we used an *ΔactA* mutant, denoted *Lm*, which contains the primary attenuating mutation used in many *Listeria* vaccine trials in patients[164]. *ΔactA Listeria* lack the ActA virulence factor, making the bacteria unable to polymerize host

actin and spread from cell to cell, but importantly, *Lm* can still escape from phagosomes and grow in the host cell cytosol, making it a potent inducer of MHC-I-restricted CD8 T cell responses. The *ΔactA* mutation renders *Lm* 1,000-10,000-fold less virulent than WT *L. monocytogenes* without decreasing its capacity to induce CD8 T cells[259]. This feature has led to its use in many *Listeria*-based clinical trials due to its increased safety in patients [172,174,197,243]. However, the direct injection of *Listeria* into tumors of cancer patients has never been tested. We performed dose-escalating experiments with IT *Lm*, from $5x10^5$-$5x10^7$ CFUs, into tumors 11 days post tumor inoculation, when tumors just became palpable, and found no lethality at a dose of $5x10^7$ *Lm*. Therefore, we used $5x10^7$ CFUs for IT *Lm* injections throughout subsequent experiments. IT injection of *Lm* did not result in tumor control in either MC38 or B16F10 tumor models but instead led to a significant increase in tumor growth **(Figures 1A-1C).** This result was unexpected, as *Lm* has been widely tested in experimental mouse models and was not observed to cause increased tumor growth. However, IT injection of *Lm* was never tested, so we hypothesized that this route of administration might be responsible for increased tumor growth. Therefore, we tested the impact of *Lm* administration on tumor growth after intravenous (IV) delivery, the most commonly used method of administration, of $1x10^6$ *Lm* (a lower dose was required to prevent lethality by an IV route)[237]. In this scenario, we found no increase in tumor growth of B16F10 or MC38 tumors, nor did we observe tumor growth control using the *Lm* strain **(Figures S1A-1C)**. This finding is significant, as the route of *Lm* inoculation, IT versus IV, appears to convert *Lm* from either being detrimental or potentially therapeutic for the control of cancer.

*Lm* colonizes tumors and persists in immunocompetent mice.

We hypothesized that the varying impacts on tumor growth after IT compared to IV *Lm* routes of administration could be due to varying *Lm* colonization in lymphoid organs or the tumor itself. To examine the localization of *Lm* in tumor-bearing mice after IV or IT *Lm* administration, we measured colony forming units (CFUs) for living bacteria from various tissues harvested from tumor-bearing mice at several time points after *Lm* administration. We found that *Lm* was cleared from spleens, lymph nodes, and liver by 5-10 days post injection in tumor-bearing mice, regardless of the route of delivery **(Figures 1D-E)**. However, while *Lm* numbers peaked at $10^5$-$10^6$ CFU in the spleen and liver shortly after IV injection, *Lm* CFUs were much lower in these organs after IT injection, despite a 50-fold greater number of bacteria administered in IT-injected mice. These results confirm that the different routes of *Lm* administration and different doses of *Lm* injected lead to a different biodistribution of *Lm* in mice. Nevertheless, *Lm* injected either IV or IT colonized both tumor models for up to 15

days, when experiments were ended due to uncontrolled tumor growth **(Figures 1D-E, and S1D)**. The persistence of live *Lm* within tumors long-term (more than 3-5 days) in immunocompetent mice has not been documented. Furthermore, the colonization and persistence of *Lm* in tumors after IV administration revealed that systemic routes of *Lm* administration can lead to seeding, growth, and long-term colonization of *Lm* in tumors, making *Lm* deliverable to tumors that are not easily accessible for direct IT injection. However, whereas IV leads to an increased early exposure of *Lm* to lymphoid tissues, IT injection leads to a greater bacterial load early within tumors, potentially impacting the antitumor immune response.

Figure 2. *Lm* recruits and localizes in PMNS during intratumoral infection.

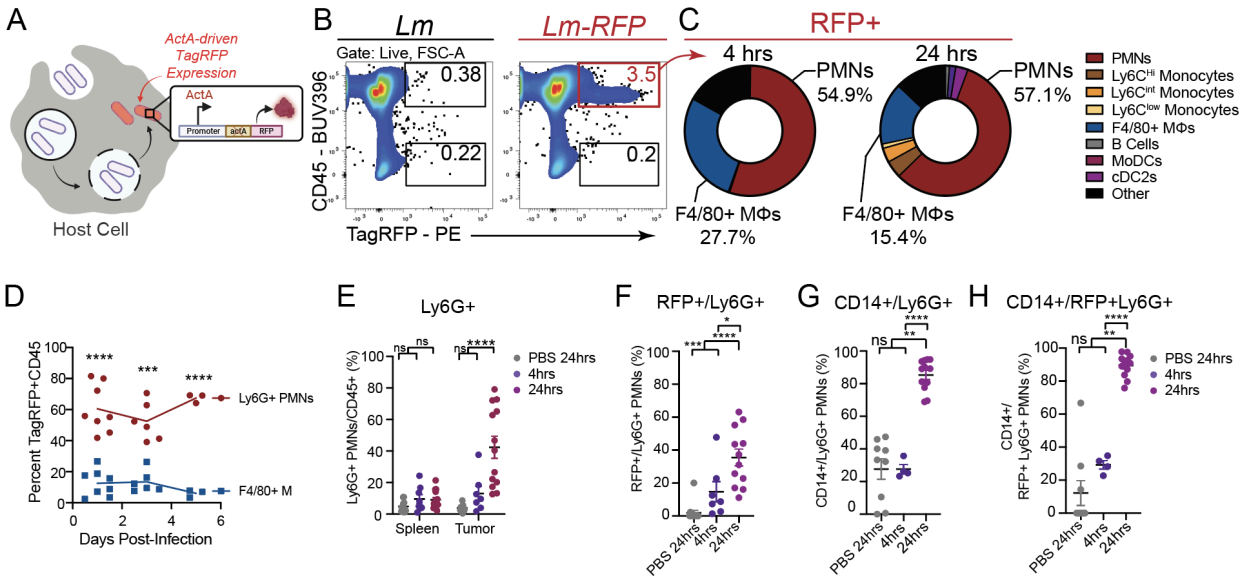


**Figure 2 | *Lm* localizes in intratumoral PMNs.**

(A) Schematic of Tag-RFP expression from *Lm-RFP*.

(B) Representative flow plots of single cell suspensions from MC38 tumors 4 hours post IT of *Lm* (left) or *Lm-RFP* (right) showing CD45 staining versus Tag-RFP fluorescence.

(C) Proportion of RFP$^+$ cells (C) from MC38 tumors 4 hours (left) or 24 hours (right) after IT *Lm* (representative data from three experiments with n= 3-6 mice/group).

(D) Flow analysis of Tag-RFP localization in PMNs and TAMs at 1, 3, and 5 days after IT *Lm* in MC38 tumors (representative data from two experiments with n= 3-6 mice/group).

(E) Frequency of Ly6G$^+$ PMNs in spleen and MC38 tumors 4 and 24 hours after IT *Lm* or IT PBS.

(F-G) Frequency of intratumoral Ly6G$^+$ PMNs that were RFP$^+$ (F) or CD14$^+$ (G) at 4 and 24 hours after IT *Lm*.

(H) Frequency of RFP⁺Ly6G⁺ PMNs that were CD14⁺ at 4 and 24 hours after IT *Lm*.
Results for D-H are pooled from three experiments with n= 2-5 mice/group per experiment.
For all plots, mean ± s.e.m. and *P<0.05, **P<0.01, ***P<0.001 by two-way ANOVA (D-E) or student t-test (F, G, H).

*Supplementary Figure 2. Population analysis of intratumoral APC subsets post Lm i.t.*

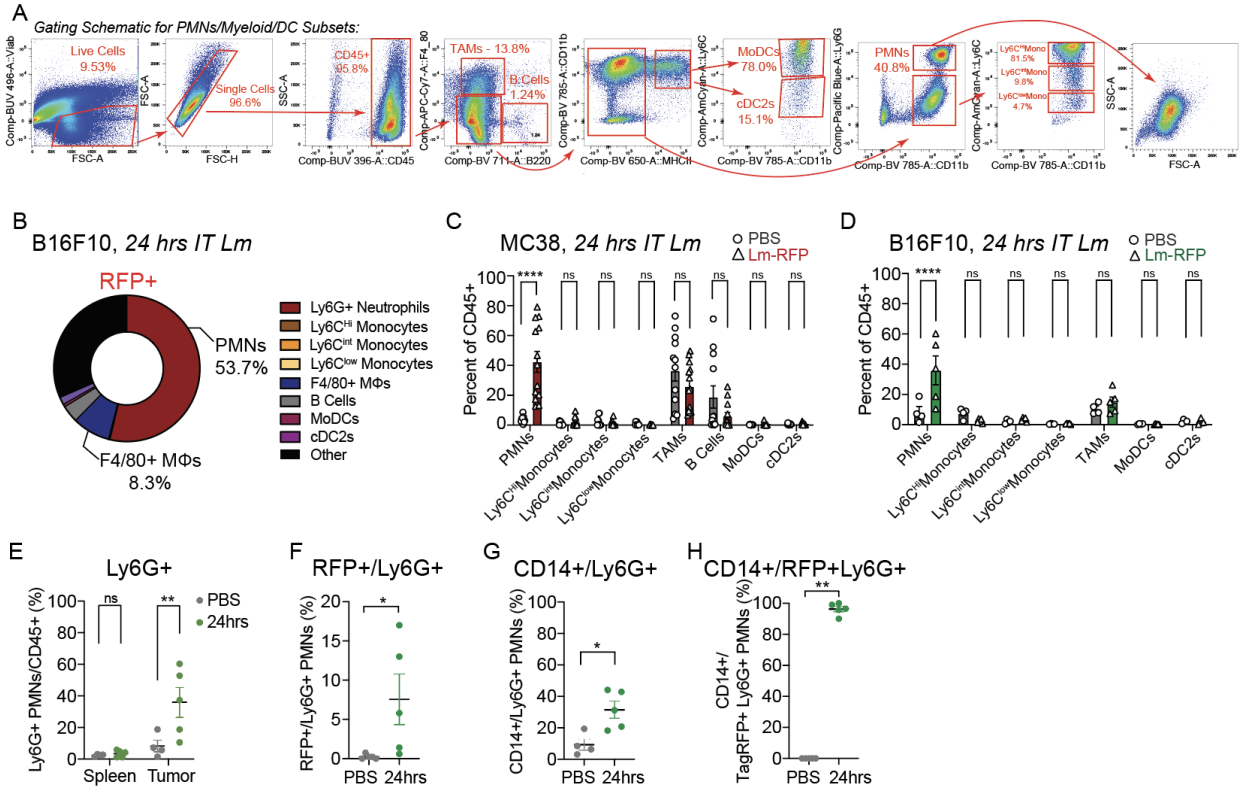


**Figure S2 | Population analysis during intratumoral *Lm* infection in MC38 and B16F10 tumors.**

(A) Gating strategy for identifying immune cell subsets from single cell suspension of MC38 tumor.

(B) Proportion of immune cell types that were CD45⁺RFP⁺ from B16F10 tumors 24 hours after IT *Lm* (n= 4-5 mice/ group).

(C-D) Frequency of intratumoral immune cell populations as percent of all CD45⁺ cells 24 hours after IT *Lm* injection in MC38 (C) or B16F10 (D) tumors (n=4-5 mice/group).

(E) Frequency of Ly6G⁺ PMNs in spleen and B16F10 tumors 24 hours after IT PBS or IT *Lm* (n=4-5 mice/group).

(F-G) Frequency of intratumoral Ly6G⁺ PMNs that were RFP⁺ (F) or CD14⁺ (G) at 24 hours after IT *Lm* in B16F10 tumors.

