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Uncovering the Role of Dendritic Cell-Mediated Modulation of T Cells in Driving Responses to
Bacterial Immunotherapy and Toll-Like Receptor Agonists

By

Timothy F. Campbell

A dissertation submitted in partial satisfaction of the

requirements for the degree of
Doctor of Philosophy

in
Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in Charge:

Professor Michel DuPage, Chair
Professor Gregory Barton
Professor Ellen Robey
Professor Astar Winoto

Fall 2025

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Abstract

Uncovering the Role of Dendritic Cell-Mediated Modulation of T Cells in Driving Responses to Bacterial Immunotherapy and Toll-Like Receptor Agonists

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Timothy F. Campbell

Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor Michel J. DuPage, Chair

The past several decades have seen a paradigm shift in our understanding of the role played by the immune system in cancer. Perhaps the most impactful discoveries have related to basic mechanisms by which T cells drive antitumor immune responses, which in turn have led to an explosion of interest in immunotherapies that seek to promote antitumor T cell responses. This effort has produced several massive successes—most notably the pioneering of checkpoint blockade—but nevertheless most patients' cancers remain recalcitrant to immunotherapy. This suggests that a deeper understanding of the mechanisms that regulate antitumor T cell responses will further increase the efficacy of cancer immunotherapy. Contributing to this effort was the major driving aim of my thesis work, which focused on the role played by upstream regulators of T cells, namely antigen presenting cells, in driving productive antitumor immune responses.

My thesis work encompassed two broad but related approaches. The first approach was to determine how attenuated *Listeria monocytogenes* (*Lm*), a promising vector for cancer vaccines, modulated antitumor T cell-mediated immunity by modulating innate immune cells in tumors. This led to the discovery that *Lm* could either moderately inhibit tumor growth following intravenous infection or promote tumor growth following intratumoral infection. These effects were due to recruiting anti-*Lm* CD8⁺ T cells or polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) into tumors, respectively. Injection of intravenous followed by intratumoral *Lm* elicited greater tumor control by first priming anti-*Lm* CD8⁺ T cells and then drawing these cells into tumors to kill PMN-MDSCs. This increased cell death and decreased proliferation by tumor cells, increased tumor antigen presentation by conventional type 1 dendritic cells (cDC1s) in tumors, and ultimately promoted tumor antigen-specific CD8⁺ T cell immunity. These findings are significant for cancer vaccine development, which until now has utilized vectors expressing either common tumor antigens—used in generic vaccines—or antigens specific to a single patient. These strategies create barriers to the wide use of cancer vaccines in patients, which we suggest can be circumvented by instead driving *Lm*-specific immune responses within tumors.

My second approach was to identify how innate immune stimulation from *Lm* or from individual Toll-like receptor (TLR) ligands impacted the regulation of pro- and anti-tumor T cells by antigen presenting cells. I found that tumor control by IV+IT *Lm* was dependent upon TLR2 stimulation by *Lm*-derived lipoproteins. TLR2 signaling and anti-*Lm* CD8⁺ T cells

together led cDC1s to promote antitumor CD8⁺ T cell immune responses. TLR2 signaling in conventional type 2 dendritic cells (cDC2s) abrogated immune suppression by regulatory T cells (Tregs) within tumors, which was required for the protective effects of IV+IT *Lm*. Individual ligands for TLR2 and TLR9 mediated tumor control both individually and, when combined, synergistically by reducing Treg frequency and increasing CD8⁺ T cell frequency. These findings are significant because they suggest that CD8⁺ T cell responses benefit from the coordinated activity of multiple cDC types acting on multiple T cell populations.

Overall, my work fits into recent trends in the field of cancer immunology which have ascribed specific pro- and anti-tumor effects to individual cell subsets. However, my findings also emphasize the interconnectivity of these populations and their mutual regulation within tumors. My findings suggest that a more holistic approach to immunotherapy development that considers multiple antigen presenting cell and T cell subsets can improve the efficacy of cancer immunotherapies, including those which are thought to act through CD8⁺ T cells alone.

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Dedication

I dedicate this dissertation to Eileen Rohmer, who was the first person to suggest that I pursue science more seriously. Her influence led me to discover a passion for science and initiated the chain of events that culminated in my completion of this degree. I cannot overstate the effect that her mentorship had on me, and I will always remember her fondly. Rest in peace, Ms. Rohmer.

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in an era when I was struggling to do so, and in the subsequent years I have often found myself asking how each of you might react to a new idea or question that I have. Likewise, I want to thank David Raulet for providing me with constructive feedback at all stages of my PhD. I also want to thank my undergraduate and Fulbright research mentors, including Dr. Rino Rappuoli of GlaxoSmithKline Vaccines; Dr. Caitlin Esoda and Dr. Meta Kuehn of Duke University; and Dr. Enrico Luchinat and Prof. Lucia Banci of the University of Florence. Without the opportunities and mentorship that they gave me, I may never have been able to pursue this career.

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Chapter 1

Introduction

1.1 Historical background of cancer immunology and immunotherapy

The history of cancer immunology and immunotherapy is deeply connected to the history of the broader field of immunology. Indeed, the first recorded use of what would today be termed cancer immunotherapy came before even Elie Metschnikoff's 1887 discovery of macrophages and Paul Ehrlich's side chain theory of immune recognition in 1901, two critical steps in the development of our current conception of the immune system.^{1,2} This was the work of Profs. Wilhelm Busch and Frederick Fehleisen, who in 1867 and 1882 independently observed the spontaneous remission of tumors in patients who became infected with *S. pyogenes* and later replicated this occurrence via the deliberate infection of other patients.^{3,4} However, the person most frequently cited as the pioneering figure of cancer immunotherapy was Dr. William Coley, who beginning in 1891 deliberately infected sarcoma patients with a formulation of heat-killed *Streptococcus pyogenes* and *Serratia marcescens* that has since come to be known as Coley's Toxins.^{5,6} Unlike his antecedents, who quickly moved on from their discoveries, Dr. Coley became a strong advocate for his treatment, thus earning his title as the primary founder of cancer immunotherapy.⁷ At the time, Coley's work was met with skepticism and even downright hostility by his disbelieving peers, and was nearly forgotten as radiotherapy underwent its own explosion in popularity around the same time.^{7,8} However, in light of over a century of developments in cancer immunology and immunotherapy, Coley and his Toxins have come to be understood as ahead of their time.^{7,9} Indeed, my own work involving the deliberate infection of tumors with bacteria and molecules derived from them strongly parallels Coley's work.

The discovery of histocompatibility is another major link between the history of immunology as a whole and cancer immunity specifically. Working off of the pioneering tumor transplantation techniques of Prof. Carl Jensen, Prof. Leo Loeb demonstrated that tumors derived from inbred strains of mice could engraft into mice of the same strain but not other strains in the first decade of the 20th century.¹⁰ A decade later, Prof. Clarence Little extended this idea by proposing a multigenic model for the heritability of tumor and tissue compatibility in mice.^{11,12} The rejection of tumors by unrelated mice was connected to immune responses by P. A. Gorer in 1937, about a decade before George Snell coined the term "histocompatibility" in 1948 to describe the compatibility of transplanted tissues.^{13,14} In the same year, Gorer, Snell, and S. Lyman released a paper detailing the use of tumor transplantation to infer the existence of at least 12 genetic loci related to histocompatibility in mice (hence termed major histocompatibility genes, or MHC).^{15,16} This would prove to be an underestimate, likely made due to the close genetic linkage of individual MHC genes, but nevertheless remarkable for its accuracy given the tools available at the time.¹⁵⁻¹⁷ The early 1960s saw both the discovery of the role of the thymus in producing mature lymphocytes (which would later be named T cells) and of cellular cytotoxicity, one of the major functions of T cells and perhaps the role most important to cancer.^{18,19} These discoveries were later connected to MHC in 1974, for the first time connecting the idea of immunological compatibility to the cytolytic activity of T cells.²⁰ Each of these discoveries—tumor transplantation in mice, the importance of MHC to tumor rejection, and the identification of T cells as the primary mediators of this effect—set the stage for a period of massive advancements in immunology in the late 1970s and 1980s as these findings were contextualized and extended upon, and their translational potential came to be understood.^{9,21,22} Indeed, without these advances my own research, which focuses largely on the signals and cell types that regulate T cell immune responses in cancer, would not have been possible.

Since then, cancer immunology and immunotherapy has undergone a renaissance, buoyed technical and conceptual advancements in cellular and molecular biology and immunology.^{9,21} As our understanding of the fundamental mechanisms governing immune responses to cancer have grown more sophisticated, they have produced insights into how molecular therapies can intervene in these processes, culminating in the current success of a novel class of antitumor drugs termed checkpoint inhibitors.^{9,21,23} Checkpoint inhibitors are monoclonal antibodies that bind to inhibitory receptors or their ligands on immune cells—most frequently T cells or cells that regulate them—in a process termed checkpoint blockade.^{9,21,23} By blocking inhibitory receptors that negatively regulate antitumor adaptive immune responses, such as Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4) and Programmed Death protein 1 (PD-1), checkpoint blockade enhances the body's capacity to fight cancer, effectively unleashing the immune system to combat neoplastic growth.²⁴ The advent of checkpoint blockade has led to a sea change in the treatment of cancer, with months- to years-long increases in overall survival among patients with cancers including melanoma, renal cell carcinoma (RCC), head and neck squamous cell carcinoma (HNSCC), colorectal cancer (CRC), and other malignancies.^{9,21,23,25–29} These incredible results led to the much-deserved awarding of the 2018 Nobel Prize in Physiology or Medicine to Drs. Jim Allison and Tasuku Honjo for their fundamental contributions to checkpoint blockade immunotherapy for cancer, and it was into this environment that I stepped as I began my PhD in the same year. In turn, these successes have led to an explosion of interest in extending the uses of checkpoint blockade by developing new checkpoint inhibitors directed against other inhibitory receptors and agonists that activate stimulatory receptors (**Table 1.1**).^{21,23,30,31} Nevertheless, while the surge of interest in checkpoint blockade and its associated funding has created myriad opportunities for both basic and translational investigation, the pace of advancement has paradoxically slowed in recent years.^{21,23,30}

Table 1: Second- and Third-Generation Checkpoint Inhibitors			
Target	Category	Development Stage	Ref.
LAG3	Inhibitor	Stage I/II/III clinical trials, approved (melanoma)	32,33
TIGIT	Inhibitor	Stage II/III clinical trials	33,34
ICOS	Agonist	Stage I/II clinical trials	35,36
GITR	Inhibitor, Agonist	Stage I/II clinical trials	37
TIM-3	Inhibitor	Stage I/II/III clinical trials	33
CD40	Agonist	Phase I/II clinical trials	38
4-1BB	Agonist	Phase I/II clinical trials	39,40
BTLA	Inhibitor	Phase I/II clinical trials	41
OX40	Agonist	Phase I/II clinical trials	42

In part, this slowing has been the result of new checkpoint inhibitors failing to match the successes of anti-CTLA-4 and anti-PD-1/PD-L1.^{21,30,31,43} Immune-mediated side effects have also played a role in slowing progress, as both approved and novel drugs have been shown to cause autoimmune toxicity by leading to excessive T cell activation.^{21,30,31,44,45} Finally, and most directly relevant to my work, checkpoint inhibitors have been found to have inconsistent effects on immunosuppressive regulatory T cells (Tregs), in some cases decreasing their frequency and functionality and in others having exactly the opposite effects.⁴⁶⁻⁴⁹ As a result, interest has shifted towards demonstrating synergy between existing immunotherapies and other drugs.^{21,30,31,43} As I prepared to enter into the field of cancer immunology, wary of chasing a train that had already left the station, I instead felt that I could best contribute to the field by investigating how different innate immune stimuli impacted the balance of inhibitory or stimulatory receptor signaling in T cells. Cognizant of the impactful but poorly understood role played Tregs in checkpoint blockade therapy, I reasoned that by better understanding how these signals naturally regulate the balance between tumor-inhibitory conventional T cells tumor-supporting Tregs, we could gain greater insight into the factors that control the course of tumor progression.

While my work ultimately strayed from this topic to a broader focus on how innate immune stimuli regulate T cells in cancer, this goal was a major factor in guiding my initial work as a graduate student in the lab of Dr. Michel DuPage. In this doctoral thesis, I will lay out my investigations into the innate immune signals that mediate tolerance versus immunity in cancer. I will begin with my first published first-author work, which uncovered a mechanism by which anti-*Listeria* T cell-mediated immunity can both directly (via bystander effects) and indirectly (via the elicitation of stronger tumor-specific immune responses) combat cancer. Next, I will continue with my second first-author manuscript, which investigated the innate immune signals that partially underlie the ability of *Listeria* therapy to combat cancer, with a particular focus on the role played by Toll-like receptor 2 in negatively regulating Treg-mediated immune suppression in tumors. I will then summarize my ongoing work on the differences in mechanism by which Toll-like receptors can promote antitumor immunity by acting on different innate immune cells to differentially impact effector T cells and Tregs. Finally, I will conclude with a discussion of how these findings can be contextualized in the current research landscape and how my doctoral work might help to uncover novel pathways that might be utilized to combat cancer.

1.2 T cells in cancer

T cells are key mediators of cellular immunity and are among the most important cell types in cancer immunology and immunotherapy.⁵⁰ T cells are characterized by their expression of the T cell receptor (TCR), a transmembrane multiprotein complex that enables T cells to recognize non-self antigens (molecules recognized by cells of the adaptive immune system) and respond to immunological insults.⁵¹ The TCR complex includes germline-encoded proteins involved in signal transduction (e.g. CD4 and CD8), and, critically, a heterodimer of TCR gene fragments that are unique to a given cell clone due to their rearrangement during T cell development in the thymus.^{52,53} The TCR is composed of either a TCR α/β or TCR γ/δ heterodimer; however, α/β T cells comprise 95% of all T cells and will be the subject of the remainder of this work.^{52,53} By binding to peptides derived from antigens (epitopes) presented on major histocompatibility complex (MHC) molecules by antigen-presenting cells (APCs), the α/β TCR heterodimer allows T cells to sense the presence of an antigen to initiate an immune response against the antigen's source.⁵³ Negative selection of self-reactive T cells during thymic development normally ensures

that T cells should only react to foreign antigens (e.g., those derived from a pathogen).⁵⁴ However, mutations and errors in protein synthesis during tumor development lead to the formation of tumor neoantigens that are recognized as non-self and thus can initiate a T cell immune response.⁵⁴ T cells are therefore well-suited to mediate specific antitumor immune responses, and mounting evidence suggests that T cells play an important role in combating early-stage neoplasias as they develop.⁵⁵ This also makes T cells attractive candidates for the development of immunotherapies, and T cells are the cell type that most frequently mediate the antitumor effects of checkpoint inhibitors.^{24,50} However, this basic tenet of cancer immunology was discovered only relatively recently, and the development of the first and most successful generation of checkpoint inhibitors was fueled by basic studies into the mechanisms regulating T cell activation and function.^{9,23} This topic, especially the mechanisms by which APCs and innate immune receptors differentially regulate conventional T cells and Tregs, is central to my thesis work. I will therefore proceed with an overview of key aspects of T cell biology and highlight the ways in which these features intersect with cancer immunology and immunotherapy.

1.2.1 T cell activation

As mentioned above, T cells are defined by their expression of the TCR, which confers antigen specificity.⁵¹ However, T cells also require additional signals to be activated and differentiate into functional subsets specific to different immunological insults.⁵⁶ These signals are grouped into three categories that serve distinct but necessary functions: Signal 1. TCR signaling by binding to peptide-MHC complexes (pMHC); Signal 2. Costimulation through costimulatory receptors such as CD28; and Signal 3. Cytokine signaling.⁵⁶ While the role of these three signals are reviewed frequently and at length elsewhere, all three are required to elicit T cell responses.⁵⁰ I will therefore briefly review the role of these signals in T cell activation and note where they are relevant to my own work presented here.

Signal 1: TCR-pMHC signaling

During thymic T cell development, T cell progenitors undergo a highly regulated process of gene recombination at the *Tcrα* and *Tcrβ* genetic loci to produce rearranged, non-germline-encoded α and β TCR genes that are unique to an individual T cell clone.⁵⁷ During and after this process, T cells undergo a two-stage selection process of positive selection in the thymic cortex followed by negative selection in the thymic cortex and medulla.^{57,58} In positive selection, which occurs in the thymic cortex, developing T cells upregulate both CD4 and CD8 coreceptors to test for sufficient affinity for pMHC-II or pMHC-I complexes.^{52,57,58} At this stage, cells with insufficient pMHC affinity undergo death by neglect, while those with excess affinity undergo an initial round of negative selection resulting in apoptotic cell death.⁵⁸ Cells with low-to-intermediate affinity for pMHC-II or pMHC-I commit to the CD4⁺ or CD8⁺ lineages, respectively.^{52,58} These newly CD4⁺ or CD8⁺ single-positive T cells then proceed to the thymic medulla where they sample additional self antigens, undergoing an additional round of negative selection.⁵⁸ Tregs are an exception to this process and usually possess TCRs with intermediate affinity to self pMHC-II (**1.2.3 Treg Development**).⁵⁸ Negative selection helps stave off autoimmune disease by preventing mature T cells from reacting to self antigens in peripheral tissues.^{58,59} Thus, mature T cells having undergone positive selection possess an intermediate affinity for pMHC.⁵⁸ This allows them to undergo transient, low-affinity interactions with pMHC complexes upon their exit from the

thymus and circulation through secondary lymphoid organs (SLOs) such as lymph nodes (LNs) and the spleen, where mature, naive T cells first encounter their antigen.⁵⁸

TCR signaling (signal 1) occurs when the TCR binds to a pMHC complex of sufficient affinity or avidity to elicit its downstream signaling pathway.^{53,56} In broad terms, TCR α/β ligation elicits conformational changes and/or clustering of germline-encoded signaling adapters to initiate multiple parallel signaling cascades.^{53,60} Concurrent binding of CD4 or CD8 to MHC-II or MHC-I, respectively, contributes to this process by recruiting intracellular components of the TCR signaling complex.^{53,60} Through signaling cascades downstream of these adapters, TCR signaling activates the transcription factors Activator Protein 1 (AP-1), Nuclear Factor of Activated T cells (NFAT), and Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF- κ B), although the latter two transcription factors are not fully engaged in the absence of costimulation (**Signal 2: Costimulation**).⁵³ TCR signaling leads to transcriptional changes, cell proliferation, and myriad other effects that mediate the expansion and effector functions of activated T cells, which will be discussed below (**1.2.2 Conventional T cell subsets and their roles in cancer, 1.2.3 CD4⁺ Regulatory T cells and their roles in cancer**).^{53,60} Nevertheless, TCR signaling alone is insufficient to elicit T cell activation or the activity of each of these transcription factors. Instead, rather than becoming activated, T cells that receive TCR stimulation in the absence of costimulation enter a hyporesponsive state termed anergy.^{56,61,62} Anergy is presumed to be an additional mechanism of enforcing self-tolerance outside of the thymus, as the presence of accessory signals is often predicated on signaling through inflammatory receptors expressed on APCs during the uptake of pathogen-derived antigens.⁶³ In my thesis, I will discuss how the activation of *Listeria*-specific CD8⁺ T cells and proinflammatory TLR2 signaling contribute to reinvigoration of tumor-specific T cell responses, likely due in part to enhanced TCR signaling enabled by enhanced tumor antigen uptake and presentation (**Chapters 2 and 3**).

Signal 2: Costimulation

Costimulation is required for T cells to become fully activated in response to TCR stimulation and avoid anergy.⁶⁴ Costimulation is perhaps best exemplified by signaling through CD28, the first-discovered and prototypical T cell costimulatory receptor.⁶⁵ CD28 is constitutively expressed by all CD4⁺ and CD8⁺ T cells, and in most cases signaling through CD28 is required for the initial activation of naive T cells.⁶⁵ Homodimeric CD28 interacts with its ligands CD80 and CD86 (aka B7.1 and B7.2), which are expressed by APCs and are upregulated in response to innate immune stimuli.⁶⁵ Binding of CD80 or CD86 to CD28 recruits proteins involved in TCR signal transduction, strongly amplifying TCR signaling.^{65,66} In particular, CD28 signaling helps to activate NFAT and NF- κ B, but also exerts TCR-independent functions.⁶⁴⁻⁶⁶ Together, these effects exemplify the critical role of costimulation in T cells activation.

In addition to CD28, T cells express a variety of other costimulatory receptors that exert both stimulatory and inhibitory effects on T cells, typically but not always after their initial activation by TCR and CD28 signaling.⁶⁴ These receptors and their ligands primarily fall into either the IgG superfamily (CD28, inducible costimulator (ICOS)) or the tumor necrosis factor receptor (TNFR) superfamily (TNFR2, CD27, 4-1BB, Glucocorticoid-induced TNFR-related protein (GITR), and OX40).^{67,68} While each receptor is unique in its specific signaling cascades, co-stimulatory receptors of both the IgG and TNFR superfamilies both amplify and diversify the effects of TCR signaling.^{64,68,69} Signaling through co-stimulatory receptors also have different

effects in conventional T cells and Tregs.^{64,67,68} From a clinical perspective, these differences provide opportunities to leverage signals to drive specific T cell responses and potential hazards due to unintended amplification of deleterious T cell responses.⁶⁹ This was a major driver of my initial interest in studying how TLR signaling changes the expression of ligands for these receptors to differentially impact Tregs and other T cell types (**Chapter 4**).

The counterpart of costimulatory receptors are coinhibitory receptors, which include members of both the IgG superfamily (e.g., CTLA-4 and PD-1) and several noncanonical coinhibitory receptors (e.g., T cell Immunoglobulin and mucin domain-containing protein 3 (TIM-3) and Lymphocyte-Activation Gene 3 (LAG3)) play similar roles in regulating activated T cells.⁶⁹ Negative regulation of T cell responses by these receptors occurs by diverse mechanisms.⁶⁴ Among the better-characterized mechanisms are those that directly counteract effects of costimulation, including outcompetition for ligand binding (e.g., CTLA-4 binds to CD80/CD86 with higher affinity than CD28 and can mediate the removal of these molecules from APCs)⁷⁰ and inhibition of intracellular signaling cascades (e.g., PD-1 recruits phosphatases to dephosphorylate TCR signaling proteins).⁷¹ However, some co-inhibitory receptors participate in *bona fide* signaling in addition to inhibiting costimulatory signals, although these mechanisms have not been fully elucidated.^{32,72,73}

The balance between costimulation and coinhibition plays a critical role in maintaining T cell homeostasis, and disruptions to this balance can play roles in driving both autoimmunity (in the case of insufficient coinhibitory signals and/or excess costimulatory signals) and cancer (excess coinhibitory signals and insufficient costimulatory signals).^{64,69,74} These receptors are therefore of immense interest for therapeutic development, and a wide array of inhibitory, stimulatory, and depleting antibodies directed against these molecules are being investigated (**1.1 Historical background of cancer immunology and immunotherapy**).^{21,23,30,31} Similarly, inflammatory signals sensed by cells of the innate immune system can drastically change costimulatory ligand expression to influence T cell responses indirectly, providing an additional route for cancer immunotherapy development (**1.3.2 Regulation of antigen presentation and APC function by PRRs**).^{75,76} A desire to explore how changes in costimulatory signaling regulate T cells played a major role early in my thesis research, ultimately leading me to explore how different TLRs affect Foxp3 expression in regulatory T cells (**Chapter 4**).

Signal 3: Cytokines

Cytokines comprise the final signal in T cell activation.^{77,78} Cytokines are secreted glycoproteins that carry signals between cells, helping to coordinate immune responses at the cellular, tissue, and organismal level.^{79,80} To fulfill this role, cytokines are diverse in their structure, signaling cascades, regulation, and effects.^{81,82} Major cytokine families relevant to T cell-mediated immunity include interleukin-1 (IL-1), IL-6, IL-10, IL-12, interferon (IFN), common gamma chain (γ_c), and transforming growth factor beta (TGF- β) families, which are defined by shared structural and functional features.⁸²⁻⁸⁹ The differences in signaling mediated by cytokines are further increased by the diversity of cofactors and transcription factors elicited by each cytokine.^{78,81,82} Thus, cytokine signaling provides critical contextual information that helps to tailor immune responses to specific immunological challenges.^{81,82}

Cytokines play similar, partially-overlapping roles in the activation and differentiation of CD4⁺ and CD8⁺ T cells.^{77,81,90,91} Upon undergoing TCR stimulation and costimulation, CD4⁺ T

cells begin to differentiate into one of several specialized subsets (**1.2.2 Conventional T cell subsets and their roles in cancer: Conventional CD4⁺ T cells**).⁹² This process is tightly regulated by cytokines, which lead to the expression of lineage-specific transcription factors that further reinforce cell subset identity by driving the expression of cytokines characteristic of each CD4⁺ T cell subset.^{91–93} In contrast, CD8⁺ T cells were long believed to rely on signaling via specific cytokines, namely type I and II interferons and/or IL-12, to activate and differentiate into effector cells.^{77,94} However, emerging evidence suggests that CD8⁺ T cells, like CD4⁺ T cells, may undergo differentiation into distinct functional subsets under the influence of polarizing cytokines.⁹⁵ Nevertheless, upon activation, both CD4⁺ and CD8⁺ T cells become dependent upon the γ c family cytokines IL-2, IL-7, and/or IL-15 for survival, proliferation, and memory cell formation.^{87,90,93,96,97} Thus, cytokine signaling is essential for both the initiation and maintenance of both CD4⁺ and CD8⁺ T cell immune responses. My thesis will touch on the important role played by IL-2 signaling in reinvigorating tumor-specific CD8⁺ T cells in response to *Listeria* immunotherapy (**Chapter 3**), the ability of Tregs to inhibit this process by outcompeting CD8⁺ T cells for IL-2 (**Chapter 3**), and a potential role for cytokines in driving differential responses to Toll-like receptor stimulation by CD8⁺ T cells and Tregs (**Chapter 4**).

1.2.2 Conventional T cell subsets and their roles in cancer

As outlined above, T cells can be grouped into phenotypic and functional categories defined by shared expression of characteristic markers and transcription factors.⁵¹ My thesis will focus on the roles of CD4⁺Foxp3⁺ regulatory T cells and CD8⁺ T cells in two model systems, *Listeria* immunotherapy and intratumoral adjuvant delivery (**Chapters 2, 3, and 4**). I will therefore first proceed by describing these T cell subsets and the roles they play in antitumor immunity.

CD8⁺ T cells

CD8⁺ T cells are the primary mediators of direct T cell-mediated cytotoxicity.⁹⁸ CD8⁺ T cell cytotoxicity is mediated by recognition of peptide-bound MHC-I.⁹⁸ MHC-I is specialized for the presentation of intracellular antigens and is expressed by all nucleated cells in the body (**Section 1.3.1 The basics of antigen presentation**).^{99,100} Thus, CD8⁺ T cells are critical for combating infections caused by intracellular pathogens, whose antigens are accessible to the MHC-I antigen presentation pathway, and can theoretically kill any cell presenting their cognate antigen.^{99,100} CD8⁺ T cells therefore play a role in combating infections not only by viruses, which by necessity undergo replication only within host cells, but also by bacteria that possess an intracellular stage in their life cycle, such as *Listeria monocytogenes*, *Salmonella enterica*, and *Mycobacterium tuberculosis*.^{101,102} Likewise, their ability to recognize intracellular antigens, their potent cytotoxic capacity, and the universal expression of MHC-I by host cells makes CD8⁺ T cells highly suited for combating cancer.^{103,104}

Initial activation of CD8⁺ T cells, known as priming, occurs upon concurrent recognition of an antigen presented by an APC, costimulation, and cytokine signaling.^{56,98} Priming most often occurs in SLOs, which serve as focal points for the immune system where lymphocytes can interact with each other and their antigens.⁹⁸ Upon activation, CD8⁺ T cells undergo metabolic and transcriptional change, proliferate, and upregulate effector molecules.⁹⁸ Activated CD8⁺ T cells are guided to the source of their cognate antigen by a combination of adhesion molecules and chemokines, whereupon they respond to antigen recognition by several effector mechanisms.¹⁰⁵ One major arm of this effector response are direct cytotoxic mediators, including

both cytotoxic granules (e.g., perforin and granzymes) and death receptors (e.g., FasL), which together enable CD8⁺ T cells to precisely eliminate cells presenting their cognate antigen.^{98,106} Another major arm of the CD8⁺ T cell effector response are indirect mediators of cytotoxicity, including proinflammatory cytokines (e.g., IL-2, TNF- α , and IFN- γ) that promote the activity of effector cells including NK cells, macrophages, and other T cells.^{98,107} By utilizing a combination of these mechanisms, CD8⁺ T cells kill cells presenting their cognate antigen to eliminate infection or, in the case of cancer, neoplastic cells.^{100,104,106}

Nevertheless, antitumor CD8⁺ T cells encounter myriad challenges.^{95,103,104,108} Many of these challenges can be attributed in part to a lack of microbially-provided PAMPs that normally play critical roles in driving effective CD8⁺ T cell responses **(1.3.2 Regulation of APCs by PRRs)**.^{95,103,104,108} This can result in a lack of factors required for T cell function like IL-2¹⁰⁹ or pMHC expression.⁵⁵ Conversely, TCR signaling without sufficient costimulatory signaling can promote CD8⁺ T cells exhaustion, a hyporesponsive state characterized by reduced cytokine production and killing capacity.¹¹⁰ Exhausted CD8⁺ T cells can upregulate coinhibitory receptors such as PD-1 and LAG3, further inhibiting their function.¹¹¹ CD8⁺ T cell-directed immunotherapies like checkpoint blockade seek to strengthen antitumor immune responses by shifting the balance of these signals **(1.1 Historical background of cancer immunology and immunotherapy)**.^{69,95,111} Cytokine therapy, which can entail agonists of cytokine receptors that boost CD8⁺ T cell function (such as IL-2 and IL-12) or blockade of inhibitory cytokines, is a similar strategy.¹¹² Likewise, therapeutic cancer vaccines seek to circumvent ineffective or absent TCR signaling in tumor-specific CD8⁺ T cells by using vectors engineered to express tumor antigens to increase the abundance and diversity of tumor-specific CD8⁺ T cells.¹¹³ Finally, adjuvants have been proposed to offer a supplemental or alternative approach to these strategies **(1.3.4 Targeting APCs for cancer immunotherapy)**.¹¹⁴ My thesis will specifically focus on the role of antigen presentation and cytokine signaling in driving CD8⁺ T cell responses in both a *Listeria*-based model of cancer immunotherapy **(Chapters 2 and 3)** and in response to intratumoral adjuvants **(Chapter 4)**.

Conventional CD4⁺ T cells

Conventional CD4⁺ T cells (i.e., non-regulatory CD4⁺ T cells) play varied roles in antitumor immune responses (Tregs will be discussed in the following section).^{51,115} CD4⁺ T cells express class II MHC-restricted TCRs and are capable of mediating cytotoxicity via recognition of antigen presented by MHC-II⁺ cancer cells in some cases.^{116,117} However, while MHC-II expression has been observed to correlate with responsiveness to checkpoint blockade immunotherapy in patients across a variety of tumor types, its expression by tumor cells is varied and much less common than MHC-I.^{118–122} Rather, MHC-II is typically expressed on a narrow subset of MHC-II⁺ “professional” APCs including B cells, monocytes/macrophages, and dendritic cells (DCs).¹²³ Nevertheless, even in tumors that lack tumor cell MHC-II expression, CD4⁺ T cells can exercise important effects on antitumor immunity through interactions with APCs and indirect effects on surrounding cells.^{115,124} In lymphatic tissues, activated CD4⁺ T cells provide “help” during CD8⁺ T cell priming in the form of signals that promote APC function (e.g., CD40L) and cytokines that promote CD8⁺ T cells proliferation and effector function acquisition (e.g., IL-2).^{115,124} In tumors, CD4⁺ T cells secrete cytokines that promote the polarization and antitumor function of both CD8⁺ T cells and innate cells like macrophages and NK cells.^{125–127} While the full extent of the contribution of CD4⁺ T cells to antitumor immunity is

still unknown, mounting evidence suggests that these mechanisms may play a more important role than previously thought.^{51,115,124} My thesis work only briefly touches on conventional CD4⁺ T cells in the context of *Listeria* immunotherapy (**Chapters 2 and 3**). The following section is therefore brief and included solely to provide context for the cell types which were the primary focus of my thesis work, namely dendritic cells, CD8⁺ T cells, and Tregs.

CD4⁺ T cell activation mirrors that of CD8⁺ T cells in that they require concurrent TCR stimulation by an APC presenting their cognate antigen on MHC-II and costimulation to be primed, usually within SLOs.^{56,128} However, while cytokine signaling is important in both CD8⁺ and CD4⁺ T cell activation, CD4⁺ T cells critically rely upon specific cytokines during priming to undergo differentiation into functional subsets with distinct nonredundant functions^{1,129,130}. Once a cell fate decision is made, mutually inhibitory interactions between the cytokines and transcription factors that define each CD4⁺ T cell subset further reinforce cellular identity.^{129,130} Nevertheless, CD4⁺ T cells do exhibit a degree of lineage plasticity in response to strong inflammatory signals, which may be critically important in the context of cancer immunity and immunotherapy.^{126,129–131} A brief description of the conditions that give rise to these CD4⁺ T cell subsets, their characteristics, and their impact on antitumor immunity follows.

Th1 cells

Th1 cells are induced by the cytokines IL-12 and IFN- γ and express the transcription factor T-box expressed in T cells (T-bet).^{129,130,132} Th1 cells produce large quantities of IFN- γ , which drives M1 macrophage polarization, promotes autophagy, upregulates MHC-I, and promotes the proliferation and effector function of CD8⁺ T cells.^{129,130,133} Th1 cells are especially potent inhibitors of intracellular bacterial pathogens including *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Salmonella typhimurium*.^{129,130} Th1 cells are often beneficial to antitumor immunity, in part due to their production of high amounts of IL-2 and IFN- γ .^{124,126,131}

Th2 cells

Th2 cells are induced by IL-4 and IL-2 and express the transcription factor GATA-binding protein 3 (GATA-3) and the cytokine IL-4.^{129,134} Th2 differentiation is also driven by weak TCR signaling and costimulation through OX40.^{134,135} Through their production of IL-4, Th2 cells promote immunity against extracellular parasites and cause allergic diseases.^{136,137} Th2 cells also contribute to tissue repair.¹³⁶ While Th2 cells have been shown to play antitumor roles in some contexts, more often their mutual antagonism with Th1 and CD8⁺ T cells and promotion of M2 macrophage polarization is detrimental to antitumor immunity.^{126,138}

Th9 cells

Th2 and Th9 development are similar, but Th9 cells also require TGF- β and express the transcription factor purine-rich box 1(PU.1) and the cytokine IL-9.^{129,139} Through production of IL-9 and IL-21, Th9 cells participate in immune responses against a wide range of infectious diseases, in particular helminthic and fungal infections.¹⁴⁰ IL-9 can promote T activation by recruiting DCs and increasing their antigen presentation capacity, while IL-21 can boost proliferation and cytotoxicity of CD8⁺ T cells.^{140,141} Th9 cells promote antitumor immunity in

¹Recent evidence suggests that similar cell fate decisions play a role in CD8⁺ T cell priming, , the importance of this process in CD8⁺ T cells is less well understood.⁹⁵ The CD8⁺ T cell differentiation into exhausted, effector, memory, and stem-like cell fates has been the focus of more intense research efforts, particularly in the context of cancer.¹⁰⁸

some cancers, but correlate with poor survival in others.^{126,139,140} Therefore, additional studies are needed to better define the role of this subset in antitumor immune responses.

Th17 cells

Th17 cells development is driven by IL-6, TGF- β , and in some cases IL-21.^{129,142} Th17 cells express the transcription factor ROR γ T and the cytokines IL-17A and IL-17F.¹⁴³ IL-1 β and IL-23 can drive Th17 cells into a proinflammatory phenotype marked by expression of IL-22, and IFN- γ , IL-21, and Granulocyte and Macrophage Colony-Stimulating Factor (GM-CSF).¹⁴⁴ Th17 cells plays a key role in autoimmunity and defense from extracellular bacterial and fungal pathogens.^{129,142} Th17 cells phenotype is remarkably plastic, leading Th17 cells to play a complex role in cancer.^{126,144,145} This is further complicated by the interplay between Th17 and Treg cell fates and the mutual inhibition of these populations **(1.2.3 CD4⁺ Regulatory T cells)**.¹⁴⁶

Tfh cells

T follicular helper cells (Tfh) are induced by strong TCR signaling and the cytokines IL-6 and IL-21 and express the transcription factor B-cell lymphoma 6 (Bcl6) and the chemokine receptor CXCR5.^{130,147} CXCR5 recruits Tfh cells to B cell follicles within SLOs, where Tfh cells drive germinal center formation and B cell affinity maturation via the costimulatory ligand CD40L and the cytokines IL-4 and IL-21.^{130,147} Tfh serve unconventional but important roles in antitumor immunity by promoting tumor-specific antibody production and cooperating with B cells to form tumor-associated tertiary lymphoid structure (TLSs), supporting antitumor CD8⁺ T cell function.^{148–150}

1.2.3 CD4⁺ Regulatory T cells and their roles in cancer

Regulatory T cells represent a distinct, specialized population of CD4⁺ T that play vital roles in immune homeostasis **(Canonical Treg Functions)**.^{151,152} Treg identity is conferred by the transcription factor Foxp3, which enforces the Treg inhibitory gene program and suppresses proinflammatory gene expression.^{153–155} Despite composing only ~5-10% of total CD4⁺ T cells in steady-state conditions, Tregs are heterogeneous and exhibit a great deal of plasticity.¹⁵⁶ This diversity in part stems from Treg ontogeny—Tregs can be generated by multiple mechanisms **(Treg Development)**—and in part from activated Tregs undergoing differentiation into functional subsets that “mirror” conventional CD4⁺ T cell types.^{156–158} Nevertheless, Tregs share certain core features in their development, mechanisms of action, and roles in health and disease, including cancer. The role of Tregs in cancer is a central theme in my work, which through multiple avenues has converged on the role played by TLR2 signaling in reversing this function to promote antitumor immunity mediated by CD8⁺ T cells. Therefore, several features of Tregs are discussed below, with a particular focus on their relevance to Tregs in the setting of cancer.