(H) Frequency of RFP⁺Ly6G⁺ PMNs that were CD14⁺ 24 hours after IT *Lm* into B16F10 tumors.

For all plots, mean ± s.e.m. and *P<0.05, **P<0.01, ***P<0.001 by student t-test (B, C, E, F, G, H).

*Lm* infects PMN-MDSCs in tumors.

We hypothesized that the distribution of *Lm* in specific cells of the tumor microenvironment after IT administration shaped the immune response to promote tumor growth. Because *Lm* is a facultative intracellular pathogen which primarily resides in host cells after infection, we sought to identify which cells harbor bacteria in the tumors of *Lm*-treated mice. To monitor intracellular infection of host cells by *Lm*, we used a fluorescent-protein based system wherein a Tag-RFP reporter was expressed under the control of the *actA* promoter in *ΔactA Listeria*, denoted *Lm-RFP* **(Figure 2A)**[260]. Since the *actA* promoter is induced more than 100-fold *when Lm* enters the host cell cytosol, expression of Tag-RFP indicates that the bacteria have entered the cytosol of a cell[261]. We observed co-localization of the RFP signal exclusively in $CD45^+$ immune cells from single cell suspensions of MC38 or B16F10 tumors infected with *Lm-RFP*. Notably, we did not observe RFP fluorescence in $CD45^-$ cells, which includes tumor cells (**Figure 2B**).

Next, we tracked which types of $CD45^+$ immune cells harbored *Lm* in their cytosol. At 4-and 24-hours post-infection, we observed an enrichment of RFP-expressing bacteria in phagocytic immune cells, especially $Ly6G^+$ polymorphonuclear (PMN) cells (**Figures 2C and S2A-D**)**.** To a lesser extent, *Lm-RFP* was detected in $F4/80^+$ macrophages, $Ly6C^+$ monocytes, and $CD11c^+MHC\text{-}II^+$ dendritic cells (**Figures 2C and S2B**). This distribution of *Lm* in phagocytic immune cells, primarily PMNs and macrophages, was maintained after the initial infection for up to five days (**Figure 2D**). However, in comparison to tumors at steady state, IT *Lm* only induced a significant influx of PMNs into tumors, whereas other phagocytic cell frequencies were unchanged (**Figures 2E and S2C-E)**. The $RFP^+$ PMNs also increased between 4 and 24 hours after IT delivery (**Figure 2F**). Altogether, these results demonstrated that PMNs are the primary reservoir for *Lm* in the TME. This result was unanticipated, as we expected PMNs would rapidly kill phagocytosed *Lm*, preventing their escape into the cytosol and the expression of RFP [262–264].

We hypothesized that the escape of *Lm* into the cytosol of PMNs and the long term colonization of *Lm* in the TME was the result of PMN conversion into PMN-MDSCs[265,266]. Myeloid cells, including PMNs, have been shown to differentiate into myeloid derived suppressor cells (MDSCs) in tumors, which have the capacity to promote tumor development and metastasis by inhibiting T cell responses against tumor cells [246]. MDSCs are identified as a population of $CD45^+$ immune cells that stain

positively with anti-CD11b and anti-Gr-1 antibodies [246]. The anti-Gr-1 antibody is dually reactive to Ly6G and Ly6C, and by using antibodies to each of these specific proteins, two populations of MDSCs, monocytic (M-MDSCs) and granulocytic (G- or PMN-MDSCs), respectively, have been described [267–269]. Markers to distinguish suppressive PMN-MDSCs from their non-suppressive PMN counterparts have been well-characterized, e.g. expression of CD14, PD-L1, Arg1, and CD300ld are associated with a MDSC phenotype [267–270]. Phenotyping of PMNs between 4 and 24 hours after IT *Lm* revealed a dynamic increase in PMNs expressing the prototypical PMN-MDSC marker CD14, with more than 90% of PMNs expressing CD14 by 24 hours after IT *Lm* in MC38 tumors (**Figures 2G and S2G)**. Interestingly, *Lm* localized preferentially in the $CD14^+$ PMN-MDSCs as compared to $CD14^-$ PMNs (**Figure 2H**). In B16F10 tumors, *Lm* was also found almost exclusively in $CD14^+$ PMN-MDSCs, revealing a strong selectivity for *Lm* in the cytosol of PMN-MDSCs in two tumor models (**Figure S2E-H**). These results fit a scenario in which PMNs recruited by *Lm* to tumors rapidly adopt a MDSC phenotype and become a reservoir for *Lm* colonization of their cytosol.

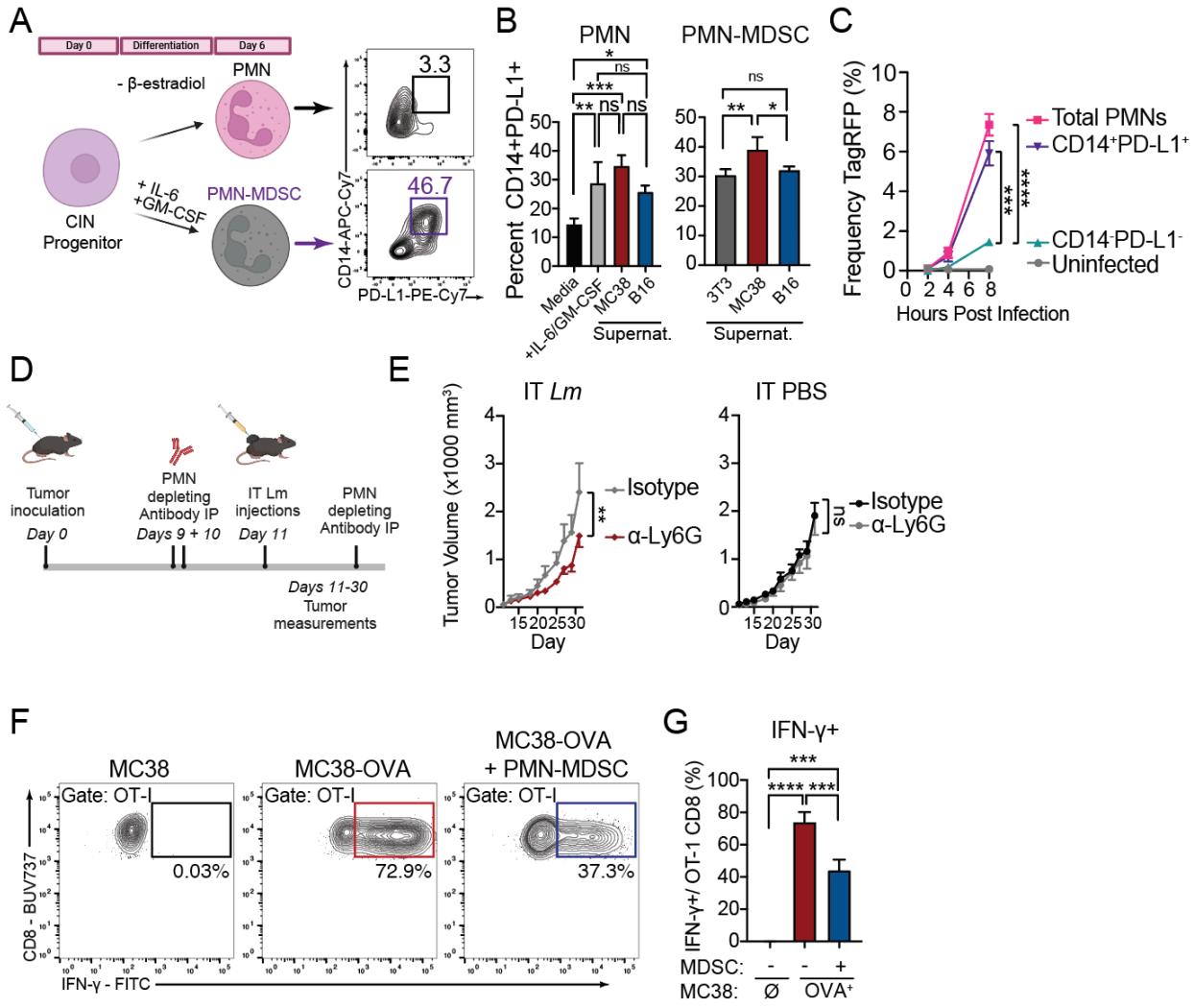


**Figure 3 | PMN conversion into MDSCs permits *Lm* infection and promotes tumor growth.**

(A) Experimental strategy and representative flow plots for *in vitro* differentiation of conditionally immortalized neutrophil (CIN) progenitors into PMNs or PMN-MDSCs.

(B) PMNs (left) or PMN-MDSCs (right) derived from CINs were cultured in supernatants from 3T3, MC38, or B16F10 cells for 24 hours *in vitro* and the frequency of $CD14^+PD\text{-}L1^+$ cells was assessed (representative of two experiments).

(C) Heterogeneous PMN-MDSCs differentiated from CINs as in (A) were infected with *Lm-RFP in vitro* and Tag-$RFP^+$ cells were identified 2, 4, or 8 hours later in all cells (Total PMNs), $CD14^+$ cells, or $CD14^-$ cells by flow cytometry (representative of three experiments).

(D) Experimental design to deplete PMNs prior to IT PBS or IT *Lm* and measure tumors.

(E) Tumor growth measured in mice injected IT with PBS (left) or IT *Lm* (right) that were treated with isotype control or anti-Ly6G-depleting antibodies prior to tumor inoculation and throughout the experiment (n=5 mice/group).
(F) Representative flow plots of IFNγ production from OT-I CD8 T cells.
(G) Percent of IFNγ$^+$ OT-1 CD8 T cells after 24 hours of co-culture with MC38 or MC38-OVA tumor cells in the presence or absence of FACS-purified PMN-MDSCs from IT *Lm* MC38 tumors. Cells were plated at a 1:1 ratio of OT-1 T cells to PMN-MDSCs.
For all plots, *P<0.05, **P<0.01, ***P<0.001 by one-way Anova (B, F) or two-way ANOVA (C, E), mean ± s.e.m for *in vivo* experiments (E) or mean ± s.d. for *in vitro* experiments (B-C, G).

IT *Lm* recruits PMNs that differentiate into pro-tumorigenic PMN-MDSCs within the tumor microenvironment.

We hypothesized that PMNs become permissive to *Lm* escape into and growth in the cytosol due to their differentiation into an immunosuppressive cell type in the TME. To test this, we used conditionally immortalized neutrophils (CINs) or primary bone marrow progenitors differentiated into PMNs *in vitro* (**Figures 3A and S3A**)[271,272]. PMNs cultured in supernatants from MC38 or B16F10 tumor cells increased their expression of the PMN-MDSC markers CD14 and PD-L1 (**Figures 3B**). As a positive control, PMNs were differentiated into PMN-MDSCs by adding IL-6 and GM-CSF, which led to a similar induction of CD14 and PD-L1 in CINs and bone marrow derived PMNs[271]. To evaluate whether the adoption of the MDSC phenotype was associated with increased susceptibility to *Lm* infection, we infected these heterogeneous cells *in vitro* with *Lm-RFP* and monitored infection by flow cytometry. The CD14$^+$ PMNs were almost exclusively associated with *Lm* cytosolic infection as determined by RFP expression (**Figures 3C and S3B**). These results strongly suggest that IT *Lm* recruits PMNs to tumors where they become converted into PMN-MDSCs that allow for *Lm* to infect and persist intracellularly in PMN-MDSCs.