Treg Development

The broadest categorization of Tregs divides them into two pools based on their ontogeny. The first pool is composed of Tregs that differentiate in the thymus (aka thymic Tregs; tTregs), while the second is composed of conventional CD4⁺ Tregs that differentiate into Tregs upon activation in peripheral tissues (aka peripheral Tregs; pTregs).^{154,155,159,160} tTregs differ from other CD4⁺ T cells and pTregs in that they exit the thymus committed to their specific lineage. This is partially driven by TCR signal strength during thymic development, although costimulation and cytokine

signaling are also involved.^{161–164} As previously discussed (**Signal 1: TCR-pMHC signaling**), T cells with high self-pMHC affinity are eliminated by negative selection, while T cells with low self-pMHC affinity develop into CD8⁺ and conventional CD4⁺ T cells.⁵⁸ Conversely, tTreg development requires an intermediate degree of self-MHC reactivity between that of conventional CD4⁺ T cells and cells that are eliminated by negative selection.^{161,162} In particular, this favors the development of Tregs with TCR specificity to rare self antigens (often tissue- and cell type-specific).¹⁶⁵

pTreg development differs greatly from tTreg development in several ways, including in the epigenetic and transcriptional states that coordinate tTreg and pTreg cell identity.^{159,160,166,167} Indeed, despite some similarities including a shared dependence on Foxp3 and overlapping suppressive mechanisms, pTregs are in other respects more analogous to the development of conventional CD4⁺ Th subsets as discussed above.¹⁵⁹ pTregs development requires TGF- β and IL-2 and typically occurs in mucosal immune sites.¹⁶⁰ pTregs prevent excess inflammation and limit immune responses to commensal microbiota, allergens, and other innocuous nonself antigens.^{168,169} Accordingly, pTreg TCRs are generally held to be specific to nonself antigens, unlike tTregs.¹⁵⁴ pTregs also less stably express Foxp3, perhaps as an adaptive mechanism in the event of infection, and serve complementary roles to tTregs to maintain tolerance.^{154,160}

Canonical Treg Functions

The best-known (and first identified) function of Tregs is to inhibit autoimmune disease.¹⁵³ The discovery of Tregs was enabled by several closely related discoveries, which recently earned three of the key scientists in this process the 2025 Nobel Prize in Physiology or Medicine.¹⁷⁰ First, Sakaguchi *et al.* reported that CD4⁺ T cells expressing CD25 prevented a wide variety of autoimmune pathologies in mice.¹⁷¹ Next, Brunkow, Ramsdell, *et al.* reported that *Scurfy*, a recessive, X-linked allele in mice that led to multiorgan autoimmunity, was caused by a mutation in the gene *Foxp3*.¹⁷² Additional reports made by Ramsdell's group in the same year also identified that the disorder immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) was the human equivalent of the *Scurfy* phenotype, which could be abrogated by complementation of *Scurfy* mice with the WT *Foxp3* allele.^{173,174} Soon after, the Sakaguchi and Ramsdell groups independently verified the importance of *Foxp3* in Treg development.^{175,176} Multiple lines of inquiry have subsequently shown that *Foxp3* expression is not only required for initiating Treg lineage commitment, but rather that continued expression of Foxp3 is required to sustain Treg suppressive function. This has been well documented by the Rudensky group, who showed that Foxp3⁺ Tregs are required throughout the lifetime of mice to enforce self-tolerance, confirming that peripheral tolerance is an active and dynamic process.¹⁷⁷ More recent work from the Rudensky group has shown that while the Foxp3-enforced gene program in Tregs does not require constant Foxp3 expression in steady-state conditions, Foxp3 is required to maintain Treg fitness in response to inflammation and in tumors.¹⁷⁸ This final point is particularly relevant to my own work, for which Treg lineage stability in tumor tissue has been a major theme. Namely, I have found that TLR2-mediated destabilization of Foxp3 expression in Tregs reduces Treg-mediated immunosuppression in tumors (**Chapters 3 and 4**).

In addition to inhibition of autoimmunity, Tregs also play roles in infection and wound healing.^{179,180} The contribution of Tregs to infection is multifaceted and includes both beneficial and deleterious effects. On the one hand, Tregs-mediated suppression of anti-pathogen effector cells can limit the magnitude or efficacy of T cell responses against foreign antigens.^{181–184} This

can lead to defective clearance of pathogens in the short term, and in the long term can contribute to defective memory cell formation, limiting the efficacy of recalled memory T cell responses upon secondary infection.^{185–189} On the other hand, Tregs limit tissue damage caused by excess inflammation during infection.^{188,190} Similarly, Tregs prevent the off-target activation of autoreactive effector T cells during infection, which can be caused by the concurrent release of self-antigens and inflammatory signals by dying cells.^{191,192} Finally, in some cases Tregs assist in the resolution of innate and adaptive immune responses after pathogen clearance without affecting disease burden.^{189,193} The push and pull between these dual effects of Tregs in the context of infection necessitates that Treg suppression be tightly regulated (**1.2.3 Treg Activation Stability**). Signaling through pattern recognition receptors (PRRs), which recognize microbially derived pathogen-associated molecular patterns (PAMPs), plays an important role in regulating this balance. My own work will focus extensively on this topic, especially with respect to how *Listeria*-derived ligands for TLR2 (**Chapter 3**) and components of bacterial membranes (**Chapter 4**) mediate the destabilization of Tregs in tumors to enable CD8⁺ T cell-mediated control of *Listeria* in the tumor microenvironment (TME).

This damage-limiting role in the context of infection intersects with another key function of Tregs that has been identified in Tregs in recent years, namely their roles in wound healing and tissue repair.¹⁹⁴ This was first observed by the Mathis lab in the context of muscle injury, and was attributed to a clonally-expanded Treg population expressing the cytokine amphiregulin.¹⁹⁵ This was soon followed by work from the Rudensky lab demonstrating a key role for IL-18 and IL-33 signaling in driving amphiregulin expression by a population of Tregs that expressed ST2 (i.e., IL-33R).¹⁹⁰ While not the sole mechanism by which Tregs contribute to healing, the IL-33-ST2-amphiregulin axis nevertheless has been widely reported to participate in Treg-mediated tissue repair across multiple tissues and injury types.^{196–198} However, this axis is not always beneficial, as IL-33-ST2 signaling in Tregs has also been linked to immune suppression in multiple cancer models.^{199–203} This observation is in line with the characterization of cancer as “a wound that will not heal”,²⁰⁴ although this platitude does not fully capture the multifaceted role of Tregs in cancer and antitumor immunity.

Mechanisms of Treg Suppression

The heterogeneity of Tregs, and the number of functions that they exercise, necessitates that the mechanisms by which Tregs suppress effector T cells are also varied and complex, and as such are not yet fully understood.^{156,205,206} However, several core mechanisms have been identified which can be broadly categorized along several dividing based on whether they affect effector T cells directly (i.e., by acting in an effector T cell-intrinsic manner) or indirectly (i.e., effector T cell-extrinsic), although in many cases the same Treg effector mechanism can act both directly and indirectly in different settings.^{156,205,206}

Direct T cell suppression comprises a mixture of contact-dependent and -independent mechanisms.^{156,205,206} Contact-independent suppression includes Treg production of immunosuppressive cytokines including IL-10, TGF- β , and IL-35, which exert pleiotropic effects that include upregulating coinhibitory receptors and inhibiting proliferation.^{207–210} Tregs can also suppress cells at a distance by catalyzing the breakdown of extracellular ATP to AMP then adenosine through the activity of CD39 and CD73, respectively; adenosine subsequently inhibits effector T cells upon binding to A2AR.^{211–213} The mechanisms of contact-dependent, direct Treg inhibition are more eclectic. For example, Treg-expressed PDL1 has also been shown

to limit T cell responses via PD1 signaling in T cells.²¹⁴ Tregs can also suppress T cells via direct cytosolic transfer of the secondary messenger cAMP through gap junctions, which inhibits effector T cell proliferation and IL-2 production.²¹⁵ On the extreme end of the suppressive spectrum, Tregs can mediate the direct cytolysis of effector lymphocytes via both the PRF-GzB-caspase and TRAIL-DR5 signaling pathways.^{216–220}

Indirect T cell suppression by Tregs comprises a similar diversity of mechanisms.^{156,205,206} Among these include Tregs outcompeting effector T cells for access to proinflammatory signals like IL-2, which Tregs selectively take up with greater efficacy than other T cells due to their constitutive expression of the high-affinity IL-2R α chain CD25.^{221–223} Similarly, Tregs outcompete effector T cells when aggregating around DCs.²²⁴ While this preferential occupation of DC contact area may function in part by simply blocking access to ligands needed for activation such as pMHC and CD80/86, it also enables Tregs to deplete these ligands from DCs via trogocytosis mediated by TCR and CTLA-4, respectively.^{225,226} However, Tregs also use more conventional mechanisms to mediate indirect T cell suppression by acting on DCs, including PDL1 and TGF- β .^{214,227,228} Together, these mechanisms provide myriad ways for Tregs to limit the functionality of APCs, thereby indirectly limiting the scope of effector T cell responses.²²⁹ My work in will touch on some of these roles, namely Tregs outcompeting CD8⁺ T cells for IL-2 and inhibiting antigen presentation to CD8⁺ T cells by APCs (**Chapter 3**).

Treg Activation and Stability

As a subset of CD4⁺ T cells, Tregs require the same three signals to become activated as their conventional counterparts—namely, TCR signaling (signal 1), costimulation through CD28 (signal 2), and cytokine signaling (signal 3). tTregs undergo higher levels of tonic TCR signaling than CD8⁺ or conventional CD4⁺ T cells, resulting in a somewhat different activation process that is nevertheless dependent upon TCR and especially CD28 signaling.^{230–235} Analogously to conventional T cells, the absence of these signals can lead to Treg dysfunction.^{222,236,237} Differences in the quality and quantity of these signals can also lead Tregs to downregulate Foxp3 expression, becoming so-called “ex-Tregs”, a process featured extensively in my work (**Chapters 3 and 4**).^{235,238–241} This process, termed Treg destabilization, involves changes in transcription factor expression and the Treg epigenome.^{166,167,242–244} Treg destabilization results in reduced Treg immunosuppression, and in some cases can lead Tregs to upregulate inflammatory genes or even to adopt characteristics of conventional CD4⁺ T cells.²⁴⁵ For example, IL-6 and IFN- γ signaling can cause Tregs to convert to an IL-17⁺ or IFN- γ ⁺ phenotype resembling Th17 and Th1 cells, respectively.^{246–249} However, this does not always occur, as cytokines involved in destabilization can also play a role in driving Tregs into functionally stable subsets that mirror and suppress specific CD4⁺ Th subsets.^{156,157,245,250} Thus, while Tregs (or at least tTregs) are nominally committed to their lineage, their dual ability to suppress—or, conversely, to reprogram into—other CD4⁺ T cell subsets confers remarkable plasticity to Tregs.^{245,250,251} This flexibility suggests that the deliberate modulation of Treg stability may offer opportunities for translational development, either to favor stability in inflammatory or autoimmune diseases or to decrease it in cancer.^{130,252,253} This will necessitate a greater understanding of the ways in which identical costimulatory and cytokine signals have similar or dissimilar effects on Tregs and conventional CD4⁺ T cells, many of which have already been described but not all of which are fully understood.^{254,255} My thesis will focus extensively on the role of Treg destabilization in mediating tumor control by a model of *Listeria* immunotherapy, showing that *Listeria*-dependent, TLR2-

and cDC2-mediated destabilization is functionally equivalent to partial Treg depletion (**Chapter 3**). The combination of reduced Treg immunosuppression and increased CD8⁺ T cell-mediated tumor control that we observe in our models would further suggest that differential impacts of costimulatory and cytokine signals on Tregs and other T cells play a role in our tumor models.

Tregs functions in cancer and opportunities in cancer immunotherapy

Tregs play a central role in the immune response against cancer, although the nature of their contribution can vary.^{256,257} Tregs are particularly impactful in solid tumors, in which Tregs often constitute a significant component of the TME.²⁵⁸ One meta-analysis of patient data showed that Tregs correlated with worse survival in cervical, renal, ovarian, melanoma, pancreatic, hepatocellular, gastric, and breast cancers.²⁵⁹ Later studies found similar prognostic value in non small-cell lung, prostate, and colorectal cancers and soft-tissue sarcomas.^{260–262} While the specific of Tregs in these malignancies can differ, the consensus from these studies is that Tregs can play a substantial role in supporting tumor development by inhibiting antitumor immunity.

Tregs accomplish this feat via a wide suite of effector mechanisms (**Mechanisms of Treg Suppression**) that act both locally and in tumor-associated lymphatic tissues, inhibiting both effector function and priming of antitumor T cells.^{257,258} Within tumors, Tregs suppressive mechanisms can target effector T cells directly to inhibit cytokine secretion or cytolytic function.^{263,264} Tregs in tumor tissue can also inhibit effector T cells indirectly by selective uptake of inflammatory signals like CD80/86 and IL-2, the latter of which has been demonstrated by myself and other groups (**Chapter 3**).^{226,265} Tregs also modulate tumor APCs; for example, our lab showed that Tregs inhibit the cross-presentation of tumor antigen by type I conventional DCs (cDC1s) within tumor tissue.²⁶⁶ Within SLOs, Tregs inhibit tumor-specific T cell priming by employing many of the same mechanisms—both T cell- and APC-intrinsic—utilized in autoimmunity and infectious disease.^{257,258} A notable mechanism of Treg suppression, featured in my work, is the inhibition of antigen transport to lymph nodes by dendritic cells (**Chapters 2 and 3**).^{267–269} Altogether, these mechanisms create a formidable barrier to antitumor immunity.

Understanding how therapies modulate Treg-mediated immune suppression has become a major goal in cancer immunotherapy.^{258,270,271} However, the first generation of checkpoint inhibitors—namely, α -PD-1/ α PD-L1/ α -CTLA-4—have inconsistent and sometimes opposing effects on Tregs, which may contribute to the lack of efficacy of these drugs in some cases.^{48,272,273} While some studies have shown that PD-1 blockade can inhibit Treg function, others have shown that α -PD-1 and α -PD-L1 can increase the frequency and suppressive activity of Tregs in tumors to enhance immune suppression.^{46,274–277} Conversely, α -CTLA-4 has been theorized to function in part by depleting CTLA-4⁺ Tregs from tumor tissue based on findings in mice, although the validity of this mechanism in human patients is contested.^{278–280} These findings suggest that abrogating Treg-mediated immunosuppression may be one of the critical functional determinants of checkpoint blockade.^{48,281} Indeed, the efficacy of Treg depletion in mice suggests that inhibiting regulatory T cells in cancer patients—or even better, depleting tumor Tregs—is a viable strategy to promote antitumor immunity.²⁸¹ This is in agreement with my own work, which has shown that TLR-mediated Treg destabilization and partial Treg depletion by artificial means are both methods of achieving tumor control that are at least in part functionally interchangeable (**Chapters 3 and 4**).

The goal of predictably inhibiting Tregs in cancer has contributed to efforts to develop novel checkpoint inhibitors,^{258,270,272,282} including blocking and depleting antibodies.^{48,281} Among the former category are antibodies specific to the coinhibitory receptors TIGIT, TIM-3, and LAG-3, which have often been tested in combination with approved checkpoint inhibitors and shown synergistic inhibition of tumor growth (**Table 1.1**).^{33,43,273,283–285} Several drugs that deplete regulatory T cells also show promise, but this approach is complicated by the fact that systemic Treg depletion causes autoimmunity in both preclinical mouse models and human patients.^{286–290} To circumvent this, investigators have searched for targetable surface markers on tumor Tregs to enable localized depletion without affecting systemic immune tolerance.⁴⁸ Chemokine receptors are a promising candidate for this approach, as several chemokine receptors are expressed at higher levels on tumor Tregs than other T cells or Tregs in other tissues.^{266,291–293} CCR4 and CCR8 are of particular interest due to strong effects in preclinical models, and clinical trials using α -CCR4 and α -CCR8 depleting antibodies are underway.^{281,294–298}

Targeting Treg lineage stability offers an alternative to directly inhibiting or depleting Tregs.^{244,252} Some blocking antibodies—including α -CTLA-4, α -LAG-3, and α -CD25—have the capacity to elicit Treg destabilization in some settings.^{285,299,300} Agonistic antibodies against the coreceptors GITR and OX40 can elicit Treg reprogramming in cancer.^{301–304} However, whether destabilization of *Foxp3* expression was the ultimate cause of tumor control was not proven with either the blocking or agonistic antibody therapies, which may also act directly on effector T cells independently of Tregs.^{285,299–304} The capacity for Treg destabilization to mediate antitumor immune has been more directly supported by studies using genetically modified mice to enable Treg-specific deletion of genes involved in the positive regulation of *Foxp3* expression.^{178,305–315} Nevertheless, one caveat in attributing these effects solely to destabilization of *Foxp3* expression in Tregs is the fact that many of the targeted pathways also regulate other transcription factors and cellular processes.³¹⁵ However, three recent reports showed that *Foxp3* is dispensable for Treg stability in the steady state but is required to prevent Treg reprogramming in response to inflammation, including in the setting of cancer.^{178,316} Together, the cumulative evidence from these studies suggests that destabilization of *Foxp3* expression in Tregs should be a target for cancer immunotherapy development. Indeed, several findings from my own work are in line with this conclusion, showing that strong inflammatory signals can destabilize *Foxp3* expression in tumor Tregs, critically contributing to antitumor immune responses (**Chapters 3 and 4**).

1.3 Antigen presenting cells in cancer

As outlined above (**1.2.1, T cell activation**), virtually all T cell responses are predicated upon T cell activation.⁵⁰ The expression of MHC-I on almost all nucleated cells, and of MHC-II on select nonprofessional APCs, enables a variety of cell types to stimulate CD8⁺ and CD4⁺ T cells, albeit less potentially than professional APCs.^{317,318} Professional APCs are a category of cells that excel at priming T cells due to their high capacity to take up and present antigens on MHC and their upregulation of costimulatory molecules and cytokines upon activation by inflammatory signals.^{319,320} Professional APCs drive T cell priming in the vast majority of cases, including cancer.^{321–324} Interactions between T cells and professional APCs within tumors can promote or inhibit antitumor T cell function and Treg-mediated immunosuppression.^{266,325–328} The ability of intratumoral APCs to sustain T cell responses within tumors can be especially critical in cases of tumor cells downregulating antigen presentation, a common occurrence in response to strong antitumor T cell responses.^{329,330} The biology of APCs is therefore inextricably linked to T cells

in cancer, and attempts to engineer T cell-based immunotherapies will likely benefit greatly from considering how APCs contribute to or inhibit their efficacy. In this thesis, I will touch on critical roles played by APCs in mediating priming of CD8 T cells (**Chapter 3**), supporting CD8 T cell function in tumors (**Chapters 2, 3, and 4**), and modulating immune suppression by tumor Tregs (**Chapters 3 and 4**). Therefore, this section will contain a summary of the basics of antigen presentation, major APC types, their functions in health and infectious disease, and their role in cancer. Finally, I will conclude with a survey of APC-based cancer immunotherapies.

1.3.1 The basics of antigen presentation

The process by which antigens are presented on MHC-I and MHC-II differ substantially but share several core features.³³¹ The first step in either pathway is the acquisition of antigens, followed by proteolytic processing into short polypeptides, then delivery and loading of peptides onto MHC molecules residing in the ER-Golgi network (MHC-I) or endosomal network (MHC-II), and finally the display of these pMHC complexes on the cell surface.³³¹ This highly coordinated process allows T cells to survey protein composition of both intracellular and extracellular environments and detect when nonself antigens.³³¹

MHC-II is the primary mechanism for the surveillance of extracellular antigens (although MHC-I serves a specialized role in this process; see below).^{332,333} Antigens presented by MHC-II are acquired through mechanisms including receptor-mediated endocytosis, macropinocytosis, and phagocytosis.³³² Internalized antigens proceed through the endolysosomal network and are cleaved by proteases to release short peptides.³³⁴ Peptides are then loaded onto empty MHC-II molecules with by chaperone proteins.³³²

In contrast to MHC-II, MHC-I is mostly involved in regulating responses to intracellular antigens, befitting its role in activating CD8⁺ T cells.³³¹ The majority of MHC-I peptides are produced by proteasomal protein degradation in the cytosol, whereupon they are fed into the ER by the transporter associated with antigen presentation (TAP) complex.^{335,336} Most TAP-dependent peptides originate from cytosolic proteins, with a fraction also derived from secretory or membrane-bound proteins synthesized in the endoplasmic reticulum (ER) and translocated to the cytosol.³³⁷ Other peptides enter the MHC-I pathway via TAP-independent mechanisms.³³⁸ TAP-independent peptides largely originate in the ER, although some appear to be derived from the cytosol via an unidentified mechanism.³³⁸ TAP-dependent and -independent peptides are then loaded onto MHC-I molecules by the peptide loading complex (PLC) before trafficking to the cell surface.^{331,339} My work touches on endogenous MHC-I antigen presentation of *Listeria* antigens in the context of *Listeria* immunotherapy (**Chapter 2**).

While endogenous peptides comprise the majority of pMHC-I complexes on most cells, some cell types are also capable of presenting exogenous antigens on MHC-I via a process termed cross-presentation.^{333,340} Cross-presentation enables CD8⁺ T cells to respond to infected or neoplastic cells with abnormal features (e.g., that have downregulated MHC-I) or to nonself antigens produced by nonprofessional APCs.³³³ Cross-presentation therefore plays a major role in generating CD8⁺ T cell responses in cancer and infectious disease.^{330,341,342} My work found a role for antigen cross-presentation in driving tumor-specific CD8⁺ T cell immunity in the context of *Listeria* immunotherapy and innate immune stimulation of APCs (**Chapters 2 and 3**).

1.3.2 Regulation of APCs by PRRs

Most professional APCs present antigens on both MHC-I and MHC-II at steady state.³⁴³ However, APCs integrate extracellular signals to regulate antigen processing and presentation machinery and costimulatory ligand and cytokine expression at both the transcriptional and posttranslational level.^{344–347} This intersects with regulation of APC localization; some APCs constitutively localize to SLOs, while others reside in non-lymphatic tissues and migrate to lymphatic tissues upon sensing inflammatory stimuli.^{348,349} As each of these aspects of APC biology are key determinants of APC function, understanding how different extracellular signals differentially regulate them is critical to understanding T cell-mediated immune responses.

Perhaps the most important category of signal regulating APC function are signals through pattern recognition receptors (PRRs).^{75,350} PRRs recognize conserved molecular moieties derived from pathogens or damaged cells termed pathogen- and damage-associated molecular patterns respectively (PAMPs and DAMPs).⁷⁵ PRRs enable rapid innate immune responses against a wide array of pathogens.⁷⁵ My thesis will repeatedly return to the topic of how sensing of bacterial and viral PAMPs by PRRs in APCs differentially affects APC regulation of T cells in cancer by influencing their expression of pMHC (**Chapters 2 and 3**), costimulatory ligands (**Chapters 3 and 4**), and cytokines (**Chapter 4**).

Non-TLR PRRs in immunity

Pattern recognition receptors are primarily categorized on the basis of shared structural features, with secondary groupings by subcellular localization and signaling cascades.³⁵¹ The primary PRR families are Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), AIM2-like receptors (ALRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and C-type lectin receptors (CLRs).³⁵¹ Members of these families typically differ in their ligand-binding domains to enable the detection of different PAMPs and DAMPs while sharing largely homologous signaling domains.³⁵² Consequently, there is often significant overlap in the signaling cascades elicited by each PRR within a given PRR family, although significant differences due to the location of signaling and the specific molecules that mediate signal transduction also exist.^{351,352} A brief description of NLRs, ALRs, RLRs, CLRs, and the unrelated cGAS-STING pathway follows. As TLRs are a primary focus of my work, they will be discussed separately in the following section.

NLRs reside in the cytosol, where they are capable of recognizing a wide variety of PAMPs.^{351,353} Ligand binding by NLRs leads to the oligomerization of multiple NLR molecules via their CARD or PYD domains, at which point they can either drive the expression of proinflammatory genes through the NF- κ B and MAPK pathways or mediate the formation of inflammasome complexes.³⁵³ ALRs, another family of cytosolic PRRs, recognize intracellular DNA, also leading to CARD-mediated oligomerization and inflammasome formation.³⁵¹ Inflammasomes are large multimeric complexes that assemble around ALR and NLR oligomers to recruit and cleave pro-caspase-1 into its active form.³⁵⁴ Through a series of intermediaries, Caspase-1 cleavage leads to pyroptosis, an inflammatory form of programmed cell death involving the secretion of IL-1 β , IL-18, and HMGB1.³⁵⁵

Like ALRs, RLRs and the cGAS-STING pathway are cytosolic PRRs that recognize nucleic acids.^{356,357} RLRs and cGAS drive cytokine expression, particularly type I interferons (TI IFN).^{356,357} However, these receptors differ in several respects. RLRs recognize viral RNA,

leading to CARD-mediated oligomerization in a manner similar to NLRs and ALRs but leading to the activation of the transcription factors NF- κ B, interferon regulatory factor 3 (IRF3), and IRF7.³⁵⁶ The STING pathway possesses multiple signaling cascades, only one of which employs cGAS.^{357–359} In the well-known cGAS-dependent pathway, cGAS oligomerizes upon binding to DNA, leading cGAS to synthesize the signaling intermediate cyclic GMP-AMP (cGAMP) which binds to STING to activate NF- κ B and IRF3.³⁵⁷ The cGAS-independent STING pathway is less well-defined, and it is possible that STING signaling has more than two distinct pathways.^{358,359}

Apart from TLRs, the final group of PRRs is CLR.³⁵¹ CLRs share partial structural homology with C-type lectins, from which they derive their name.³⁶⁰ CLRs recognize a wide array of ligands, both exogenous and endogenous, including components of essentially all phyla of pathogens (i.e., viruses, bacteria, fungi, protozoa, and helminths), dead cells, and in some cases multiple ligands.³⁶⁰ CLR ligation can mediate signaling alone or in conjunction with receptor-mediated endocytosis, although the precise relationship between signaling and endocytosis remains unclear.^{360–362} Their capacity to signal and mediate endocytosis upon ligand binding can enable the uptake and presentation of antigens on both MHC-I and MHC-II.^{360,362}

TLRs

Toll-like receptors (TLRs) are a major PRR family with important roles in innate and adaptive immunity.^{363–365} The best-known function of TLRs—and the one most important to my work—is the activation of APCs to drive T cell priming.^{75,366} Indeed, TLR4 was the first identified PRR, and its discovery and recognition of its binding to bacterial LPS were a major breakthrough in understanding how innate and adaptive immune responses were linked.^{367–369} Since these initial discoveries, countless studies have reaffirmed the importance of TLRs in initiating adaptive immune responses, while seemingly establishing that the recall of previously primed T cells upon secondary infection does not require TLRs.^{370–372} However, more advanced methods and model systems have partially challenged our understanding of how these receptors exert control over immune responses.^{75,366,373} A key component of my graduate work was showing that TLR signaling is required to reactivate preexisting *Listeria*-specific memory T cells within the suppressive TME (**Chapter 3**). This is likely due to immunosuppression in the TME, which may necessitate a higher threshold of inflammatory signals to reactivate T cells than other model systems in which T cell responses have been studied.

Like other PRR families, TLRs share common structural features that enable their function while also enabling recognition of a wide variety of ligands (**Table 1.2**).^{363,374} TLRs are dimeric transmembrane proteins with an extracellular ligand-binding domain composed of leucine-rich repeats (LRRs) and an intracellular Toll IL-1 receptor (TIR) signaling domain.³⁷⁴ Ligand binding to LRRs elicits a conformational change which brings the TIR domains of each TLR monomer into close proximity.³⁷⁴ Most TLRs form homodimers, with the exception of TLR1/2/6, which form TLR2/1 and TLR2/6 heterodimers.³⁷⁴ The TIRs of dimerized TLRs then recruit intracellular adaptor proteins to mediate signal transduction.^{363,375} In the canonical view of TLR signaling, TLR2/1, TLR2/6, TLR5, TLR7, and TLR9 signal via TIRAP and MyD88, which through a series of intermediaries, leads to the activation of AP-1 and NF- κ B.³⁷⁵ TLR7 and TLR9 also activate IRF7 via TIRAP/MyD88, while TLR2/1, TLR2/6, and TLR5 do not.³⁷⁵ By contrast, TLR3 signals through the adaptors TRAM and TRIF to activate AP-1, NF- κ B, and IRF3, while TLR4 uniquely activates both pathways.³⁷⁵ These transcription factors connect TLR signaling to APC function; IRF3 and IRF7 are both strong inducers of T1 IFNs, AP-1 and NF- κ B drive the

expression of proinflammatory cytokines and costimulatory ligands, and IRF3, AP-1, and NF- κ B upregulate components of MHC presentation pathways.^{344,375}

Table 1.2. Summary of TLR expression, specificity, and signaling

TLR	Expressed		Known Ligands	Known Pathogens	Primary Location	Primary Adaptor(s)	Primary Coreceptor(s)	Ref
	Mo	Hu						
2	✓	✓	lipoproteins, zymosan, viral proteins, β -Glu can, tGPI-mutin	bacteria, viruses, fungi, parasites	cell surface	MyD88	TLR1, TLR6, TLR10, CD14, CD36	376–378
3	✓	✓	dsRNA	viruses	endosomes	Trif	n/a	378
4	✓	✓	LPS, viral proteins, mannan, GPI	bacteria, viruses, fungi, parasites	cell surface	MyD88, Trif	LBP, CD14, MD-2	378,379
5	✓	✓	bacterial flagellin	bacteria	cell surface	MyD88	n/a	378
7	✓	✓	ssRNA	bacteria, viruses, fungi	endosomes	MyD88	CD14	378,380
8	✗	✓	ssRNA	bacteria, viruses	endosomes	MyD88	n/a	378
9	✓	✓	CpG DNA motifs	bacteria, viruses, fungi, parasites	endosomes	MyD88	CD14	378,380
10	✗	✓	Shared w/ TLR2 (?)	bacteria, viruses	cell surface	MyD88, Trif	TLR2, other TLRs (?)	381
11	✓	✗	bacterial flagellin, <i>T. gondii</i> profilin	bacteria, parasites	endosomes	MyD88	TLR12	378,382
12	✓	✗	<i>T. gondii</i> profilin	parasites	endosomes	MyD88	TLR11	382
13	✓	✗	bacterial rRNA, viral ssRNA	bacteria, viruses	endosomes	MyD88	n/a	378,383,384

While the signaling cascades downstream of TLR ligation largely overlap with one another, their effects on APCs can differ substantially.^{375,385} Differential expression of TLRs by APC subsets is a major factor underlying these differences, but another is the signaling pathways engaged by each receptor.^{75,385,386} Using T1 IFN as an example, TLR9 elicits T1 IFN production through IRF7, while TLR2 does not.^{375,387} However, factors including the location (i.e., at the cell surface or from endosomes) and cell type can reshape the outcome of TLR signaling.³⁷⁵ Continuing with the example of T1 IFN, TLR9 signaling occurs in both plasmacytoid DCs and conventional DCs (pDCs and cDCs), but TLR9 elicits much higher T1 IFN in pDCs than in cDCs.^{387,388} Conversely, while convention dictates that TLR2 signals from the cell surface and does not activate T1 IFNs, reports have shown that TLR2 can drive T1 IFN expression via signaling in endosomes in inflammatory monocytes, but not cDCs or macrophages.^{389–393} However, other reports have shown TLR2-mediated T1 IFN production in other cell types, in some cases from the cell surface, and have variously attributed this effect to MyD88 or TRIF pathways.^{389–394} The diversity of mechanisms by which TLR signaling elicits—or fails to elicit—T1 IFNs exemplifies the context-dependent nature of TLR signaling, in part explaining how similar PAMPs can have dissimilar effects in different tissues, cell types, and infection models.^{375,385} These subtle differences played a role in my thesis, in particular with respect to the distinct effects of different TLRs on Treg stability *in vitro* and *in vivo* (**Chapters 3 and 4**).

1.3.3 APC types and their roles in cancer

The initial activation of T cells by APCs is the critical first step in driving T cell adaptive immune responses (**1.2.1 T cell activation, 1.3.1 The basics of antigen presentation**). Several immune cell populations have evolved alongside T cells to play this role.³⁹⁵ Several of these populations, namely macrophages and B cells, also exert critical immune effector functions to mediate the neutralization and killing of invading pathogens and the clearance of infectious material from surrounding tissues in addition to their APC functions.^{396–398} Dendritic cells (DCs), another APC population, primarily function by controlling T cell immune responses.³⁹⁹ However, while APC subsets are ontogenetically distinct and their functions generally nonredundant, *in vivo* T cell responses are coordinated by heterogeneous populations of APCs.^{400,401} My work emphasizes this fact by showing that antitumor immunity elicited by *Listeria* immunotherapy and TLR ligands requires the coordinated activity of multiple DC types (**Chapters 2, 3, and 4**). While the following will introduce separate APC types and discuss their roles in cancer as distinct entities, it is important to remember that the situation is nuanced and complex *in vivo*.

Dendritic cells

Dendritic cells (DCs) are a rare and specialized APC subset that, despite their relative lack of abundance, are critical in driving T cell immune responses.⁴⁰² DCs, so named for their unique, branched dendrite morphology, were discovered by Ralph Steinman in 1973.⁴⁰³ Subsequent work by Steinman and colleagues went on to show that DCs are the APC type most able to prime T cells *in vitro*.^{404–406} The functional importance of DCs as APCs was later confirmed by studies which showed that DCs not only initiated T cell activation *in vivo* but also influenced the polarization of T cells into distinct functional subsets.^{321,407–413} This function of DCs is mediated by their sensing of signals derived from specific tissue environments, PAMPs, and cytokines, which in turn enable DCs to tailor T cell immune responses to specific immunological conditions.^{78,399,414–416} DCs are therefore incredibly important for linking the innate and adaptive

arms of the immune system, and recognition of this importance ultimately led to Steinman sharing the 2011 Nobel Prize in Physiology or Medicine for the key conceptual advancements in immunology that the discovery of DCs enabled.^{417,418} My first introduction to DCs was during the introductory course for the UC Berkeley MCB graduate program, and from the moment I learned about them I became enthralled with their capacity to regulate adaptive immune responses. This fascination was a driving factor throughout my PhD, and is reflected in my work both with *Listeria* immunotherapy (**Chapters 2 and 3**), TLR ligands (**Chapters 3 and 4**), and work not included in this thesis related to CXCR3⁺ Tregs in cancer.²⁶⁶ My work has repeatedly returned to the topic of how individual DC subsets influence Tregs (cDC2s) and CD8⁺ T cells (cDC1s), and the following review of DCs is critical to understanding my work in the context of the field.

Dendritic cell development

Like T cells, DCs can be divided into subsets by their ontogeny, phenotype and function.^{399,419–423} The broadest categories are plasmacytoid DCs (pDCs) and myeloid DCs (mDCs).⁴²⁰ mDCs possess the typical “dendritic” morphology originally described by Steinman, while pDCs are morphologically similar to plasma cells.^{424,425} pDCs can arise from either lymphocytic or myeloid progenitors and express the transcription factor TCF4.^{426,427} In some respects, pDC development mirrors mDC development (e.g., both rely on FMS-like tyrosine kinase 3 (Flt3) signaling), but ultimately these subsets differ in their expression of transcription factors like PU.1.^{427–429} mDC subsets share morphological features and high expression of the surface markers CD11c and MHC-II and are further subdivided into monocyte-derived DCs (moDCs), which differentiate from monocytes, and conventional DCs (cDCs), which arise from committed DC progenitors.^{419,420,425} moDCs rely upon unique signals during their development (Macrophage Colony-Stimulating Factor (M-CSF) or IL-4 and GM-CSF, but not Flt3) but ultimately express similar transcription factors to cDCs, particularly cDC2s (e.g., Zbtb46, IRF-4).^{421,422,430,431} moDCs play a limited role in repopulating tissue-resident DCs under homeostatic conditions, but are strongly induced by inflammation.^{420–422,432}

Excluding RORγT⁺ DCs, which were recently described and do not feature in this work, the other major DC lineage cDCs.^{433,434} cDCs are the main APC type to prime T cells under most conditions and play important roles in infectious disease, autoimmunity, and cancer.^{435,436} Lineage-tracing and gene knockout mouse models have led cDC development to be relatively well understood, which in turn has facilitated the development of tools to study their functions in various diseases.^{420,427,435,437} cDCs arise from a common dendritic cell progenitor (CDP) under the influence of Flt3 signaling.⁴³⁸ At some point during their development, pre-DCs begin to develop into type 1 and type 2 cDCs (cDC1s and cDC2s, respectively).^{420,438,439} cDC1s express the transcription factors Batf3 and IRF8, and in mice cDC1s can be distinguished by the cell-surface markers XCR1 and either CD8 (for SLO-resident cDC1s) or CD103 (for non-SLO-resident cDC1s, discussed below).^{437–440} cDC2s express Zeb2 and IRF4 and can be distinguished from cDC1s by the surface markers CD11b and SIRPα (cell-surface markers to distinguish between SLO-resident and -nonresident cDC2s have not been defined).^{437–442} cDC2s can be further subdivided into anti-inflammatory cDC2A and pro-inflammatory cDC2B, which are identified by T-bet and ESAM expression, respectively.^{443–445} While my work does not distinguish between cDC2As and cDC2Bs, it will touch on the vital role played by cDC2s generally in regulating Tregs in the context of cancer (**Chapter 3**) and TLR stimulation (**Chapters 3 and 4**).

Dendritic cell localization, activation, and antigen presentation

DC localization plays an important role in regulating T cell responses.⁴⁰² DC localization is tightly regulated, and localization patterns of DC subsets may play a role in their preferential activation of distinct T cell subsets (e.g., CD4⁺ vs. CD8⁺) *in vivo*.^{446–449} Constitutive expression of select chemokine and sphingosine receptors (e.g., CCR7, S1P1) helps to delineate a pool of SLO-resident DCs.^{437,450–456} Both SLO-resident and nonlymphoid tissue-resident DCs contribute to T cell priming; generally, SLO-resident DCs capture and present antigens borne into SLOs by blood, lymph, or migratory APCs, while migratory nonlymphoid tissue-resident DCs traffick antigens from their tissue of origin to SLOs.^{457–461} While DCs constitutively traffick between SLOs and other tissues during homeostasis, the antigen uptake and migration by nonlymphoid tissue-resident DCs is enhanced by inflammatory signals (e.g., PAMPs).^{75,448,462} This process, variously termed activation or maturation,² plays a key role in priming T cell responses in infection, autoimmunity, and certain forms of cancer immunotherapy.^{75,448,453,465} The influence of DC activation on T cell responses in infection and cancer will feature prominently in my work (**Chapters 3 and 4**). I will therefore briefly discuss the major APC functions, specific localization patterns, signaling pathways controlling activation of individual DC subsets below.

moDCs are an adaptable DC cell type that can serve various functions depending on the signals that they receive during and after their development.^{421,422,466} This adaptability enables moDCs to play roles in response to viral, bacterial, fungal, and protozoal infections.^{467–471} Despite this versatility, moDCs cannot fully substitute for cDCs in all cases, but rather appear to serve complementary functions.⁴⁷² This may be in part due to differences in the migratory capacity, although moDCs regulate T cell responses both in SLOs to and in nonlymphoid tissues.^{422,473–475} moDCs express TLR1/2/4/6/7/8/9, and signaling by these TLRs plays a major role in moDC development and function, including by driving T cell differentiation.^{422,438,474,476} moDC diversity enables these cells to serve both proinflammatory and antiinflammatory functions and to prime both CD8⁺ and CD4⁺ T cell responses (including Th1, Th2, Th17, and Treg).^{421,422,466,477–479}

cDC1s excel at the cross-presentation of antigens on MHC-I and IL-12 expression and play a central role in driving CD8⁺ T cell and CD4⁺ Th1 responses.⁴⁸⁰ This makes cDC1s critical for immunity against intracellular pathogens and cancer (discussed below).^{480–488} Both CD8⁺ SLO-resident cDC1s and migratory CD103⁺ nonlymphoid tissue cDC1s can be vital for these functions, depending on the location of infection and access of these DCs to antigens.⁴⁸⁰ The ability of cDC1s to respond to pathogens is in part due to their expression of TLRs, although expression by mouse and human cDC1s does not align perfectly; while mouse cDC1s express TLR2/3/4/6/7/9, human cDC1s express TLR1/2/3/6/8.^{75,438} My thesis will return repeatedly to the role of cDC1s in cross-presentation to CD8⁺ T cells (**Chapters 2, 3, and 4**) and the role played by TLR signaling in this process (**Chapter 3**).

cDC2s specialize in the presentation of antigens to both Tregs and conventional CD4⁺ T cells.^{443–445} cDC2As and cDC2Bs preferentially drive the differentiation of specific Th subsets; however, the use of different model systems and phenotypic definitions across reports makes it difficult to draw definitive conclusions about which Th subsets each type favors.^{443–445} Overall, the ability of cDC2s to prime CD4⁺ T cells implicates cDC2s in immunity against essentially all categories of pathogen, both intracellular and extracellular, given the critical supporting roles

²I prefer the term activation, as non-activated DCs are developmentally mature and play multiple roles in coordinating adaptive immunity.^{463,464} I will use the term activation for the remainder of this work.

played by Th cells in infectious diseases.^{129,489} cDC2s also play a role in peripheral tolerance by priming of tTregs and polarizing naive CD4⁺ T cells into pTregs.^{323,445,490} The regulation of Tregs by cDC2s—which both mediate Treg accumulation in tumors under steady-state conditions and destabilize Tregs upon TLR2 stimulation—is prominent in my work (**Chapters 3 and 4**).

pDCs are less effective than other DC subsets at the presentation of antigens on MHC, but nevertheless contribute substantially to T cell priming during viral infections.^{424,491} pDCs are found in SLOs and the blood, but are particularly important in mucosal barrier tissues (e.g., lungs and intestines) where they are positioned to respond quickly to viral pathogens.^{424,491} pDCs highly express TLR7/9 and produce large quantities of type 1 and 3 interferons.^{424,491} pDC migration is distinct from cDC migration; pDCs primarily migrate through the blood rather than lymph, but also migrate to sites of inflammation and LNs upon activation.^{492,493} pDC-produced T1 and T3 IFNs contribute to the priming of virus-specific CD8⁺ T and Th1 cells.^{491,494–496}

To close, I will note that while drawing distinctions between DC subsets and their functions is useful, their functional specializations are not set in stone. While recent research has led to a trend of ascribing a deterministic role of ontogeny in DC function, I believe that this is an oversimplification, as every categorical distinction can be met with a counterexample.^{323,419,489} For example, while cDC1s are often casually referred to as the sole cross-presenting DC type *in vivo*, evidence from multiple model systems refutes this simplistic characterization and shows that cDC2s can cross-present effectively in specific circumstances.^{497–500} It is therefore critical to remember that environmental signals, notably including TLRs, play vital roles in driving DCs into different functional states that can drastically impact T cell-mediated immune responses.^{75,366} Simultaneous interactions between T cells multiple DC types further complicate the relationship between DC ontogeny and the outcome of T cell priming.⁴⁰⁰ This theme will feature prominently in my work with *Listeria monocytogenes* and small-molecule TLR ligands (**Chapters 3 and 4**).

Dendritic cells in cancer

DCs play important roles in regulating antitumor T cells, with their best-characterized role being the priming of *de novo* T cell responses.^{324,501,502} CD8⁺ T cells are thought to be the most potent mediators of antitumor immunity, and thus cross-presentation by cDC1s has been highlighted as a critical step in generating productive antitumor immune responses.^{503,504} Nevertheless, cDC2 can also contribute to this process via mechanisms including secreting inflammatory cytokines, priming Th cells that assist CD8⁺ T cell priming, and priming cytotoxic CD4⁺ T cells.^{269,502,505–507} DCs also regulate T cells after priming, which can occur via the provision of all three canonical T cell-activating signals (pMHC, costimulation, and cytokines), the recruitment of activated T cells, and the generation of niches that enable T cell function.^{266,326,473,508–517} Accordingly, a high frequency of tumor-infiltrating DCs—particularly activated DCs and/or cDC1s—is a beneficial prognostic marker in several cancers.^{512,518,519} Nevertheless, our understanding of the roles played by DCs within tumors—as opposed to in priming T cells in SLOs—is relatively limited. My work will return to this topic repeatedly, focusing in particular on the role played by DCs in reversing CD8⁺ T cell exhaustion within tumors both by increasing the presentation of tumor antigens on MHC-I (**Chapters 2 and 3**) and by destabilizing tumor Tregs to alleviate immune suppression in the TME (**Chapters 3 and 4**).

However, DCs are often incapable of exerting these antitumor functions in the absence of therapeutic intervention.^{324,502,520} A combination of intrinsic and TME-derived factors can drive

DC dysfunction to inhibit antitumor T cells or even to actively promote tumor growth.^{324,502,520} DC dysfunction in cancer is often mediated by cytokines, including both inhibitory (e.g., IL-10) and inappropriate levels of proinflammatory (e.g., IL-6) cytokines.^{324,502,520,521} Dysfunctional DCs can exhibit defects in all T cell-activating signals (i.e., signals 1, 2, and 3), migration, or a combination of these factors.^{324,502,517,521–523} A subset of DCs, termed tolerogenic DCs, also drive tolerance to tumor antigens.^{324,502,520,524,525} Tolerogenic DCs display reduced costimulatory and increased coinhibitory ligand expression and secrete inhibitory cytokines, but retain the capacity to present antigens and to migrate into and out of tumors and SLOs.^{464,526,527} This can result in either anergy or the differentiation of CD4⁺ T cells into pTregs, further reinforcing immune suppression.^{229,528–530} DCs can also utilize these mechanisms to inhibit T cell responses locally within tumors.^{326,531,532} While my thesis does not directly investigate DC suppressive function of in tumors, I will address the inverse question of tumor-infiltrating DCs inhibiting intratumoral Tregs in response to TLR2 signaling in the TME (**Chapters 3 and 4**).

Macrophages

Macrophages (MΦ) were the first immune cell type to be identified, a feat accomplished by Elie Metschnikoff in 1887.¹ Metschnikoff also was the first person to identify the role of MΦs in cellular immunity, describing how they took in pathogenic fungal cells, a process he later termed phagocytosis.¹ Since these discoveries, MΦs have come to be understood to play multiple roles in both immune and nonimmune processes, rendering them extremely important in maintaining homeostasis at the cellular, tissue, organ, and organismal level.⁵³³ However, my own work has not focused extensively on macrophages or their function as APCs. The following section is included to complete the picture of the major APC types that can play roles in controlling T cells in cancer, but is not strictly necessary to understand my work.

Macrophage development

MΦs are a diverse cell type that play critical roles at all stages of life including development, maintenance of homeostasis, tissue repair, and immunity.⁵³⁴ The wide array of processes in which MΦs participate necessitates a similar diversity of MΦ subsets, with specialized MΦ populations residing in almost all organ systems in the body.⁵³⁴ This is only complicated further when the origins and phenotypic plasticity of MΦ are considered, and a proper accounting of these dynamics is beyond the scope of my thesis.^{535–537} Some MΦ subsets can prime T cells, although their low ability to circulate between nonlymphoid tissues and SLOs by these ensures that they mostly present antigens borne through the lymph or blood or trafficked to SLOs by other cell types.^{538–540} MΦs also can present antigens to activated T cells within tissues following their initial priming, providing continuous input into T cell responses, including in cancer.^{328,538,541,542}

MΦ activation usually occurs directly within tissues.^{543,544} MΦ activation incorporates signals from PRRs, cytokines, and other receptors (e.g., tissue-specific integrins) to coordinate a response appropriate to the specific immunological challenge and tissue environment.^{543–546} There is thus no uniform result of macrophage activation; rather, different signals lead to the polarization of MΦs into distinct functional subsets.^{543–548} The classical definition of these subsets was into a binary distribution of “classically” (M1) or “alternatively” (M2) activated MΦs, which were conceived of as having pro- or anti-inflammatory properties respectively.^{549–551} More recent evidence using high-dimensional analyses and incorporating up-to-date knowledge of immune cell populations has challenged this paradigm, but as cancer immunology has been slower to adopt this more nuanced position, I will proceed with the archaic definitions of these

cells.^{547–552} M1 MΦs develop in response to Th1/CD8⁺ T cell-produced cytokines (e.g., IFN-γ, TNF-α) and TLR stimulation, while M2 MΦs develop in response to Th2 and Treg cytokines (e.g., IL-4, IL-10, TGF-β).^{397,544,553} M1 and M2 MΦs then engage in functions related to these T cell lineages; M1 MΦs produce IL-12 and proinflammatory cytokines to drive effector T cell responses, while M2 macrophages promote vascularization and repair and remodel tissues and stroma.^{397,544,553} While both M1 and M2 MΦs can present antigens, M1 MΦs typically exhibit higher expression of MHC-II, CD80, and CD86.^{397,544,553} Along with their phagocytic capacity and production of T cell-activating cytokines, these features render M1 MΦs generally more effective at promoting CD4⁺ Th1 and CD8⁺ T cell-mediated immunity.^{397,544,553}

Macrophages in cancer

Given the often key role played by CD4⁺ Th1 and CD8⁺ T cells in antitumor immunity, and historical results showing that activated macrophages contributed to tumor control, the hypothesis that M1 MΦs are antitumoral while M2 macrophages are protumoral was expressed from the outset of the M1/M2 MΦ paradigm.^{551,554,555} Experience would show this prediction to be largely accurate; while tumor-associated MΦs (TAMs) generally are predictive of poor survival, studies where M1 and M2 markers were taken into account have generally showed that higher frequencies of M1 MΦs or a higher ratio of M1:M2 MΦs were associated with longer survival and better responses to immunotherapy, while M2 MΦs exerted the opposite effect.^{556–563} Accordingly, targeting MΦs to skew the M1:M2 balance—and to enhance MΦ APC function—has become a major goal in cancer immunotherapy.^{556,564–566} However, several fundamental questions about TAMs remain unanswered, among which is the question of TAM ontogeny.^{567,568} While many studies have characterized TAMs as arising primarily from MoMΦs, others have demonstrated critical roles for TRMs in both primary and metastatic tumors.^{567–574} Ultimately, both MoMΦs and TRMs appear to participate in tumor development and progression, with the tissue and cell type of tumor origin likely contributing to this balance, and consideration of these factors may be important for MΦ-directed cancer immunotherapies.^{366,566–568}

B cells

Along with T cells, B cells constitute one of the primary arms of adaptive immunity.⁵⁷⁵ B cells are also the most abundant APC type in SLOs and the second most common in blood.^{576–578} Unlike other APC types but similarly to T cells, B cells express a non-germline encoded antigen receptor (BCR) that is generated by the rearrangement of BCR gene fragments during B cell development in the bone marrow.⁵⁷⁹ Antibodies are soluble, secreted forms of the BCR, and they play major roles in adaptive immunity.⁵⁷⁹ However, neither this nor function nor their role as APCs is relevant to my thesis work. I will therefore pass over other aspects of B cell biology and only briefly overview how B cells contribute to cancer.

B cells are a favorable prognostic indicator in several cancers.⁵⁸⁰ B cells in cancer both act as APCs and produce tumor-specific antibodies, the latter of which will not be discussed.⁵⁸¹ Some evidence suggests that B cells can function APCs within the TME.⁵⁸¹ B cells in cancer can help to form tertiary lymphoid structures (TLSs), SLO-like clusters of lymphatic tissue that form around developing tumors.^{581,582} By bringing together and organizing T cells and APCs within the broader tumor environment, TLSs can dramatically shape T cell responses to cancer.^{582–584} However, B cells also can play inhibitory roles in antitumor immunity, best exemplified by regulatory B cells (Bregs).⁵⁸⁵ Like Tregs, Bregs employ immunosuppressive mechanisms

including inhibitory cytokines (e.g., IL-10, IL-35, TGF- β) and coinhibitory ligands (e.g., PD-L1, CTLA-4).^{581,585} Bregs can also convert conventional CD4⁺ T cells to Tregs, reinforcing immune suppression in the TME.⁵⁸⁴ Conventional B cells can contribute to tumor progression via IL-4, which can inhibit tumor cell apoptosis and drive Th2 polarization.^{586,587} B cells in the TME can also promote angiogenesis by producing VEGF.⁵⁸⁸ Therefore, while these findings suggest that B cells may be an underappreciated target for cancer immunotherapy, greater understanding of their contributions to antitumor immunity will likely be required before this goal can be achieved.^{587,589}

1.3.4 Targeting APCs for cancer immunotherapy

Given the paramount importance of APCs in driving T cell-mediated immunity, targeting APCs presents an alternative path to improving T cell responses against cancer without targeting T cells directly.⁵⁹⁰ This concept is supported by epidemiological evidence showing a prognostic benefit of several T cell-stimulatory APCs in cancer (**1.3.3 APC types and their roles in cancer**). The validity of an APC-focused approach is further evidenced by the large number of preclinical studies that have demonstrated improved antitumor immunity by using techniques to increase APC number and functionality and drive the presentation of tumor antigens, as discussed below. These techniques have drawn on a wide range of concepts based in essentially all aspects of APC function, including several that I will not cover here (e.g., cytokine therapy, APC engineering, and others). Instead, I will limit my discussion to the two approaches most directly relevant to my own work, therapeutic cancer vaccines and PRR agonists. In particular, I will focus on microbial vaccines and TLR ligands, concepts which appear repeatedly in my thesis work (**Chapters 2, 3, and 4**).

Cancer vaccines

The broad goal of most cancer vaccines is to elicit protective adaptive immune responses to tumor antigens, with the goal of providing specific, long-lasting protection against cancer.^{113,591} In broad terms, these vaccines can be categorized as prophylactic or therapeutic. Prophylactic cancer vaccines are administered before the onset of carcinogenesis.^{591,592} However, these vaccines did not feature in my graduate work and will not be discussed further. In contrast, therapeutic cancer are administered after tumors have developed, with the goal of improving T cell responses to tumor antigens.¹¹³ Therapeutic cancer vaccines come in diverse forms ranging from purified macromolecules to microbial vectors.^{113,593} Macromolecular vaccines include nucleic acids encoding tumor antigens (both DNA and RNA) and purified tumor antigen peptides.^{593–596} DNA and RNA vaccines work by APCs (especially DCs) taking up these molecules, producing the antigens that they encode, and presenting these antigens to T cells.^{113,593–595} Peptide vaccines operate by a similar principle, but provide ready-made portions of tumor antigens.^{593,596} An extension of this principle are DC vaccines, in which DCs are differentiated from PBMCs *ex vivo*, loaded with tumor antigens, and reinfused into the patient.^{597,598} Sipuleucel-T, which uses DCs treated with antigen-GM-CSF fusion proteins, is the prototypical DC vaccine and one of only three approved therapeutic cancer vaccines.⁵⁹⁹ However, this drug has limited approvals, and ongoing preclinical and clinical studies are attempting to refine the DC vaccine approach and extend its utility to other indications.^{597–601} A difficulty in accomplishing this is the identification of common tumor antigens shared by numerous patients (which inherently limits its utility to those patients whose tumors express the antigen), or that the vaccine be personalized to a specific patient's tumor antigens (which is

slower and more expensive than an off-the-shelf vaccine).⁵⁹⁰ A possible solution to this problem is to target elements of the tumor other than tumor antigens themselves.^{602,603} My own work has shown that intravenous followed by intratumoral injection of attenuated *Listeria* can promote tumor control by eliciting an immune response against *Listeria* within the TME (**Chapters 2 and 3**). Tumor control is initially mediated by bystander effects of *Listeria*-specific CD8⁺ T cells (**Chapter 2**), but ultimately these cells and TLR2 signaling lead to enhanced tumor-specific CD8⁺ T cell-mediated immunity (**Chapters 2 and 3**).

The final category of therapeutic cancer vaccines, microbial cancer vaccines, has historical roots that can be traced back to the work of Dr. Coley.^{9,604,605} The remaining two approved therapeutic cancer vaccines are microbial cancer vaccines; these are talimogene laherparepvec (T-VEC), a modified Herpes simplex virus (HSV) that is both directly oncolytic and drives expression of GM-CSF, and Bacillus Calmette-Guerin (BCG), a non-cytolytic mutant of *Mycobacterium bovis* originally and still employed as a vaccine for tuberculosis.^{606–610} Like other cancer vaccines, microbial cancer vaccines seek to elicit tumor-specific T cell-mediated immunity, but they are unique in that they employ bacteria or viruses as vectors.^{611–614} These microbes usually are attenuated to limit their virulence while maximizing immunogenicity.^{611–614} The complexity of live microbial vectors offers several potential advantages, including the ability to activate multiple extracellular and intracellular PRRs.^{611,615} Microbes can also employ virulence mechanisms that allow for efficient antigen delivery.^{611,613,616,617} Most preclinical and clinical studies of microbial cancer vaccines have used vectors engineered to express tumor antigens.^{54,611–614} This strategy appears sensible, as well-designed vaccines should be able to elicit an optimal and predictable T cell response.⁶¹⁸ However, in practice, tumors may downregulate either the target antigen or antigen presentation entirely, such that cancer vaccines may require the engagement of T cells specific to other antigens (i.e., epitope spreading) in order to be effective.^{619,620} Notably, neither BCG nor T-VEC are engineered to express tumor antigens, but are the only approved microbial cancer vaccines^{606–609} Moreover, both vaccines are delivered to tumors directly and exert their effects via effector CD4⁺ and/or CD8⁺ T cells, suggesting that microbes in the TME are capable of driving tumor-specific immunity without expression of tumor antigens themselves.^{606–609} This is in line with my own observations in the context of *Listeria* cancer vaccines, particularly with observations that I made regarding the capacity of this therapy to improve tumor-specific T cell responses (**Chapters 2 and 3**). Furthermore, my work shows that this capacity is due in part to TLR2 stimulation by *Listeria* (**Chapters 3 and 4**); I will return to this idea in the following section (**PRR agonists**).

Listeria monocytogenes is one of the most extensively studied platforms for microbial cancer vaccines.^{621–623} Several aspects of the *Listeria* life cycle cause it to be a strong inducer of CD8⁺ T cell-mediated immunity: *Listeria* is largely taken up by phagocytes; it can escape from phagosomes into the cytosol, facilitating presentation of antigens by MHC-I; and, it elicits a CD8⁺/Th1-activating cytokine milieu.^{624–633} Cytokine-mediated CD8⁺ T cell responses to *Listeria* are, at least in part, promoted by PRR signaling, including a combination of TLRs, NLRs, and cGAS-STING.^{624,634} However, the extent to which each PRR contributes to *Listeria*-specific CD8⁺ T cell priming and whether these PRRs are redundant is controversial, which motivated me to study the role of TLR signaling in *Listeria* immunotherapy (**Chapter 3**).^{624,634} Secondly, *Listeria* virulence factors like *actA* and *inlB* can be deleted to decrease pathogenicity without affecting the immunogenicity of *Listeria* cancer vaccines.^{635–637} *Listeria* with mutations in both genes—*L. monocytogenes* $\Delta actA \Delta inlB$, usually referred to as live, attenuated, double-deleted

(LADD)—has been extensively tested in clinical trials, although other similar strains have also been studied.^{621–623,638} Finally, *Listeria* is genetically tractable, facilitating the generation of new mutant strains and the overexpression of tumor antigens in order to optimize its utility as a cancer vaccine vector.^{621–623} LADD cancer vaccines have been engineered to express a variety of TSAs and TAAs including HER2 (osteosarcoma, other malignant solid tumors), PSA (prostate cancer), mesothelin (mesothelioma, prostate cancer), HPV oncoprotein E7 (cervical cancer) or a multiple antigens (NSCLC, prostate cancer; separate antigen combinations).^{639–649} These drugs were administered intravenously (IV), following the long history of IV *Listeria* in preclinical cancer vaccine models.^{621–623,637} However, while these trials showed a generally acceptable safety profile, several immune-related adverse events were noted, and none showed sufficient efficacy to move forward to phase 3 clinical trials.^{639–649} This lack of efficacy, despite eliciting high frequencies of CD8⁺ T cells in SLOs, suggests that using IV *Listeria* alone with the sole goal of priming T cells may be insufficient for these drugs to exert their intended function.^{621–623} Greater insight into the mechanisms by which *Listeria* modulates host immunity—both within the TME and SLOs—is required. This was the primary factor motivating our initial comparison of IV and intratumoral (IT) *Listeria* injection in tumor-bearing mice (**Chapter 2**).

PRR agonists

By some definitions, PRR agonists were the first cancer immunotherapies. Coley's toxins are understood to function in part by stimulating TLRs, while BCG is characterized by some as more of a TLR agonist than a vaccine, as it lacks tumor antigen expression and is known to stimulate several TLRs including TLR2/4/9.^{114,650–652} More recently, BCG has been joined by the TLR agonists imiquimod (a TLR7 agonist approved for topical use in the treatment of skin cancer), and monophosphoryl lipid A (MPLA, a TLR4 agonist approved for use as an adjuvant).^{653,654} Each of these PRR ligands is speculated to work at least in part by modulating the activity of APCs to drive better T cell responses.^{652–654} It is therefore unsurprising that ligands for several PRRs have been extensively studied for cancer immunotherapy.^{114,655}

Among the cytosolic PRRs, STING agonists (and to a much lesser extent NLR and RLR agonists) have seen a surge in interest over the last decade.^{114,655} This has followed from the identification of STING as the major PRR for cytosolic DNA, and especially from the identification of cyclic dinucleotide (CDN) 2'3'-cGAMP produced by cGAS as the direct source of STING ligands *in vivo*.^{656,657} Artificial CDNs have generated substantial interest, and clinical trials for some of these are ongoing.^{658,659} This interest is due in large part to the ability of STING agonists to induce strong T1 IFN responses, which have been associated with strong antitumor CD8⁺ T cell immunity.^{658–660} RLRs, which are also potent inducers of T1 IFNs, have been also been targets for drug development for this reason, although only one of these selectively targets RLRs without also activating RNA-sensing TLRs.^{661,662} In contrast, NLRs have been relatively less intensively studied as cancer immunotherapeutics. Mifamurtide, a synthetic muramyl dipeptide (MDP) NOD2 agonist, has been approved for the treatment of osteosarcoma in the EU and is under investigation as an adjuvant for HER2 peptide vaccines in the USA.^{663,664} NLR agonists have a more complicated relationship to T1 IFN signaling than STING and RLRs, and neither MDP (NOD2 agonist) nor NLRP3 agonists mediate T1 IFN production in the absence of accessory ligands.^{665–667} Instead, NLR agonists activate inflammasomes in immunosuppressive TAM, causing the pyroptosis of these cells and releasing IL-1 β and IL-18 into the TME.^{114,665} This demonstrates that while T1 IFN-inducing PRRs are often touted as the gold standard of

PRR agonists in cancer therapy, this feature is not the sole determinant of antitumor efficacy. The ability of non-T1 IFN-producing TLRs to drive antitumor T cell-mediated immunity will feature in my work, which identified Treg destabilization as a necessary component of TLR2-mediated tumor control (**Chapters 3 and 4**). Nevertheless, my work supports the notion that T1 IFN-producing TLRs play a role in CD8⁺ T cell priming, which synergistically boosts antitumor immunity when combined with Treg depletion by TLR2 (**Chapter 4**).

TLR agonists are among the most well-studied drugs for cancer immunotherapy.^{668–670} Three of the four PRR agonists that have been approved for use in the treatment of cancer (including all three approved in the USA) are TLR agonists. BCG (TLR2/4 and perhaps others) is the standard of care for noninvasive bladder cancer; imiquimod (TLR7) supplements surgical excision in the treatment of skin cancers; and, MPLA has earned approval for use as an adjuvant in cancer and vaccines.^{653,654,671–676} TLR agonists have been found to play numerous beneficial roles in cancer. Essentially all aspects of APC biology from T cell activation (i.e., signals 1, 2, and 3) to phagocytosis, metabolism, and migration can be improved by TLR signaling in tumor APCs.^{669,677–681} In a broad sense, TLR signaling reconfigures the TME from an immunologically “cold” environment dominated by inhibitory signals to a “hot” one replete with costimulatory ligands and proinflammatory cytokines.^{668–670} This ability to modulate the TME locally, and not only impact T cell-mediated immunity systemically in SLOs, has led to a recent increase in interest in delivering TLR agonists directly to tumors via IT injection.⁶⁸² This will feature prominently in my own work, which extensively employed IT TLR agonists (**Chapters 3 and 4**).

Additional considerations behind the use of TLR agonists in cancer immunotherapy include the well-characterized specificity of TLR for their ligands and agonists, the breadth of ligands recognized by TLRs, and their broad, variegated expression by immune and nonimmune cells.^{75,668–670} These features make TLR agonists highly adaptable to different malignancies, as reflected by the breadth of agonists and tumor types currently being tested in clinical trials (TLR2/3/4/5/7/9 have all been tested for a range of malignancies).^{683–685} Nevertheless, the perceived importance of T1 IFN in driving CD8⁺ T cell-dominated immune responses is also a major factor shaping the body of work surrounding TLR agonists for cancer therapy.^{686,687} This has led to endosomal TLRs (TLR3/7/8/9) being increasingly favored for clinical development due to their activation of T1 IFN via IRF3/7.^{682–688} However, many studies that claim the superiority of agonists for endosomal TLRs fail to account for several factors that may be important when considering the utility of these drugs in cancer immunotherapy. First, many studies do not characterize TLR agonists in parallel, instead focusing on a single (often proprietary) agonist with other agonists used as negative controls. This approach can be used to bias results in favor of a novel compound. Second, the effect of TLR agonists on the priming of T cells is often used as the primary criterion for determining efficacy. This fails to account for effects that TLRs might have on other immune cell populations after the priming stage of T cell activation, which my own work has shown to be impactful (**Chapters 3 and 4**). Finally, CD8⁺ T cells are often the primary focus of any TLR agonist, with changes in the cellular composition of the TME treated as secondary to improved CD8⁺ T cell responses. This fails to account for the direct regulation of Tregs by APCs downstream of TLR signaling. While I do not address these shortcomings in full, my thesis will repeatedly return to these issues as they relate to the often-overlooked capacity of TLR2 to promote antitumor immune responses (**Chapters 3 and 4**).

Chapter 2

Cellular mechanisms underlying beneficial versus detrimental effects of bacterial antitumor immunotherapy

2.0 Authors

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2.1 Abstract

Bacteria engineered to express tumor antigens as cancer vaccines have produced mixed results. Here, we used an attenuated strain of *Listeria monocytogenes* (Δ actA, *Lm*) that lacks tumor antigens to examine the immune response to *Lm* itself in tumor-bearing mice following intravenous (IV), intratumoral (IT), or combined IV+IT *Lm* delivery. Unexpectedly, IT *Lm* alone recruited neutrophils to tumors, which became immunosuppressive, provided an intracellular reservoir for long-term persistence of *Lm* in tumors, and promoted tumor growth. In contrast, prior IV *Lm* administration generated anti-*Lm* cytotoxic CD8⁺ T cells that infiltrated tumors upon IT *Lm* delivery. These *Lm*-specific CD8⁺ T cells controlled tumors by inducing cancer cell apoptosis, limiting cancer cell proliferation, and enhancing tumor antigen cross-presentation to tumor-specific T cells. Thus, an anti-*Lm* CD8⁺ T cell response against *Lm* colonized tumors can control cancer, offering a paradigm for cancer immunotherapy that leverages systemic CD8⁺ T cell immunity targeting intra-tumoral bacteria.

2.2 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.^{689–691} Today, intravesical administration of Bacillus Calmette-Guérin (BCG), a live attenuated strain of the Gram-positive intracellular pathogen *Mycobacterium bovis*, remains the standard of care for non-muscle-invasive bladder cancer and the only FDA-approved bacterial cancer therapy.⁶⁹² Bacteria may naturally colonize many tumors, offering new opportunities for drug delivery systems or cancer detection probes.^{693–704} Advances in genetic manipulation also enables the engineering of bacteria that express tumor antigens, cytokines, or cytotoxic proteins to treat cancer.^{705–709} This progress has renewed interest in bacterial-based cancer immunotherapies.

Listeria monocytogenes engineered to express tumor-associated antigens and administered intravenously effectively controls multiple cancer models in mice and is the most tested bacterial immunotherapy in patients.^{621,710–713} Despite generating tumor antigen-specific CD8⁺ T cells and improving patient outcomes in otherwise untreatable cancers, overall, responses have been variable.^{640,646,648,649,714,715} However, the direct colonization of tumors by *Listeria* has not been explored. A deeper understanding of bacterial interactions with host immune cells, its migration, and its persistence in tumors may reveal vulnerabilities of current bacterial-based strategies and guide improved uses of bacteria as direct intratumoral adjuvants.

Effective antitumor immunity requires a robust anti-tumor immune cell response and a permissive tumor microenvironment (TME).^{244,716–718} However, the TME is often immunosuppressive due to high frequencies of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs).^{719–724} The sole use of *Listeria* as a vaccine to generate tumor-specific CD8⁺ T cells may underlie its variable efficacy. *Listeria* could be used to engage innate immunity in tumors to reverse immunosuppression⁷²⁵ by directly colonizing tumors.⁷²⁶ Clinical and preclinical studies provide clear evidence that intratumoral injection of bacterial adjuvants that activate host pattern recognition receptors (PRRs) and stimulate innate immunity can control not only injected tumors but also disseminated tumors.^{707,727–729}

Here, we show that intratumoral (IT) or intravenous (IV) injection of Δ actA-attenuated *Listeria monocytogenes* (*Lm*) lacking tumor antigen expression fails to control tumor growth when administered alone. IT *Lm* recruits polymorphonuclear cells (PMNs; neutrophils) into the TME, polarizing them into suppressive PMN-MDSCs that inhibit antitumor CD8⁺ T cells.^{730,731} In contrast, IV *Lm* vaccination prior to IT administration generated anti-*Lm* cytotoxic CD8⁺ T cells that eliminated *Lm*-harboring PMN-MDSCs, restricted tumor proliferation, enhanced cancer cell apoptosis, and enhanced tumor-specific CD8⁺ T cell infiltration by increasing tumor antigen cross-presentation by type I dendritic cells (DC1s). Our findings redefine bacterial cancer therapies by highlighting the importance of direct T cell targeting of conserved bacterial antigens, which is a more broadly applicable and rapidly translatable strategy compared to engineering bacteria to express patient-specific neoantigens. These insights are fundamental for developing improved bacterial-based cancer therapies.

2.3 Results

2.3.1 Intratumoral injection of attenuated *Listeria* increases tumor growth.

Immune adjuvants (i.e. PRR agonists) alone can be effective against cancers when delivered directly into tumors without the addition of engineered tumor antigens.^{729,732} To test whether antitumor responses could be generated by direct intratumoral (IT) injection of an attenuated strain of *L. monocytogenes* lacking tumor antigen expression, we used a $\Delta actA$ mutant, denoted *Lm*, which is an attenuating mutation used in many *Listeria* vaccine clinical trials.⁶²³ $\Delta actA$ *Listeria* lacks the ActA virulence factor, blocking the bacteria's ability to spread from cell to cell via actin-based motility while retaining its capacity to induce MHC-I-restricted CD8⁺ T cell responses by escaping from phagosomes and growing in host cells' cytosol.⁶³⁷ The $\Delta actA$ mutation renders *Lm* 1,000-10,000-fold less virulent than wildtype *L. monocytogenes* without decreasing its capacity to induce CD8 T cells, making it an ideal vaccine strain that is safe in patients.^{648,649,685,715} However, direct injection of *Listeria* into tumors has not been tested. We performed dose-escalating experiments with IT *Lm* from 5×10^5 - 5×10^7 CFUs into tumors 11 days post tumor inoculation, when tumors just became palpable (50-100mm³). We found no lethality at a dose of 5×10^7 *Lm*, which we used for all IT injections in this study (data not shown). 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 2.1A-C and S2.1A-B**). *Lm* has not been observed to cause increased tumor growth, although it has not been tested by IT injection. Thus, the IT route of administration might cause increased tumor growth. In line with this hypothesis, growth of B16F10 or MC38 tumors was not increased by *Lm* administration (1×10^6) by an intravenous (IV) route, which is the most common method of administration (a lower *Lm* dose was required to prevent lethality by the IV route) (**Figures S2.1C-F**).⁷¹⁰ This finding is significant, as the route of *Lm* inoculation, IT versus IV, appears to convert *Lm* from being detrimental to potentially therapeutic for controlling cancer.

2.3.2 *Lm* colonizes tumors 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, we measured colony forming units (CFUs) for living bacteria in various tissues harvested from tumor-bearing mice at several time points after IT or IV *Lm* administration. We found that *Lm* was cleared from spleens, lymph nodes, and livers by 5-10 days post injection in tumor-bearing mice, regardless of the route of delivery (**Figures 2.1D-E**). However, *Lm* colonized subcutaneous MC38 and B16F10 tumors for up to 15 days, when experiments ended due to uncontrolled tumor growth (**Figures 2.1D-E**). *Lm* also colonized orthotopic pancreatic ductal adenocarcinomas (PDAC) after IV injection (**Figure 2.1F**).⁷³³ While other bacterial species have been shown to persist in tumors of mice, the long-term (>3-5 days) persistence of live *Lm* within tumors of immunocompetent mice is not documented. The colonization and long-term persistence of *Lm* in multiple tumor types after IV administration also revealed that systemically delivered *Lm* can colonize tumors that are not easily accessible for IT injection.

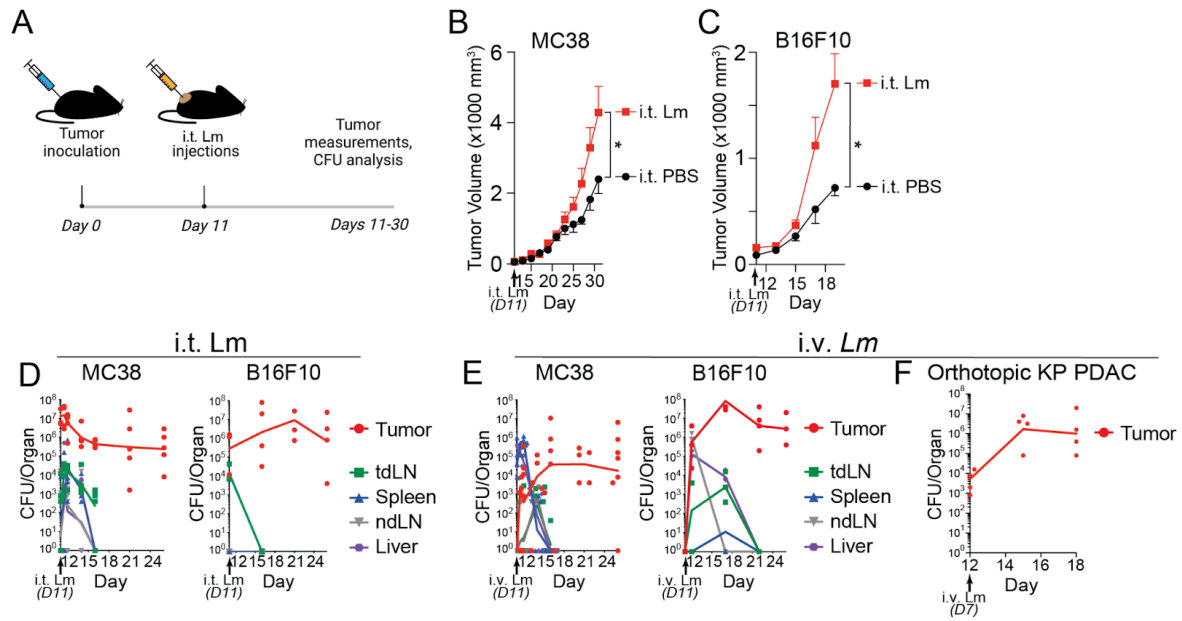


Figure 2.1: Intratumoral injection of *Lm* leads to increased tumor growth and persistent colonization of *Lm* within tumors.

(A) Experimental design.

(B-C) Tumor growth in C57BL/6J mice injected with 5×10^5 MC38 (B) or 2×10^5 B16F10 (C) tumor cells and 5×10^7 *Lm* intratumorally (IT) on day 11. Data shown from n=4-5 mice per group from one of three (B) or of two (C) experiments.

(D-E) Colony forming unit (CFU) analysis for *Lm* from indicated tissues and times after MC38 or B16F10 inoculation and injection IT (D, 5×10^7 *Lm*) or IV (E, 1×10^6 *Lm*) of *Lm* on day 11. Data shown from n=4-8 mice pooled from two experiments. tdLN = tumor-draining lymph node, ndLN = non-tumor-draining LN.

(F) CFU analysis for *Lm* from pancreatic ductal adenocarcinomas (PDAC) at indicated time points after orthotopic transplantation of FC1245 and injection of *Lm* IV on day 7 (1×10^6 , n=4 mice)⁷³⁴.

For all plots, mean $\pm$ s.e.m and *P<0.05 by two-way ANOVA (B-C).

2.3.3 *Lm* infects PMN-MDSCs in tumors.

We hypothesized that the distribution of *Lm* in specific cells of the TME after IT administration shaped the immune response to promote tumor growth. Because *Lm* is a facultative intracellular pathogen, we sought to identify which cells harbored bacteria after IT administration. To monitor intracellular infection of host cells, we used a fluorescent protein-based system wherein a Tag-RFP reporter is expressed under the control of the *actA* promoter in $\Delta actA$ *Listeria*, denoted *Lm-RFP*, leading to 100-fold induction of RFP when *Lm* enters the host cell cytosol (**Figure 2.2A**).^{735,736} We observed co-localization of the RFP signal exclusively in CD45⁺ immune cells, but not CD45⁻ (which includes tumor cells), from single cell suspensions of MC38 or B16F10 tumors infected with *Lm-RFP* (**Figure 2.2B**).

Next, we tracked which types of CD45⁺ immune cells harbored cytosolic *Lm*. At 4 and 24 hours post-infection, we observed an enrichment of RFP-expressing bacteria in phagocytic immune cells, especially Ly6G⁺ polymorphonuclear (PMN) cells, in both MC38 and B16F10 tumors (**Figures 2.2C and S2.2A-D**). To a lesser extent, *Lm-RFP* was detected in F4/80⁺ macrophages, Ly6C⁺ monocytes, and CD11c⁺MHC-II⁺ DCs (**Figures 2.2C and S2.2B**). This distribution of *Lm* in phagocytic immune cells, primarily PMNs and macrophages, was maintained five days after IT infection (**Figure 2.2D**). However, while IT *Lm* induced a significant influx of PMNs into tumors in comparison to steady-state tumors, other phagocytic cell frequencies were unchanged (**Figures 2.2E and S2.2C-E**). The frequency of RFP⁺ PMNs also increased between 4 and 24 hours after IT *Lm* (**Figures 2.2F and S2.2F**).