PMN-MDSCs are widely associated with decreased antitumor immunity and increased tumor progression. Therefore, we hypothesized that *Lm*-mediated recruitment of PMNs and their conversion into PMN-MDSCs may underlie the increased tumor growth observed after IT *Lm*. To test this hypothesis, we depleted PMNs prior to tumor development using anti-Ly6G antibodies (**Figure 3D**). In this setting, IT *Lm* no longer increased tumor growth, whereas in mice without *Lm*, anti-Ly6G had no effect on tumor growth, indicating that the increased tumor progression after IT *Lm* required Ly6G$^+$ PMNs **(Figure 3E)**. However, PMN depletion did not reduce bacterial colonization in tumors as measured by CFU analysis (**Figure S3C**). Using *Lm-RFP*, we found *Lm* was

present primarily in macrophages, whose frequency was unchanged by IT *Lm* and whose infection with *Lm* did not increase tumor growth (**Figures S3D-E**). These results showed that IT *Lm* recruits PMNs to tumors where they are converted to an MDSC phenotype that promotes increased tumor growth. MDSCs have been shown to promote tumor growth by inhibiting antitumor CD8 T cell responses [246,273]. To test this, we co-cultured tumor-specific CD8$^+$ T cells (*in vitro* activated OT-I T cells) with OVA-expressing MC38 tumor cells in the presence or absence of PMN-MDSCs isolated from MC38 tumors 24 hours after IT *Lm*. The addition of PMN-MDSCs led to a significant reduction in IFNγ cytokine production from OT-I T cells in response to direct recognition of OVA-expressing tumors (**Figure 3F-G**). Therefore, *Lm* injection into tumors can promote tumor growth by recruiting PMNs that differentiate into PMN-MDSCs that can suppress antitumor-specific CD8 T cells.

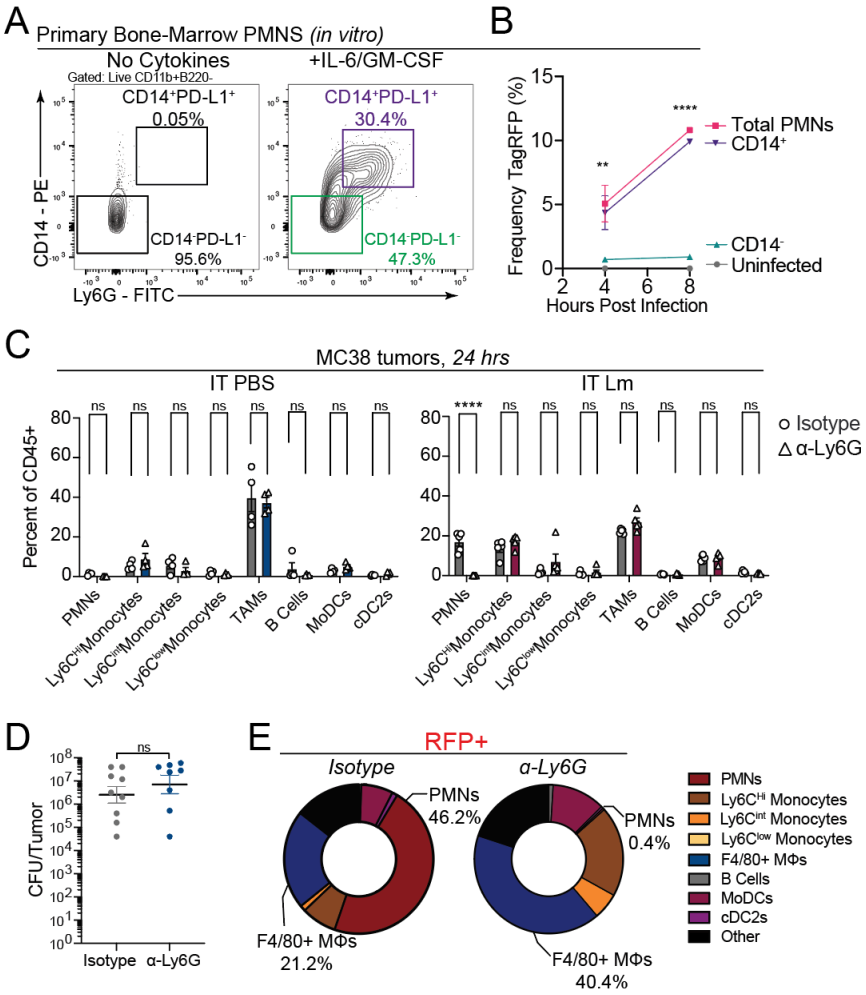


**Figure S3 | PMN-MDSCs are permissive to *Lm* infection.**
(A) Representative flow cytometry plots of the differentiation of primary bone marrow progenitors into CD14$^+$ PMN-MDSCs *in vitro* by culturing with IL-6 and GM-CSF.

(B) Heterogeneous PMN-MDSCs differentiated from bone marrow progenitors as in (A) were infected with *Lm-RFP in vitro* and Tag-RFP$^+$ cells were identified 4 or 8 hours later in all cells (Total PMNs), CD14$^+$ cells, or CD14$^-$ cells by flow cytometry.
(C) Frequency of intratumoral immune cell populations as percent of all CD45$^+$ cells 24 hours after IT *Lm* or IT PBS into MC38 tumors in mice pre-treated with isotype or anti-Ly6G (PMN-depleting) antibodies as shown in Figure 3D.
(D) *Lm* CFUs recovered from tumors 20 days after IT *Lm* in mice treated with isotype control or anti-Ly6G antibodies as shown in Figure 3D.
(E) Proportion of immune cell types that were RFP$^+$ from MC38 tumors treated with an isotype control (left) or anti-Ly6G antibody (right) 24 hours after IT *Lm*.
For all plots, *P<0.05, **P<0.01, ***P<0.001 by student t-test, mean ± s.e.m for *in vivo* experiments (C, D) or one-way ANOVA, mean ± s.d for *in vitro* experiments (B).

Figure 4. IV followed by IT injections of attenuated Listeria monocytogenes leads to better tumor control across multiple tumor models and clears Lm from tumor microenviorement

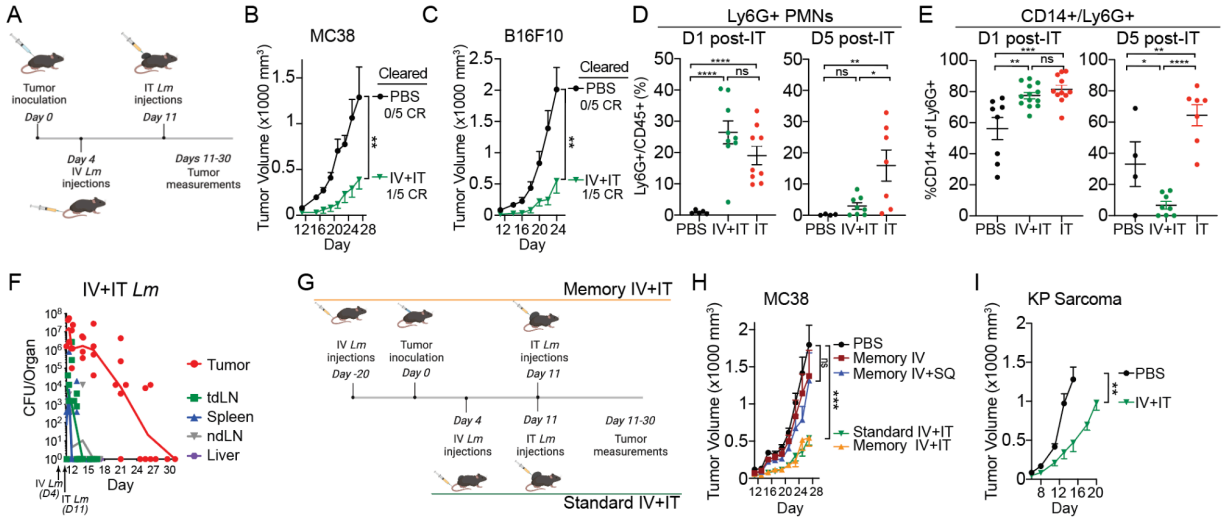


**Figure 4 | IV+IT *Lm* controls multiple tumor types while clearing intratumoral bacteria and PMN-MDSCs from tumors.**
(A) Experimental strategy for IV+IT *Lm* dosing regimen.
(B-C) IV+IT PBS versus IV+IT *Lm* regimen and growth of MC38 (B, data shown from n= 4-5 mice per group from one of three experiments) and B16F10 (C, data shown from n= 5-6 mice per group from one of two experiments) tumors.
(D) Frequency of Ly6G$^+$ PMNs of all CD45$^+$ cells one day (left) and 5 days (right) after *Lm* IT in IV+IT *Lm* regimen (data shown from n= 5 mice per group from two pooled experiments).
(E) Percent of Ly6G$^+$ PMNs that were CD14$^+$ 1 day (left) and 5 days (right) after IT *Lm* in IV+IT *Lm* regimen.

(F) CFU analysis for *Lm* after IV+IT *Lm* regimen in MC38 tumor-bearing mice. Organs were harvested 4 hours, 12 hours, and on days 1, 3, 5, 10, 15, and 20 after IT *Lm* administration.
(G) Experimental design for comparing prophylactic IV Lm (memory IV+IT) to standard IV+IT *Lm* dosing regimen.
(H) MC38 tumor growth in the setting of memory IV+IT, memory IV only, and memory IV+SubQ *Lm* (injected on opposing flank) compared to PBS treatment or standard IV+IT *Lm*.
(I) Orthotopic KP sarcoma growth with PBS versus a memory IV+IT *Lm* dosing regimen.
For all plots, *P<0.05, **P<0.01, ***P<0.001 by student t-test (C, D, E) or two-way ANOVA (B, C, H, I), mean ± s.e.m.

Figure S4.

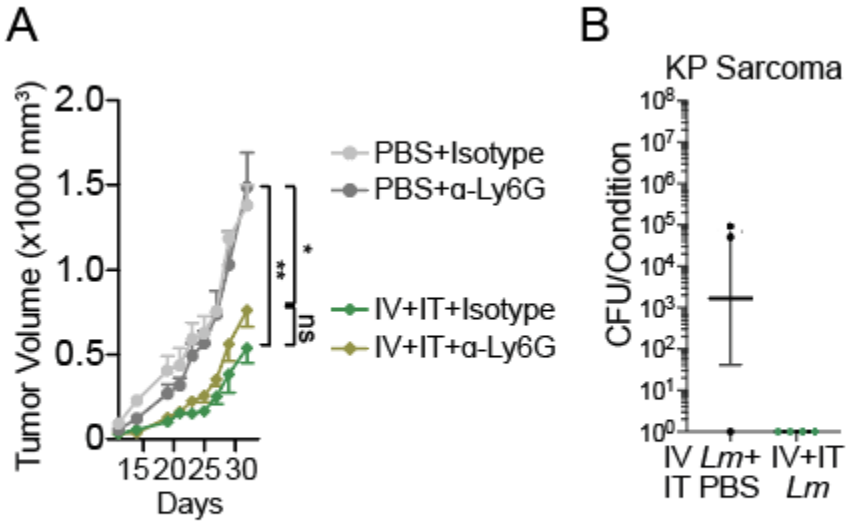


**Figure S4 | IV+IT *Lm* is not impacted by PMN depletion and IV+IT regimen leads to *Lm* clearance.**
(A) MC38 tumor growth with PBS versus IV+IT *Lm* and with or without anti-Ly6G depleting antibody treatment beginning two days prior to IT *Lm* administration (n=4-6 mice per group).
(B) CFU analysis for *Lm* in KP sarcomas from experiment depicted in Figure 4I.
For all plots, **P<0.05, **P<0.01, ***P<0.001 by two-way ANOVA (A-C), mean ± s.e.m.

A combination of IV+IT *Lm* leads to tumor control across multiple cancer models.

As neither IV nor IT administration of *Lm* alone provided strong tumor control, we considered that both routes of infection together might generate therapeutic efficacy. We reasoned that IV injection of *Lm* would effectively generate adaptive T cell responses against $Lm^{274}$, and that subsequent IT *Lm* injection would then recruit anti-*Lm* T cells into tumors to augment the antitumor immune response. To test this hypothesis, we inoculated mice with MC38 or B16F10 tumors and administered *Lm* IV four days later. At 11 days after tumor inoculation, when tumors were palpable, we injected *Lm* IT into tumors as done previously with IT *Lm* alone **(Figure 4A)**. This combination of IV *Lm* followed by IT *Lm* administration now significantly controlled both MC38 and B16F10 tumors **(Figures 4B-C)**. Importantly, tumor control occurred without the use of a *Lm* strain that expressed any tumor antigens. Tumor control was evident by 9 days after IT *Lm* injection, and tumors remained controlled throughout the experiment. Overall, of the experiments performed, only IV+IT Lm administration led to tumor rejection (20% of mice, 8 out of 40) and tumor rejection was never observed with IV or IT alone. In addition, we found that while $Ly6G^+$ PMNs were recruited equally to tumors 24 hours after IT *Lm* administration in both the IV+IT and IT-only dosing regimens, the frequency of $Ly6G^+$ cells were significantly reduced by 5 days after IT *Lm* administration in the IV+IT setting **(Figure 4D)**. Furthermore, the fraction of $CD14^+$ PMNs was almost completely abolished 5 days after IT *Lm* administration in the IV+IT *Lm* dosing regimen, a reduction even below their frequency in PBS-treated control tumors despite their robust increase 24 hours after IT *Lm* **(Figure 4E)**. Finally, when we depleted PMNs prior to tumor inoculation and then used the IV+IT *Lm* regimen, we did not observe any enhancement in tumor control, indicating that the PMN reductions with IV+IT *Lm* are sufficient to prevent PMN-MDSC promotion of tumor growth after the IT *Lm* dose in the IV+IT therapeutic regimen **(Figure S4A)**.

Next, we wanted to establish whether *Lm* persisted in tumors in the setting of IV+IT *Lm* administration. While *Lm* persisted in approximately 80% of tumors up to 10 days after IT *Lm* administration, *Lm* was ultimately cleared from tumors, with CFUs dropping to below the level of detection between days 15-20 after IT *Lm* (day 21-30 of tumor growth) **(Figure 4F)**. We hypothesized that the clearance of *Lm* from tumors was the result of *Lm*-specific T cells that were generated by IV *Lm* and then recruited to tumors when *Lm* was injected IT. Therefore, tumor control with the IV+IT *Lm* dosing could be the result of the recruitment of *Lm*-specific T cells to tumors. Alternatively, because *Lm* entered and propagated within the TME after IV administration at day 4 (**Figure S1D**), the early seeding of tumors might contribute to tumor control by changing the TME to augment the second IT injection of *Lm* in the IV+IT regimen. To address this possibility, we performed experiments in which IV *Lm* was administered

prophylactically, 20 days prior to tumor inoculation (**Figure 4G**). In this setting, *Lm* was completely cleared from mice prior to tumor inoculation and therefore absent from tumors when IT *Lm* was administered. Now we could test whether recalled *Lm*-specific adaptive immune cells, rather than changes in the distribution or amount of *Lm* in tumors, was responsible for the antitumor efficacy of the IV+IT regimen. This experiment showed clearly that mice injected with *Lm* prophylactically (memory IV+IT) controlled tumors equivalently to our standard IV+IT regimen **(Figure 4H)**. However, prophylactic IV *Lm* injection alone, or IV *Lm* followed by a subcutaneous injection of *Lm* on the contralateral flank of mice (memory IV+SQ), did not control tumors **(Figure 4H)**. These results suggested that IT administration of *Lm* mediated antitumor immunity by recalling an adaptive immune response against *Lm* locally within the tumor.

Finally, we tested the IV+IT *Lm* dosing regimen in a clinically relevant mouse sarcoma model - the tumor type most often treated by Coley, and a clinically relevant model amenable to direct IT injections[1,220221,275]. Using an aggressive *Kras*$^{G12D/+}$;*p53*$^{fl/fl}$-generated orthotopic sarcoma model[276], we found that memory IV+IT *Lm* led to significant sarcoma control as well as *Lm* clearance from *Lm*-treated sarcomas compared to tumors treated with PBS that had received IV *Lm* prophylactically **(Figures 4I and S4C)**. Taken together, these results showed that IV+IT *Lm* can clear *Lm* from tumors and control tumor growth in a manner that is not recapitulated with IV or IT *Lm* administrations alone.

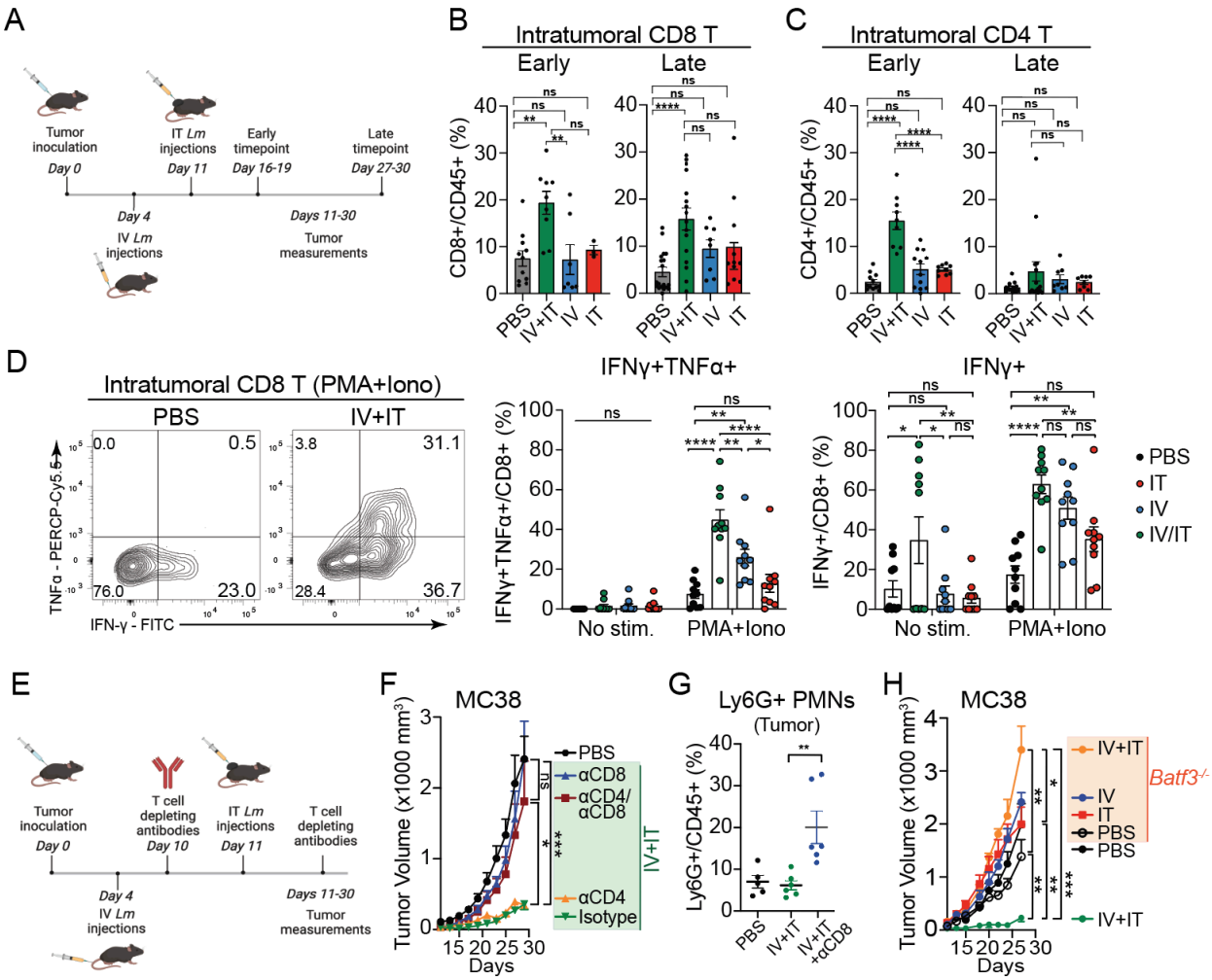


**Figure 5 | CD8⁺ T cells are required for tumor control with IV+IT *Lm*.**

(A) Experimental strategy for IV+IT *Lm* dosing regimen followed by early or late flow cytometry analysis.

(B-C) Frequency of intratumoral CD8⁺ (B) or CD4⁺ (C) T cells of CD45⁺ cells from MC38 tumors at early (left) or late (right) timepoints as specified in (A) (results pooled from four experiments with 5 mice/group).

(D) Representative flow plots for IFNγ and TNFα intracellular cytokine staining from intratumoral CD8 T cells 8 days after IT *Lm* or IT PBS administration in each *Lm* dosing regimen (results pooled from two experiments with 5 mice/group).

(E) Experimental strategy for IV+IT *Lm* dosing regimen plus anti-CD8, anti-CD4, or both depleting antibodies beginning 10 days after MC38 tumor inoculation.

(F) MC38 tumor growth with T cell depletion.

(G) Frequency of intratumoral Ly6G⁺ PMNs of CD45⁺ cells in IV+IT *Lm* treated mice +/- anti-CD8 depleting antibodies at end of experiment from (F).

(H) MC38 tumor growth in WT versus *Batf3*$^{-/-}$ mice treated with IV+IT *Lm*.

For all plots, *P<0.05, **P<0.01, ***P<0.001 by student t-test (B-C, G), one-way ANOVA (D), or two-way ANOVA (F, H), mean ± s.e.m.

CD8 T cells are required for tumor control and *Lm* clearance with the IV+IT *Lm* dosing regimen.

The reduction of tumor growth that occurred during the IV+IT *Lm* treatment regimen compared to single-dose IV or IT *Lm* administrations suggested that an adaptive immune response mediated tumor control. We hypothesized that T cells were the critical adaptive immune cells important for tumor control in the IV+IT dosing regimen due to their established role in both anti-*Lm* and antitumor immunity [274,277,278]. Indeed, flow cytometric analysis of tumors 5-8 days after IT *Lm* injection revealed an increase in the frequencies of both intratumoral CD8 and CD4 T cells in mice receiving IV+IT *Lm* compared to IV-only or IT-only *Lm* injections or PBS control injections (**Figures 5A-C, S5A-B**). Examination of tumors at the endpoint of tumor growth (15-20 days after IT *Lm* injection) also revealed a significant increase in the infiltration of CD8 T cells, but not a significant increase in CD4 T cells, in tumors of mice treated with IV+IT *Lm* compared to other groups (**Figures 5A-C, S5A-B**). Intratumoral regulatory T cells (Tregs) that are known to be immunosuppressive in the TME, were not significantly reduced with the IV+IT regimen compared to IV or IT alone, although they were reduced compared to the PBS treated mice (**Figures S5C-D**)[233,245–247,279–281]. Next, we assessed the functionality of the CD8 T cells by IFNγ and TNFα production 8 days after IT *Lm* or PBS injection. Here, the IV+IT *Lm* dosing regimen significantly increased CD8 T cells production of IFNγ, but especially the dual production of IFNγ and TNFα compared to PBS, IT *Lm* only, or IV *Lm* only (**Figure 5D**).