We performed several experiments to rule out that *Lm* was phagocytosed during the single cell preparation for flow cytometry. First, we used confocal microscopy of frozen tumor sections and observed cytosolic RFP⁺ *Lm* within Ly6G⁺ PMNs, which aggregated in clusters within tumors (**Figures 2.2G and S2.2G**). Second, we prepared single cells from tumors in the presence of the antibiotic gentamicin to kill any extracellular *Lm*, FACS purified Ly6G⁺ PMNs, and performed microscopic analysis of cytopun cells (**Figure 2.2H**). We observed cytosolic RFP⁺ *Lm* in Ly6G⁺ PMNs by fluorescence microscopy with anti-Ly6G, and bacteria inside of cells by Gram staining (**Figure 2.2I**). Altogether, these results demonstrate that PMNs served as a reservoir for *Lm* in tumors after IT injection. These results were unanticipated, as we expected PMNs would rapidly kill phagocytosed *Lm*, preventing their escape into the cytosol and RFP expression.⁷³⁷⁻⁷³⁹

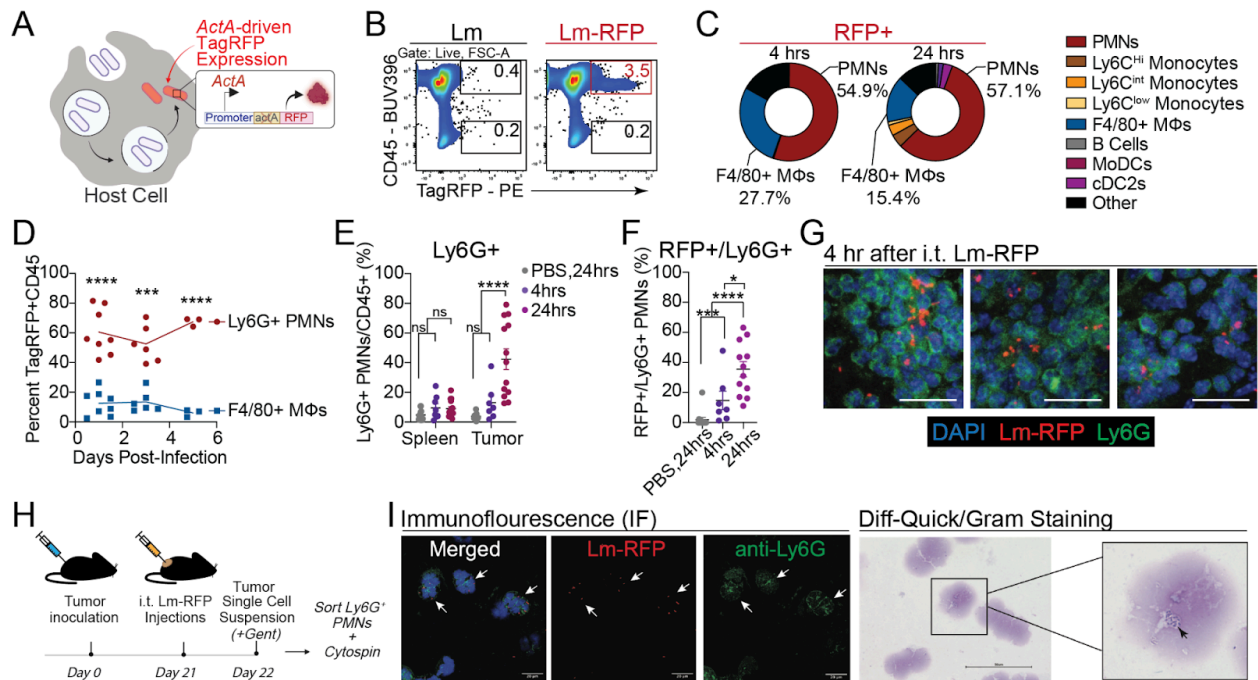


Figure 2.2: *Lm* localizes in intratumoral PMNs.

- (A) Schematic of cytosolically induced Tag-RFP expression with *Lm-RFP*.
- (B) Representative flow plots from single cell suspensions of MC38 tumors 4 hours post IT *Lm* (left) or IT *Lm-RFP* (right) showing CD45 staining versus Tag-RFP fluorescence.
- (C) Distribution of RFP⁺ immune cell types from MC38 tumors at 4 (left) or 24 (right) hours after IT *Lm*. Representative data from three experiments with n= 3-6 mice/group.
- (D) Analysis of the fraction of PMNs or macrophages that were RFP⁺ at 1, 3, and 5 days after IT *Lm-RFP* in MC38 tumors. Representative data from two experiments with n= 3-6 mice/group.
- (E) Frequency of Ly6G⁺ PMNs in spleens or tumors from MC38-bearing mice at 4 and 24 hours after IT *Lm* or IT PBS.
- (F) Frequency of intratumoral Ly6G⁺ cells that were RFP⁺ at 4 and 24 hours after IT *Lm* or IT PBS. Results in E-F are pooled from three experiments with n= 2-5 mice/group per experiment.
- (G) Images of frozen sections of MC38 tumors 4 hours after IT *Lm*. DAPI (blue), *Lm-RFP* (red), Ly6G (green). Scale bar = 20 μ m.
- (H) Schematic for cytopspin imaging analysis of sorted intratumoral Ly6G⁺ PMNs after IT *Lm*.
- (I) Immunofluorescence (left) and Diff-Quick gram staining (right) of sorted intratumoral Ly6G⁺ PMNs 24 hours after IT *Lm*. For immunofluorescence: DAPI(blue), *Lm-RFP* (red), Ly6G (green). Scale bar = 20 μ m. For Diff-Quick: Scale bar = 50 μ m.
- For all plots, mean $\pm$ s.e.m. and *P<0.05, **P<0.01, ***P<0.001, ****<0.0001 by two-way ANOVA (D-E) or one-way ANOVA (F).

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

We hypothesized that the colonization and persistence of *Lm* in the cytosol of PMNs was the result of PMN differentiation into myeloid derived suppressor cells (MDSCs) within tumors, which could promote tumor progression by inhibiting T cell responses.^{269,648,740,741} Phenotyping of PMNs at 4 and 24 hours after IT *Lm* revealed a dynamic increase in the frequency of PMNs expressing the prototypical PMN-MDSC marker CD14, which was enriched in RFP⁺ cells (**Figures 2.3A-B and S2.3A-B**).⁷⁴² To confirm differentiation of PMNs into MDSCs within tumors, we sorted Ly6G⁺ PMNs from MC38 tumors and analyzed the expression of genes upregulated in MDSCs. We observed that Ly6G⁺ cells sorted from tumors had higher expression of *Cd274*, *Cd14*, and *Vegfa* transcripts in comparison to Ly6G⁺ cells sorted from the spleen or bone marrow (**Figure 2.3C**). These results suggest that *Lm* infection within tumors recruited PMNs that then differentiated into an MDSC phenotype in tumors, which may suppress antitumor immune responses while also creating a reservoir for *Lm* colonization.

To test whether PMN-MDSCs are more permissive to cytosolic *Lm* growth than non-tumor-infiltrating PMNs, we used conditionally immortalized neutrophils (CINs) or primary bone marrow progenitors differentiated into PMNs *in vitro* (**Figures 2.3D and S2.3C**).^{743,744} CIN-derived PMNs treated with conditioned supernatants from MC38 or B16F10 tumor cells increased expression of CD14 and PD-L1 relative to noncancerous, immortalized NIH 3T3 cells, indicative of a PMN-MDSC phenotype (**Figures 2.3D-E**). As a positive control, PMNs were differentiated into PMN-MDSCs by adding IL-6 and GM-CSF, which induced CD14 and PD-L1 in CINs and bone marrow derived PMNs (**Figures 2.3D and S2.3C**).⁷⁴³ To test whether the adoption of this MDSC phenotype increased susceptibility to *Lm* infection, we infected heterogeneous PMN cultures *in vitro* with *Lm*-RFP and monitored infection by flow cytometry. *Lm* cytosolic infection was almost exclusively associated with CD14⁺ PMNs (**Figures 2.3F and S2.3D**). These results strongly suggest that IT *Lm* recruits PMNs to tumors where they become converted into PMN-MDSCs that allow *Lm* to infect and persist intracellularly.

PMN-MDSCs are widely associated with decreased antitumor immunity and increased tumor progression, primarily by inhibiting antitumor CD8⁺ T cell responses.^{717,745} To test whether PMN-MDSCs inhibited CD8⁺ T cells to promote cancer progression, we co-cultured *in vitro* activated OT-I T cells with OVA-expressing MC38 tumor cells in the presence of PMNs sorted from spleens or from MC38 tumors. The addition of Ly6G⁺ PMNs from tumors (PMN-MDSCs), but not spleens, led to a significant reduction in IFN γ cytokine production from OT-I T cells in response to direct recognition of OVA-expressing MC38 tumors (**Figures 2.3G-H**). Next, we tested whether the depletion of all PMNs could prevent increased tumor growth with IT *Lm* infection (**Figure 2.3I**). Indeed, depletion of Ly6G⁺ cells prevented increased tumor growth (**Figures 2.3J and S2.3E**). Notably, anti-Ly6G had no effect on tumor growth in mice not receiving IT *Lm*. Interestingly, PMN depletion did not reduce *Lm* colonization of tumors (**Figure S2.3F**), but *Lm*-RFP now resided in tumor-associated macrophages (TAMs) (**Figure S2.3G**). This was not due to an increased TAM frequency following PMN depletion, as macrophage frequency was unaffected by anti-Ly6G treatment (**Figure S2.3H**). These results support that IT *Lm* recruits PMNs to tumors where they are converted to a MDSC phenotype that promotes increased tumor growth. Therefore, *Lm* injection into tumors can promote tumor growth, warranting caution when using bacterial cancer therapies.

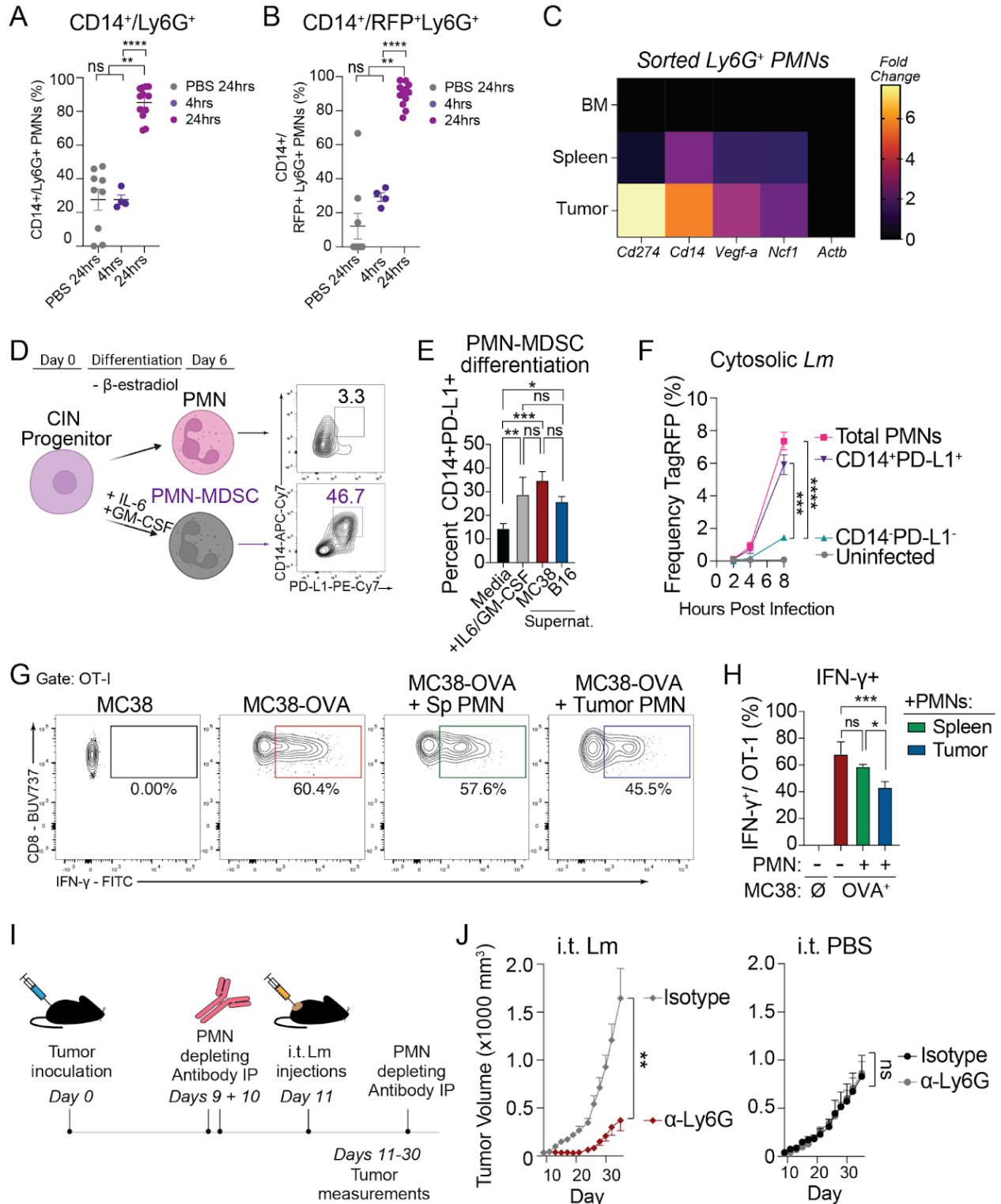


Figure 3. PMN conversion into MDSCs permits *Lm* infection and promotes tumor growth.

(A-B) Frequency of CD14⁺ cells of Ly6G⁺ (A) or of RFP⁺Ly6G⁺ cells (B) from MC38 tumors 4 and 24 hours after IT PBS or IT *Lm*. Results pooled from three experiments with n= 2-5 mice/group per experiment.

(C) Expression of indicated genes by qPCR analysis from bone marrow (BM), spleen, or tumor of MC38-bearing mice. Results from n= 6 mice.

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

(E) PMNs derived from CINs were cultured with conditioned supernatants from NIH3T3, MC38, or B16F10 cells for 24 hours *in vitro* and the frequency of CD14⁺PD-L1⁺ cells was assessed compared to unconditioned media or IL-6+GM-CSF treatment. Representative of two experiments.

(F) Heterogeneous CD14⁺ PMNs 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⁺PD-L1⁺ cells, or CD14⁺PD-L1⁻ cells by flow cytometry. Representative of three experiments.

(G) Representative flow plots of IFN γ production from OT-I CD8⁺ T cells after 24 hour co-culture with MC38 or MC38-OVA tumors in the presence or absence of FACS-purified PMNs from spleens or tumors of IT *Lm*-treated MC38-bearing mice. Cells were plated at a 1:1 ratio of OT-I T cells to PMNs.

(H) Quantification of the frequency of IFN γ ⁺ OT-I CD8⁺ T cells (G). Results from two independent experiments.

(I) Experimental design to deplete PMNs (anti-Ly6G) prior to IT PBS or IT *Lm*.

(J) Tumor growth measured in mice injected with IT *Lm* (left) or IT PBS (right) and either an isotype control or anti-Ly6G-depleting antibody prior to tumor inoculation and throughout the experiment. Data is from one of two experiments with n=5 mice/group.

For all plots, *P<0.05, **P<0.01, ***P<0.001 by one-way ANOVA (A, B, E) or two-way ANOVA (C, F), mean $\pm$ s.e.m for *in vivo* experiments (E, J) or mean $\pm$ s.d. for *in vitro* experiments (B-C, G).

2.3.5 A combination of IV+IT *Lm* leads to the control of multiple cancers.

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. IV injection of *Lm* may effectively generate adaptive T cell responses against *Lm* that could be recruited into tumors by IT *Lm* injection to augment the antitumor immune response.⁶²⁴ 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 we injected *Lm* IT into tumors as done previously with IT *Lm* alone (**Figure 2.4A**). This combination of IV *Lm* followed by IT *Lm* administration significantly controlled both MC38 and B16F10 tumors (**Figures 2.4B-C and S2.4A-B**). Of the experiments performed, only IV+IT *Lm* administration led to tumor rejection, which occurred in 20% of mice (8 out of 40). Because IV administration of *Lm* also led to colonization of tumors (**Figure 2.1E**), we tested whether a second IV administration of *Lm* could also control tumors without requiring direct IT *Lm* injection. We found that IV+IV also controlled tumors at two different doses of IV *Lm* (**Figure S2.4C**). These results indicate that a second dose of *Lm* IT, or even IV, can control tumors by *Lm* colonization of tumors and that tumor control by *Lm* can occur without engineering strains that co-express tumor antigens. In addition, we found that while Ly6G⁺ PMNs were recruited equally to tumors one day after IT *Lm* administration with IV+IT and IT-only *Lm*, the frequency of Ly6G⁺ cells was significantly reduced by 5 days after IT *Lm* administration in the IV+IT setting (**Figure 2.4D**). Furthermore, the fraction of CD14⁺ PMNs was almost completely absent 5 days after IT *Lm* administration with IV+IT *Lm*, a reduction even below their frequency in PBS-treated control tumors (**Figure 2.4E**). Finally, we did not observe any enhancement in tumor control when we depleted PMNs prior to IT *Lm* administration with IV+IT *Lm*, suggesting that the PMN reduction with IV+IT *Lm* is sufficient to prevent PMN-MDSC promotion of tumor growth after the IT *Lm* dose with IV+IT *Lm* (**Figure S2.4D**).

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 IV+IT *Lm*-treated tumors for up to 10 days after IT *Lm* administration, *Lm* was ultimately cleared from tumors by 15-20 days after IT *Lm* (day 21-30 of tumor growth) (**Figure 2.4F**). 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, which led to tumor control. Alternatively, because *Lm* colonized and persisted within the TME after IV administration at day 4 (**Figure S2.1F**), the early colonization of tumors might contribute to tumor control by changing the TME and augmenting the effect of the second IT injection of *Lm* with IV+IT *Lm*. To address this possibility, we performed experiments in which IV *Lm* was administered prophylactically, 20 days prior to tumor inoculation (**Figure 2.4G**). In this setting, *Lm* was cleared from mice prior to tumor inoculation and absent from tumors when IT *Lm* was administered. We found that mice injected with *Lm* prophylactically (memory IV+IT) controlled tumors equivalently to the standard IV+IT regimen (**Figures 2.4H and S2.4E**). However, neither prophylactic IV *Lm* injection alone nor IV *Lm* followed by a subcutaneous injection of *Lm* on the contralateral flank of mice (memory IV+SQ) controlled tumors (**Figure 2.4H**). 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 IV+IT *Lm* 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.^{691,746,747} Using a *Kras*^{G12D/+}; *p53*^{fl/fl} orthotopic sarcoma model,⁷⁴⁸ we found that memory

IV+IT *Lm* led to significant control of *Lm*-treated sarcomas compared to tumors treated with prophylactic IV *Lm* followed by IT PBS (**Figures 2.4I and S2.4F**). Together, these results show 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.

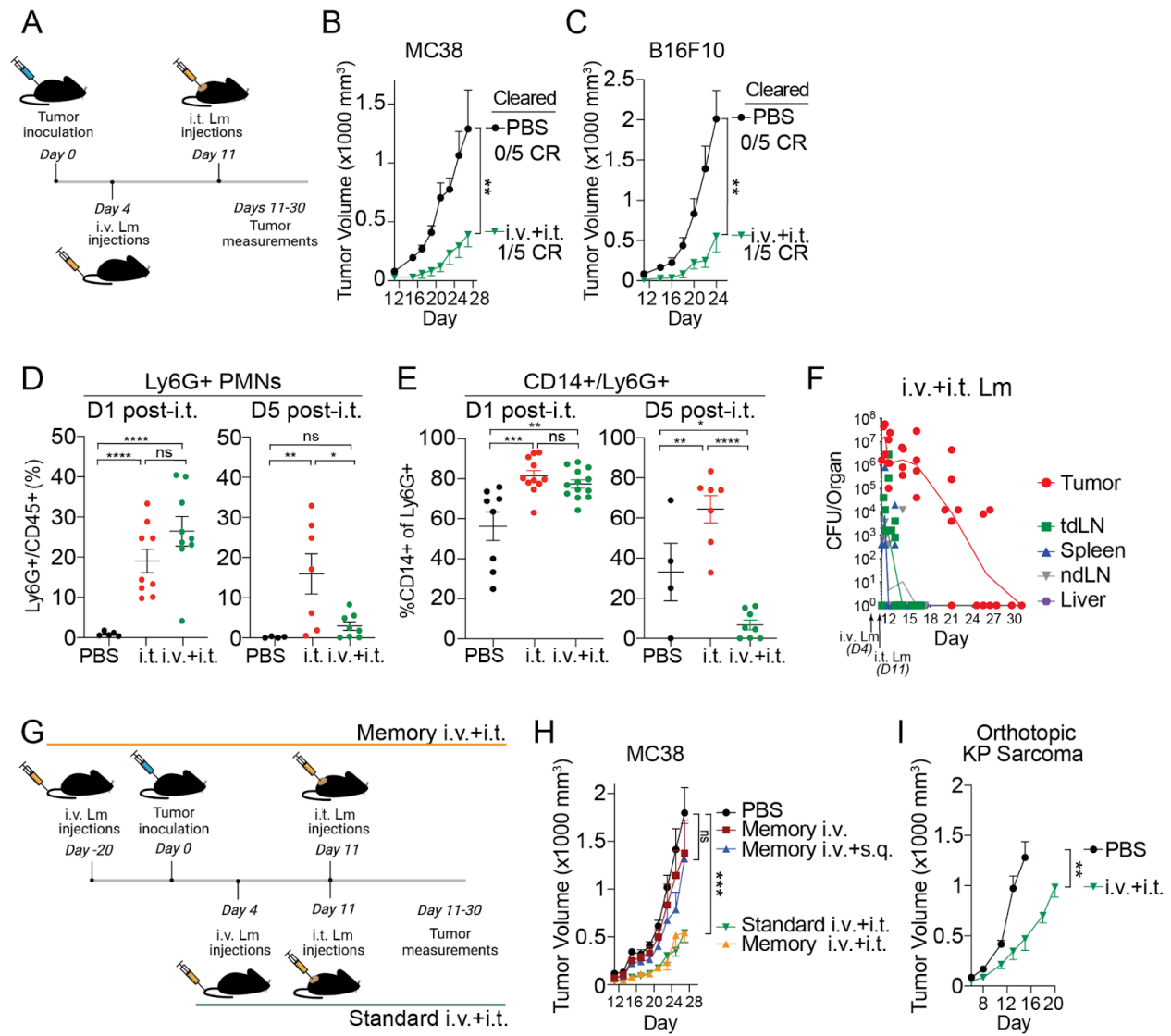


Figure 2.4: IV+IT *Lm* controls multiple tumor types while clearing intratumoral bacteria and PMN-MDSCs from tumors.

(A) Experimental strategy for the IV+IT *Lm* regimen.

(B-C) Tumor growth with IV+IT PBS versus IV+IT *Lm* of MC38 (B) and B16F10 (C). Data shown from n= 4-6 mice per group from one of three (B) or two (C) experiments.

(D-E) Frequency of Ly6G⁺ PMNs of CD45⁺ cells (D) and CD14⁺ PMNs of Ly6G⁺ cells (E) one day (left) and 5 days (right) after IT *Lm* with IV+IT *Lm* regimen. Data shown from n= 2-5 mice per group from two pooled experiments.

(F) CFU analysis for *Lm* after IV+IT *Lm* 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 to compare prophylactic IV *Lm* (memory IV+IT) to the standard IV+IT *Lm* regimen.

(H) MC38 tumor growth with memory IV+IT, memory IV only, and memory IV+SQ *Lm* (subcutaneous *Lm* injection on opposing flank) compared to PBS treatment or standard IV+IT *Lm*.

(I) Orthotopic KP sarcoma growth with PBS vs. memory IV+IT *Lm*. For all plots, mean $\pm$ s.e.m and *P<0.05, **P<0.01, ***P<0.001 by Student's t-test (D, E) or two-way ANOVA (B, C, H, I).

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

The reduction of tumor growth that occurred with IV+IT *Lm* compared to a single-dose of IV or IT *Lm* suggested that an adaptive immune response mediated tumor control. We hypothesized that T cells were the critical adaptive immune cells for tumor control with IV+IT *Lm* due to their established role in both anti-*Lm* and antitumor immunity.^{50,104,624} Flow cytometric analysis of tumors 5-8 days after IT *Lm* injection (early) 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* or PBS control injections (**Figures 2.5A-C and S2.5A-B**). Examination of tumors at the endpoint of tumor growth (late, 15-20 days after IT *Lm* injection) also revealed a significant increase in the infiltration of CD8⁺ T cells but not CD4 T cells in tumors of mice treated with IV+IT *Lm* (**Figures 2.5A-C and S2.5A-B**). Intratumoral regulatory T cells (Tregs), which are known to be immunosuppressive in the TME, were not significantly reduced with IV+IT *Lm* compared to IV or IT *Lm* alone, although they were reduced compared to the PBS treated mice (**Figures S2.5C-D**).^{244,266,305,716,718,749} Next, we assessed the functionality of the CD8⁺ T cells by IFN γ and TNF α production 8 days after IT *Lm* or PBS injection. Here, IV+IT *Lm* significantly increased CD8⁺ T cells production of IFN γ , and especially the dual production of IFN γ and TNF α , compared to other groups (**Figure 2.5D**).

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 2.5E**). Depletion of CD8⁺ T cells abrogated the efficacy of IV+IT *Lm* in MC38 and B16F10 models, indicating that CD8⁺ T cells were required for tumor control with IV+IT *Lm* (**Figures 2.5F and S2.5E-F**). In addition, CD8⁺ T cell depletion increased the frequency of Ly6G⁺ cells in tumors of mice treated with IV+IT *Lm*, suggesting that the reduction in PMN-MDSCs with IV+IT *Lm* was likely the result of CD8⁺ T cell-mediated killing of *Lm*-infected PMNs (**Figure 2.5G**). We also tested IV+IT *Lm* 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.^{750,751} IV+IT *Lm* did not lead to tumor control in *Batf3*^{-/-} mice (**Figures 2.5H and S2.5G**). Taken together, the above results suggest that IV+IT *Lm* therapy requires the activity of T cells, in particular CD8⁺ T cells. However, whether CD8⁺ T cells specific to *Listeria*, the tumor, or both were required for tumor control was not distinguished.

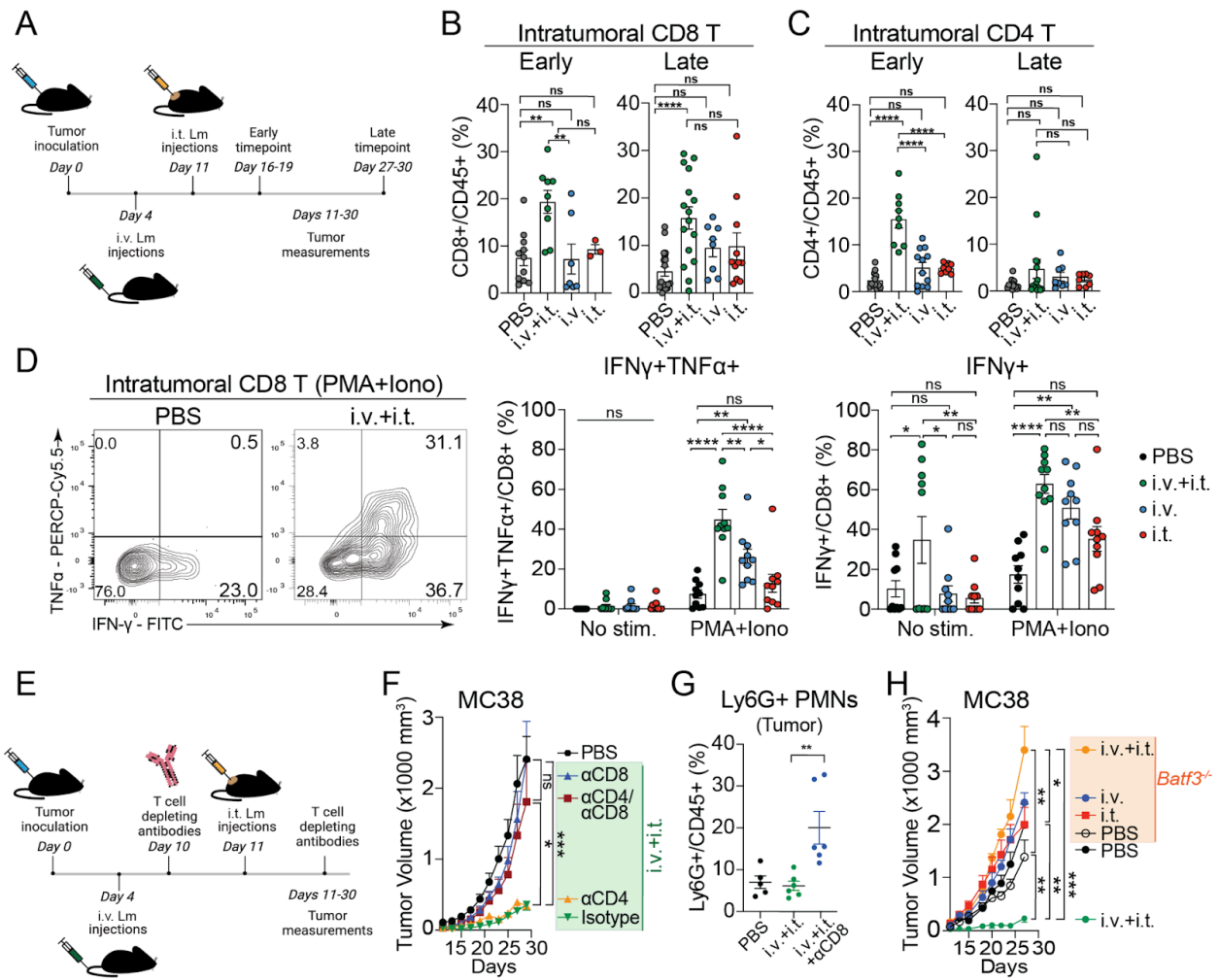


Figure 2.5: CD8⁺ T cells are required for tumor control with IV+IT *Lm*.

- (A) Experimental strategy for IV+IT *Lm* and 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 n= 5 mice/group.
- (D) Representative flow plots (left) and quantification (right) of IFN γ and TNF α intracellular cytokine staining from intratumoral CD8⁺ T cells 8 days after IT *Lm* or IT PBS administration. Results pooled from two experiments with n= 5 mice/group.
- (E) Experimental strategy for IV+IT *Lm* with anti-CD8, anti-CD4, or both depleting antibodies beginning 10 days after MC38 tumor inoculation.
- (F) MC38 tumor growth with T cell depletions.
- (G) Frequency of intratumoral Ly6G⁺ PMNs of CD45⁺ cells at end of experiment from (F).
- (H) MC38 tumor growth in WT versus *Batf3*^{-/-} mice treated with IV+IT *Lm*.
- For all plots, mean $\pm$ s.e.m and *P<0.05, **P<0.01, ***P<0.001 by Student's t-test (B-C, G), one-way ANOVA (D), or two-way ANOVA (F, H).

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

To assess whether the CD8⁺ T cells that mediate tumor control with IV+IT *Lm* were specific to *Lm* antigens, we used *Lm* engineered to express ovalbumin (*Lm-OVA*) and MHC-I (H2-K^b) tetramers loaded with SIINFEKL to track the anti-*Lm* CD8⁺ T cell response (SIIN/H2K^b) (**Figures 2.6A and S2.6A**). We observed an increased frequency of SIIN/H2K^b CD8⁺ T cells in tumors with IV alone and IV+IT *Lm*, but only a significant increase in the absolute number of these *Lm*-specific cells in tumors with IV+IT *Lm* (**Figure 2.6A**). To test whether anti-*Lm* CD8⁺ T cells alone could replace IV *Lm* administration to mediate tumor control, we used CD8⁺ T cells from OT-I mice, which have a TCR that recognizes the OVA peptide SIINFEKL presented on MHC I (SIIN/H2K^b). Adoptive transfer of activated OT-I CD8⁺ T cells into mice prior to tumor inoculation 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 (**Figures 2.4G-H**). When tumors became palpable at day 11, tumors were injected with PBS, *Lm*, or *Lm-OVA* (**Figure 2.6B**). Strikingly, only mice whose tumors were injected with *Lm-OVA* had delayed tumor growth, similar to mice that received the standard IV+IT *Lm* regimen (**Figures 2.6B and S2.6B**). Even in the aggressive orthotopic KP sarcoma model that does not express tumor-specific antigens,⁷⁵² the transfer of *Lm*-specific CD8⁺ T cells prior to sarcoma implantation and IT *Lm-OVA* treatment was sufficient to provide significant sarcoma control (**Figures 2.6C and S2.6C**). 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* regimen.

2.3.8 *Listeria*-specific CD8⁺ T cells control tumors without direct recognition of tumor cells.

It has been reported that an alternatively attenuated strain of *Listeria* can directly infect cancer cells, and this could lead to the direct killing of tumor cells by *Listeria*-specific CD8⁺ T cells.⁷⁵³ While we did not find evidence of *Lm* infecting tumor cells using the *Lm-RFP* reporter strain (**Figures 2.2A-D**), we wanted to test whether direct tumor cell killing by *Lm*-specific CD8⁺ T cells was required for tumor control. To do this, we again transferred OT-I CD8⁺ T cells into WT or *B2m*^{-/-} (MHC I-deficient) mice and implanted these mice with WT or *B2m*^{-/-} MC38 tumors (**Figures 2.6D and S2.6D**). In this scenario, we could determine whether tumor control with IV+IT *Lm* required the recognition of infected tumor cells or host cells by OVA-specific CD8⁺ T cells. OT-I T cells mediated MC38-*B2m*^{-/-} tumor control with IT *Lm-OVA*, but this only occurred in the setting of wildtype mice and not *B2m*^{-/-} mice (**Figure 2.6D**). Similarly, IT *Lm-OVA* was ineffective at controlling WT MC38 in *B2m*^{-/-} mice despite MHC-I expression on tumor cells and recruitment of OT-I T cells into both WT and *B2m*^{-/-} tumors upon IT *Lm-OVA* administration (**Figures 2.6D and S2.6D-E**). Furthermore, CFU analysis for live *Lm* demonstrated that MHC I expression on host cells, but not tumor cells, was required for *Lm* clearance from the TME, in line with *Lm* persisting in host immune cells rather than tumor cells (**Figure S2.6F**). Thus, *Lm*-specific CD8⁺ T cells require MHC I expression on host cells, but not tumor cells, to control tumors and bacterial load with IV+IT *Lm*.

To test whether the anti-*Lm* CD8⁺ T cells alone were able to mediate tumor control, we injected *in vitro* activated OT-I cells and implanted MC38 tumors into *Rag-2*-deficient mice, which lack T and B cells and therefore could not generate antitumor CD8⁺ T cells. Tumor growth was significantly reduced in *Rag-2*^{-/-} mice IT injected with *Lm-OVA*, but not when injected with

Lm not expressing OVA (**Figures 2.6E and S2.6G**). Thus, tumor control could be mediated directly by anti-*Lm* CD8⁺ T cells in the TME in the absence of host-derived T cells.

To rule out a role for OT-I transgenic T cells or the OVA antigen in mediating tumor control, we also performed standard IV+IT *Lm* against MC38-*B2m*^{-/-} tumors and still observed control of these MHC I-deficient tumors (**Figures 2.6F and S2.6H**). Furthermore, MHC I-deficiency on tumor cells did not incite NK cell-mediated tumor control, as IV+IT *Lm* controlled tumors equivalently with or without anti-NK1.1 depletion (**Figures 2.6F and S2.6H**). Finally, we tested whether the transfer of T cells specific to endogenous *Lm* antigens could drive tumor control by immunizing mice with *Lm*, expanding T cells from these mice *in vitro* with heat-killed *Lm*, and prophylactically transferring these *Lm*-specific CD4⁺ and CD8⁺ T cells into *Rag2*-deficient mice. Again, natural *Lm* antigen-specific CD8⁺ T cells were able to control tumors in the absence of host T cells (**Figures 2.6G and S2.6I**). Overall, these results indicate that T cells specific for *Lm* antigens that recognize non-tumor cells in the TME can mediate significant tumor control.

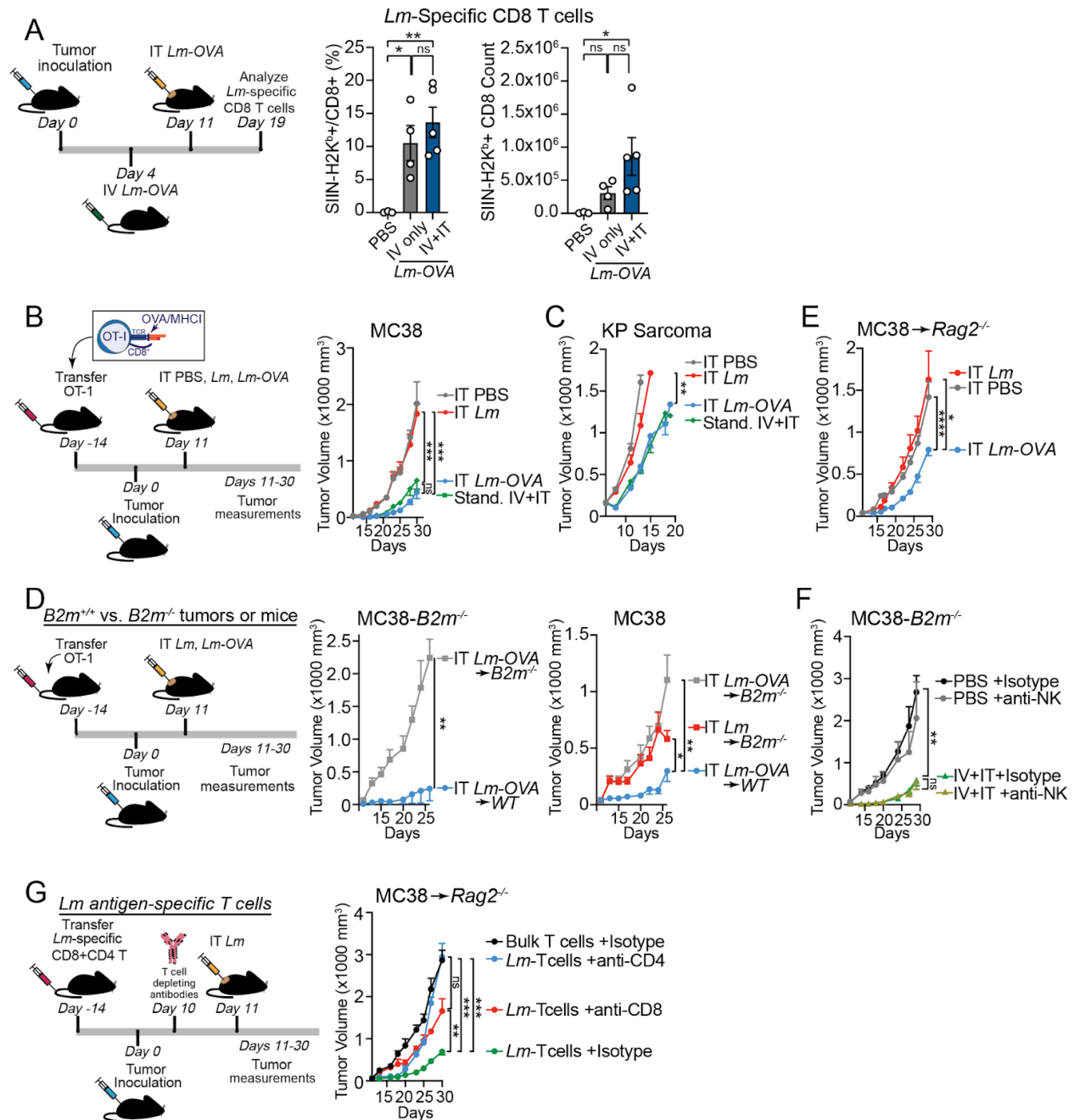


Figure 2.6: Anti-*Listeria* CD8⁺ T cell immunity mediates tumor control.

(A) Experimental design (left) to analyze the frequency (middle) or total number (right) of *Lm*-specific (SIIN-H2K^b tetramer⁺) CD8⁺ T cells from MC38 tumors 8 days after IT *Lm-OVA* in IV+IT *Lm-OVA* regimen. Data shown from one of two experiments.