To test whether $CD4^+$, $CD8^+$, or both populations of T cells were required for tumor control, we used antibody-mediated depletion of the T cells beginning one day prior to IT *Lm* injection **(Figure 5E)**. Depletion of CD8 T cells, but not CD4 T cells, abrogated the efficacy of the IV+IT *Lm* regimen, indicating that CD8 T cells were required for tumor control with IV+IT *Lm* **(Figure 5F)**. In addition, CD8 T cell depletion increased the frequency of $Ly6G^+$ cells maintained in tumors of mice treated with IV+IT *Lm*, suggesting that the reduction in PMN-MDSCs with IV+IT *Lm* is likely the result of CD8 T cell killing of *Lm*-infected PMNs (**Figure 5G**). We also tested the IV+IT dosing regimen in *Batf3*$^{-/-}$ mice that lack type I dendritic cells (DC1s), which are the primary DCs responsible for cross-presenting *Listeria* antigens to CD8 T cells [282,283]. The IV+IT dosing regimen did not lead to tumor control in *Batf3*$^{-/-}$ mice **(Figure 5H)**. Taken together, the above results suggested that IV+IT *Lm* therapy requires the activity of CD8

T cells. However, whether CD8 T cells specific to *Listeria*, the tumor, or both were required for tumor control could not be distinguished.

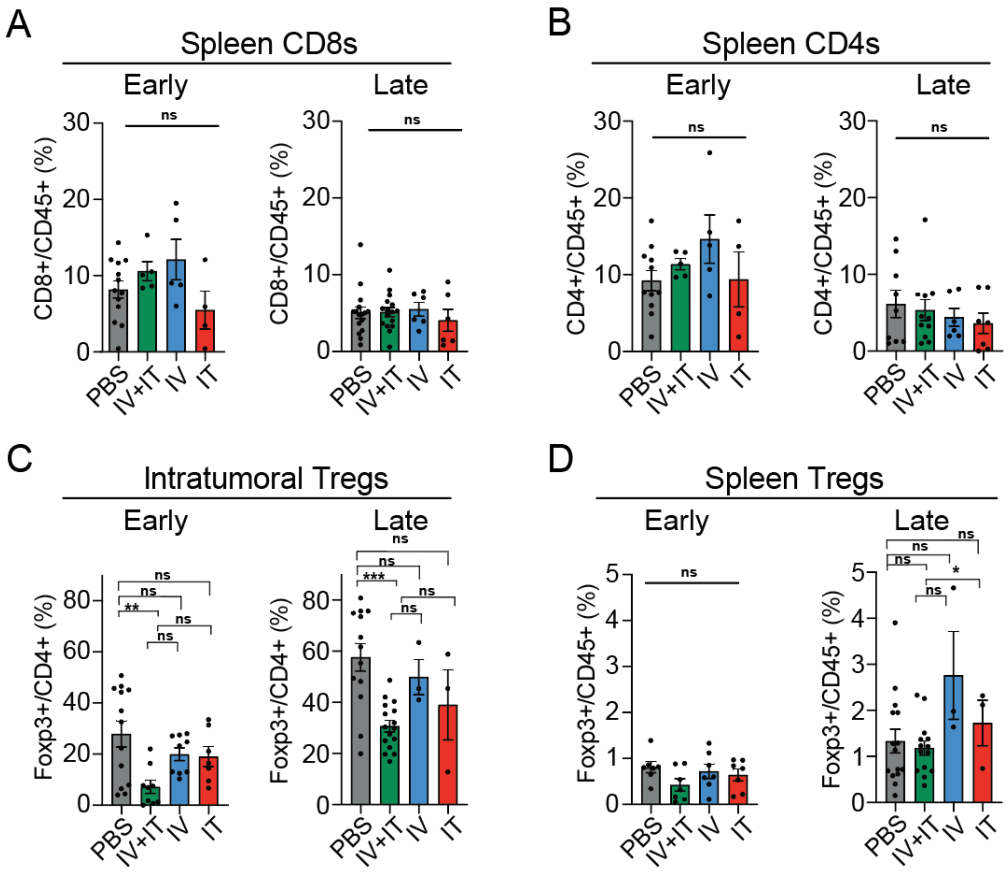


**Figure S5 | IV+IT *Lm* dosing regimen reduce intratumoral Treg frequencies but does not impact T cell frequencies in the spleen.**

(A-B) Frequency of $CD8^+$ (C) and $CD4^+$ (D) T cells from spleens at early or late timepoints.

(C) Frequency of intratumoral $Foxp3^+$ Tregs of $CD4^+$ cells from MC38 tumors at early (left) or late (right) timepoints as specified in Figure 5A.

(D) Frequency of $Foxp3^+$ Tregs of $CD4^+$ cells from spleens at early (left) or late (right) timepoints.

All data pooled from XX experiments with n=XX mice/group. For all plots timepoints are the same as what was described in Figure 5A. For all plots, *P<0.05, **P<0.01, ***P<0.001 by student t-test (A-D), mean ± s.e.m.

*Figure 6. Listeria-specific CD8 T cells drive tumor control.*

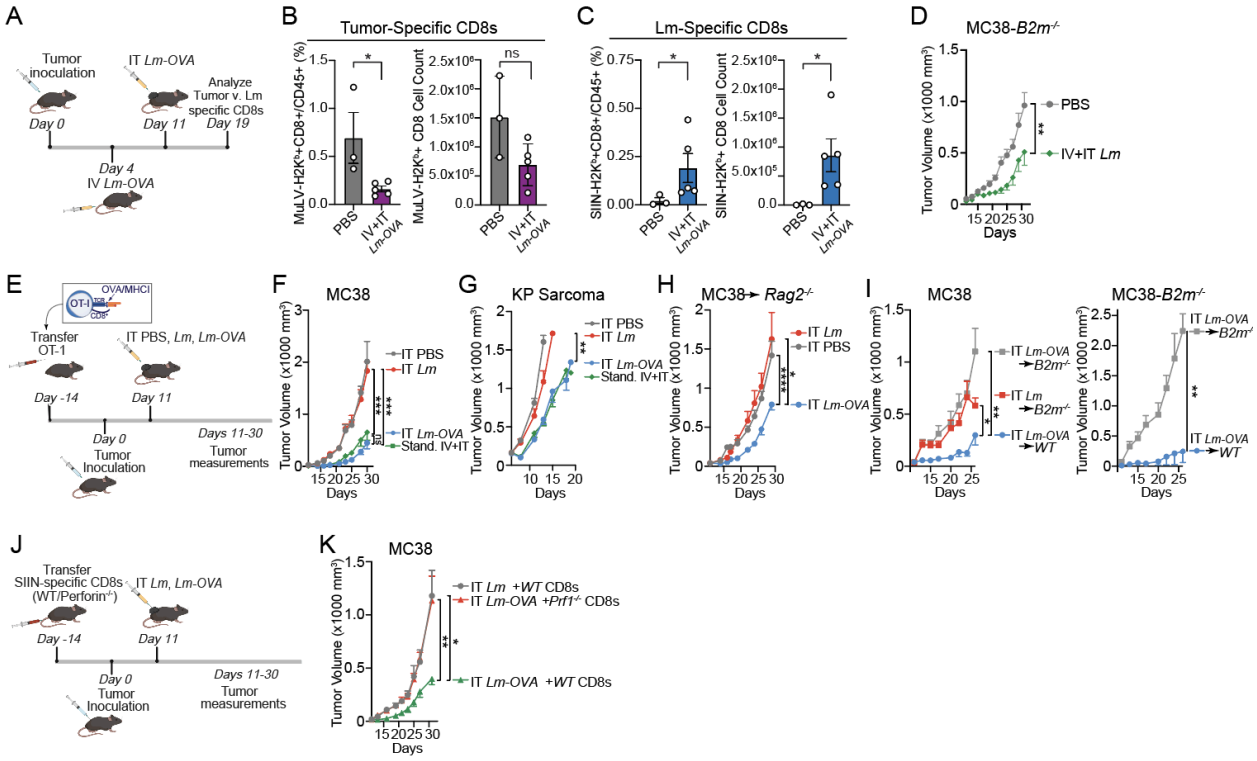


**Figure 6 | Anti-*Listeria* CD8 immunity mediates tumor control.**

(A) Experimental design to analyze tumor-specific versus *Lm*-specific CD8 T cells after IV+IT *Lm*. OVA was expressed from *Lm* as a surrogate *Lm* antigen.

(B-C) Frequency (left) and total number (right) of tumor-specific (B, MuLV-H2K$^b$ tetramer) and *Lm*-specific (C, SIIN-H2K$^b$ tetramer) CD8 T cells from MC38 tumors 8 days after IT *Lm-OVA* in IV+IT *Lm-OVA* regimen.

(D) MC38-B2m$^{-/-}$ tumor growth after IV+IT *Lm* or IV+IT PBS.

(E) Experimental strategy to adoptively transfer *in vitro*-activated OT-I CD8 T cells in place of prophylactic IV *Lm*.

(F-I) OT-I CD8 T cells were adoptively transferred and MC38 tumors measured after IT PBS, *Lm*, or *Lm-OVA*.

(F) IT PBS, *Lm*, or *Lm-OVA* were compared against standard IV+IT without OT-I transfer (n= 5-6 mice/group from one of two experiments).

(G) As in (F) but using an orthotopic KP sarcoma model (n= 5-6 mice/group).

(H) As in (F) but in *Rag2*$^{-/-}$ mice (n= 5-6 mice/group).

(I) As in (F) but IT delivery compared MC38 tumors grown in WT or β2m$^{-/-}$ mice (left) or MC38-*B2m*$^{-/-}$ tumors grown in WT or β2m$^{-/-}$ mice (right) (n= 5-6 mice per group).

(J) Experimental strategy to adoptively transfer *in vitro* expanded SIIN-specific CD8 T cells from WT or *Prf1*$^{-/-}$ mice immunized with *Lm-OVA* (see methods).

(K) MC38 tumor growth after adoptive transfer of SIIN-specific CD8 T cells from WT or *Prf1*$^{-/-}$ mice followed by IT injection of PBS, *Lm*, or *Lm-OVA* at day 11 of tumor growth (n= 6 mice/group from one of two experiments).
For all plots, *P<0.05, **P<0.01, ***P<0.001 by student t-test (B, C) or or two-way ANOVA (D, F-H, I, K), mean ± s.e.m.

Figure S6.

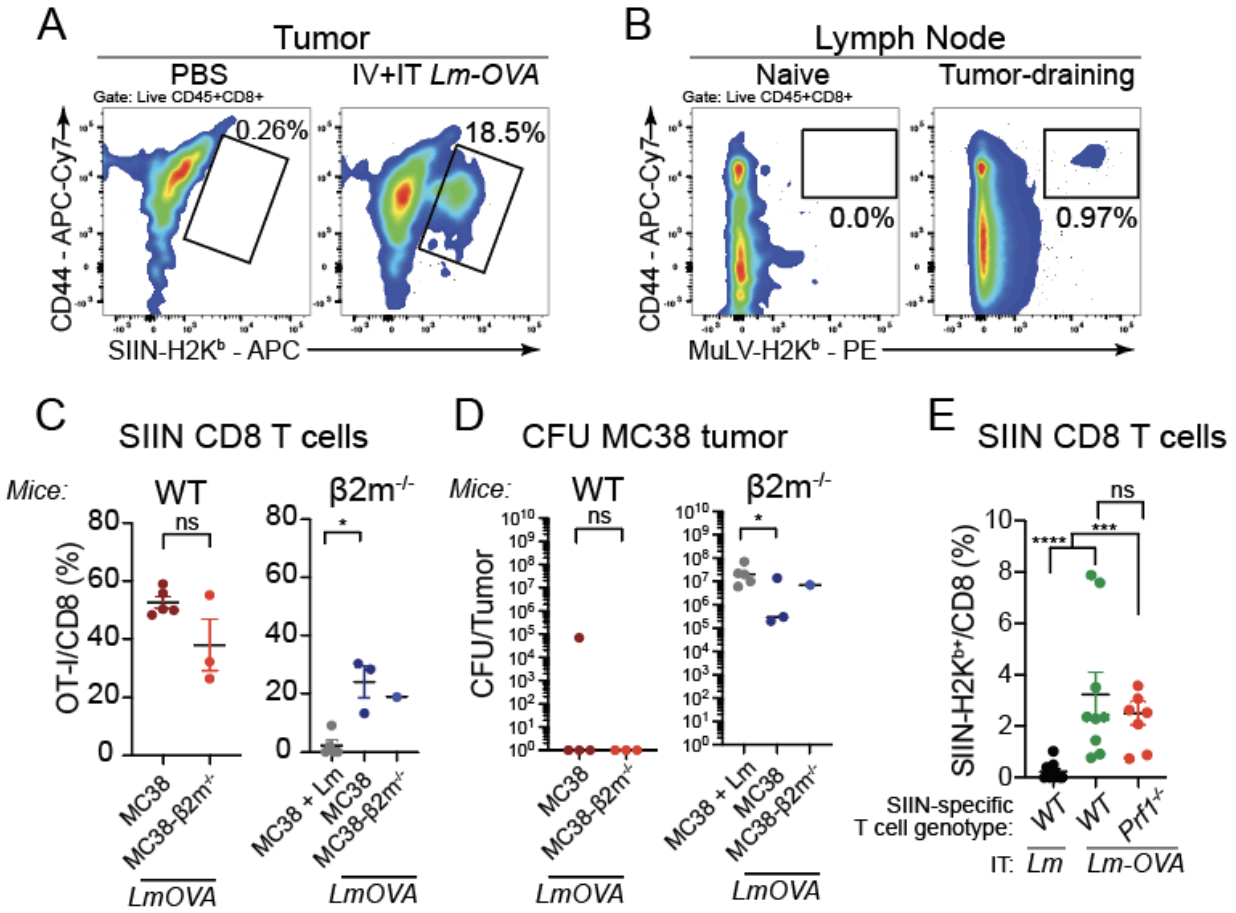


**Figure S6 | *Lm*-specific CD8 T cells are present in tumors independent of Prf1 genotype.** (A) Representative flow plots for SIIN-H2K$^{b}$ tetramer staining from tumors of mice treated with IV+IT PBS compared to IV+IT *Lm-OVA* 8 days after IT injections.
(B) Representative flow plots for MuLV-H2K$^{b}$ tetramer staining in lymph nodes from naive mice compared to MC38 tumor-bearing mice 19 days post tumor inoculation.
(C) Frequency of intratumoral OT-I CD8 T cells in mice 20 days after IT *Lm* administration from Figure 6I.
(D) CFU analysis for *Lm* in tumors from Figure 6I.
(E) Frequency of intratumoral SIIN-specific CD8 T cells in mice 20 days after IT *Lm* administration from experiments depicted in Figures 6J-K (pooled from two independent experiments).

For all plots, *P<0.05, **P<0.01, ***P<0.001 by student t-test (C,D) or one-way ANOVA (E), mean ± s.e.m.

*Listeria*-specific CD8 T cells drive tumor control.

To assess whether the CD8 T cells that mediate tumor control in response to IV+IT *Lm* treatment are specific to tumor or *Lm* antigens, we used MHC-I tetramers loaded with a tumor-derived or a *Lm*-derived peptide. We used *Lm* engineered to express ovalbumin (*Lm-OVA*) so that we could monitor SIINFEKL loaded H2-$K^b$ tetramers to track the anti-*Lm* response (SIIN/$H2K^b$) (**Figure 6A, Figure S6A**). MuLV/$H2K^b$ tetramers were used to track the tumor-specific response to an endogenous retrovirus that is reactivated in MC38 and B16F10 tumors (**Figure S6B**)[280,281]. Interestingly, there was a decrease in the frequency and number of tumor-reactive CD8 T cells in mice receiving the IV+IT *Lm-OVA* compared to PBS treated mice (**Figure 6B**). However, both the number and frequency of anti-*Lm* CD8 T cells was significantly increased in the IV+IT regimen **(Figure 6C)**.

From these results, as well as the observation that IV+IT *Lm* led to a reduction in CFUs in tumors, we hypothesized that the anti-*Lm* CD8 T cell response may be essential for the efficacy of the IV+IT *Lm* regimen. While we did not find evidence of direct tumor cell infection by *Lm* using the *Lm-RFP* reporter strain **(Figures 2A-2D**), we sought to determine whether direct tumor cell killing by *Lm*-specific CD8 T cells was required for tumor control. To test this, we inoculated mice with MC38 tumors that did not express MHC-I (*B2m*$^{-/-}$) and therefore could not be directly recognized and killed by CD8 T cells targeting either *Lm* or tumor antigens. Even in this setting, IV+IT *Lm* promoted significant control of MHC-I-deficient MC38 tumors, indicating that tumor control does not require direct tumor cell recognition by CD8 T cells (**Figure 6D**).

To further test our hypothesis that the anti-*Lm* CD8 T cell response was sufficient for tumor control, we adoptively transferred activated T cell receptor (TCR) transgenic CD8 T cells from OT-I mice, which have a TCR that recognizes the OVA peptide SIINFEKL presented on MHC-I (SIIN/$H2K^b$), into mice prior to tumor inoculation. This strategy allowed for a pool of *Lm*(SIIN)-specific memory CD8 T cells to develop in recipient mice, analogous to the expansion of *Lm*-specific memory CD8 T cells in prophylactically IV *Lm*-treated mice (**Figure 4G-H**). When tumors became palpable at day 11, tumors were injected with PBS, *Lm*, or *Lm-OVA* (**Figure 6E**). Strikingly, only mice whose tumors were injected with *Lm-OVA* had delayed tumor growth, similar to mice that received a standard IV+IT *Lm* regimen (**Figure 6F**). Even in the aggressive orthotopic KP sarcoma model that does not express tumor-specific antigens[284], the transfer of *Lm*-specific CD8 T cells prior to tumor implantation and IT *Lm-OVA*

treatment was sufficient to provide significant tumor control (**Figure 6G**). These results indicate that a CD8 T cell response specific to *Lm* antigens is sufficient to phenocopy the effect of IV *Lm* in the IV+IT *Lm* dosing regimen.

We hypothesized that the *Lm*-specific CD8 T cells were able to mediate tumor control in the context of tumor-reactive CD8 T cells that were augmented by the activity of the anti-*Lm* CD8 T cell response. However, to test whether the anti-*Lm* CD8 T cells alone were able to mediate tumor control, we injected *in vitro* activated OT-I cells into *Rag2*-deficient mice, which lack T and B cells and therefore cannot mount an antitumor CD8 T cell response. We then implanted MC38 tumors and IT injected PBS, *Lm* or *Lm-OVA* at day 11 when tumors became palpable. Similar to our observation in wild-type mice, tumor growth was significantly reduced in *Rag-2*$^{-/-}$ mice that were IT injected with *Lm-OVA*, but not when injected with *Lm* that did not express OVA (**Figure 6H**). Thus, tumor control could be mediated directly by anti-*Lm* CD8 T cells in the TME even in the absence of tumor-specific CD8 T cells.

To test whether recognition of *Lm*-antigens on non-tumor cells infected with *Lm* was required for the efficacy of IV+IT *Lm*, we continued to use the transfer of OT-I CD8 T cells, but now into wildtype versus B2m-/- mice, which lack MHC-I on all host cells. Specifically, we inoculated wildtype MC38 versus MC38-*B2m*$^{-/-}$ tumor cells into wildtype versus *B2m*$^{-/-}$ mice that had OT-1 CD8 T cells transferred two weeks prior. In this scenario, we could determine whether tumor control with IV+IT *Lm* required the recognition of infected host cells or tumor cells directly by CD8 T cells. In the setting of wildtype MC38 tumors, MHC-I expression was required on host cells to mediate tumor control, as IT *Lm-OVA* was completely ineffective in *B2m*$^{-/-}$ mice despite the presence of OT-I T cells (**Figures 6I and S6C**). Furthermore, in the setting of MC38-*B2m*$^{-/-}$ tumors, OT-I T cells still mediated tumor control with IT *Lm-OVA*, but only in the setting of wildtype mice and not *B2m*$^{-/-}$ mice (**Figures 6I**). CFU analysis for live *Lm* also demonstrated that MHC-I expression on host cells, but not tumor cells, was absolutely required for *Lm* clearance from the TME (**Figure S6D**). Thus, CD8 T cells require MHC-I for TCR recognition of host cells, but not tumor cells to mediate tumor control and bacterial clearance in the IV+IT *Lm* regimen.

Finally, we wished to determine whether killing of non-tumor cells infected with *Lm* was required for the efficacy of IV+IT *Lm*, as tumor control could also be mediated by an alternative mechanism that requires other activities of *Lm*-specific CD8 T cells. We tested whether *Lm*-specific T cells required the pore-forming effector protein Perforin to mediate tumor control by adoptively transferring activated SIIN-specific CD8 T cells generated from WT mice or *Prf1*-deficient mice vaccinated with *Lm-OVA*. Mice were then inoculated with MC38 tumors, and at day 11 when tumors were palpable, tumors were IT injected with *Lm* or *Lm-OVA* **(Figure 6J-K)**. Tumor control was lost in mice that received *Prf1*$^{-/-}$ SIIN-specific CD8 T cells. The failure of *Prf1*$^{-/-}$ SIIN-specific CD8 T cells to

mediate tumor control was not due to a failure of these cells to infiltrate the tumor, as an equivalent frequency of SIIN-specific CD8 T cells were found in tumors that received WT or *Prf1*$^{-/-}$ SIIN-specific CD8 T cells (**Figure S6E**). Taken together, these findings demonstrate that *Lm*-specific CD8 T cells that kill *Lm*-infected non-tumor cells are sufficient to mediate tumor control in the IV+IT *Lm* therapeutic treatment regimen.

Discussion:

In this study, we report two major findings. First, we showed that direct intratumoral injection of an attenuated strain of *Lm* causes increased tumor growth. This was due to *Lm*-mediated recruitment of PMNs that are converted within tumors to immunosuppressive MDSCs, which act as permissive cells for bacterial survival while suppressing antitumor CD8 T cells. Second, we showed that IT *Lm* tumor growth promotion could be converted to tumor control by prior IV *Lm* immunization, which generated anti-*Listeria* CD8 T cells whose activity alone could mediate cancer protection. Thus, depending on the context of intratumoral seeding of *Lm* into a tumor, *Listeria* can either promote or inhibit cancer progression. These results reveal new insights into the biology of *Listeria* colonization of tumors in immune competent mice as well as the potential for anti-*Lm*-specific CD8 T cells to mediate cancer control. These findings should shape future approaches using *Listeria* as an anti-cancer therapeutic.