(B) Experimental strategy to adoptively transfer *in vitro*-activated OT-I CD8⁺ T cells in place of prophylactic IV *Lm* (left) and MC38 tumor growth (right) after IT PBS, *Lm*, or *Lm-OVA*. Data from one of two experiments with n= 5-6 mice/group.

(C) As in (B) but with orthotopic KP sarcoma model. Data from one of two experiments with n= 5-6 mice/group.

(D) As in (B) but comparing MC38-*B2m*^{-/-} tumors grown in WT or *B2m*^{-/-} mice (left) or MC38 tumors grown in WT or *B2m*^{-/-} mice (right). Data from n= 5-6 mice per group.

(E) As in (B) but MC38 tumors were grown in *Rag2*^{-/-} mice. Data from n= 5-6 mice/group.

(F) MC38-*B2m*^{-/-} tumor growth after IV+IT *Lm* or IV+IT PBS +/- anti-NK1.1 antibodies at 10 days after tumor inoculation. Data from n= 5-6 mice/group.

(G) Experimental strategy for adoptive transfer of *in vitro*-activated *Lm*-specific T cells prophylactically into *Rag-2*^{-/-} mice receiving MC38 tumors. Anti-CD8, anti-CD4, or isotype antibodies were given 10 days after tumor inoculation. Data shown from one of two experiments with n= 5-6 mice/group.

For all plots, mean ± s.e.m and *P<0.05, **P<0.01, ***P<0.001 by Student's t-test (A) or two-way ANOVA (B-G).

2.3.9 *Listeria*-specific CD8⁺ T cell killing and cytokine production drive tumor control.

Next, we sought to determine how *Lm*-specific CD8⁺ T cells mediate tumor control. First, we tested whether killing of non-tumor cells infected with *Lm* was required for the efficacy of the IV+IT *Lm* regimen by prophylactically transferring activated SIIN-specific CD8⁺ T cells generated from WT versus *Prfl*-deficient (*Prfl*^{-/-}) mice, which lack the pore-forming effector protein Perforin, and vaccinating with *Lm*-OVA. Tumor control was completely abolished when the transferred *Lm*-specific T cells were *Prfl*-deficient (**Figures 2.7A-B and S2.7A**). We also tested *Lm*(SIIN)-specific T cells generated from *Ifng*^{-/-} mice, which also failed to mediate tumor control (**Figures 2.7B and S2.7B**). Notably, the failure of *Prfl*^{-/-} and *Ifng*^{-/-} CD8⁺ T cells to mediate tumor control was not due to a failure of these cells to infiltrate the tumors, as an equivalent frequency of SIIN-specific CD8⁺ T cells were found in tumors that received WT, *Prfl*^{-/-}, or *Ifng*^{-/-} T cells (**Figure S2.7C**). Since IV+IT *Lm* increased the production of IFN γ and TNF α from intratumoral CD8⁺ T cells, we also tested whether secretion of TNF α was required for the IV+IT *Lm* efficacy by using an anti-TNF- α blocking antibody. TNF- α blockade prevented tumor control with IV+IT *Lm* (**Figures 2.7C and S2.7D**). Taken together, these results show that both the killing activity and cytokine production of *Lm*-specific CD8⁺ T cells were required to mediate tumor control with IV+IT *Lm*.

2.3.10 *Listeria*-specific CD8⁺ T cells enhance antitumor CD8⁺ T cell responses.

To determine how the IV+IT *Lm* regimen controlled tumors, we performed immunofluorescence analysis on tumors one and 10 days after IT *Lm* (with or without prior IV *Lm*) or IT PBS (with prior IV PBS). We used anti-cleaved caspase 3 (CC3) staining to monitor the induction of apoptosis, as the requirement for Perforin in anti-*Lm* CD8⁺ T cells indicated an important killing activity for the effectiveness of IV+IT *Lm*. Indeed, IV+IT *Lm* induced a large amount of CC3 staining throughout the tumors one day after IT *Lm*, which was markedly reduced by depleting CD8⁺ T cells prior to IT injection (**Figure 2.7D**). The increase in CC3 staining was transient, however, as no differences were noted 10 days after IT *Lm* (**Figure S2.7E**). In addition to their potential role in promoting cell killing, the requirement for IFN γ and TNF α may be to slow tumor cell proliferation directly.⁷⁵⁴ We used anti-phospho-histone H3 (PHH3) staining to identify mitotic cells and observed a nearly complete loss of PHH3 staining in tumor cells after IV+IT *Lm*, indicative of a near complete inhibition of tumor cell cycle progression (**Figure 2.7E**). Notably, this reduced proliferation was still observed 10 days after IT injection, though less robustly (**Figures 2.7E and S2.7F**). Thus, increased apoptosis and reduced cell proliferation likely contribute to the elimination and reduced growth of IV+IT *Lm*-treated tumors.

The induction of cell death upon IV+IT *Lm* may increase the phenomenon of epitope spreading, wherein death of tumor cells leads to the increased release and presentation of tumor antigens.^{755,756} Thus, IV+IT *Lm*, while effective in controlling tumor progression with *Lm*-specific CD8⁺ T cells alone, may also enhance the host T cell response directly against tumors. Notably, tumor control with IV+IT *Lm* was better in WT compared to *Rag-2*^{-/-} mice, suggesting that host tumor-specific T cells may also contribute to the efficacy of IV+IT *Lm* (**Figures 2.6B versus 2.6E**). To assess the tumor-specific CD8⁺ T cell response with IV+IT *Lm*, we used MHC-I tetramers loaded with a tumor-derived peptide from the endogenous Murine Leukemia retrovirus, p15E (**Figures S2.7G**).^{266,749} Interestingly, while there was a decrease in the

frequency and number of tumor-reactive (MuLV/H2K^b tetramer⁺) CD8⁺ T cells in mice receiving IV+IT *Lm-OVA* compared to PBS treated mice 8 days after IT injections, the frequency and number of these tumor-specific CD8⁺ T cells was significantly increased compared to mice that only received IV *Lm-OVA* (**Figure 2.7F**). Furthermore, at later time points after IT injection (20 days), the tumor-specific T cells exhibited a phenotype of reduced T cell exhaustion (reduced PD1⁺Lag-3⁺ double and single positive cells) (**Figures 2.7G and S2.7H**). Thus, tumor-specific T cells with a less exhausted phenotype are expanded in tumors with IV+IT *Lm* compared to IV *Lm* alone.

Next we tested the importance of cross-presentation by DC1s, as we hypothesized that if anti-*Lm* CD8⁺ T cells promoted tumor-specific CD8⁺ T cell control, this would be dependent on DC1 tumor antigen cross-presentation.^{757,758} We already observed that IV+IT *Lm* tumor control was completely abrogated in *Batf3*^{-/-} mice, likely due to the inability of mice to generate an anti-*Lm* CD8⁺ T cell response after IV *Lm*.^{751,759} Therefore, we used the transfer of activated OT-I CD8⁺ T cells into *Batf3*^{-/-} mice and then IT injected *Lm* or *Lm-OVA*, reasoning that this would establish whether cross-presentation after IT *Lm* was also required to enhance that antitumor CD8⁺ T cell response and tumor control. As might be expected, *Batf3*^{-/-} mice had significantly reduced tumor control compared to *Batf3*^{+/+} mice with IT *Lm-OVA* (**Figure 2.7H and S2.7I**). However, *Batf3*^{-/-} mice treated with IT *Lm-OVA* had significantly better tumor control than *Batf3*^{-/-} mice treated with IT *Lm*, indicating that the OT-I CD8⁺ T cells targeting *Lm* provided significant tumor control in the absence of DC1s (**Figures 2.5H versus 2.7H and S2.7I**). However, the frequency of tumor-specific (MuLV/H2K^b tetramer⁺) CD8⁺ T cells was significantly increased in IT *Lm-OVA*- compared to IT *Lm*-treated mice, and this increase was dependent on the presence of *Batf3*⁺ DC1s (**Figure 2.7I**). Finally, we used the 25D1.16 monoclonal antibody, which detects SIINFEKL peptide presented on MHC-I (H2-K^b), to assess tumor antigen cross-presentation by implanting MC38-*B2m*^{-/-}-OVA tumors and treating with IV+IT *Lm*.^{244,716–718} We observed increased cross-presented tumor antigen on DC1s only with IV+IT *Lm*, but not with IV or IT *Lm* administration alone (**Figure 2.7J**). These results support the hypothesis that the *Lm*-specific CD8⁺ T cells are able to enhance the tumor-specific CD8⁺ T cell response by promoting the cross presentation of tumor antigens on *Batf3*⁺ DC1s, which may be particularly important for sustained control of tumors at later time points.

To directly test whether antitumor CD8⁺ T cells also play a role in tumor control with IV+IT *Lm*, we monitored tumor growth in the presence or absence of host T cells using the adoptive transfer of OT-I T cells and IT *Lm-OVA* administration. To do this, we generated a T cell deleter mouse by combining *CD4-Cre* and *R26*^{LSL-DTR} alleles.⁷⁶⁰ Because all host T cells upregulate CD4 during thymic development, both mature CD4⁺ and CD8⁺ T cells permanently express the diphtheria toxin receptor and are depleted with the addition of diphtheria toxin (DT) (**Figure S2.7J**).⁷⁶⁰ We adoptively transferred *in vitro*-activated OT-I CD8⁺ T cells (which are not depleted by DT) into these mice, transplanted MC38 tumors, and began DT treatment one day prior to IT injection of *Lm* or *Lm-OVA* to remove host T cells that may be specific to tumors. In this setting, we observed that the depletion of host T cells by DT treatment significantly reduced tumor control in *CD4-Cre*⁺ (host T cells depleted) compared to *CD4-Cre*⁻ (host T cells not depleted) mice (**Figures 2.7K and S2.7K-L**). However, as expected, *CD4-Cre*⁺ mice were still able to significantly control tumors without host T cells when treated with IT *Lm-OVA*, indicating that *Lm*-specific OT-I CD8⁺ T cells alone do provide significant tumor control.

Altogether, these data demonstrate that, in addition to *Lm*-specific CD8⁺ T cells controlling tumors directly, they also enhance the tumor-specific CD8⁺ T cell response to control tumors.

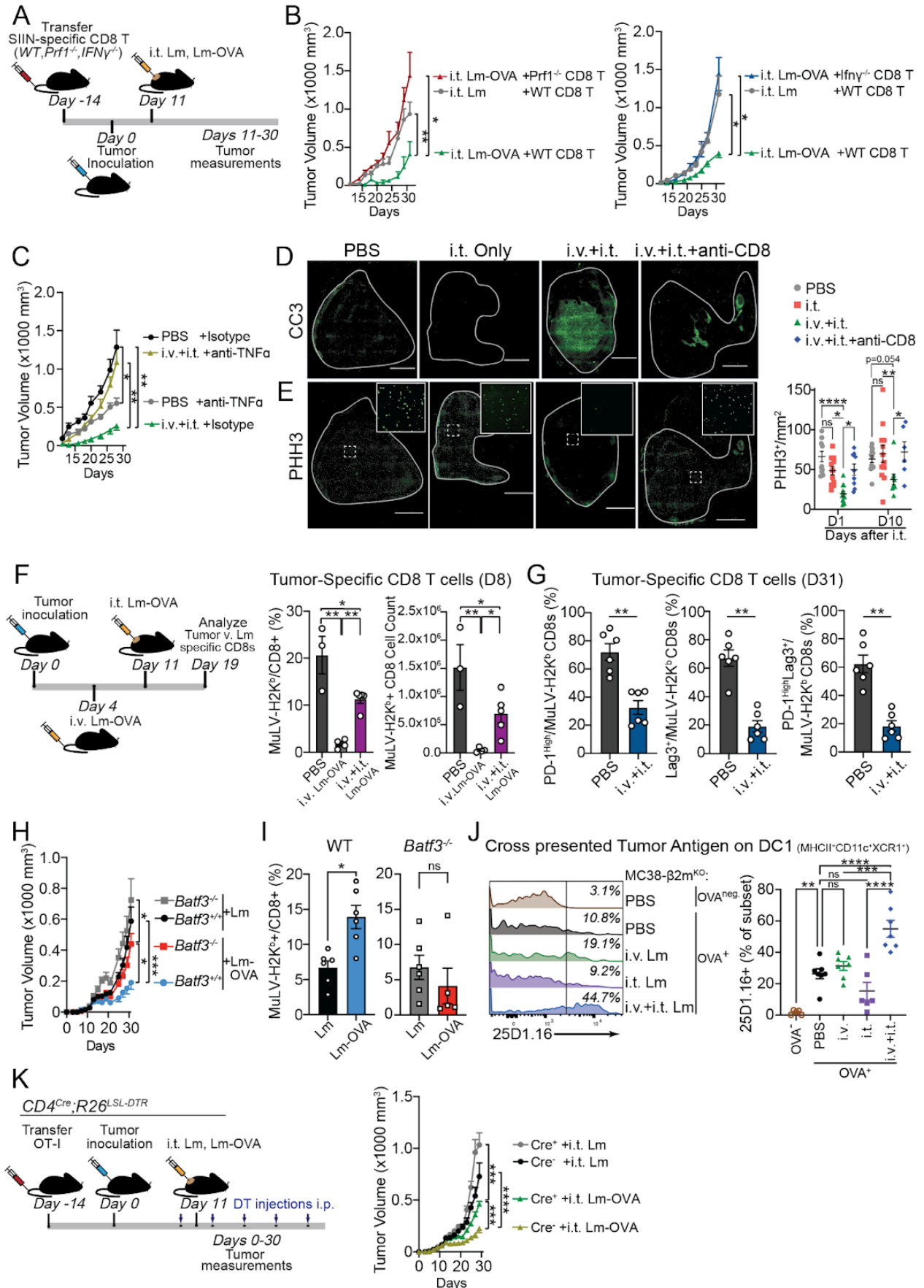


Figure 7. Anti-*Listeria* CD8⁺ T cell immunity enhances antitumor responses.

- (A) Experimental strategy to adoptively transfer *in vitro* expanded SIIN-specific CD8⁺ T cells from WT, *Prfl*^{-/-}, *Ifny*^{-/-} mice immunized with *Lm-OVA* (see methods).
- (B) MC38 tumor growth after adoptive transfer of SIIN-specific CD8⁺ T cells from WT, *Ifny*^{-/-}, or *Prfl*^{-/-} mice followed by IT injection of PBS, *Lm*, or *Lm-OVA* on day 11. Data from one of two experiments with n= 6 mice/group.
- (C) MC38 tumor growth +/- anti-TNF α 10 days after MC38-*B2m*^{-/-} tumor inoculation. Data from one of two experiments with n= 5-6 mice/group.
- (D) Representative images of Cleaved-Caspase 3 (CC3) (green) in MC38 tumors 24 hours post injection with IT PBS, IT *Lm*, or IV+IT *Lm* +/- anti-CD8. Scale bar = 2000 μ m. See Figure S7 for additional stains and day 10 images.
- (E) Representative images (left) of phospho-Histone 3 (PHH3) (green) in MC38 tumors 24 hours post injection with IT PBS, IT *Lm*, IV+IT *Lm* +/- anti-CD8 and quantification of PHH3 staining (right) in MC38 tumors at 1 day and 10 days after IT injections. Scale bar = 2000 μ m. Inset = high resolution images of boxed area of tissue. See Fig S7 for additional stains and day 10 images.
- (F) Experimental design for the analysis of frequency (left) and total number (right) of tumor-specific CD8⁺ T cells (MuLV-H2K^b tetramer) 8 days after IT *Lm-OVA* in IV+IT *Lm-OVA* regimen compared to IV+IT PBS or IV *Lm-OVA* only.
- (G) Frequency of PD-1^{High} (left), Lag3⁺ (center), and PD-1^{High}Lag3⁺ double-positive (right) tumor-specific (MuLV-H2K^b tetramer⁺) CD8⁺ T cells from MC38 tumors 31 days after PBS or IV+IT *Lm*.
- (H) *In vitro* activated OT-I CD8⁺ T cells were adoptively transferred into WT or *Batf3*^{-/-} mice, MC38 tumors inoculated, and tumors injected IT on day 11 with *Lm* or *Lm-OVA* and tumor growth monitored. Data pooled from two experiments with n= 5-6 mice per group.
- (I) Frequency of tumor-specific (MuLV-H2K^b tetramer⁺) CD8⁺ T cells in tumors from experiment in (H).
- (J) Representative histogram plots (left) and quantification (right) of 25D1.16 staining (anti-SIIN-H2K^b) on intratumoral DC1s (MHCII⁺CD11c⁺XCR1⁺) in OVA-expressing MC38-*B2m*^{-/-} tumors 24 hours after IT PBS, IT *Lm*, or IV+IT *Lm*.
- (K) *In vitro* activated OT-I CD8⁺ T cells were adoptively transferred into T cell deleter mice (*CD4-Cre;R26^{LSL-DTR}*) and treated with DT beginning 10 days after MC38 tumor inoculation and tumor growth was monitored. Data pooled from two experiments with n= 5-6 mice per group. For all plots, mean $\pm$ s.e.m and *P<0.05, **P<0.01, ***P<0.001 by Student's t-test (F-G, I) or two-way ANOVA (B-C, H, J).

2.4 Discussion

Here we report two major findings (**Figure S2.8**). First, we show that direct intratumoral injection of an attenuated strain of *Lm* caused increased tumor growth. This was due to *Lm*-mediated recruitment of PMNs that are converted within tumors into immunosuppressive MDSCs, which act as permissive cells for bacterial survival while also suppressing antitumor CD8⁺ T cells. Second, we show that IT *Lm*-mediated 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 inhibit tumor growth. Thus, depending on the context of IT *Lm* injection, *Listeria* can either promote or inhibit cancer progression. These results reveal new insights into the biology of *Listeria* colonization of tumors as well as the potential for anti-*Lm*-specific CD8⁺ T cells to contribute to cancer control. These findings refine the therapeutic mechanisms of action of bacterial cancer therapy and reveal the potential of targeting intratumoral colonizing bacteria for the treatment of cancer.

The *Lm*Δ*actA* 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 intracellular (e.g. *Salmonella*) and extracellular (e.g. *Clostridium*, *Vibrio cholerae*, *E. coli*) bacteria in tumors, which in some cases had direct tumoricidal effects.^{693,761,762} *Clostridium novyi* administered intravenously led to sporulation in the avascular regions of tumors that directly caused cancer cell death.^{700,763} Longer-term protection from cancer with *Clostridium* occurred in ~30% of mice and depended on the generation of CD8⁺ T cells, though the antigen specificity of the CD8⁺ T cells was not tested.^{700,763} *E. coli* engineered to express tumor antigens, the pore forming protein α-hemolysin, or L-arginine to promote CD8⁺ T cell function also led to improved tumor control upon bacterial colonization of tumors.^{709,764,765} The prevalence of bacteria in tumors has been attributed to the presence of nutrients or a TME that is conducive to bacterial growth.^{704,766–769} *Salmonella typhimurium* engineered to infiltrate tumors by selective auxotrophy was effective at controlling several mouse tumors.^{704,767,770} The lack of documentation of *Lm* persistence in tumors could be the result of using other attenuated strains of *Listeria* or failing to assess *Lm* persistence in previous studies. However, in the case of *Lm*Δ*actA* tumor colonization in this study, we hypothesize that bacteria migrate to tumors stochastically via the bloodstream, and upon arrival in tumors, *Listeria* is protected from immune clearance due to the immunosuppressive TME, which generates PMN-MDSCs that permit the intracellular persistence of *Lm*.^{693,771}

PMN permissiveness for cytosolic *Lm* correlated with a CD14 and PD-L1 expression, which is associated with a MDSC phenotype. This MDSC phenotype was promoted by tumor derived factors, as culturing PMNs with tumor cell supernatants was sufficient to induce CD14 and PD-L1 expression. While depletion of these PMN-MDSCs did not prevent *Lm* persistence in tumors, it prevented 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 vascularization to support tumor cell proliferation in several cancers.^{717,724,745} 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 by tumor antigen-specific CD8⁺ T

cells. Altogether, we uncovered an unexpected consequence of *Lm* 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. However, microbes have also been shown to directly promote immune tolerance, and it is possible that *Lm* directly promoted tolerance in the TME and its clearance with IV+IT *Lm* allowed for stronger tumor control.⁷⁷²

Attenuated *Listeria* vaccines generate memory CD8⁺ and CD4⁺ T cells that provide robust and long-lasting adaptive immunity.⁶²⁴ We tested whether prior IV *Lm* vaccination would impact the immunosuppressive TME and promote tumor control with a secondary IT *Lm* dose. In this setting, IT *Lm* led to the control of multiple tumor types, including an aggressive orthotopic KP sarcoma. Tumor control was also observed with prophylactic vaccination prior to tumor inoculation, ruling out a role for tumor colonization by IV *Lm* in the efficacy of IV+IT *Lm*. Our results parallel previous approaches taking advantage of TDAP vaccination and using *Listeria* as a delivery system for tetanus toxoid to elicit a CD4⁺ memory T cell response to eliminate tumor cells.⁷⁵³ However, unlike that study, we found a clear requirement for CD8⁺ T cells in mediating tumor control. Furthermore, in prior reports, it was shown that the *Listeria* infected the cancer cells directly to drive T cell recognition and control of cancer.⁷⁵³ However, in our study we used an RFP reporter to identify cytosolic *Lm* and did not find evidence of direct infection of tumor cells. Furthermore, while NK cells and MHC-I expression on tumor cells were dispensable for tumor control, MHC I expression on non-tumor host cells was essential for the antitumor efficacy of adoptive OT-I T cell transfer followed by IT *Lm*-OVA administration. Therefore, CD8⁺ T cell recognition of host cells, presumably CD45⁺ immune cells that have taken up *Lm* in their cytosol, was required for tumor control. The differences in mechanisms across studies could be the result of differences in the attenuated strains of *Listeria* used. Our study used a $\Delta actA$ mutation that prevents cell to cell spread, whereas others have used *Listeria* that retains a functional *actA* gene, allowing *Listeria* to spread to non-phagocytic cells, including tumor cells.^{706–708,713,716,753,769} Overall, while direct tumor cell infection may support immune responses to tumors, our results indicate that tumor control by *Lm*-specific CD8⁺ T cells does not require bacterial spread into tumor cells.

To test the specificity of the CD8⁺ T cells required for tumor control with IT *Lm*, we first tested the importance of *Lm*-specific CD8⁺ T cells by adoptive transfer of OT-I CD8⁺ T cells or polyclonal *Lm* antigen-specific T cells in place of IV *Lm* administration. We then used *Lm* IT delivery (without IV *Lm*) to show that T cells targeting *Lm* antigens were sufficient to mediate tumor control. Tumor control with *Lm*-specific T cells was sustained in *Rag-2*^{-/-} mice that lacked tumor-specific T cells, showing that bacteria-specific T cells responding within the TME can control cancer. 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.¹⁰³ 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 lacked pre-existing BCG-specific T cell immunity.⁷⁷³

To identify the mechanisms that anti-*Lm* CD8⁺ T cells used for tumor control, we transferred *Lm*-specific *Prfl*^{-/-} or *Ifng*^{-/-} CD8⁺ T cells or TNF- α blockade to show that killing activity, as well as IFN γ and TNF α production, were required for tumor control. We hypothesize

that killing activity by CD8⁺ T cells is required to eliminate PMN-MDSCs and the *Listeria* residing within them. This was evidenced by a high level of CC3-staining one day after IT *Lm* delivery exclusively in the IV+IT *Lm*-treated tumors that was dependent on CD8⁺ T cells. IFN γ and TNF α production may be necessary to induce MHC I expression on these cells to mediate robust killing of *Lm*-infected cells, and/or it may act directly on tumor cells to reduce tumor cell proliferation.^{774–776} In line with this, we observed a CD8⁺ T cell-dependent reduction in PHH3⁺ tumor cells with IV+IT *Lm* immediately following IT *Lm* delivery at one day and a sustained reduction in tumor cell proliferation for up to 10 days afterwards.

Finally, IV+IT *Lm* enhanced the accessibility of tumor-derived antigens for cross presentation by DC1s to tumor-specific CD8⁺ T cells by an antigen-spreading phenomenon.^{777–779} This was evidenced by both increased tumor antigen presentation on DC1s and increased tumor-specific CD8⁺ T cells infiltrating tumors with IV+IT *Lm* compared to IV *Lm* alone. We confirmed the importance of DC1s and tumor-specific T cells by genetic deficiency for DC1s (*Batf-3*^{-/-}) or tumor-specific T cells (*CD4-Cre;R26*^{LSL-DTR}, T cell deleter) and showing significant loss of tumor control in either scenario. However, the activity of anti-*Listeria* CD8⁺ T cells were still paramount to tumor control, as their depletion completely restored rapid tumor growth. Thus, the anti-*Listeria* T cell response not only controls tumors indirectly, by promoting strong, localized cell death in the tumor and inhibiting tumor proliferation, but these activities also increase epitope spreading of tumor antigens, enhancing the tumor-specific CD8⁺ T cell responses.

In conclusion, we have shown that 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 promote tumor control through direct cell killing of non-tumor cells in the TME. Because the T cell responses against certain pathogens (i.e. *Listeria*, BCG, 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 showed that methods to expand pathogen-specific T cells *ex vivo* for adoptive transfer of bacterial 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 cancer patients⁷⁸⁰. Collectively, our study demonstrates new insights into the inefficiencies of current bacterial immunotherapy approaches and reveals a tumor antigen-free bacterial dosing regimen that can control cancer independently of tumor immunogenicity or the identification of tumor neoantigens. Most importantly, the recent findings that many tumors are colonized by various bacterial species opens the door to a new alternative strategy to modify the tumor microenvironment and treat cancer by targeting intratumoral bacteria.

Limitations of the study:

This study only used one attenuated strain of *Listeria* for all investigations. While we hypothesize that the findings will be generalizable to other bacterial species, or even other attenuated strains of *Listeria*, this remains to be tested. Future studies will also need to test what specific bacterial attributes of *Listeria* are required to promote tumor control - such as the

capacity for intracellular invasion or the capacity to activate specific innate pattern recognition receptors. This will shed light on the broad applicability of these findings to other bacterial species. Next, while we showed that *Lm* colonization of a non-subcutaneous tumor model (PDAC) also occurred, further investigations to test whether the IV+IT (or IV+IV) *Lm* therapeutic strategy is also effective against internally arising epithelial cancers need to be performed. Finally, the findings here rely solely on mouse cancer models. Future studies should examine whether human tumors will yield similar results, such as with humanized mouse models or the examination of human tumor samples, especially from patients who were treated with *Listeria* vaccines.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michel DuPage (dupage@berkeley.edu).

Materials availability

Listeria monocytogenes strains used in the study will be made available from the lead contact upon request.

Data and code availability

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Acknowledgments:

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Author Contributions:

Conceptualization and Methodology, M.D., D.A.P., J.G.C., S.F., T.F.C; Investigation, J.G.C., S.F., T.F.C., J.W., D.G.V., J.Y., N.F.H., E.W., and M.D.; Writing – Original Draft, M.D., J.G.C., S.F.; Writing – Review & Editing, M.D., D.A.P., J.G.C., S.F., T.F.C; Supervision, M.D., D.A.P., J.G.C., S.F.; Funding Acquisition, M.D., D.A.P.

Declaration of Interests:

D.A.P. is on the board of directors for Laguna Therapeutics.

2.5 Methods

Experimental models and subject details:

Animal studies

C57BL/6J wildtype mice were obtained from Jackson laboratories (JAX:000664) and bred in house. OT-I transgenic mice were obtained Taconic (Catalog#: 2334) and bred in house. Prf1^{-/-} (JAX:002407) and Ifny^{-/-} (JAX:002287) mice were a gift from the Stanley lab at the University of California, Berkeley. Rag2^{-/-} (JAX:008449) and $\beta 2m^{-/-}$ (JAX:002087) mice were a gift from the Raulet lab at the University of California, Berkeley. CD4^{Cre} mice were obtained from Jackson laboratories (JAX:022071) and bred in house. R26^{DTR} mice were obtained from Jackson laboratories (JAX:008040) and bred in house. Sarcoma cell lines were generated in *Kras^{LSL-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 cultured⁷⁵². For tumor studies, syngeneic C57BL/6J mice were inoculated with 5.0x10⁵ MC38 or 2.0x10⁵ B16F10 cells in PBS subcutaneously. KP sarcoma cells were inoculated into C57BL/6J mice with 5.0x10⁵ cells by intramuscular injection of the right hind limb. For diphtheria toxin (DT) treatment for T cell depletions, we injected 1 μ g every 2 days by i.p. 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 lab⁷⁸¹. MC38- $\beta 2m^{-/-}$ cell line was kindly provided by Dr. David Raulet's lab⁷²⁸. 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°C with 5% CO₂.

Method Details:

Listeria monocytogenes strains

All strains of *L. monocytogenes* were derived from the wild-type 10403S strain. The *Lm* constructs were based on an attenuating deletion of the *actA* gene ($\Delta actA$). *Lm-OVA* expresses an LLO-OVA fusion encompassing amino acids 1-441 of Listeriolysin O (LLO) and OVA cloned in frame of LLO1-441 under the control of the LLO promoter in the pPL2 integration vector⁷⁸². *Lm-TagRFP* expresses a secreted TagRFP protein under the control of the *actA* promoter using the pPL2 integration vector.⁶³⁷ All strains were cultured in filter-sterilized nutrient-rich Brain Heart Infusion (BHI) media (BD Biosciences) containing 200 μ g/mL streptomycin (Sigma-Aldrich).

Intravenous and Intratumoral Listeria infection

Overnight cultures were grown in BHI + 200 μ g/mL streptomycin at 30°C. 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°C 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 1×10^6 CFU log-phase bacteria. For intratumoral infections, frozen stocks were diluted in PBS to infect via intratumoral injections at 5×10^7 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°C.

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, Berkeley⁷⁴⁴. 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 1×10^6 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 described⁷⁴³. To generate PMNs-MDSCs, cells were cultured in 40ng/mL IL-6 and 40ng/mL GM-CSF 1 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°C. Cell surface antigens were stained at 4°C 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, BioLegend), 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°C, 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°C, and fixed in 4% PFA. Cells analyzed by flow cytometry the following day to prevent signal loss from fluorescent protein. Cells were resuspended in PBS and filtered through a 70- μ m nylon mesh before data acquisition. Datasets were analyzed using FlowJo software (Tree Star).

Restimulation Assays

Resected tumors were minced to 1 mm³ fragments and digested in RPMI media supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 20 mg/mL DNase I (Roche), and 125 U/mL collagenase D (Roche) using an orbital shaker at 37°C. Cells from lymphoid organs were prepared by mechanical disruption pressing against a 70- μ m nylon mesh. All the cell suspensions were passed through 40 μ m filters before in vitro stimulation. Cytokine staining was performed with 3-5x10⁶ cells in Opti-MEM media supplemented with Brefeldin A (eBioscience) or 10 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma), and 0.25 μ M 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-I transgenic mice or C57BL/6J wildtype (WT) or *Prf1*^{-/-} previously vaccinated with 1.0x10⁶ CFU *Lm-OVA* 3-4 weeks prior. OT-I or OVA-responsive CD8s were activated with 1 μ g/mL SIINFEKL peptide in DMEM medium supplemented with 10% FBS, non-essential amino acids, sodium pyruvate, L-glutamine, HEPES, 55 μ M 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.

Immunofluorescence & Imaging Analysis

Tissue Sections were washed with PBS and blocked with tissue staining buffer plus 10% fetal bovine serum. Tissues were then stained with anti-Ly6G Alexa Fluor 488 antibody (Clone 1A8, Biolegend Catalog# 127624), anti- Phospho-Histone H3 (Ser10) antibody (Cell Signaling Catalog# 9701S, or anti-Cleaved Caspase-3 (Asp175) antibody (Clone 5A1E, Cell Signaling Catalog# 9664S) O/N at 4°C in tissue staining buffer (PBS with 0.2% Triton X-100, 2% FBS) and washed with PBS. For sections stained with unconjugated primary antibodies, sections were incubated with Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (Thermo Fisher Scientific, Catalog# A-11008) for 1 hour at RT followed by washes with PBS. Tissues were stained with Wheat Germ Agglutinin (WGA) Alexa Fluor 647 (Thermo Fisher Scientific, Catalog# W32466) for 45 mins at RT and washed with PBS. Stained tissue sections were mounted with Fluoromount-G Mounting Medium (Southern Biotech). Images were taken using Zeiss LSM 780 NLO microscope and Zeiss Axioscan 7 or Axioscan Z1. Imaging analysis was performed using Zeiss Zen Lite Imaging Software or Fiji/ImageJ. Quantification of PHH3 staining was performed by cropping full Axioscan images into 2 mm x 2 mm images from which 3 representative cropped images per tumor were chosen for counting. Individual PHH3+ cells in these sections were blocked out using the Fiji/ImageJ paintbrush tool until no PHH3+ cells remained visible. A click counter (Mousotron) was used to track the number of cells per section.

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 $\pm$ 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.

2.6 Supplementary Materials

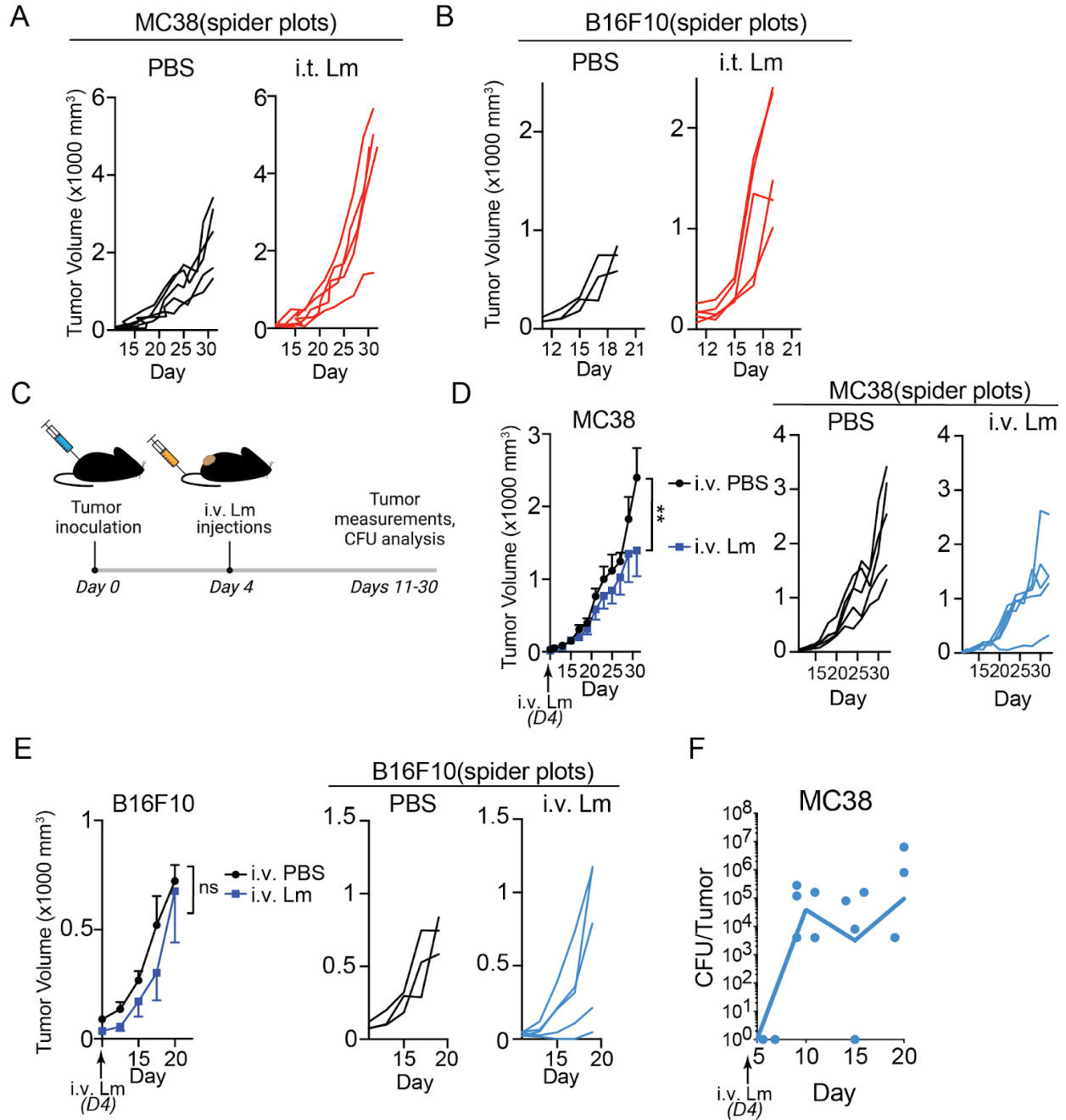


Figure S2.1 (Related to Figure 2.1)

(A) Tumor growth curves for individual mice in 1B.

(B) Tumor growth curves for individual mice in 1C.

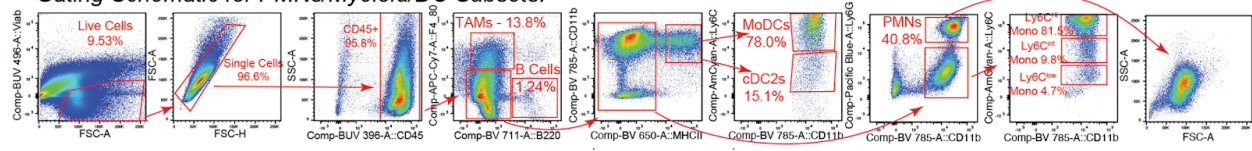
(C) Experimental design.

(D-E) C57BL/6J WT mice were injected with 5×10^5 MC38 (B, data shown from $n = 5$ mice per group from one of three experiments) or 2×10^5 B16F10 (C, data shown from $n = 5$ mice per group from one of two experiments) tumor cells and then 1×10^6 Lm CFUs i.v. 4 days later and tumors measured.

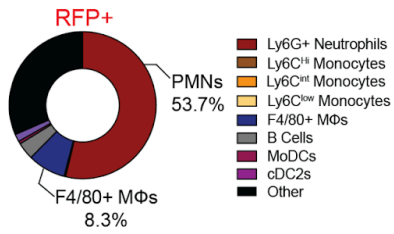
(F) CFU analysis for Lm in MC38 tumors from mice injected i.v. with 1×10^6 CFU 4 days after tumor inoculation showed seeding and persistence of Lm in tumors as in (B) ($n = 4-5$ mice per time point).

For all plots, statistical analysis was done by two-way ANOVA, mean $\pm$ s.e.m.