The *ΔactA* attenuated *Lm* strain used in this study selectively colonized and persisted in tumors when injected IT or IV. Other studies have also reported short-term persistence of bacteria in tumors, either growing intracellularly (e.g. *Salmonella*) and/or extracellularly (e.g. *Clostridium*, *Vibrio. cholerae*, *E. coli*), which in some cases had direct tumoricidal effects [47,223,285]. *Clostridium novyi* administered intravenously led to sporulation in the avascular regions of tumors that directly caused cancer cell death[64,231]. Longer term protection from cancer with *Clostridium* occurred in ~30% of mice and depended on the generation of CD8 T cells, though the specificity of the CD8 T cells, against bacteria or tumor, was never directly tested [64,231]. *E. coli* engineered to express α-hemolysin, a pore forming protein, or to deliver L-arginine to promote CD8 T cell function, both led to improved tumor control upon bacterial colonization of tumors[48,286].The prevalence of bacteria in tumors has been attributed to the presence of nutrients or an environment in tumors that is conducive to bacterial survival and growth [230,231,287–289]. *Salmonella typhimurium* engineered to infiltrate tumors by selective auxotrophy was effective at controlling several types of mouse tumors [230,287,290]. The lack of documentation of *Lm* persistence in tumors could be the result of using alternative attenuated strains of *Listeria*, or failing to look for *Lm* persistence in previous studies. However, in the case of *ΔactA*-attenuated *Listeria* colonization in this study, we

hypothesize that bacterial migration via the bloodstream is stochastic, but upon arrival in tumors, *Listeria* is protected from immune clearance due to the immunosuppressive environment created in tumors that generates PMN-MDSCs that permit the intracellular growth of *Lm*[223,291].

PMN permissiveness for cytosolic *Lm* was strongly correlated with a $CD14^+$ and $PD\text{-}L1^+$ phenotype of the PMNs, markers associated with a MDSC phenotype. We also found that this phenotype was promoted by tumor derived factors, since culture of PMNs with tumor cell supernatants was sufficient to induce their expression of CD14 and PD-L1. In our system, however, depletion of these PMN-MDSCs did not prevent *Lm* persistence in tumors, but did prevent the accelerated tumor growth observed with IT *Lm* alone. This suggests that the TME prevents *Lm* clearance in multiple phagocytic immune cell types, but that recruitment and conversion of PMNs to MDSCs specifically promotes cancer progression. PMN-MDSCs and other myeloid cells have been found to promote tumor growth by several mechanisms, such as inhibiting T cell responses as well as by promoting vasculature formation to support tumor cell proliferation in glioblastoma, pancreatic, and several other mouse tumor models [246,249,273]. Using *in vitro* co-culture of FACS-purified intratumoral PMN-MDSCs with tumor cells and antitumor CD8 T cells, we showed that PMN-MDSCs could inhibit cytokine production from CD8 T cells responding to antigen recognition on tumor cells. Altogether, we uncovered an unexpected consequence of *Lm* seeding and colonization of tumors, wherein bacterial colonization of tumors by *Lm* does not lead to direct tumoricidal effects, but rather, recruits immune cells that are subsequently converted into immunosuppressive cells that promote tumor growth. Furthermore, microbes have been shown to play a direct role in promoting immune tolerance, and so it is possible that *Lm* bacteria may also directly promote tolerance in the TME[292].

Attenuated *Listeria* vaccines generate memory CD8 and CD4 T cells that provide robust and long-lasting adaptive immunity [239,274]. We tested whether prior IV *Lm* administration to generate anti-*Lm* CD8 T cells would impact the immunosuppressive TME and promote tumor control with a secondary IT *Lm* dose. In this setting, IT *Lm* led to tumor control across multiple tumor models, including an aggressive orthotopic KP model of sarcoma. Tumor control was also observed if mice were prophylactically vaccinated prior to tumor inoculation, ruling out a role for tumor colonization with IV *Lm* in the efficacy of this IV+IT *Lm* regimen. Our results parallel previous approaches taking advantage of TDAP vaccination and using *Listeria* as a delivery system to introduce tetanus toxoid into tumors to elicit a $CD4^+$ memory T cell response to eliminate tumor cells[293]. However, in these reports, it was shown that the *Listeria* infected the cancer cells directly to drive T cell recognition and control of cancer[293]. However, using a TagRFP reporter of cytosolic *Lm*, we did not find evidence of direct infection of tumor cells. Nor was direct recognition of tumor cells by anti-*Lm* CD8

T cells required for tumor control, as MHC-I-deficient tumors were also controlled by the IV+IT *Lm* regimen. Differences in the attenuated strains of *Listeria* used in other studies compared to this study likely underlie these differences in tumor cell infiltration by bacteria. While our study uses an *ΔactA* mutation to attenuate the bacteria by preventing cell to cell spread, others have used attenuated *Listeria* that retains a functional *actA* gene, thus allowing for the spread of *Listeria* to non-phagocytic cells, including tumor cells [233–236,238,289,293,294].

To test the role of anti-*Lm*-specific CD8 T cells directly in tumor control with IT *Lm*, we used adoptively transferred OT-I CD8 T cells in place of IV *Lm* administration. We then used *Lm-OVA* IT delivery to show that OT-I T cells targeting only a surrogate *Lm* antigen (OVA), and incapable of recognizing tumor antigens, were able to mediate tumor control. Of note, in clinical trials using BCG treatment for bladder cancer, patients with a positive skin tuberculosis test (PPD) due to previous vaccination with BCG, responded better to the BCG treatment and had longer recurrence-free survival than patients who did not establish pre-existing BCG-specific T cell immunity[45]. In our study, we ruled out that *Lm* directly infected cancer cells to mediate tumor control by showing that the IV+IT *Lm* regimen was effective in controlling MHC-I-deficient tumors that cannot be directly recognized by CD8 T cells. Furthermore, MHC-I-deficient tumors that did not express OVA were controlled by transfer of OT-I T cells followed by IT *Lm-OVA* delivery. However, MHC-I expression on non-tumor cells was essential for tumor control, as *B2m*-deficient mice receiving anti-*Lm* OT-I T cells were unable to control tumors after IT *Lm-OVA* administration. Therefore, CD8 T cell recognition of host cells, presumably $CD45^+$ immune cells that have taken up *Lm* in their cytosol, including PMN-MDSCs, was required for tumor control. Finally, we showed that anti-*Lm* CD8 T cells required killing of host cells, rather than the tumor cells directly, to mediate tumor control. Using prophylactic transfer of *Lm*(OVA)-specific CD8 T cells generated in *Prf1*$^{-/-}$ mice, we found that tumors were no longer controlled with IT *Lm-OVA* administration. These results are contrary to the established dogma that an increase in tumor-specific CD8 T cells is required for the efficacy of bacterial-based cancer immunotherapies[295]. These results also indicate that tumor control by CD8 T cells does not necessitate bacterial spread into tumor cells.

While further investigation of the mechanisms underlying tumor control in the IV+IT *Lm* regimen is needed, our results raise the tantalizing potential of a universal therapeutic vaccine for cancer[296]. The introduction of a microbe into a tumor may lead to tumor control if patients already have CD8 T cells targeting microbial antigens. Indeed, direct IT injection of an FDA-approved seasonal influenza vaccine demonstrated antitumor activity in a mouse cancer model[297]. Alternatively, the generation of CAR T cells against *E. coli* in combination with the seeding of *E. coli* into the TME was shown to robustly control tumors[298]. Mechanistically, the infiltration of a large number of

*Lm*-specific CD8 T cells that cannot directly target and kill tumor cells still could control tumors by inducing local tissue damage or impacting the blood vasculature that supports tumor growth [299,300,230,237,287]. Inflammation may also reprogram other immune cells in tumors to become more tumoricidal, which was observed in mouse lung tumor models where influenza infection of the lung conferred reprogramming of alveolar macrophages to protect against tumor development[301]. If such generalized strategies can be effective, it obviates the need to engineer microbes to overexpress patient-specific neoantigens. This will not only save time and reduce costs, but also improve the safety of the therapy by using a reliably tested and generalized biologic. Great potential also exists to improve the effect of the local infection with specially engineered bacteria to boost the inflammatory response against *Lm* to more effectively spread against tumor-specific antigens, as well as combine bacterial-based approaches with other immunomodulatory therapies.

In conclusion, we have shown bacterial immunotherapy can have unintended negative outcomes by driving the accumulation of immunosuppressive cell types within tumors. However, the tumor promoting impact of *Lm* can be overcome by using a *Lm* dosing regimen that first generates anti-*Lm* CD8 T cells before direct intratumoral administration. The CD8 T cell response against *Lm* can not only remove the immunosuppressive cells from tumors, but also through direct cell killing of non-tumor cells in the TME, promote tumor control. Because the T cell responses against certain pathogens (i.e. *Listeria*, influenza, etc.) have been heavily studied and characterized, these findings have far-reaching consequences and may reveal new applications for existing therapeutics and vaccines. In addition, we show that methods to expand pathogen-specific T cells *ex vivo* for adoptive transfer of bacteria or viral antigen-specific T cells are effective and may be safer than attempting to generate memory T cells by active vaccination in potentially immunocompromised individuals, including cancer patients[302]. Collectively, our study demonstrates new insights into the inefficiencies of current bacterial immunotherapy approaches and reveals a tumor antigen-free, bacterial-based dosing regimen that can control cancer independently of its immunogenicity or the identification of neoantigens.

**Methods**:

**Animal studies**

C57BL/6J wildtype mice were obtained from Jackson laboratories (JAX:000664) and bred in house. OT-1 transgenic mice were obtained Taconic (Catalog#: 2334) and bred in house. Prf1$^{-/-}$ mice were a gift from the Stanley lab at the University of California, Berkeley (JAX:000274). Rag2$^{-/-}$ (JAX:008449) and β*2m2m*$^{-/-}$ (JAX:002087) mice were a gift

from the Raulet lab at the University of California, Berkeley. Sarcoma cell lines were generated in *Kras$^{LSL\text{-}G12D/+}$;p53$^{fl/fl}$ mice* by intramuscular injection of the left hind limb with replication-incompetent lentiviruses expressing Cre recombinase as reported previously, harvested, and cultured[284]. For tumor studies, syngeneic C57BL/6J mice were inoculated with 5.0x10$^5$ MC38 or 2.0x10$^5$ B16F10 cells in PBS subcutaneously. KP sarcoma cells were inoculated into C57BL/6J mice with 5.0x10$^5$ cells by intramuscular injection of the right hind limb. Tumor measurements were performed blindly across the entire experiment by a single operator measuring three dimensions of the tumor with calipers three times per week. All the experiments were conducted according to the Institutional Animal Care and Use Committee guidelines of the University of California, Berkeley.

**Cell lines**

MC38 and B16F10 cell lines were kindly provided by Dr. Jeff Bluestone's lab[303]. MC38-β2m$^{-/-}$ cell line was kindly provided by Dr. David Raulet's lab[254]. All cell lines were maintained in DMEM (GIBCO) supplemented with 10% FBS, sodium pyruvate (GIBCO), 10mM HEPES (GIBCO), and penicillin-streptomycin (GIBCO). Tumor cells were grown at 37℃ with 5% $CO_2$.

***Listeria monocytogenes* strains**

All strains of *L. monocytogenes* were derived from the wild-type 10403S strain. The Lm constructs were based on Lm ΔactA. Lm-OVA expresses a secreted ActA-OVA fusion as described under the control of the *actA* promoter in a derivative of the pPL2 integration vector that was used to stably integrate each antigen cassettes at the tRNA$^{Arg}$ locus on the bacterial chromosome[259]. Lm-TagRFP expresses a secreted TagRFP protein as described under the control of the *actA* promoter using the pPL2 integration vector as described above. All strains were cultured in filter-sterilized nutrient-rich Brain Heart Infusion (BHI) media (BD Biosciences) containing 200 μg/mL streptomycin (Sigma-Aldrich)

**Intravenous and Intratumoral *Listeria* infection**

Overnight cultures were grown in BHI + 200 μg/mL streptomycin at 30℃. The following day, bacteria were grown to logarithmic phase by diluting the overnight culture in fresh BHI + 200 μg/mL streptomycin and culturing at 37℃ shaking. Log-phase bacteria were washed and frozen in 9% glycerol/PBS. For intravenous infections, frozen stocks were diluted in PBS to infect via the tail vein with 1 x 10$^6$ CFU log-phase bacteria. For intravenous infections, frozen stocks were diluted in PBS to infect via intratumoral infections at 5 x 10$^7$ CFU log-phase bacteria. The mice were

euthanized 8-20 days after intratumoral injections and organs were collected for flow analysis.

**Colony forming unit assays from tissues**
Tissues were collected in 0.1% NP40 buffer diluted in PBS. Organs were homogenized and serially diluted on non-TC treated 96-well plates (Genesee). Serial dilutions were plated on BHI + 200 µg/mL streptomycin plates. Plates were incubated overnight at 37℃.

**CIN/Primary bone-marrow derived PMN Maintenance and Differentiation:**
Cas9+ ER-Hoxb8 conditionally immortalized neutrophils (CINs) were a gift from the Stanley lab at the University of California, Berkeley[272]. CINs were expanded as progenitors in Optimem with 10% FBS, 1% L-glutamine, 30µM 2-mercaptoethanol, and 1% Stem-cell factor (SCF)-producing CHO cell supernatant (CIN Media) that also contained 1µM beta-estradiol in non-TC treated flasks (Genesee) maintaining a concentration less than $1x10^6$ cells/mL. Upon reaching the desired quantity, non-adherent progenitors were harvested, washed twice in cold PBS 1x, and plated in non-TC treated flasks containing CIN media lacking beta-estradiol. To generate PMNs, CINs were differentiated until Day 6 in CIN Media + 5ng/mL GM-CSF. To generate PMN-MDSCs, CINs were differentiated in CIN media + 5ng/mL GM-CSF, 40ng/mL IL-6, and 40ng/mL GM-CSF. For primary bone marrow PMNs and PMN-MDSCs, cells were isolated and cultured as described[271]. To generate PMNs-MDSCs, cells were cultured in 40ng/mL IL-6 and 40ng/mL GM-CSF1 day post isolation for 6 days.

**In vitro infections:**
Cells were infected with *Lm-TagRFP* at an MOI that resulted in 30% of the cells being infected. At 1hr post infection, 50µg/mL Gentamicin was added to kill all extracellular Listeria. For flow analysis, cells were collected at time points indicated, stained, and fixed with 4% PFA.

**Cancer Supernatant Assays**
Cancer cell supernatant was generated via the expansion of MC38, B16F10, and 3T3 cell lines in TC-treated flasks in Optimem + 10% FBS, followed by collection of supernatant fluid when cells reached near ~100% confluency. After filtration, all supernatants were frozen down at -20°C. Surveying cancer supernatants involved a 24h incubation period where cells were either re-seeded into TC-treated 24 well plates or non-TC treated flasks in fresh CIN media with a 50:50 ratio of MC38, B16F10, or 3T3 supernatant. Cells were collected at 24h, stained, fixed with 4% PFA, and analyzed by flow cytometry.

**Tissue Collection and preparation for Flow cytometry**

Flow cytometry was performed on an BD LSR Fortessa X20 (BD Biosciences), CyTEK Aurora (CyTEK Biosciences) or LSRFortessa (BD Biosciences) and datasets were analyzed using FlowJo software (Tree Star). Single cell suspensions were prepared in ice-cold FACS buffer (PBS with 2mM EDTA and 1% BS) and subjected to red blood cell lysis using ACK buffer (150mM NH4Cl, 10mM KHCO3, 0.1mM Na2EDTA, pH7.3). Dead cells were stained with Live/Dead Fixable Blue or Aqua Dead Cell Stain kit (Molecular Probes) in PBS at 4℃. Cell surface antigens were stained at 4℃ using a mixture of fluorophore-conjugated antibodies. Surface marker stains for murine samples were carried out with anti-mouse CD3 (17A2, BioLegend), anti-mouse CD4 (RM4-5, BioLegend), anti-mouse CD8a (53-6.7, BioLegend), anti-mouse, CD44 (IM7, BioLeged), anti-mouse CD45 (30-F11, BioLegend), anti-H-2Kb MuLV p15E Tetramer-KSPWFTTL (MBL), anti-H-Kb-A2/SIINFEKEL tetramer (NIH tetramer core), anti-IAb/NEKYAQAYPNVS tetramer (NIH tetramer core) in PBS, 0.5% BSA. Cells were fixed using the eBioscience Foxp3/Transcription Factor staining buffer set (eBioscience), prior to intracellular staining. Intracellular staining was performed using anti-mouse Foxp3 (FJK-16S, eBioscience), anti-mouse TNF-α (MP6-XT22, BioLegend), anti-mouse IFN-γ (XMG1.2, eBioscience), at 4℃, according to manufacturer's instructions. For Lm-TagRFP infected tissues, single cell suspensions were prepared as above, stained using a mixture of fluorophore-conjugated antibodies at 4℃, and fixed in 4% PFA. Cells analyzed by flow cytometry the following day to prevent signal loss from flourescent protein. Cells were resuspended in PBS and filtered through a 70-µm nylon mesh before data acquisition. Datasets were analyzed using FlowJo software (Tree Star).

**Restimulation Assays**

Resected tumors were minced to 1 $mm^3$ fragments and digested in RPMI media supplemented with 4-(2-hydroxyethyl)-1-piperazi-neethanesulfonic acid (HEPES), 20 mg/mL DNase I (Roche), and 125 U/mL collagenase D (Roche) using an orbital shaker at 37℃. Cells from lymphoid organs were prepared by mechanical disruption pressing against a 70-µm nylon mesh. All the cell suspensions were passed through 40 µm filters before in vitro stimulation. Cytokine staining was performed with 3-5x$10^6$ cells in Opti-MEM media supplemented with Brefeldin A (eBioscience) or 10 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma), and 0.25 µM ionomycin (Sigma). Fixation/permeabilization of cells was conducted for intracellular staining using the eBioscience Foxp3 fixation/permeabilization kit (BioLegend) or Tonbo Foxp3 / Transcription Factor Staining Buffer Kit.

**Adoptive transfer experiments**

For in vitro T cell culture, spleens and lymph nodes were collected from OT-1 transgenic mice or C57BL/6J wildtype (*WT*) or *Prf1*$^{-/-}$ previously vaccinated with 1.0x10$^{6}$ CFU *Lm-OVA* 3-4 weeks prior. OT-1s or OVA-responsive CD8s were were activated with 1µg/mL SIINFEKL peptide in DMEM medium supplemented with 10% FBS, non-essential amino acids, sodium pyruvate, L-glutamine, HEPES, 55 µM β-ME and 200 IU/ml recombinant human IL-2 (TECIMTM, Hoffman-La Roche provided by NCI repository, Frederick National Laboratory for Cancer Research). T cells were transferred into WT or Rag2$^{-/-}$ or *B2m*$^{-/-}$ mice by intravenous injection two weeks prior to MC38 or KP sarcoma inoculations.

***In vivo* antibody-mediated cell depletion**

For tumor progression studies, CD8 depletion was achieved by intraperitoneal injection of 200 µg per mouse of the anti-CD8 monoclonal antibody clone YST-169 (Leinco Technologies, Catalog # C2442) two days prior to tumor inoculation, followed by additional doses every 6 days thereafter. For CD4 depletion, intraperitoneal injection of 200 µg/mouse of clone GK1.5 (Leinco Technologies, Catalog: C1333) was done two days prior to tumor inoculation, followed by additional doses every 6 days thereafter. PMN depletion was done by intraperitoneal injection of 200 µg per mouse of the anti-Ly6G monoclonal antibody clone IA8 (Leinco Technologies, Catalog: L280) twice prior to tumor inoculation, followed by additional doses every 2 days.

**Statistical Methods**

p values were obtained from unpaired two-tailed Student's t tests for all statistical comparisons between two groups, and data were displayed as mean ± SEMs. For multiple comparisons, one-way ANOVA was used. For tumor growth curves, two-way ANOVA was used with Sidak's multiple comparisons test performed at each time point or by multiple regression analysis p values are denoted in figures by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

Conclusions and future perspectives.

Bacterial derived immunotherapies against cancer have shown great promise in the past few decades, due to our growing understanding of the innate and adaptive immune system, combined with recent advances in cancer immunotherapies, especially with checkpoint blockade therapy. Furthermore the BCG vaccine is used in the clinic as the standard of care to treat high-risk, non-muscle-invasive bladder cancer. And many bacteria mediated immunotherapies have been used in clinical trials, involving

*Salmonella, Clostridium and Listeria* among others. Meaning that research is now past the preclinical stage in many of the aforementioned bacterial species. Preclinical studies involving mice have shown great promise when combining bacterial immunotherapies with existing chemotherapy and checkpoint blockade, however these preliminary results have failed to completely recapitulate in human clinical trials leading to modest, statistically insignificant, or no positive results at all in patients. Lack of positive results stem from a number of factors that can be improved upon, these include; (1) The need of the immune system to mount a strong reaction to bacterial immunotherapies, which can be challenging, especially in patients undergoing treatments that can ultimately suppress the immune response (e.g. most chemotherapeutic drugs). This is especially challenging in human clinical trials since usually these trials involve patients that have exhausted previous treatment modalities. Ultimately this can be ameliorated by including bacterial immunotherapies in combination with early cancer treatments in clinical trials. (2) The balance between virulence and attenuation is an area where improvements can be more easily achieved, because when bacterial immunotherapies are too attenuated, they fail to activate the innate and adaptive immune systems to mount the necessary response[233]. Conversely, when bacterial immunotherapies are under-attenuated, they can cause severe immune side effects which can in some cases lead to patient deaths. Recognizing virulence factors in bacteria is crucial for the safety of bacterial immunotherapies. (3) A better understanding of the tumor microenvironment as a niche for bacteria is needed to develop better strains that will only be "active" once they have infiltrated tumors and have low adverse effects in healthy tissues. It is already recognized that the tumor microenvironment of some tumors contain anerobic zones that some bacterial strains are able to grow[304]. Furthermore, selecting from strains that can turn "cold" tumors into "hot" tumors can be of great benefit, especially in combination with checkpoint blockade, but further studies are needed to scale up to human trials. (4) Current studies lack the understanding behind the mechanism of action of bacterial immunotherapies, especially when looking at the phenotype of the immune system when treating with aforementioned bacterial therapies. There almost seems to be a disconnect between microbiologists and immunologists when it comes to setting up experiments and interpreting data. It is known that these therapies can control and clear tumors in animal models, but there is a lack of fundamental understanding of how these therapies work, and more studies are needed to elucidate the interactions between the bacteria, the immune response and the tumor microenvironment, especially when it comes to characterizing NK cells, T cells and MDSCs in the tumor microenvironment with bacterial cancer therapies. Without this fundamental knowledge, it is more difficult to scale from animal models to humans. (5) Better tumor models used in preclinical studies are needed to understand the interplay between the immune response and what

it is observed in the clinic, completely homogeneous malignancies are rarely seen in the clinic, because the cancer cell lines used in the lab do not always recapitulate what it is seen in cancer patients. In recent years GEM cancer models have gained popularity because they better represent human cancer at the molecular and phenotypic level[305]. Few bacterial immunotherapy studies have taken advantage of GEM mice. (6) Dosage plays an important role in the efficacy of bacterial immunotherapies, and it can be intrinsically hard to administer a standard dose, ultimately there is more research to be done and perhaps dosage must be correlated to tumor grade[306]. In conclusion, there have been major advances in bacterial immunotherapies in recent decades, regarding gene engineering, virulence attenuation, bacterial delivery, and tumor colonization, this has paved the way for synergy between existing chemotherapies, radiotherapies, and other immunotherapies. These bacterial cancer immunotherapies show great promise in the lab and have been scaled up to numerous clinical trials. However, numerous hurdles still need to be overcome before full-scale adoption of bacterial cancer immunotherapies can be achieved.

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