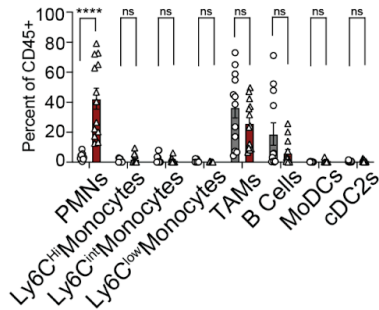
A Gating Schematic for PMNs/Myeloid/DC Subsets:



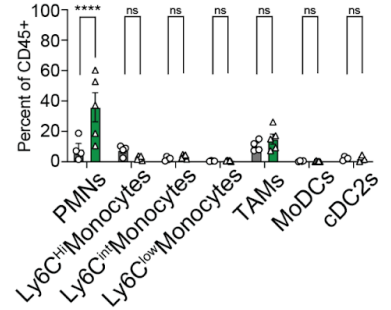
B B16F10, 24 hrs i.t. Lm



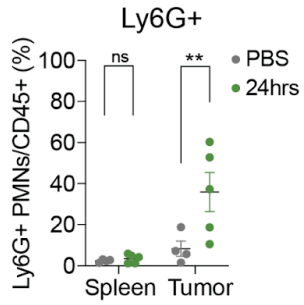
C MC38, 24 hrs i.t. Lm



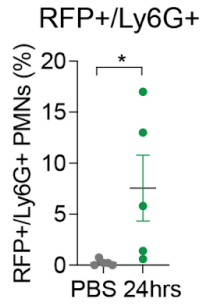
D B16F10, 24 hrs i.t. Lm



E



F



G

Treat mouse (in vivo)

Isotype control

anti-Gr1 depletion

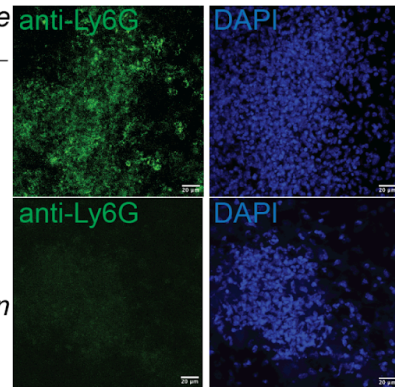


Figure S2.2 (Related to Figure 2.2)

(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 i.t. Lm (n= 4-5 mice/ group).

(C-D) Frequency of intratumoral immune cell populations as percent of all CD45⁺ cells 24 hours after i.t. PBS or 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 i.t. PBS or i.t. Lm (n=4-5 mice/group).

(F) Frequency of intratumoral Ly6G⁺ PMNs that were RFP⁺ 24 hours after i.t. Lm in B16F10 tumors.

(G) Validation of anti-Ly6G staining on frozen tumor sections by testing sections from mice treated with an isotype control antibody or an anti-Gr1 antibody that depletes all Ly6G⁺ cells.

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

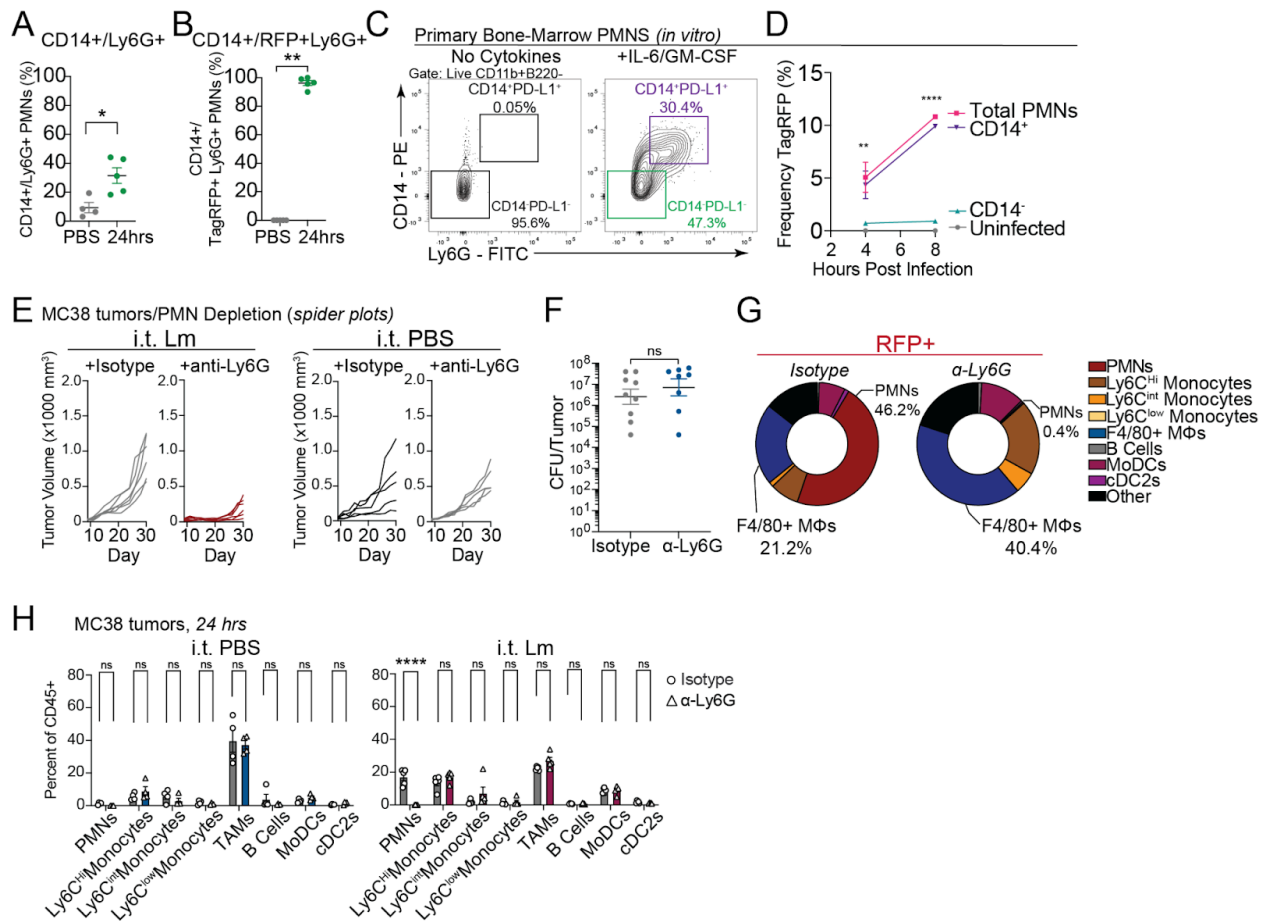


Figure S2.3 (Related to Figure 2.3)

(A) Frequency of CD14⁺ PMNs 24 hours after i.t. Lm in B16F10 tumors. Results from n= 4-5 mice/group

(B) Frequency of intratumoral CD14⁺Ly6G⁺ cells that were RFP⁺ 24 hours after i.t. PBS or i.t. Lm.

(C) 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.

(D) 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.

(E) Tumor growth curves for individual mice in 3J.

(F) Lm CFUs recovered from tumors 20 days after i.t. Lm in mice treated with isotype control or anti-Ly6G antibodies as shown in Figure 3J.

(G) 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 i.t. Lm.

(H) Frequency of intratumoral immune cell populations as percent of all CD45⁺ cells 24 hours after i.t. Lm or i.t. PBS into MC38 tumors in mice pre-treated with isotype or anti-Ly6G (PMN-depleting) antibodies as shown in Figure 3I.

For all plots, *P<0.05, **P<0.01, ***P<0.001 by student t-test (A-B, F, H), or one-way ANOVA (D), mean $\pm$ s.d for in vitro experiments. Two-way ANOVA (J), mean $\pm$ s.e.m for in vivo experiments

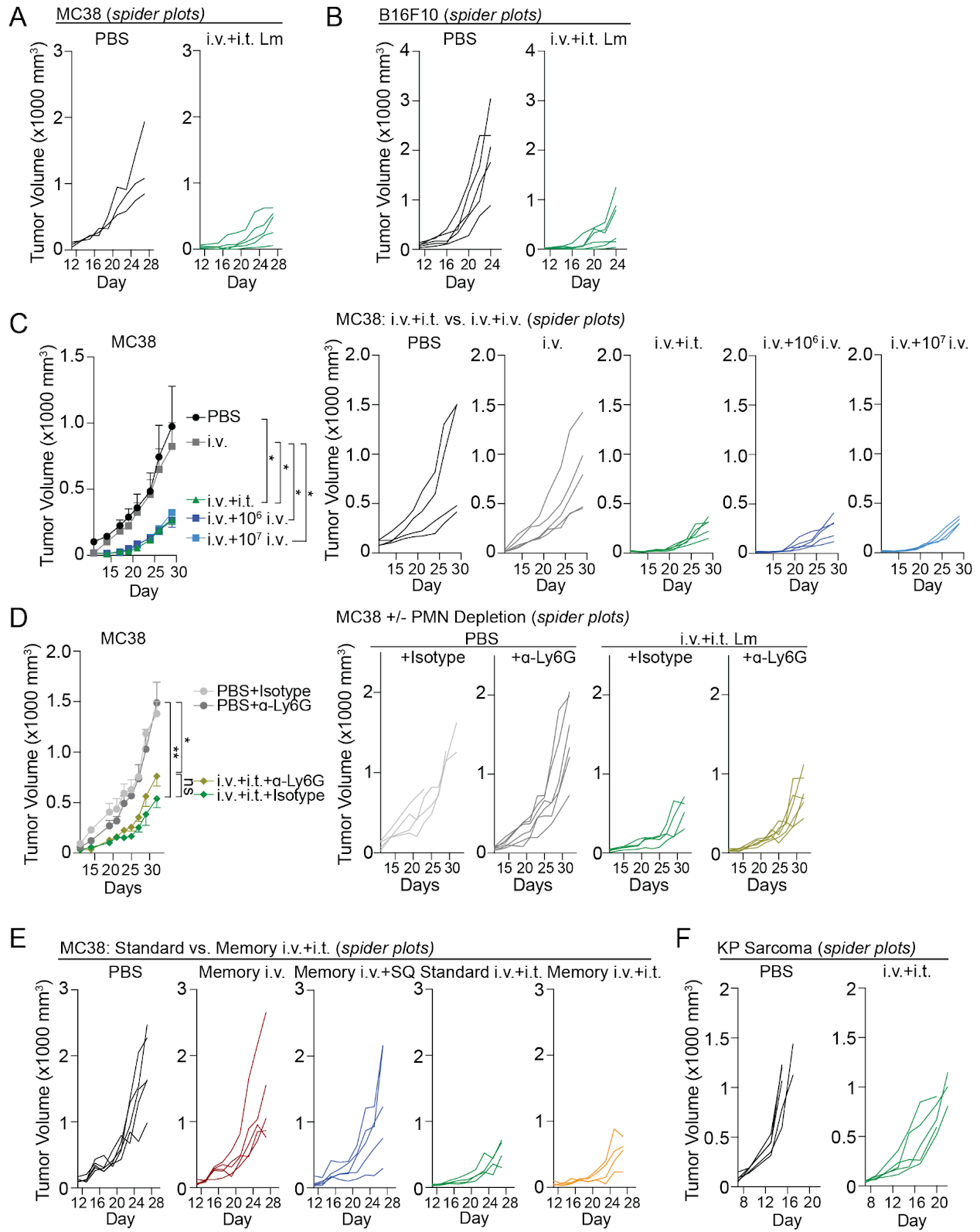


Figure S2.4 (Related to Figure 2.4)

(A) Tumor growth curves for individual mice in 4B.

(B) Tumor growth curves for individual mice in 4C.

(C) Average (right) and individual tumor growth (left) for MC38 with i.v.+i.v. PBS versus i.v.+i.v. Lm versus i.v.+i.t. Lm regimen (data shown from n= 5-6 mice per group from one of two experiments).

(D) Average (right) and individual tumor growth (left) for MC38 tumor growth with PBS versus i.v.+i.t. Lm and with or without anti-Ly6G depleting antibody treatment beginning two days prior to i.t. Lm administration (n=4-6 mice per group).

(E) Tumor growth curves for individual mice in 4H.

(F) Tumor growth curves for individual mice in 4I.

For all plots, **P<0.05, **P<0.01, ***P<0.001 by two-way ANOVA (C-D), mean $\pm$ s.e.m.

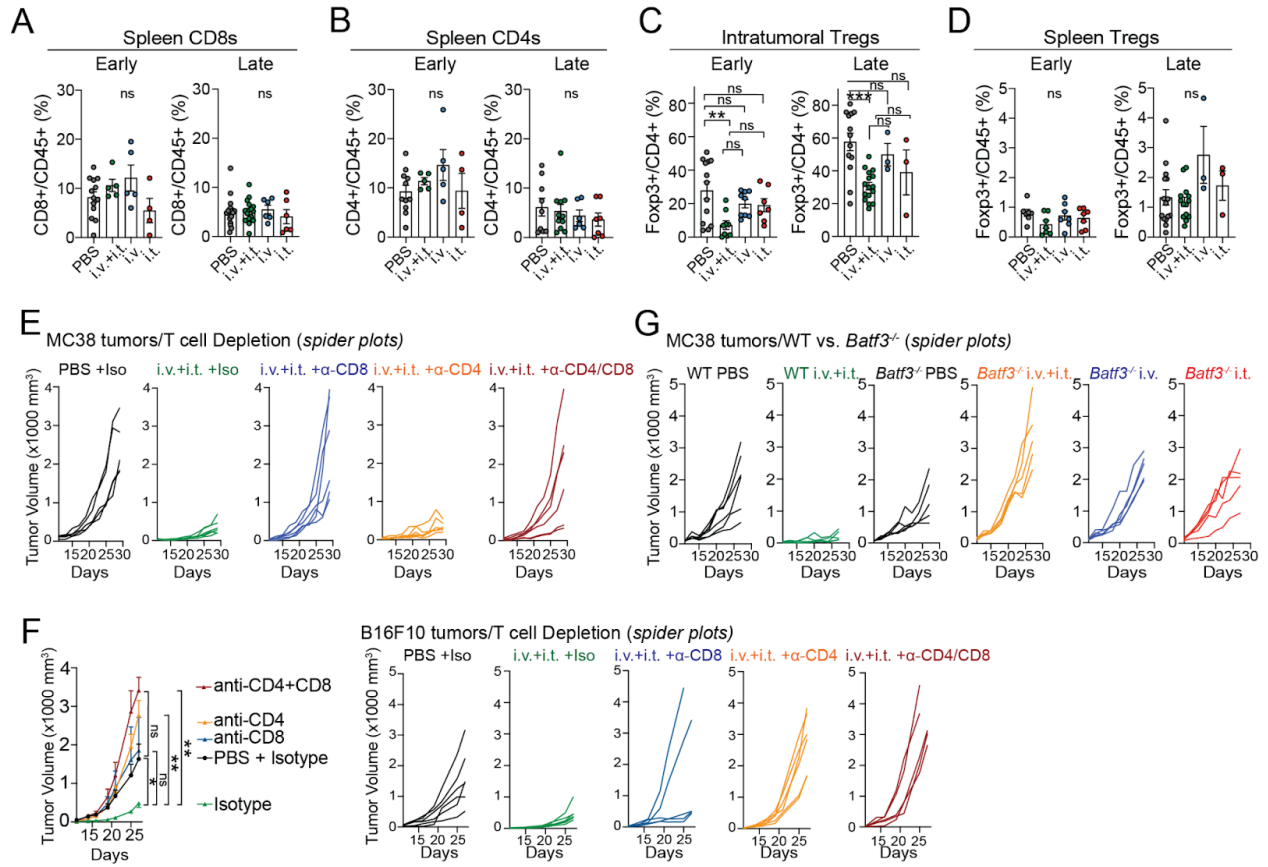


Figure S2.5 (Related to Figure 2.5)

(A-B) Frequency of CD8⁺ (C) and CD4⁺ (D) T cells from spleens at early or late timepoints. Data pooled from four experiments with 5 mice/group.

(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.

(E) Tumor growth curves for individual mice in 5F.

(F) Average (right) and individual tumor growth (left) for B16F10 with i.v.+i.t. Lm regimen plus anti-CD8, anti-CD4, or both depleting antibodies beginning 10 days after B16F10 tumor inoculation.

(G) Tumor growth curves for individual mice in 5H.

For plots in S5A-S5D, 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) or two-way ANOVA (F), mean ± s.e.m.

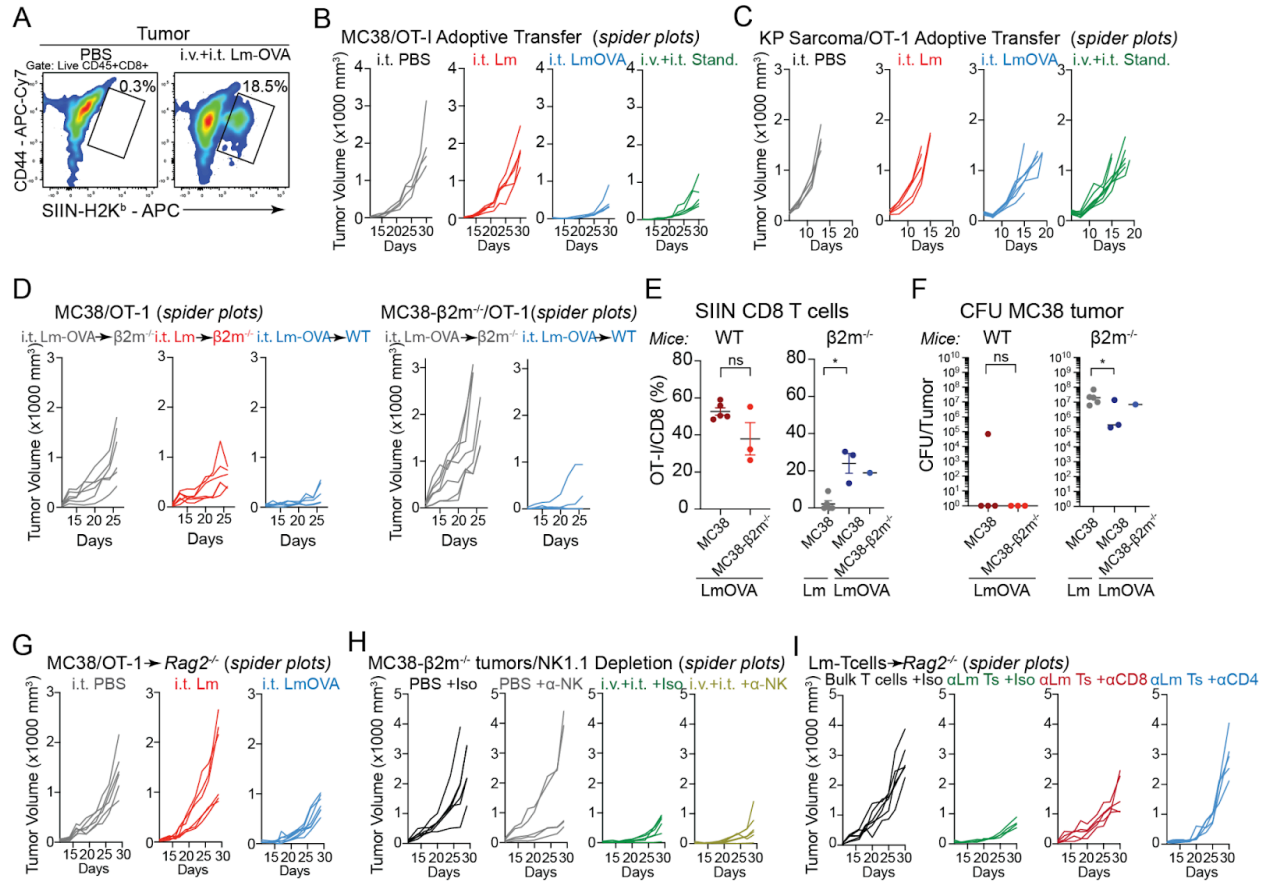


Figure S2.6 (Related to Figure 2.6)

(A) Representative flow plots for SIIN-H2K^b tetramer staining from tumors of mice treated with i.v.+i.t. PBS compared to i.v.+i.t. Lm-OVA 8 days after i.t. injections.

(B) Tumor growth curves for individual mice in 6B.

(C) Tumor growth curves for individual mice in 6C.

(D) Tumor growth curves for individual mice in 6D.

(E) Frequency of intratumoral OT-I CD8 T cells in mice 20 days after i.t. Lm administration from Figure 6D.

(F) CFU analysis for Lm in tumors from Figure 6D.

(G) Tumor growth curves for individual mice in 6E.

(H) Tumor growth curves for individual mice in 6F.

(I) Tumor growth curves for individual mice in 6G.

For all plots, *P<0.05, **P<0.01, ***P<0.001 by student t-test (E-F), mean $\pm$ s.e.m.

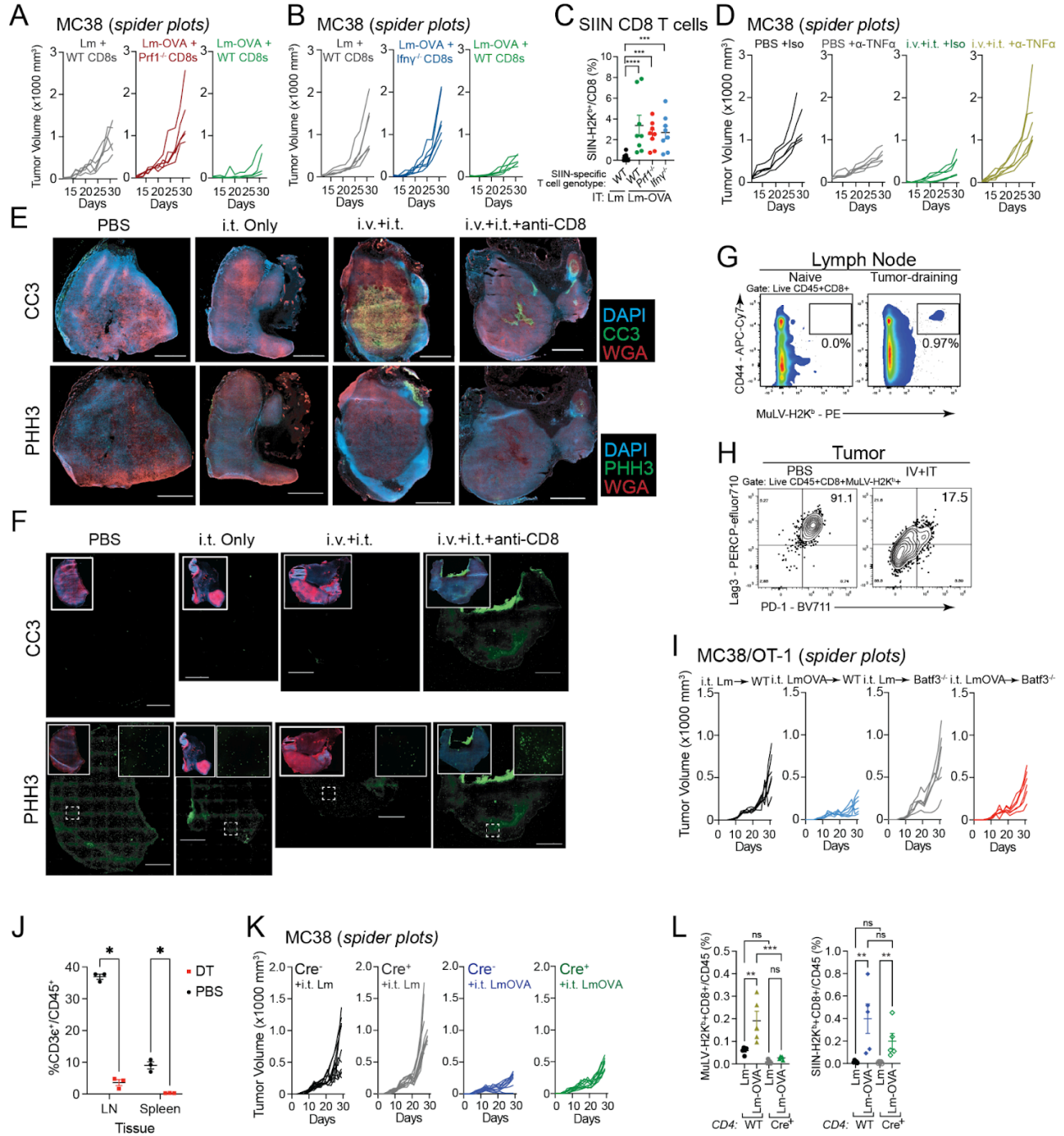


Figure S2.7 (Related to Figure 2.7)

(A-B) Tumor growth curves for individual mice in 7B.

(C) Frequency of intratumoral SIIN-specific CD8 T cells in mice 20 days after i.t. Lm administration from experiments depicted in Figures 6J-K (pooled from two independent experiments)

(D) Tumor growth curves for individual mice in 7C.

(E) Images of MC38 tumors 24hrs post injection with i.t. PBS, i.t. Lm, or given i.v.+i.t. regimen +/- anti-CD8 i.p: phospho-Histone 3 (PHH3) or cleaved-caspase 3 (CC3) (green), WGA (red) and DAPI (blue). Scale bar = 2000 μ m.

(F) Images of MC38 tumors 10 days post injection with i.t. PBS, i.t. Lm, or given i.v.+i.t. regimen +/- anti-CD8 i.p: phospho-Histone 3 (PHH3) or cleaved-caspase 3 (CC3) (green), WGA (red) and DAPI (blue). Scale bar = 2000 μ m.

(G) 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.

(H) Representative flow plots for PD-1+Lag3+ MuLV-H2K^b CD8 T cells for 7G.

(I) Tumor growth curves for individual mice in 7H.

(J) Depletion of T cells in T cell deleter mice (CD4^{Cre}; R26^{LSL-DTR}) in non-tumor bearing mice. Mice were injected twice with 1 μ g DT i.p. every two days and inguinal lymph nodes and spleen collected for flow analysis.

(K) Tumor growth curves for individual mice in 7K.

(L) Frequency of intratumoral tumor-specific (MuLV-H2K^b tetramer) CD8 T cells (right) and OT-I CD8 T (left) cells in mice 20 days after I.T. Lm administration from Figure 7J.

For all plots, *P<0.05, **P<0.01, ***P<0.001 by student t-test (C,M) or two-way ANOVA (H), mean $\pm$ s.e.m.

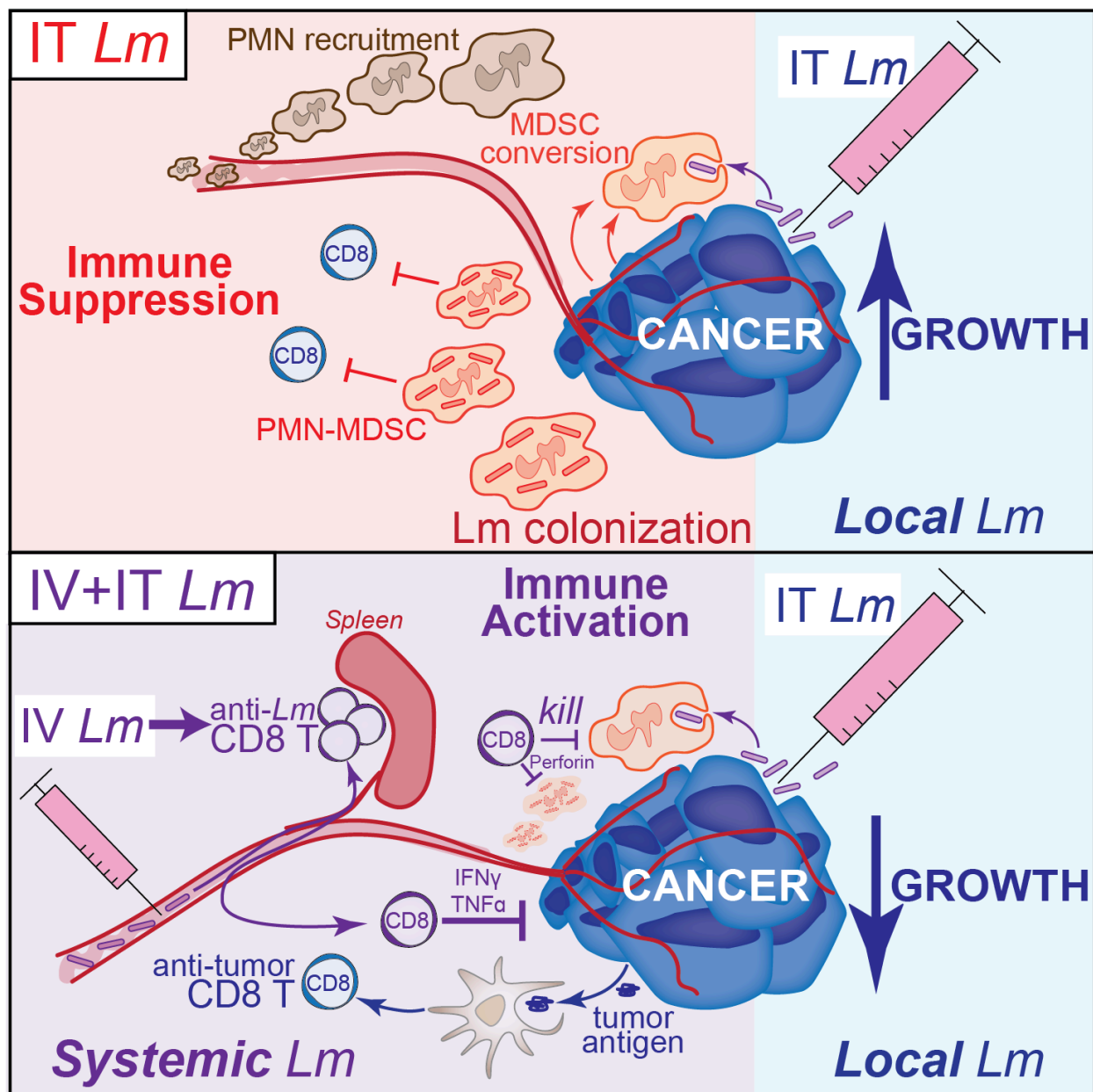


Figure S2.8 (Graphical Abstract)

IT *Lm*: recruits PMNs to the TME, which then are converted to immunosuppressive PMN-MDSCs.

IV *Lm*: generates *Lm*-specific CD8⁺ T cells.

IV+IT *Lm*: recalls *Lm*-specific CD8⁺ T cells to the TME, killing *Lm*-infected PMN-MDSCs via Perforin, suppressing tumor growth via TNF- α and IFN- γ , and leading to uptake and cross presentation of tumor antigens to drive tumor-specific CD8⁺ T cell immunity.

Chapter 3

TLR2 signaling unleashes multiple mechanisms to reduce Treg immunosuppression and enhance immunity to cancer

3.0 Authors

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3.1 Abstract

Toll-like receptors (TLRs) play known roles in innate immunity and priming adaptive responses, but their roles in cancer and secondary infection remain unclear. To investigate this, we used an attenuated *Listeria monocytogenes* (*Lm*) strain that promotes CD8⁺ T cell-mediated tumor control upon intravenous then intratumoral (IV+IT) infection. We found TLR2 was uniquely important for tumor control by IV+IT *Lm*. While TLR2 supported T cell priming during primary IV infection, surprisingly, TLR2 was also critical during secondary IT infection. This effect was partly due to TLR2 signaling in type II dendritic cells (cDC2s), which destabilized Foxp3 expression and depleted regulatory T cells (Tregs) from tumors. Treg destabilization correlated with enhanced tumor antigen cross-presentation by type I dendritic cells (cDC1s) and stronger CD8⁺ T cell responses. These findings reveal a novel role for TLR2 in reshaping the immune landscape of tumors during secondary *Lm* infection by coordinating cDC subsets to reduce immunosuppression and enhance adaptive responses.

3.2 Introduction

Recent advancements in cancer immunotherapy have focused on targeting T cells that kill cancer cells.^{783,784} Therapeutic cancer vaccination, which uses microbial vectors like attenuated *Listeria monocytogenes* (*Lm*) engineered to express tumor antigens, is one such strategy that aims to elicit antitumor T cell mediated immunity.^{611,623,637} While bacterial therapeutic vaccines have shown promise in preclinical models, most have not demonstrated sufficient efficacy in clinical trials.^{611,623} An advantage of microbial cancer vaccines is that pattern recognition receptor (PRR) ligands produced by microbial vectors promote tumor-specific T cell priming, allowing a single agent to serve as both antigen and adjuvant.^{611,623} However, beyond their role in T cell priming, the effects of PRR stimulation by cancer vaccine vectors remain poorly understood.⁷⁸⁵ Furthermore, in vectors like *Lm* that activate multiple PRRs, it is unclear whether a single PRR ligand provides unique adjuvant function or whether redundant pathways collectively drive antitumor efficacy.⁶³⁴ The failure of prior clinical trials suggests that successful therapeutic cancer vaccination will require not only optimizing tumor-specific T cell priming but also taking full advantage of PRR signaling to enhance antitumor immunity.^{611,623,637}

Toll-like receptors (TLRs), a well-characterized PRR family, are among the first innate receptors to be engaged during infection.⁷⁵ TLR signaling in antigen-presenting cells (APCs) such as dendritic cells (DCs) upregulates genes involved in antigen presentation and costimulation that are crucial for initiating T cell-mediated immune responses.⁷⁵ TLRs and APCs thus play key roles in shaping antitumor T cell responses elicited by cancer vaccines.^{75,786–788} Accordingly, intratumoral injection of TLR ligands can inhibit tumor growth in mice, presumably by increasing T cell priming against tumor antigens.^{655,785,786} Clinical testing of TLR ligands has emphasized the viral-sensing endosomal TLRs (TLR3, 7/8, and 9), leading to the approval of the TLR7 agonist imiquimod for treating superficial basal cell carcinoma.^{655,669,682,685,686,786,789} However, how TLR signaling affects antitumor immunity beyond its role in *de novo* T cell priming is poorly understood.⁷⁸⁵ Moreover, whether bacteria-sensing cell-surface TLRs (TLR1/2, 2/6, 4, and 5) can promote antitumor CD8⁺ T cell responses similarly to endosomal TLRs remains underexplored.

Several findings provide evidence that bacterially-mediated cell-surface TLR stimulation within tumors can benefit cancer immunotherapy. Intravesical BCG, which activates TLR2 and 4, is a standard treatment for noninvasive bladder cancer.⁹ Similarly, the first recorded form of cancer immunotherapy, Coley's Toxins, employed direct injection of heat-killed *Streptococcus pyogenes* and *Serratia marcescens* (activating TLR2, TLR4, and TLR5) into sarcomas.^{7,790–793} *Lm* can likewise stimulate TLR2, 5, and 9, and TLR signaling is essential for controlling *L. monocytogenes* in non-tumor tissues, prompting speculation that TLR signaling by *Lm* cancer vaccines is beneficial.^{372,634,794–800} However, studies of anti-*Lm* immunity outside the context of cancer vaccination suggest that anti-*Lm* CD8⁺ T cell immunity during secondary infection is largely TLR-independent.^{372,634,795,797,800,801} Likewise, TLR signaling-deficient patients, while more susceptible to bacterial infections early in life, appear to develop effective adaptive immunity to bacteria late in life, implying a limited role for TLRs beyond the control of primary infections and *de novo* T cell priming.^{802,803} Consequently, the effects of *Lm*-mediated TLR signaling on antitumor T cell responses are understudied, leaving several fundamental questions about the role of TLR signaling by *Lm* cancer vaccines unanswered.

One open question is whether TLR signaling by *Lm* cancer vaccines modulates tumor antigen presentation. While *Lm* cancer vaccines deliver tumor antigens to the MHC-I presentation pathway upon entry into the cytosol, their effect on the presentation of unlinked, endogenous tumor antigens (i.e., cross-presentation) remains unclear.^{611,623,634} In steady-state conditions, tumor antigen cross-presentation by Batf3⁺ cDC1s is the primary mechanism of priming antitumor CD8⁺ T cells.⁵⁰⁴ Intriguingly, cross-presentation is also required to prime *Lm*-specific CD8⁺ T cells despite the ready access of *Lm* antigens to the endogenous MHC-I pathway.^{804–806} We recently found that anti-*Lm* T cell responses in tumors can facilitate the cross-presentation of tumor antigens.⁸⁰⁷ *Lm*-mediated TLR signaling within tumors may underlie this effect.^{340,808} Indeed, TLR ligands linked to tumor antigens can enhance cross-presentation and bolster antitumor T cell responses *in vivo*.^{808,809} However, whether TLR signaling regulates *Lm*-mediated cross-presentation - and if this affects endogenous tumor antigens in addition to *Lm* antigens - remains unknown.

Another unanswered question is how TLR stimulation by *Lm* affects immune suppression within the tumor microenvironment (TME), in particular the frequency and functionality of regulatory T cells (Tregs), which are a major impediment to CD8⁺ T cell-mediated antitumor immunity.⁸¹⁰ Tregs have been shown to inhibit antitumor T cell responses by limiting tumor antigen cross-presentation,^{267,811} preferentially binding the T cell mitogenic cytokine IL-2,⁸¹² and expression of immunosuppressive genes such as CTLA-4, IL-10, and TGF- β .²⁵⁸ Tregs have been reported to be inhibited both directly and indirectly by TLR signaling,^{250,252,813} and infection of mice with *L. monocytogenes* and other microbes can reduce Treg frequency and inhibitory function.^{182,814–816} However, direct evidence to link reduced Treg function and frequency to TLR signaling by *L. monocytogenes* is lacking, and it is not known whether Treg modulation by TLRs plays a role in antitumor immunity in the context of *Lm*-based cancer immunotherapies.

Given the multiple unresolved questions about the role played by TLR signaling in antitumor immunity elicited by *Listeria* cancer vaccines, we set out to identify whether any of the TLRs known to be involved in combating *Listeria* infection (TLR2, TLR5, and TLR9) play a role in our recently published model of dual intravenous and then intratumoral *Lm* administration (IV+IT *Lm*). IV+IT *Lm* controls tumor growth in a Batf3⁺ type I dendritic cell (cDC1)- and CD8⁺ T cell-dependent manner by enhancing the CD8⁺ T cell response against *Lm* and tumor antigens, but the contribution of TLR signaling to this system is unknown.⁸⁰⁷ Here we show that TLR2 is required for the efficacy of IV+IT *Lm* via several interlocking mechanisms. As expected, TLR2 supported the priming of *Lm*-specific CD8⁺ T cells during IV *Lm* administration. However, TLR2 signaling also exerted unanticipated effects during IT *Lm* administration that were distinct from its role in priming anti-*Lm* T cells. First, TLR2 contributed to the recall of *Lm*-specific CD8⁺ T cells to tumors following IT *Lm* administration. Second, TLR2 mediated tumor antigen cross-presentation by cDC1s, rapidly reversing tumor-specific CD8⁺ T cell exhaustion in tumors and driving *de novo* antitumor T cell priming in the tumor-draining lymph node. Third, TLR2 signaling in type II dendritic cells (cDC2s) mediated the destabilization of Foxp3 expression in tumor-infiltrating Tregs, which reduced immune suppression within tumors and potentiated the antitumor effects of IV+IT *Lm*. Importantly, only TLR2 signaling, and not any other TLR tested, impacted Treg function in this manner, thus explaining the unique contribution of TLR2 during IT *Lm* administration. Together, these results demonstrate an unexpected role for TLR2 signaling in CD8⁺ T cell-mediated immunity during both primary and secondary immune responses that can be leveraged to enhance the efficacy of microbial cancer immunotherapies.

3.3 Results

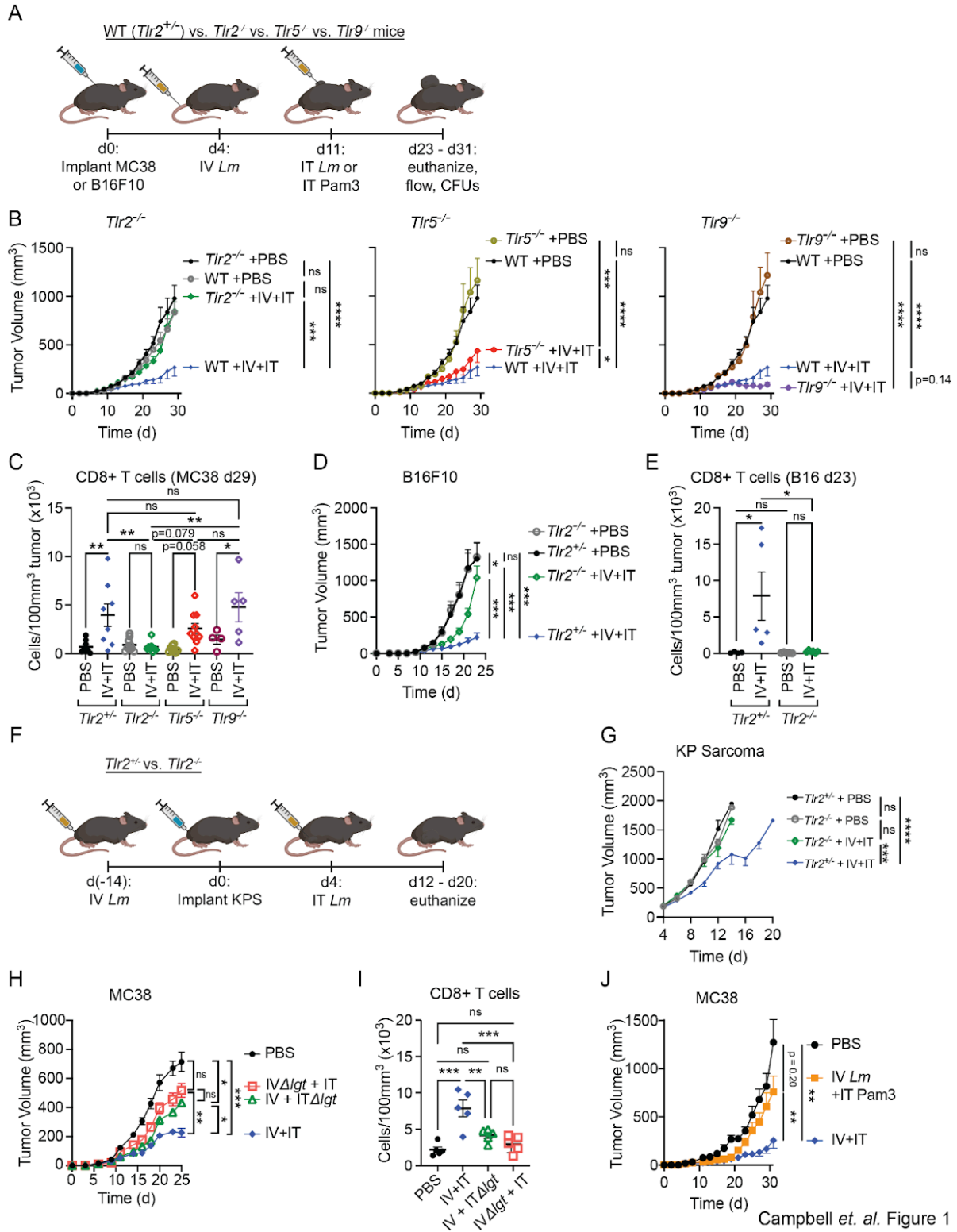
3.3.1 TLR2 signaling is necessary for tumor control during both IV and IT stages of IV+IT *Lm* immunotherapy.

To test the impact of TLR signaling on tumor control with IV+IT *Lm*, we assessed the ability of the IV+IT *Lm* therapeutic regimen to mediate tumor control in MC38 tumor-bearing mice that were deficient for each of the TLRs known to be stimulated by *Lm* (TLR2, TLR5, and TLR9) (**Figure 3.1A**).^{797,798,800,801,807,817,818} *Tlr2*^{+/-} mice were used as WT controls, as preliminary experiments using BMDCs generated from *Tlr2*^{+/+}, *Tlr2*^{+/-}, and *Tlr2*^{-/-} mice showed that heterozygous and homozygous cells responded with equal potency to TLR2 stimulation *in vitro*, in agreement with the established haplosufficiency of TLR2 in human PBMCs (data not shown).⁸¹⁹ We found that while *Tlr5*-deficient mice exhibited a modest but significant reduction in tumor control, both *Tlr5*- and *Tlr9*-deficient mice were still able to control tumors (**Figure 3.1B**). In contrast, IV+IT *Lm* did not control tumors in *Tlr2*-deficient mice (**Figure 3.1B**). Interestingly, tumor control in *Tlr2*^{-/-} mice was already reduced by the day of IT *Lm* treatment (day 11, 7 days after IV *Lm*), suggesting that TLR2 signaling was important at the IV phase of *Lm* delivery (**Figure S3.1A**). Since anti-*Lm* CD8⁺ T cells were the primary mediators of tumor control in response to IV+IT *Lm* and are primed by IV *Lm* infection,⁸⁰⁷ we hypothesized that the loss of tumor control in *Tlr2*^{-/-} mice was due to a reduced CD8⁺ T cell response. Indeed, while the absolute number and/or the frequency of CD8⁺ T cells was increased in IV+IT *Lm*-treated WT, *Tlr5*^{-/-}, and *Tlr9*^{-/-} mice, this effect was lost in *Tlr2*^{-/-} mice (**Figure S3.1B-C**). Accompanying the loss of tumor control and reduced CD8⁺ T cell frequency was a significant increase in the CFU of *Lm* recovered from tumors in *Lm*-treated *Tlr2*^{-/-} mice compared to WT and *Tlr9*^{-/-} mice (**Figure S3.1D**). A similar trend towards heightened *Lm* CFUs was observed when compared to *Tlr5*^{-/-} mice, although the latter also had a significant increase in CFU compared to WT and *Tlr9*-deficient mice (**Figure S3.1C**). Altogether, these findings suggested that TLR2, and to a lesser extent TLR5, plays a significant role in tumor control by IV+IT *Lm*. Given the greater impact of TLR2 on tumor growth, CD8⁺ T cells, and control of *Lm* burden within tumors, we therefore undertook further investigation of the specific effects of TLR2 signaling to determine why this receptor exerted these effects in the IV+IT *Lm* model.

To determine whether the dependence of IV+IT *Lm* upon TLR2 signaling was applicable beyond the MC38 tumor model, which is highly immunologically active and readily treated by immunotherapy, we next used IV+IT *Lm* to treat *Tlr2*^{+/-} and *Tlr2*^{-/-} littermate mice bearing B16F10 tumors, which are less immunogenic and less responsive to immunotherapy.⁸²⁰ We again found that tumor control was significantly attenuated in *Tlr2*^{-/-} mice, that CD8⁺ T cell number and frequency was reduced, and that *Lm* CFUs were increased in tumors of *Tlr2*^{-/-} mice (**Figures 3.1C-1E and S3.1E-F**). Finally, we tested the requirement for TLR2 signaling in IV+IT *Lm*-mediated control of the orthotopically implanted *Kras*^{G12D};*p53*^{-/-} (KP) sarcoma model, which is influenced by muscle tissue-specific environmental factors that can affect responsiveness to immunotherapy and therefore better model human malignancies than subcutaneous tumors.⁸²¹⁻⁸²³ Importantly, we again found that TLR2 was essential for tumor control with IV+IT *Lm* (**Figure 3.1F-G and S3.1G**). Together, these findings suggest that TLR2 plays a critical and conserved role in limiting *Lm* burden in tumor tissue and in mediating tumor control elicited by IV+IT *Lm* across multiple tumor types and anatomical locations.

To test whether TLR2 signaling was required during the IV or IT stage of the IV+IT *Lm* regimen, we used an *Lm* Δ *lgt* strain, which does not generate the bacterial lipoprotein ligands for TLR2, during either IV or IT administration of *Lm* to MC38 tumor-bearing mice.^{797,799} Surprisingly, mice that were treated with *Lm* Δ *lgt* at either the IV or IT stage failed to fully control tumor growth with IV+IT *Lm* administration (**Figure 3.1H**). While IV *Lm* Δ *lgt* failed to slow tumor growth to the extent of IV *Lm* in the days preceding IT *Lm* administration (day 11, 7 days after IV *Lm* Δ *lgt*) (**Figure 3.1I**), substituting *Lm* Δ *lgt* for either IV or IT *Lm* ultimately significantly reduced tumor control (**Figure 3.1H**). Finally, as with *Tlr2*^{-/-} mice, IV *Lm* Δ *lgt* significantly reduced the frequency of tumor-infiltrating CD8⁺ T cells. Finally, as with *Tlr2*^{-/-} mice, IV *Lm* Δ *lgt* significantly reduced the frequency of tumor-infiltrating CD8⁺ T cells, while both IV and IT *Lm* Δ *lgt* failed to increase CD8⁺ T cell numbers but did exhibit increased *Lm* CFUs in tumors (**Figures 3.1J and S3.1H-I**). Thus, TLR2 signaling is necessary during both the IV and IT phase of *Lm* administration for the efficacy of IV+IT *Lm*.

To test whether TLR2 stimulation alone was sufficient to phenocopy IT *Lm* in the IV+IT *Lm* regimen, MC38 tumor-bearing mice were treated with IV *Lm* followed by either IT *Lm* or Pam3CSK4 (Pam3), a synthetic TLR2 ligand. IT Pam3 treatment initially controlled tumors but ultimately did not provide long-term control compared to IT *Lm* or completely clear *Lm* CFUs from tumors (**Figures 3.1K and S3.1J-K**). Intriguingly, supplementing IT *Lm* Δ *lgt* with Pam3 produced a strong trend towards greater tumor control than either *Lm* Δ *lgt* or Pam3 alone, but did not affect control of *Lm* CFUs (**Figures S3.1L-M**). Altogether, these results demonstrate that IT TLR2 signaling is necessary, but not sufficient, for tumor control and the clearance of *Lm* from tumors.



Campbell *et. al.* Figure 1

Figure 3.1: TLR2 signaling is required for the efficacy of IV+IT *Lm* therapy.

- (A) WT (*Tlr2*^{+/+}), *Tlr2*^{-/-}, *Tlr5*^{-/-}, and *Tlr9*^{-/-} mice were implanted subcutaneously with 5x10⁵ MC38 or B16F10 tumor cells (day 0). On day 4, mice received 1x10⁶ CFU IV *Lm*. On day 11, mice received 5x10⁷ CFU IT *Lm*.
- (B) MC38 tumor growth in WT (*Tlr2*^{+/+}), *Tlr2*^{-/-}, *Tlr5*^{-/-}, and *Tlr9*^{-/-} mice receiving IV+IT *Lm* or PBS.
- (C) Number of CD8⁺ T cells per 100 mm³ of MC38 tumor tissue from experiment in Figure 1B on day 29 of tumor growth.
- (D) B16F10 tumor growth in *Tlr2*^{+/+} and *Tlr2*^{-/-} littermates receiving IV+IT *Lm* or PBS.
- (E) Number of CD8⁺ T cells per 100 mm³ of B16F10 tumor tissue from experiment in Figure 1D on day 23 of tumor growth.
- (F) *Tlr2*^{+/+} and *Tlr2*^{-/-} littermate mice received orthotopically implanted KP sarcomas. Two weeks prior to tumor growth, mice were treated with IV *Lm* (1x10⁶ CFU) or PBS (mock infection). 4 days after implantation, mice were treated with IT *Lm* (5x10⁷ CFU) or PBS.
- (G) KP sarcoma growth in *Tlr2*^{+/+} and *Tlr2*^{-/-} mice receiving IV+IT *Lm* or PBS.
- (H) MC38 tumor growth in WT mice receiving *Lm*Δ*lgt* during the IV stage (red squares), the IT stage (green triangles), or *lgt*-competent *Lm* at both stages of administration (blue diamonds).
- (I) Number of CD8⁺ T cells per 100 mm³ of MC38 tumor tissue from experiment in Figure 1H on day 25 of tumor growth.
- (J) Tumor growth in WT mice receiving IV *Lm* and IT *Lm* or IT Pam3 (100 ug). Mean ± s.e.m. and one-way ANOVA (C, E, I) or two-way ANOVA (B, D, G, H, J).

3.3.2 TLR2 stimulation plays multiple roles in supporting CD8⁺ T cells in IV+IT *Lm* immunotherapy.

Next, we sought to determine how TLR2 signaling impacted the CD8⁺T cell response at each stage of the IV+IT *Lm* regimen. We hypothesized that TLR2 signaling could contribute to the expansion of *Lm*-specific CD8⁺ T cells during IV *Lm* administration, which has not been previously described but has been observed in multiple murine models of viral infection.^{824,825} Therefore, we infected WT (*Tlr2*^{+/+}), *Tlr2*^{-/-}, *Tlr5*^{-/-}, and *Tlr9*^{-/-} mice with *Lm-OVA* and analyzed the frequency and activation of *Lm-OVA*-specific CD8⁺ T cells in the spleen 5 days later using H-2K(b)-SIINFEKL tetramer staining (**Figure 3.2A**). The frequency of *Lm-OVA*-specific (tetramer⁺) CD8⁺ T cells was significantly reduced in *Tlr2*^{-/-} mice compared to WT, *Tlr5*^{-/-}, and *Tlr9*^{-/-} mice (**Figures 3.2B-C**). Similarly, the frequency of I-A(b)-NEKYAQAYPNVS tetramer⁺ CD4⁺ T cells (recognizing an epitope derived from the *Lm* virulence factor LLO) was decreased in *Tlr2*^{-/-} mice, as was the frequency of activated SIINFEKL- and NEKYAQAYPNVS-nonspecific (i.e., tetramer⁻) CD8⁺ and CD4⁺ T cells (**Figures S3.2A-D**). Overall, these findings demonstrate that TLR2 signaling was required for the optimal priming of *Lm*-specific T cells during IV *Lm* administration.

We then asked whether TLR2 signaling also played a role in supporting the CD8⁺ T cell response during IT *Lm* administration. To do this, we adoptively transferred *in vitro*-activated OT-I CD8⁺ T cells into naïve *Tlr2*^{+/+}, *Tlr2*^{-/-}, *Tlr5*^{-/-}, and *Tlr9*^{-/-} mice to serve as a surrogate for *Lm-OVA*-specific CD8⁺ T cells, circumventing the defect in CD8⁺ T cell priming observed in *Tlr2*^{-/-} mice. Two weeks later (d0), we implanted MC38 tumors before injecting tumors IT with *Lm-OVA* on d11 of tumor growth, which phenocopies tumor control elicited by IV+IT *Lm* (**Figure 3.2D**).⁸⁰⁷ Consistent with our previous results using IV *Lm*+IT *LmΔlgt*, OT-I-bearing *Tlr2*^{+/+}, *Tlr5*^{-/-}, and *Tlr9*^{-/-} mice but not *Tlr2*^{-/-} mice controlled tumors in response to IT *Lm-OVA* (**Figure 3.2E**). Endpoint analysis of tumors revealed a significant reduction in the frequency and number of OT-I T cells in *Tlr2*^{-/-} mice treated with *Lm-OVA* in comparison to WT and *Tlr9*^{-/-} mice, and a similar trend when compared to *Tlr5*^{-/-} mice (**Figures 3.2F and S3.2E**). Similarly, endogenous CD8⁺ T cells in *Tlr2*^{-/-} mice treated with *Lm-OVA* failed to downregulate the inhibitory receptors Lag3 and PD1 as we had observed in our previous work,⁸⁰⁷ while transferred OT-I T cells in these mice exhibited heightened expression of these markers, suggesting a heightened state of CD8⁺ T cell exhaustion in the absence of TLR2 signaling (**Figures 3.2G-H**). Control of *Lm-OVA* CFUs in tumors was also significantly reduced in *Tlr2*^{-/-} mice (**Figure S3.2F**). Finally, to address the possibility that TLR2 signaling was exercising its effects via signaling in either tumor cells or transferred OT-I CD8⁺ T cells, we repeated these experiments in *Tlr2*^{+/+} and *Tlr2*^{-/-} littermate mice using *Lm-OVA* (which could still stimulate TLR2 on tumor cells and/or transferred OT-I T cells but not host cells in *Tlr2*^{-/-} mice) or *LmΔlgt-OVA* (which cannot stimulate TLR2 tumor, host, or transferred OT-I T cells). We found no significant difference in tumor control or *Lm* clearance from tumors between *Tlr2*^{+/+} mice that received IT *LmΔlgt-OVA* or *Tlr2*^{-/-} mice that received either *Lm-OVA* or *LmΔlgt-OVA* (**Figure S3.2G-H**). Together, these findings indicate that TLR2 signaling in host immune cells, but not tumor cells, is required at the IT stage of IV+IT *Lm*. This requirement is at least in part for promoting the function of anti-*Lm* CD8⁺ T cells within tumors, despite no requirement for CD8⁺ T cell-intrinsic or tumor cell-intrinsic TLR2 signaling. Nevertheless, TLR signaling within tumor cells themselves may also be important and warrants further experimentation.

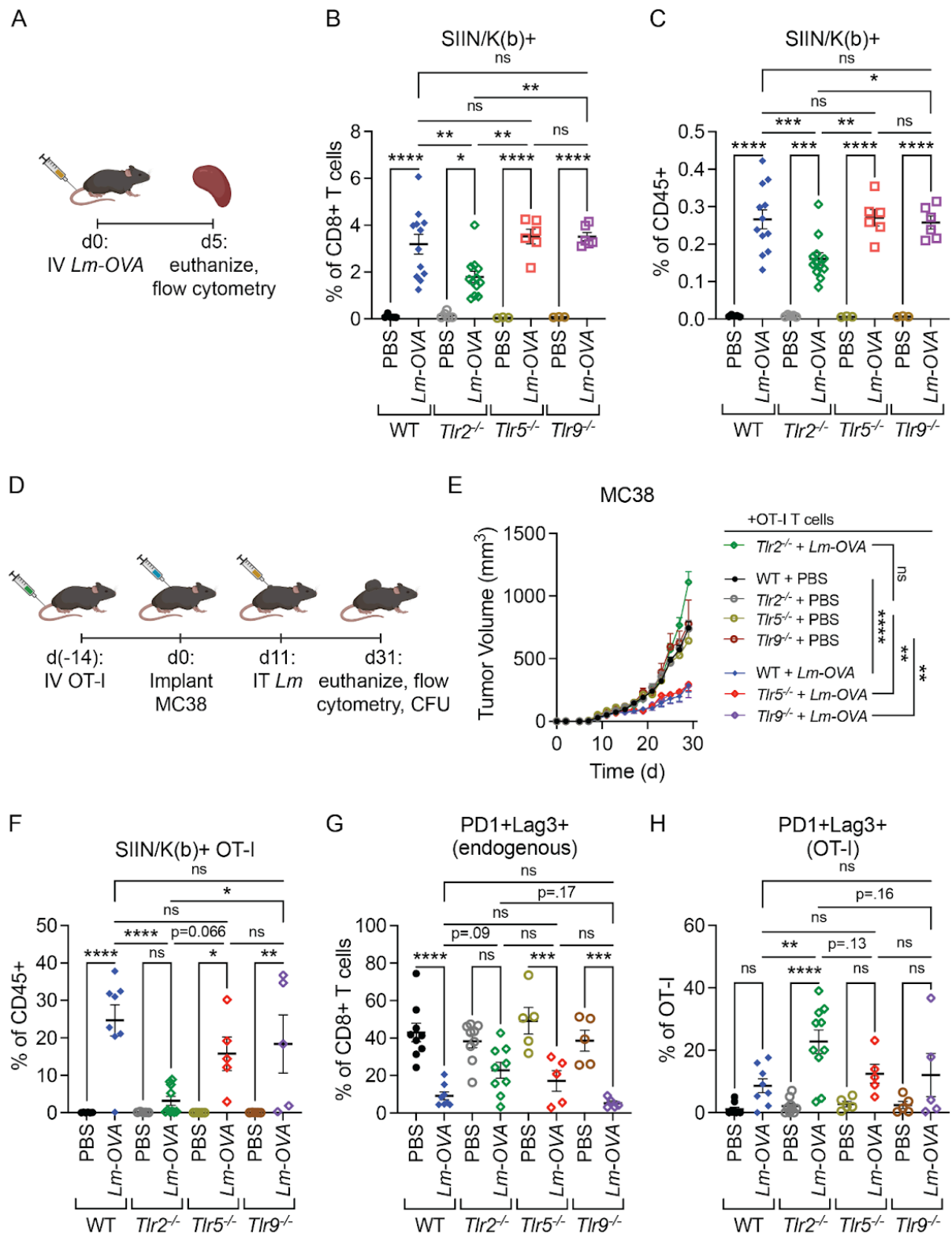


Figure 2. Campbell et al.

Figure 3.2: TLR2 controls the expansion and function of CD8⁺ T cells in IV+IT *Lm* therapy.

- (A) WT (*Tlr2*^{+/-}), *Tlr2*^{-/-}, *Tlr5*^{-/-}, or *Tlr9*^{-/-} mice were infected IV with 1x10⁶ CFU of *Lm-OVA* and splenocytes were analyzed by flow cytometry five days later.
- (B, C) Quantified frequencies of SIIN/K(b) tetramer-specific CD8⁺ T cells in spleens five days after *Lm-OVA* infection presented as the frequency of CD8⁺ T cells (B) or of CD45⁺ cells (C).
- (D) 2x10⁶ *in vitro* activated OT-I T cells were adoptively transferred into WT (*Tlr2*^{+/-}), *Tlr2*^{-/-}, *Tlr5*^{-/-}, or *Tlr9*^{-/-} mice. Two weeks later (day 0), mice were implanted with 5x10⁵ MC38 cells and on day 11 were treated IT with 5x10⁷ CFU *Lm-OVA* or PBS.
- (E) Tumor growth through day 31 in mice receiving OT-I transfer and IT *Lm-OVA* or PBS.
- (F) SIIN/K(b) tetramer⁺ OT-I T cell frequency among total CD45⁺ T cells in tumors on day 31.
- (G) Co-expression of PD1 and Lag3 on endogenous CD8⁺ T cells on day 31.
- (H) Co-expression of PD1 and Lag3 on transferred OT-I T cells on day 31. Mean ± s.e.m. and one-way ANOVA (B, C, F, G, H) or two-way ANOVA (E).

3.3.3. *Lm* activates TLR2 signaling in myeloid cells within MC38 tumors

We therefore sought to identify the immune cells that respond to *Lm* bacterial lipoproteins in tumors to determine how TLR2 signaling affects CD8⁺ T cell-mediated responses in the IV+IT *Lm* regimen. To do this, we developed a fluorescent tracking approach to identify cells in tumors that: (1) express TLR2, (2) harbor *Lm*, and (3) have taken up tumor antigen. We used *Tlr2*^{GFP} mice to identify TLR2-expressing cells.⁸²⁶ We injected tumors with *Lm-tagBFP*, wherein BFP expression is driven by the *actA* promoter leading to BFP fluorescence in cytosolically localized *Lm*.⁸²⁷ This approach allowed us to identify cells that harbored *Lm* and, by proxy, the cells most likely to trigger TLR2 signaling during phagocytosis of *Lm*, which can occur during the uptake of *Lm* and other TLR2 ligands.^{391,828–830} Finally, we implanted mCherry-expressing MC38 tumors to identify mCherry⁺ immune cells that had taken up mCherry from tumors (**Figures 3A-B**).

Analysis of the cell types expressing *Tlr2*^{GFP} in MC38 tumors revealed that cDCs and monocytes almost uniformly expressed TLR2 (80-100%), but that cDCs expressed the highest levels of TLR2 by mean fluorescence intensity (**Figures 3.3C, S3.3A-B, and S3.4A-C**). The overall frequency of *Tlr2*^{GFP+} cells was increased by both IT and IV+IT *Lm-tagBFP* injection, which was in part due to upregulation of *Tlr2*^{GFP} by cDCs and monocytes upon IT and especially upon IV+IT *Lm-tagBFP* treatment (**Figures 3.3B and S3.4A-C**). This accords with previous findings that TLR2 expression can be upregulated by TLR2 signaling in cDCs and monocytes.^{831,832} Lower fractions of macrophages and polymorphonuclear neutrophils (PMNs) also expressed TLR2 (30% and 5%, respectively), such that the influx of PMNs into tumors elicited by IT or IV+IT *Lm-tagBFP* treatment described in our earlier work also contributed to increased *Tlr2*^{GFP+} cell frequency (**Figures 3.3C and S3.4A-C**).⁸⁰⁷ In contrast, neither Tregs, Tcon, nor CD8⁺ T cells expressed detectable levels of *Tlr2*^{GFP} (**Figures 3.3C and S3.4B-C**). This strongly suggests that the effects of TLR2 signaling on CD8⁺ T cells in IV+IT *Lm*-treated tumors are mediated by myeloid cells.

3.3.4 IV+IT *Lm*-mediated TLR2 signaling drives the uptake of *Lm* and tumor antigen by cDCs

We previously observed that tumor antigen uptake and presentation by cDCs was enhanced in mice treated with IV+IT *Lm*, but did not identify the signals that drove this response.⁸⁰⁷ As cDCs were highly enriched for *Tlr2*^{GFP} expression in MC38 tumors, and TLR2 is known to drive antigen uptake and presentation,^{833,834} we hypothesized that the TLR2-dependent CD8⁺ T cell response following IV+IT *Lm* might be driven by increased antigen presentation by cDCs. We therefore assessed the uptake of tumor antigen (mCherry) and *Lm* (*tagBFP*) by APCs within tumors. While neither IT *Lm* alone nor IV+IT *Lm* led to an increase in total mCherry⁺CD45⁺ immune cells in tumors, IV+IT *Lm* specifically increased mCherry uptake in cDC1s and cDC2s (**Figures 3.3D and S3.4D-F**). IV+IT *Lm* also increased the frequency of *tagBFP*⁺CD45⁺ immune cells (**Figures 3.3B and S3.4G**). This was in part due to *Lm-tagBFP* uptake by cDC1s and cDC2s with IV+IT *Lm-tagBFP*, which did not occur with IT *Lm-tagBFP* alone (**Figure S4H-I**). IV+IT *Lm-tagBFP* also led to the emergence of a substantial population of cells that took up both mCherry and *tagBFP* (**Figure 3.3B**). These mCherry⁺*tagBFP*⁺ cells were mostly *Tlr2*^{GFP+} (**Figure S3.4J**), and *Tlr2*^{GFP+}mCherry⁺*tagBFP*⁺ cells were significantly enriched for cDC1s and cDC2s (27.9x and 33.7x enriched relative to cDC1 and cDC2 frequency among total CD45⁺, respectively) (**Figure 3.3E and S3.4K**). Together, these results suggest that TLR2 signaling may

drive the uptake of *Lm* and tumor antigens by cDCs in response to IV+IT *Lm* but not IT *Lm* alone, perhaps because of a lack of TLR2 signaling in these cells with IT *Lm* alone or due to the absence of other signals that require prior IV *Lm* injection.

Prior work from our lab established that IV+IT *Lm* leads to the Batf3⁺ cDC1-dependent enhancement of tumor-specific CD8⁺ T cell responses,⁸⁰⁷ which corresponded to increased uptake of mCherry by cDC1s only in IV+IT *Lm*-treated MC38-mCherry⁺ tumors (**Figure 3.3D and S3.4E-F**). We therefore next asked whether increased mCherry uptake by tumor cDCs was driven by direct TLR2 signaling accompanying the uptake of *Lm*. By stratifying tumor cDCs from IV+IT *Lm*-tagBFP-treated tumors on the basis of tagBFP fluorescence, we observed that mCherry uptake was increased only in *Lm*-tagBFP⁺ cDCs (**Figure 3.3F**). Similarly, only *Tlr2*^{GFP+} cells took up more mCherry in IV+IT *Lm*-treated tumors (**Figure S3.4L**). These results suggest that increased tumor antigen uptake by cDCs in IV+IT *Lm*-tagBFP-treated tumors is driven by cDC-intrinsic TLR2 signaling, but do not exclude the possibility that tumor antigen and *Lm* uptake in these cells is driven by secondary signals produced by a different TLR2-expressing cell type. To address this, we implanted mCherry-expressing MC38 tumors into mixed bone marrow chimeric mice reconstituted with congenically marked *CD45*^{1/1};*Tlr2*^{+/+} and *CD45*^{2/2};*Tlr2*^{-/-} cells and treated tumors with IT Pam3 (**Figure S3.4M**). We found that mCherry uptake was increased only in *Tlr2*^{+/+} cDCs upon IT Pam3 treatment, supporting our hypothesis that antigen uptake in response to IV+IT *Lm* is driven by cDC-intrinsic TLR2 signaling (**Figure S3.4M-N**). Similarly, BMDCs cultured with lysate from MC38 or MC38-mCherry⁺ tumors took up significantly more mCherry upon Pam3 stimulation *in vitro* (**Figure S3.4O**). Altogether, these findings support our hypothesis that *Lm*-mediated TLR2 signaling directly modulates tumor antigen uptake, which may contribute to the TLR2-dependent effects of IV+IT *Lm* on T cells in tumors by enabling antigen presentation.

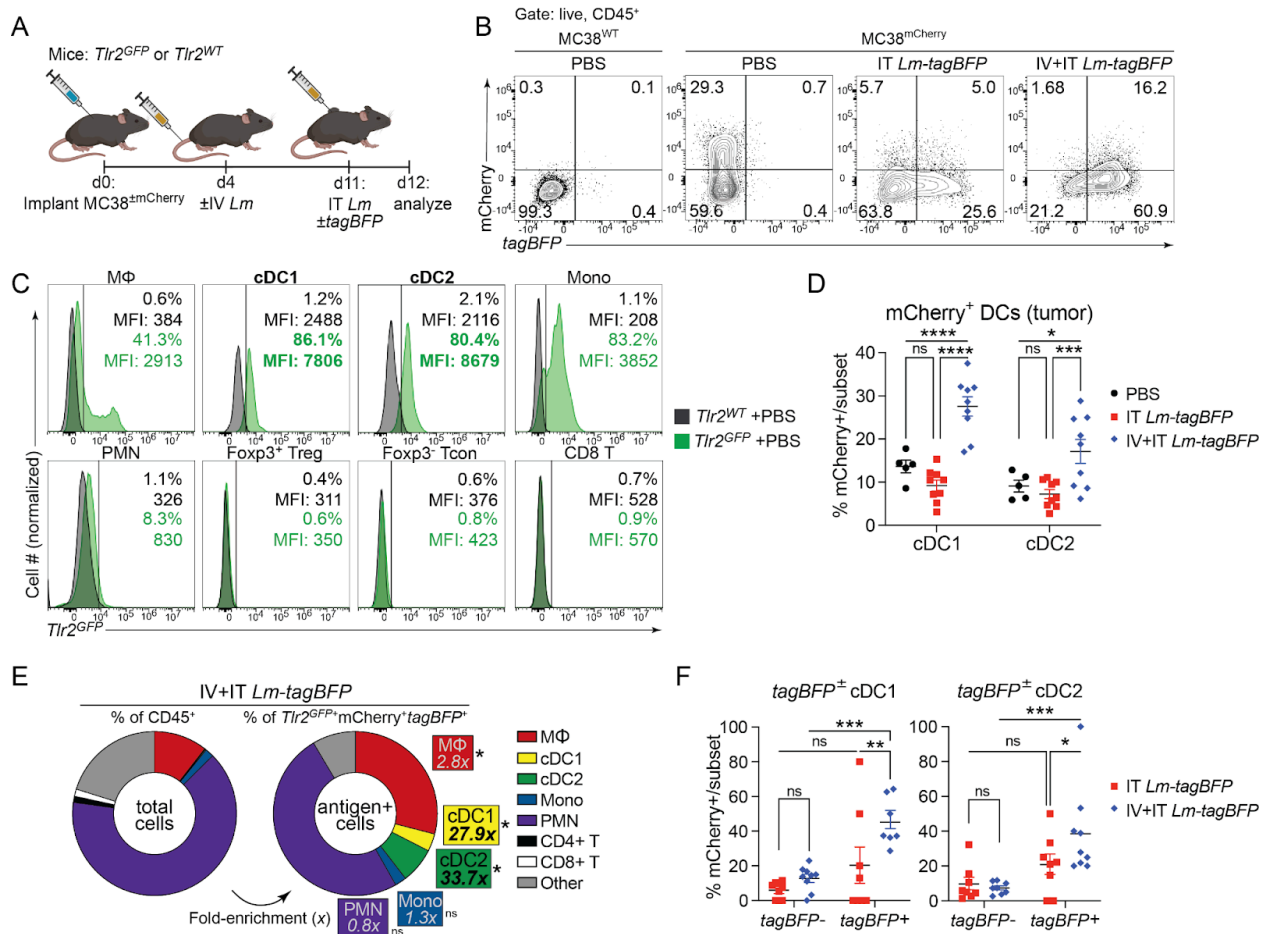


Figure 3. Campbell et al.

Figure 3.3: *Lm*-triggered TLR2 signaling enhances tumor antigen uptake by cDCs.

(A) *Tlr2^{GFP}* or WT mice were implanted with 5×10^5 MC38 cells $\pm$ mCherry (day 0) and were treated IV with 1×10^6 CFU *Lm* $\pm$ *tagBFP* or PBS (day 4) followed by 5×10^7 CFU *Lm* $\pm$ *tagBFP* or PBS IT (day 11) 24 hours prior to euthanasia and flow cytometry analysis (day 12).

(B) Representative flow cytometry histograms showing mCherry and *tagBFP* fluorescence by CD45⁺ cells from MC38 tumors of *Tlr2^{GFP}* mice treated with PBS, IT *Lm-tagBFP*, or IV+IT *Lm-tagBFP*.

(C) Representative flow cytometry histograms showing GFP fluorescence by immune cell subsets in MC38 tumors of *Tlr2^{GFP}* or *Tlr2^{WT}* mice.

(D) Frequency of mCherry⁺ cDC subsets in MC38 tumors.

(E) Cell type composition of total CD45⁺ versus *Tlr2^{GFP}*⁺mCherry⁺*tagBFP*⁺CD45⁺ immune cells in IV+IT *Lm-tagBFP*-treated MC38 tumors showing fold-enrichment of macrophages, cDC1s, and cDC2s among triple-positive cells relative to their frequency among total CD45⁺ cells.

(F) Frequency of mCherry⁺ among *tagBFP* $\pm$ cDC1s (left) and cDC2s (right) in MC38 tumors of mice treated with IT *Lm-tagBFP* or IV+IT *Lm-tagBFP*.

Mean $\pm$ s.e.m. and one-way ANOVA (D, E) or two-way ANOVA (F).

3.3.5 TLR2 signaling and recognition of *Lm* antigens by CD8⁺ T cells drive tumor antigen cross-presentation

Because our experiments with fluorescent *Lm* and tumor antigens did not directly address the impact of TLR2 on antigen presentation, which is often but not always tied to antigen uptake,³³⁰ we adjusted our approach by employing IV+IT *Lm* in MC38-β2m^{KO}-OVA tumors. The incorporation of OVA into a MHC-I antigen presentation-deficient tumor allows for both the monitoring of antigen cross-presentation (by the staining for the OVA-derived peptide SIINFEKL bound to H-2K(b) using the 25D1.16 monoclonal antibody) and tumor-specific CD8⁺ T cells (transferred OT-I and endogenous SIIN/K(b) tetramer⁺ T cells) to monitor tumor antigen-specific responses without any direct antigen presentation by tumor cells.^{807,835}

We first tested the requirement for TLR2 in IV+IT *Lm*-mediated cross-presentation by treating MC38-β2m^{KO}-OVA[±] tumor-bearing mice with IV+IT *Lm* and analyzing cross-presentation by flow cytometry 24 hr later (**Figures 3.4A-B**).⁸⁰⁷ As we had observed previously, CD103⁺XRC1⁺ cDC1s had the highest frequency of SIIN/K(b)⁺ in tumors, and this was further increased by IV+IT *Lm* (**Figure 3.4C**).⁸⁰⁷ While the fraction of SIIN/K(b)⁺ cDC2s also increased significantly with IV+IT *Lm* (**Figure 3.4D**), cDC1s remained the cell type with the highest degree of tumor antigen cross-presentation. In contrast, few to no macrophages, PMNs, or monocytes cross-presented tumor antigen (**Figures S3.5A-C**). In line with our results using MC38-mCherry tumors, increased tumor antigen cross-presentation by IV+IT *Lm* was abrogated in *Tlr2*^{-/-} mice (**Figures 3.4EF and S3.5D-F**). Together, these findings demonstrate that IV+IT *Lm* acts at least in part via TLR2 to increase tumor antigen cross-presentation by cDCs.

Nevertheless, TLR2 signaling alone was insufficient to elicit tumor antigen cross-presentation, as we found that IT Pam3 alone was insufficient to enhance tumor antigen cross-presentation by cDCs (**Figures S3.5G-H**). Similarly, previous work from our lab showed that only mice that received both IV and IT *Lm* increased cross-presentation of tumor antigen, while mice that received IT *Lm* alone did not.⁸⁰⁷ Furthermore, tumor-specific CD8⁺ T cell responses were enhanced in WT but not in *Batf3*^{-/-} mice that received adoptively transferred OT-Is followed by IT *Lm*-OVA but not IT *Lm*.⁸⁰⁷ Together, these results suggest that tumor antigen cross-presentation following IV+IT *Lm* may also require antigen recognition by *Lm*-specific CD8⁺ T cells generated by IV *Lm* injection.⁸⁰⁷ To test this hypothesis, we repeated our MC38-β2m^{KO}-OVA⁺ cross-presentation experiments, but this time delivered a single dose of anti-CD4, anti-CD8, or isotype control depleting antibody 24hr prior to IT *Lm* (**Figure 4G**). Indeed, depleting CD8⁺ T cells prior to IT *Lm* injection abrogated the enhanced tumor antigen cross-presentation normally seen with IV+IT *Lm* (**Figure 4H-I**). Thus, both TLR2 stimulation and antigen recognition by *Lm*-specific CD8⁺ T cells within tumors following IT *Lm* are required for cross-presentation elicited by IV+IT *Lm*.

3.3.6 TLR2 and *Lm*-specific CD8⁺ T cells enhance tumor-specific CD8⁺ T cell priming

We observed in our fluorescent tumor antigen experiments that the frequency of *Tlr2*^{GFP+}mCherry⁺ cells was increased in tumor-draining (tdLN) but not non-draining (ndLN) lymph nodes in MC38-mCherry tumor-bearing *Tlr2*^{GFP} mice upon IV+IT *Lm*-tagBFP injection

(**Figure S3.4P**). Together with our cross-presentation results and our earlier finding that tumor-specific T cell responses are enhanced by IV+IT *Lm*,⁸⁰⁷ this led us to hypothesize that IV+IT *Lm* would lead to a TLR2- and *Lm* CD8⁺ T cell antigen recognition-dependent increase in tumor-specific T cell priming in the tdLN. To test this hypothesis, we transferred naive, CFSE-labeled OT-I CD8⁺ T cells into congenic CD45.1⁺ MC38- β 2m^{KO}-OVA⁺ tumor-bearing mice one day after IV+IT *Lm* administration (**Figure 3.4J**). After three days, tumor, tdLN, and ndLN were harvested and OT-I CD8⁺ T cells in these tissues were assessed by flow cytometry (**Figure 3.4K**). In addition, the frequency of endogenous SIIN-specific CD8⁺ T cells was monitored. IV+IT *Lm* led to a significant increase in the frequency of both endogenous tumor-specific CD8⁺ T cells and transferred OT-I T cells in the tdLN but not the ndLN (**Figures 3.4L-M, and S3.5H**). Importantly, neither IV *Lm* alone, IT *Lm* alone, nor IV *Lm*+IT *Lm* Δ lgt increased the frequencies of endogenous or transferred tumor-specific CD8⁺ T cells compared to PBS treated mice (**Figures 3.4L-M**). The increased frequency of OT-I CD8⁺ T cells in the tdLN with IV+IT *Lm* resulted from increased OT-I priming as determined by increased CFSE dilution (**Figures 3.4N and S3.5I**), and ultimately coincided with CD45.1⁺CFSE⁺CD44⁺ OT-I CD8⁺ T cells entering the tumor in 8/10 IV+IT *Lm*-treated mice (**Figure 3.4O**). Therefore, the improved tumor antigen-specific CD8⁺ T cell response elicited by IV+IT *Lm* is at least in part due to the TLR2- and anti-*Lm* CD8⁺ T cell-dependent priming of tumor antigen-specific CD8⁺ T cells.

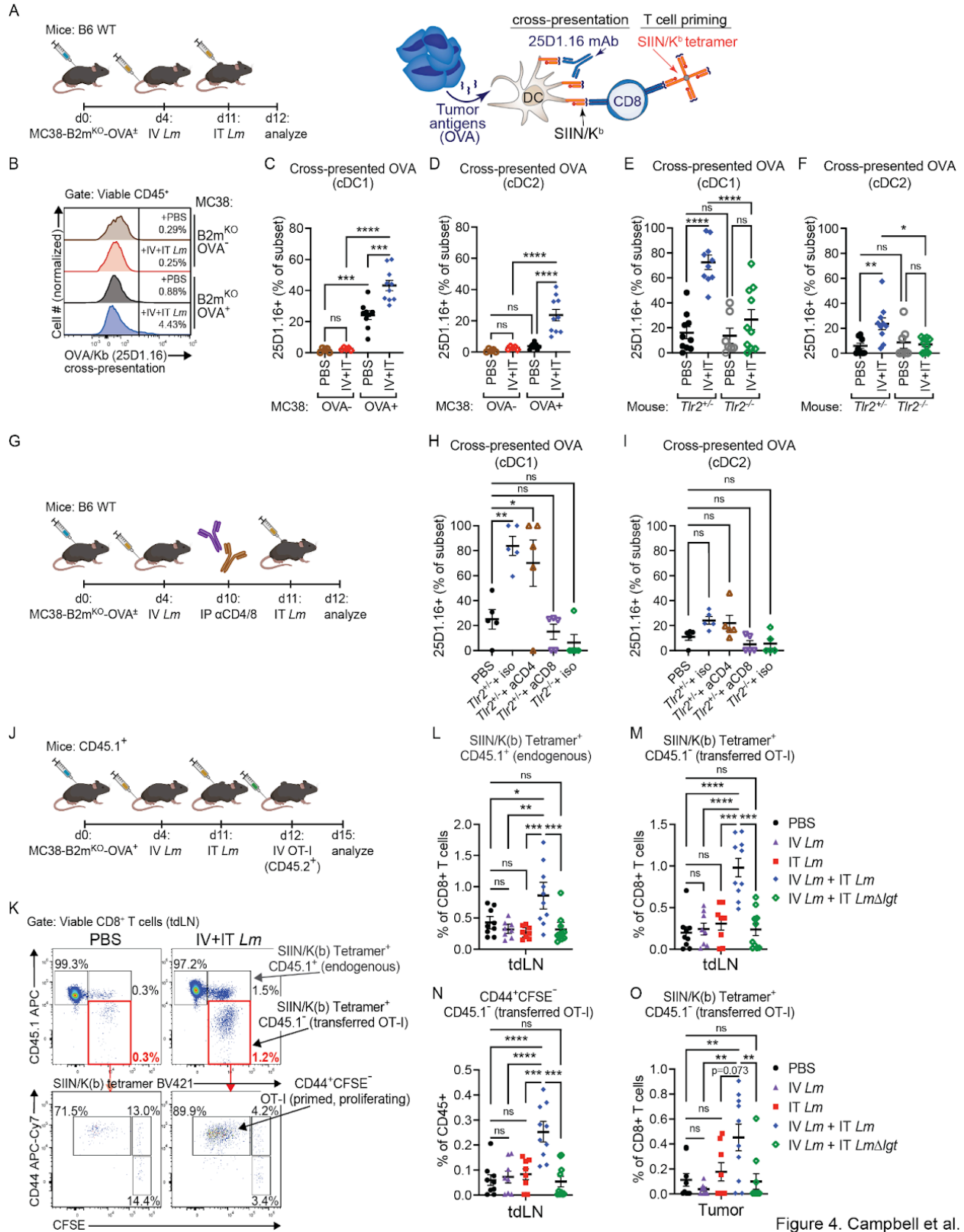


Figure 4. Campbell et al.

Figure 4: IV+IT *Lm* acts via *Lm*-specific T cells and TLR2 to induce the cross-presentation of tumor antigen and boost tumor-specific CD8⁺ priming.

(A) *Left*: WT or *Tlr2*^{-/-} B6 mice were implanted with 1x10⁶ MC38-β2m^{KO} cells ±OVA (day 0) and were treated with 1x10⁶ CFU *Lm* IV (day 4) followed by 5x10⁷ CFU *Lm* IT (day 11) or PBS 24 hours prior to euthanasia and analysis. *Right*: diagram depicting how surface SIIN/K(b) complexes are detected by 25D1.16 mAb staining to identify tumor antigen cross-presentation, while SIIN/K(b)-reactive TCRs are stained with SIIN/K(b) tetramer to identify tumor-specific CD8⁺ T cells.

(B) Representative flow cytometry histograms showing SIIN/K(b) staining in total CD45⁺ TILs in MC38.

(C, D) Frequency of SIIN/K(b)⁺ cells among cDC1s (D) and cDC2s (E) detected by 25D1.16 staining in tumors of MC38-β2m^{KO} control or MC38-β2m^{KO}-OVA⁺ tumor-bearing mice.

(E, F) Frequency of SIIN/K(b)⁺ cells among cDC1s (E) and cDC2s (F) detected by 25D1.16 staining in tumors of MC38-β2m^{KO}-OVA⁺ tumor-bearing *Tlr2*^{+/-} and *Tlr2*^{-/-} littermate mice.

(G) WT or *Tlr2*^{-/-} were implanted with MC38-β2m^{KO}-OVA⁺ tumors and treated with IV+IT *Lm*, but received anti-CD4, anti-CD8b, or isotype control depleting antibody IP on d10, 24 hr prior to IT *Lm* injection.

(H, I) Frequency of SIIN/K(b)⁺ cells among cDC1s (H) and cDC2s (I) detected by 25D1.16 staining in tumors of mice receiving anti-CD4, anti-CD8b, or isotype control depleting antibody on d10.

(J) CD45^{1/1} B6 mice were implanted with 1x10⁶ MC38-β2m^{KO}-OVA⁺ cells (day 0) and were treated with 1x10⁶ CFU *Lm* IV (day 4) followed by 5x10⁷ CFU *Lm* IT (day 11). 24 hr later (day 12), 5x10⁵ naive, CFSE-labeled, CD45^{2/2} OT-Is were transferred by IV injection. After 3 days (day 15) mice were euthanized and tissues were analyzed by flow cytometry to assess OT-I infiltration and priming.

(K) Representative flow cytometry plots demonstrating the gating strategy used to distinguish transferred OT-I (CD45.1-SIIN/K(b)⁺ from endogenous tumor-specific T cells (CD45.1⁺SIIN/K(b)⁺) and to assess OT-I priming and proliferation (CD44⁺CFSE⁻).

(L) Frequency of endogenous SIIN/K(b)-specific CD8⁺ T cells among CD8⁺ T cells in the tdLN.

(M) Frequency of transferred OT-I among total CD8⁺ T cells in the tdLN.

(N) Frequency of primed, proliferating OT-I among total CD45⁺ lymphocytes in the tdLN.

(O) Frequency of transferred OT-I among CD8⁺ T cells in MC38 tumors. Mean +/- s.e.m. and one-way ANOVA (C, D, E, F, H, I, L, M, N, O).

3.3.7 TLR2 signaling decreases Foxp3 in Tregs and leads to Treg depletion in tumors.

Tregs can restrict T cell priming in tumors and the tdLN by limiting tumor antigen presentation to T cells.^{268,298,811,836–838} Because TLR2 was required to elicit priming of tumor-specific T cells in response to IV+IT *Lm*, we hypothesized that TLR2 stimulation by IT *Lm* may modulate antigen presentation in part via effects on Tregs. Indeed, endpoint analysis of tumor-bearing mice treated with IV+IT *Lm* revealed a significant decrease in the frequency and number of tumor Tregs in WT, *Tlr5*^{-/-}, and *Tlr9*^{-/-} mice that did not occur in *Tlr2*^{-/-} mice (**Figure 3.5A**). Combined with the previously-described increase in CD8⁺ T cells (**Figures S3.1B and S3.1D**), IV+IT *Lm* led to a significantly increased ratio of CD8⁺ T cells to Tregs, which serves as a prognostic indicator of beneficial responses to immunotherapy across multiple cancer types (**Figure 3.5B**).^{839,840} Reduced Treg frequency and increased CD8:Treg ratio were also weakened by substituting *LmAlgt* at the IT stage of *Lm* administration (**Figures S3.6A-C**). However, substituting *LmAlgt* during IV *Lm* administration did not impact tumor Treg frequency to the same degree as substituting *LmAlgt* during IT *Lm* administration (**Figure S3.6C**). Similarly, we found no difference in splenic Treg frequency in WT, *Tlr2*^{-/-}, *Tlr5*^{-/-}, or *Tlr9*^{-/-} mice 5d after IV *Lm*-OVA administration (from Figures 2A-C, data not shown). This suggested that TLR2 signaling during IT *Lm* administration was principally responsible for reduced Treg frequencies in tumors with IV+IT *Lm*.

Increased tumor antigen cross-presentation and trafficking to the tdLN occurred within 24 hr of treatment with IV+IT *Lm* (**Figures 3.4B-F and S3.4J**), leading us to hypothesize that the impact of TLR2 on tumor Tregs would be similarly rapid. To investigate the immediate impact of TLR2 signaling on tumor Tregs, we treated MC38 tumor-bearing mice with *Lm*, *LmAlgt*, or Pam3 and analyzed the tumors by flow cytometry 24 hr later (**Figure 3.5C**). IT *Lm* led to a marked reduction in the frequency and number of tumor-infiltrating Tregs and a significant reduction in the MFI of Foxp3 within remaining Foxp3⁺ Tregs, which is often associated with reduced Treg suppressive function (**Figure 3.5D-E**).⁸⁴¹ These changes were confined to the tumor, as the frequency of Tregs was not reduced by IT *Lm* in proximal (tdLN) or distal (ndLN and spleen) lymphatic tissues (**Figure S3.6D**). While IT Pam3 was sufficient to phenocopy Treg frequency reduction and Foxp3 downregulation observed with IT *Lm*, these effects were significantly muted with IT *LmAlgt* (**Figures 3.5D-E and S3.6E**). Similarly, *Tlr2*^{-/-} mice failed to exhibit reduced tumor Treg frequency or Foxp3 expression following IT *Lm* administration (**Figures 3.5E-G**). These results indicate that TLR2 signaling in the TME is both necessary and sufficient to reduce Foxp3 expression and Treg frequency in response to IT *Lm*, which may underlie the specific dependence of IV+IT *Lm* upon TLR2.

We next asked if *Lm*-mediated TLR2 stimulation led to increased generation of “ex-Tregs”, Tregs that lose their suppressive function due to transcriptional reprogramming upon loss of Foxp3.^{241,242} To test this possibility, we used Foxp3 lineage-trace mice in which the *Foxp3* promoter drives the expression of a GFP-Cre fusion protein, which labels Foxp3⁺ cells with GFP and excises a *lox-stop-lox* cassette upstream of an IRES-RFP element at the constitutively expressed *Rosa26* locus (**Figure 3.5H**).^{241,842} IT Pam3 led to decreased MFI for GFP and Foxp3 antibody staining, which corresponded to an increase in the formation of GFP-RFP⁺ ex-Tregs in tumors and a decrease in the total frequency of both stable and ex-Tregs within tumors (**Figures 3.5I-K and S3.6F**). These findings confirm that TLR2 signaling in tumors disrupts Foxp3 expression and lineage stability in Tregs.

Figure 5: TLR2 stimulation by *Lm* mediates the depletion of Tregs from MC38 tumors.

(A) Frequency of Tregs as % of CD4⁺ T cells (left) or number of Tregs per 100mm³ tumor tissue (right) on day 29 of tumor growth in WT, *Tlr2*^{-/-}, *Tlr5*^{-/-}, or *Tlr9*^{-/-} mice treated with PBS or IV+IT *Lm* (see Fig. 1B).

(B) Ratio of CD8⁺ T cells:Tregs on day 29 of tumor growth in WT, *Tlr2*^{-/-}, *Tlr5*^{-/-}, or *Tlr9*^{-/-} mice treated with PBS or IV+IT *Lm*.

(C) WT (D-E), or WT and *Tlr2*^{-/-}, *Tlr5*^{-/-}, and *Tlr9*^{-/-} (F-G) mice were implanted with 5x10⁵ MC38 cells (day 0) and were treated with 5x10⁷ CFU *Lm* or *Lm*Δ*lgt*, 100 ug Pam3, or PBS 24 hours prior to euthanasia and flow cytometry analysis.

(D) Frequency of Tregs as % of CD4⁺ T cells (left) or number of Tregs per 100mm³ tumor tissue (right) 24 hr post-IT PBS, *Lm*, *Lm*Δ*lgt*, or Pam3.

(E) Foxp3 fluorescence within Foxp3⁺CD25⁺ tumor Tregs 24 hr post-IT PBS, *Lm*, *Lm*Δ*lgt*, or Pam3.

(F) Frequency of Tregs as % of CD4⁺ T cells (left) or number of Tregs per 100mm³ tumor tissue (right) 24 hr post-IT PBS or *Lm*.

(G) Foxp3 fluorescence within Foxp3⁺CD25⁺ tumor Tregs 24 hr post-IT PBS or *Lm*.

(H) Representative flow cytometry plot showing CD4⁺ T cells in MC38 tumors of Treg lineage trace mice with conventional CD4⁺ T cells (Tcon), stable Tregs, and ex-Tregs labeled.

(I) Foxp3-GFP fluorescence within RFP⁺CD25⁺ tumor Tregs 24 hr post-IT PBS or Pam3.

(J) Anti-Foxp3 antibody fluorescence within RFP⁺CD25⁺ tumor Tregs 24 hr post-IT PBS or Pam3.

(K) Frequency of GFP⁻RFP⁺ ex-Tregs among total RFP⁺ cells 24 hr post-IT PBS or Pam3.

Mean +/- s.e.m. and one-way ANOVA (A, B, D, E, F, G) or Student's t-test (I, J, K).

3.3.8 TLR2 uniquely destabilizes tumor Tregs without depleting other T cell subsets

The dependence of *Lm*-mediated Treg destabilization in tumors upon TLR2, but not TLR5 or TLR9, led us to ask whether TLR2 exhibits a unique capacity among TLRs to destabilize Treg Foxp3 expression. We therefore repeated our IT experiments using ligands for TLR2-9 (excluding TLR8, which is not functional in B6 mice) (**Figure 3.6A**).^{843,844} We found that while both TLR2 and TLR4 reduced the number of Tregs and their frequency among CD4⁺ T cells and led to the downregulation of Foxp3, TLR4 also significantly decreased the number of tumor CD8⁺ T cells (**Figure 3.6B-D**). The reduction in T cell number with TLR4 stimulation coincided with a high degree of cell death in these tumors that occurred across a wide range of IT LPS doses, suggesting that this is a general effect of IT TLR4 and not a result of the 20ug dose specifically (**Figures S3.7A-C**). Indeed, the unique impact of TLR2 on tumor Tregs could not be ascribed solely to differences in the degree of APC activation elicited by each TLRL, because all tested TLRL led to the maturation of at least one major APC subset in tumors (cDC1s, cDC2s, macrophages, and monocytes) as measured by the upregulation of CD40, CD86, and MHC-II (**Figures S3.7D-E**). Similarly, all TLRL significantly downregulated ICOSL on tumor APCs, while PDL1 was almost universally upregulated on these cells, further supporting the bioactivity of the 20 ug dose for each TLRL (**Figure S3.7E**). Together, these results suggest that the divergent effects of TLR2 versus other TLRs on tumor Tregs were not due to quantitative differences in the degree of stimulation elicited by the 20ug TLRL dose, but instead were due to qualitative differences in signaling mediated by each receptor. Therefore, TLR2 stimulation appears to have a unique capacity to elicit the destabilization of tumor Tregs.

3.3.9 TLR2 signaling in cDCs mediates the destabilization of Foxp3 in Tregs.

Multiple reports using *in vitro* and *in vivo* systems have identified various Treg-intrinsic effects of TLR2 stimulation.^{824,845–848} While the absence of GFP expression in Tregs from *Tlr2*^{GFP} mice suggested that direct TLR2 signaling was not responsible for tumor Treg destabilization (**Figure 3.3C**), we nevertheless sought to test this mechanism by employing a reductionist approach in which sorted, CTV-labeled naive Tregs were co-cultured *in vitro* with sources of Treg-intrinsic or Treg-extrinsic TLR stimulation. To model Treg-intrinsic TLR stimulation, Tregs were cultured with anti-CD3/anti-CD28-coated microbeads with or without TLR ligands. To model Treg-extrinsic TLR stimulation, Tregs were mixed with bone marrow-derived dendritic cells (BMDCs) which had been stimulated with TLRL for 24 hr and washed extensively to remove unbound TLRL (**Figure 3.6E**). Consistent with our findings *in vivo*, Tregs cultured with TLR2-stimulated BMDCs downregulated GFP and converted to ex-Tregs to a significantly greater extent than Tregs cultured with unstimulated BMDCs (**Figures 3.6F-G**). TLR2-stimulated BMDCs also induced Tregs to downregulate the expression of Nrp1 and GITR, two markers of functionally suppressive Tregs whose loss is associated with Treg lineage destabilization (**Figure 3.6G**).^{849,850} Notably, these effects were specific to TLR2, as BMDCs stimulated by any other TLR failed to induce downregulation of Foxp3, Nrp1, or GITR (**Figures 3.6F-G**). While TLR2-stimulated BMDCs also increased ICOS expression in Tregs, TLR5-stimulated BMDCs had the same effect despite not inducing loss of Foxp3, suggesting that this change was not related to Treg lineage stability (**Figure 3.6G**). Importantly, Treg proliferation (quantified by CTV dilution) did not differ between Tregs cocultured with BMDCs stimulated by different TLRL, ruling out the possibility that TLR2-mediated changes in Treg

phenotype were due to differences in Treg activation (**Figure 3.6G**). Finally, direct TLR2 stimulation of Tregs had no impact on Treg phenotype, suggesting that Treg-intrinsic TLR2 signaling was not responsible for changes in Treg phenotype seen in our co-culture system (**Figures 3.6F-G**). To confirm that cell-intrinsic TLR2 signaling in Tregs also did not mediate Foxp3 destabilization in tumors, we treated tumor-bearing mixed bone marrow chimeric mice reconstituted with congenically marked *Tlr2*^{+/+} and *Tlr2*^{-/-} cells with IT Pam3 (**Figure S3.6F-G**). In accordance with our *in vitro* results, we found that reduced Treg frequency and Foxp3 expression in tumors was independent of Treg genotype (**Figures S3.6F-I**). Altogether, our results from both *in vivo* and *in vitro* systems establish that *Lm*-mediated TLR2 signaling in APCs, but not directly in Tregs, uniquely drives tumor Treg destabilization in response to IT *Lm* administration.

3.3.10 cDC2s mediate Treg destabilization in response to TLR2 signaling in tumors.

We next sought to determine which APC type was responsible for mediating Treg destabilization by TLR2 *in vivo*. Our co-culture system demonstrated that TLR2 signaling in cDCs can destabilize Tregs *in vitro*, which aligned with our previous finding that cDCs highly expressed *Tlr2*^{GFP} in tumors (**Figure 3.3**). We therefore hypothesized that cDCs were the cell type to mediate Treg depletion from tumors. To test this hypothesis, we repeated our IT Pam3 experiments using *Itgax*^{DTR} mice, in which CD11c⁺ cells express DTR to enable the conditional depletion of cDCs.³²¹ In support of our hypothesis, the ability of Pam3 to mediate the depletion of Tregs from tumors or their downregulation of Foxp3 was significantly attenuated in *Itgax*^{DTR} mice (**Figures 3.6H-I, left**). However, while DT treatment efficiently depleted cDCs from tumors of *Itgax*^{DTR} mice, it also significantly depleted macrophages and monocytes, making it impossible to ascribe the reversal of TLR2-mediated Treg destabilization to cDCs specifically (**Figure S3.7F**). To refine our approach, we next employed *CD64*^{Cre};*Rosa26*^{Isl-DTR} (“*CD64*^{DTR}”) mice, in which all CD64⁺ cells (conventionally thought to be primarily composed of macrophages) permanently express DTR.⁸⁵¹ Both macrophages and monocytes were significantly depleted from the tumors of these mice upon IP DT treatment, while cDC1s and cDC2s remained unperturbed (**Figure S3.7G**). Treg destabilization by TLR2 was unimpacted by the depletion of monocytes and macrophages, ruling out a contribution of these cell types to this effect and suggesting that cDCs were indeed responsible (**Figures 3.6H-I, center**). To validate this conclusion, we generated *Zbtb46*^{DTR} bone marrow chimera mice, which enable the specific depletion of cDCs, and repeated our IT Pam3 experiments (**Figures S3.7H-I**).⁸⁵² DT treatment of these mice abrogated the effects of IT TLR2 on tumor Tregs while only depleting cDCs from tumors and leaving other APC populations intact, confirming that cDCs are the cell type to mediate Treg destabilization by TLR2 (**Figures 3.6H-I, right, and S3.7J**). Finally, to establish the specific subset of cDCs responsible for these effects, we repeated our experiments with *Batf3*^{-/-} and *Zeb2*^{3δ/3δ} mice, which are congenitally deficient for cDC1s and cDC2s respectively (**Figure S3.7K**). Strikingly, while *Batf3*^{-/-} mice still significantly destabilized Tregs in response to TLR2, this effect was entirely lost in *Zeb2*^{3δ/3δ} mice (**Figures 3.6J-K**). Altogether, these results establish that cDC2s comprise the cell type primarily responsible for Treg destabilization and subsequent reduction in Treg tumor frequency in response to IT TLR2 stimulation.

Figure 3.6: TLR2 signaling in cDC2s destabilizes Foxp3 expression in tumor Tregs.

(A) WT mice were implanted with 5×10^5 MC38 cells (day 0) and were treated IT with PBS or 20 ug Pam3 (TLR2), poly(I:C) (TLR3), LPS (TLR4), flagellin (TLR5), R848 (TLR7), or CpG DNA (TLR9) 24 hours prior to euthanasia and flow cytometry analysis (day 15).

(B, C) Frequency of Tregs among $CD4^+$ T cells (B) and Foxp3 fluorescence within Foxp3⁺CD25⁺ tumor Tregs (C) 24 hr post-IT PBS or TLRL.

(D) Number of Tregs and $CD8^+$ T cells per 100mm³ tumor tissue 24 hr post-IT PBS, Pam3, or LPS.

(E) Experimental outline for Treg:BMDC co-culture to test the ability of TLRL to destabilize Tregs *ex vivo* via direct (Treg-intrinsic) or indirect (Treg-extrinsic) TLR stimulation.

(F) Frequency of GFP-RFP⁺ ex-Tregs among total RFP⁺ Tregs cultured with TLR-stimulated or unstimulated BMDCs (left) or α CD3/CD28 beads with or without Pam3 (right) after 72 hr co-culture.

(G) Fold-change in MFI relative to PBS-stimulated BMDC-cocultured Tregs (above) or to PBS-stimulated bead cocultured Tregs (below) of *Foxp3*^{GFP-hCre}, GITR, Nrp1, ICOS, and CTV after 72 hr co-culture.

(H, I) Frequency of Tregs among $CD4^+$ T cells (H) and Foxp3 fluorescence within Foxp3⁺CD25⁺ Tregs (I) in $CD11c^+$ cell-depleted (*Itgax*^{DTR}; left), $CD64^+$ cell-depleted (*Fcgr1*^{DTR}; middle), and cDC-depleted (*Zbtb46*^{DTR} bone marrow chimera; right) mice 24 hr post-IT PBS or Pam3.

(J, K) Frequency of Tregs among $CD4^+$ T cells (H) and Foxp3 fluorescence within Foxp3⁺CD25⁺ Tregs (I) in cDC1-deficient (*Batf3*^{-/-}) and cDC2-deficient (*Zeb2* ^{$\Delta 1+2+3$}) mice 24 hr post-IT PBS or Pam3. Mean $\pm$ s.e.m. and one-way ANOVA (B, C, F [left], G [above], H, I, J, K), two-way ANOVA (D), or Student's t-test (F [right], G [below]).

3.3.11 TLR2-mediated reduction of tumor Tregs is required for the efficacy of IV+IT *Lm* therapy.

The unique impact of TLR2 stimulation on Treg lineage stability *in vivo* and *in vitro* led us to hypothesize reducing tumor Treg frequency was part of the mechanism by which TLR2 signaling exerted its effect on tumor control during IT *Lm* administration in IV+IT *Lm*-treated mice. To test this model, MC38 tumors were implanted into *Foxp3^{DTR}* mice to enable conditional depletion of Foxp3⁺ Tregs by DT administration, independently of TLR2 signaling.¹⁷⁷ Next, tumor-bearing mice received IV *Lm* followed by one of four IT injections: 1) IT *Lm* to provide *Lm* antigens to reactivate *Lm*-specific CD8⁺ T cells and deplete Tregs via TLR2 (positive control); 2) IT *LmΔlgt*, which provides *Lm* antigens but fails to deplete Tregs (negative control); 3) a single, one-time low dose (50 ng) of IT DT to transiently and incompletely deplete tumor Tregs without *Lm* antigens present (**Figure S3.8A**), or 4) IT *LmΔlgt* plus a single low dose of DT to transiently deplete tumor Tregs and provide *Lm* antigens (**Figure 3.7A**). Strikingly, only mice treated with IT *LmΔlgt*+DT phenocopied tumor control by IT *Lm* (**Figure 3.7B**). This confirmed that TLR2-mediated Treg depletion by IT *Lm* was indeed required for tumor control, establishing a mechanism for the specific requirement for TLR2 signaling during IT *Lm* administration to control tumors in response to IV+IT *Lm*.

In addition to tumor control, the reduced Tregs and increased CD8⁺ T cell frequencies and increased CD8⁺ T cell:Treg ratio observed with IT *Lm* were partially restored with IT *LmΔlgt*+DT (**Figures S3.8B-6D**). However, mice treated with IT *LmΔlgt*+DT did not control *Lm* CFUs in tumors independently of only Treg depletion, which is expected due to TLR2 stimulation's known impact on enhancing the intrinsic activity of phagocytic cells to kill bacteria (**Figure S3.8E**).^{828,853,854} Notably, the failure of IT DT alone to slow tumor growth demonstrates that Treg depletion by TLR2 is necessary but not sufficient for the activity of IV+IT *Lm*, analogous to the inability of IV *Lm*+IT Pam3 to fully control tumors which is partially restored upon treatment with IT *LmΔlgt*+Pam3 (**Figures 3.1H and S3.1L**). This underlines the fact that both Treg destabilization by TLR2 and the reactivation of *Lm*-specific CD8⁺ T cells in tumors by *Lm* antigens during IT *Lm* administration are required for tumor control.

3.3.12 IV+IT *Lm* enhances IL-2 signaling in tumor-infiltrating CD8⁺ T cells to mediate tumor control

We previously observed that tumor-specific CD8⁺ T cells exhibited reduced PD1 and Lag3 expression during endpoint analysis of tumors treated with IV+IT *Lm*.⁸⁰⁷ While this is consistent with TLR2 driving *de novo* tumor-specific CD8⁺ T cell priming (**Figures 3.4J-O**), we hypothesized that the TLR2-mediated reduction in Treg-mediated suppression and increased tumor antigen cross-presentation would act synergistically to reduce exhaustion in preexisting tumor-specific CD8⁺ T cells. We therefore measured PD-1 and Lag3 expression on tumor-specific T cells 24 hours after IT *Lm* injection in MC38-β2m^{KO}-OVA[±] tumor-bearing mice (**Figures 3.4A and S3.9A**). This revealed that IV+IT *Lm* rapidly reduced the expression of both PD1 and Lag3 on tumor-specific CD8⁺ T cells (**Figures S3.9B-E**). In addition, expression of the high-affinity IL-2Rα chain (CD25) was increased on both tumor-specific and bulk CD8⁺ T cells with IV+IT *Lm* (**Figures S3.7F-G**), a phenotype associated with increased antitumor cytotoxic T cell activity.⁸⁵⁵ Together, these changes in tumor-specific CD8⁺ T cell phenotypes suggest that

IV+IT *Lm* rapidly reprograms preexisting tumor-specific CD8⁺ T cells in tumors in addition to promoting *de novo* priming.

CD25 is upregulated by TCR signaling along with its ligand IL-2, which together create an autocrine signaling loop that supports T cell proliferation and effector function.⁸⁵⁶ Treg competition for IL-2 can limit IL-2 signaling to CD8⁺ T cells, leading to reduced CD25 expression and inhibition of CD8⁺ T cell responses.⁸⁵⁶ Thus, we hypothesized that IV+IT *Lm* may boost IL-2 production in tumor-infiltrating CD8⁺ T cells by reducing tumor Treg frequency and increasing antigen presentation to CD8⁺ T cells. Indeed, IV+IT *Lm* significantly increased IL-2 production by tumor CD4⁺ and CD8⁺ T cells (**Figures 3.7C-E and S3.7H**). This aligned with our previous finding that IV+IT *Lm* also increased secretion of IFN- γ and TNF- α by tumor CD4⁺ and CD8⁺ T cells at this timepoint.⁸⁰⁷ IFN- γ and TNF- α secretion by *Lm*-specific CD8⁺ T cells was found to be necessary for the efficacy of IV+IT *Lm*, leading us to hypothesize that IL-2 secretion would also contribute to tumor control.⁸⁰⁷ Confirming the importance of this mechanism to the effects of IV+IT *Lm*, administering anti-IL-2 antibody blockade beginning the day before IT *Lm* injection completely abrogated tumor control and led to a trend towards increased *Lm* CFUs recovered from tumors (**Figures 3.7I-J, and S3.7I**). Altogether, these results indicate that IL-2 signaling is essential for eliciting tumor control with IV+IT *Lm*. Mechanistically, these results are consistent with *Lm*-mediated TLR2 signaling reducing tumor Treg frequency and increasing tumor antigen cross-presentation, which together increase IL-2 production and signaling by tumor-specific CD8⁺ T cells to enhance their capacity to control tumors.

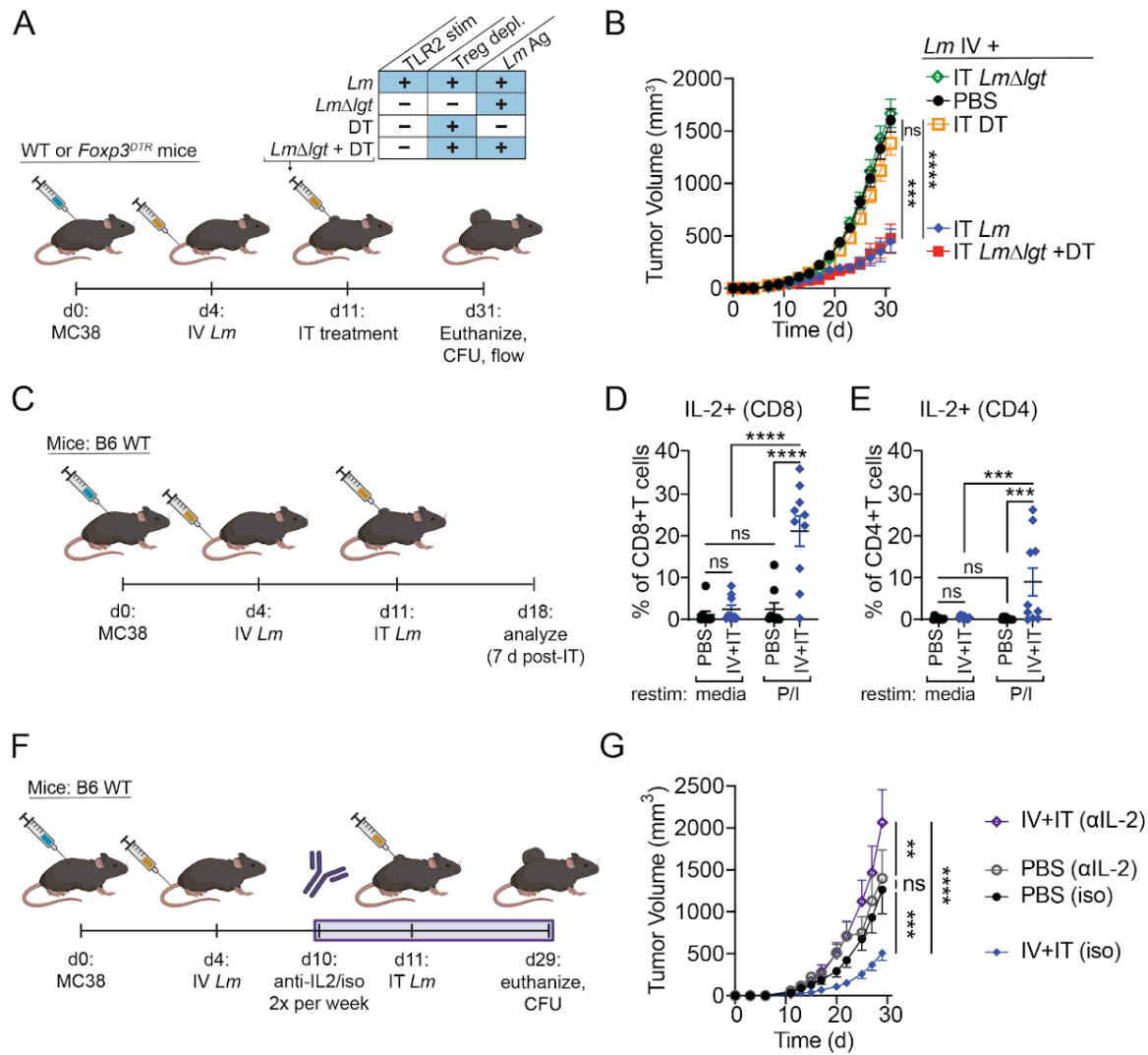


Figure 7: Treg depletion by TLR2 is essential for IV+IT *Lm* efficacy.

- (A) Experimental outline for determining whether Treg depletion by IT *Lm* is required for tumor control in response to IV+IT *Lm*.
- (B) MC38 tumor growth following treatments indicated in A.
- (C) Experimental outline for testing if IV+IT *Lm* boosts IL-2 production in tumor T cells.
- (D, E) IL-2 production in tumor-infiltrating CD8⁺ T cells (D) and CD4 T cells (E) following 6 hr restimulation with media or PMA+ionomycin.
- (F) Experimental outline for determining the necessity of IL-2 for tumor control in IV+IT *Lm*.
- (G) MC38 tumor growth in the presence of an IL-2 depleting antibody or isotype control. Mean $\pm$ s.e.m. and two-way ANOVA (B, G) or one-way ANOVA (D, E).

3.4 Discussion

Although TLRs are widely appreciated to enhance adaptive immune responses, the mechanisms by which they do so, and the specific effects of signaling by different TLRs on adaptive immunity within the TME, remain incompletely understood. Here, using an IV+IT *Lm* regimen to elicit a potent CD8⁺ T cell-dependent anti-tumor response, we uncovered an essential and multifactorial role for TLR2 signaling at both the IV and IT phases of *Lm* administration (**Figure S3.10**). During IV *Lm* administration, TLR2 contributed to the priming of *Lm*-specific CD8⁺ T cells, in line with the well-characterized role of TLRs in promoting the priming of pathogen-specific T cells (**Figure S3.10A**). However, to our surprise, TLR2 was also critical during IT *Lm* administration. This was due to TLR2 signaling in cDC2s uniquely driving the destabilization of Foxp3 expression in tumor Tregs during IT *Lm* administration, leading to reduced Treg frequency and immunosuppression in tumors (**Figure S3.10B**). Treg depletion enhanced the frequency and functionality of *Lm*-specific CD8⁺ T cells within tumors and reversed CD8⁺ T cell exhaustion. These effects synergized with direct TLR2 signaling to enhance the uptake of both bacteria and tumor antigens and increased cross-presentation of tumor antigens on cDC1s and cDC2s, leading to increased priming of tumor-specific CD8⁺ T cells in the tDLN, enhanced CD8⁺ T cell recruitment to tumors, and decreased expression of PD-1 and Lag-3 on CD8⁺ T cells in tumors (**Figure S3.10C**). These effects were due, at least in part, to increased IL-2 production and signaling in tumor CD8⁺ T cells, likely also as a result of reduced tumor Treg competition for IL-2 (**Figure S3.10C**). Thus, TLR2 signaling has the unique ability to promote CD8⁺ T cell-dependent antitumor immunity by destabilizing Tregs within tumors, revealing a novel adjuvant function of TLR2 in the context of cancer immunotherapy.

The requirement for TLR2 signaling by *Lm* to mediate tumor control with IV+IT *Lm* provides clear experimental evidence demonstrating that TLR stimulation by *L. monocytogenes* contributes to its antitumor activity.^{622,634} Until now, the importance of TLR signaling for bacterial cancer therapy was most strongly supported by the clinical success of BCG, which also strongly activates TLR2 but appears to require the activation of multiple TLRs to elicit tumor control.^{857,858} Interestingly, the only other study examining *L. monocytogenes*-mediated TLR2 signaling in the setting of cancer, which utilized BALB/C mice and a different tumor model, found that TLR2 signaling in tumor cells, and not immune cells in the TME, impacted tumor progression.⁸⁵⁹ Nevertheless, our results generated using *LmΔlgt* and *Tlr2*^{-/-} mice are very similar, suggesting that tumor cell-intrinsic TLR2 signaling is not a major contributor to tumor growth in the context of IV+IT *Lm*. In line with our results demonstrating antitumor effects of TLR2 signaling, another study found that *Akkermansia*-mediated TLR2 stimulation increased the efficacy of IL-2 cancer therapy.⁸⁶⁰ A variety of small-molecule TLR2 agonists have also been shown to inhibit tumor growth in preclinical mouse cancer models, bolstering interest in the anti-cancer efficacy of TLR2-activating drugs.^{861–863} Our results expand upon these findings by demonstrating that TLR2 signaling mediated by *Lm* lipoproteins provides immune-mediated control of tumors in the absence of additional therapy such as IL-2. Together, results from BCG, *Akkermansia*, and *Lm* indicate that bacterial TLR2 ligands can mediate antitumor immunity and make a compelling case to broaden the direction of clinical investigations from the activation of endosomal TLRs to also include surface TLRs, which are currently not under investigation as adjuvants for antitumor immunity.^{669,682,685,686,789}

TLR2 signaling is known to be important for combating primary *L. monocytogenes* infection in the liver and the spleen, but its role in CD8⁺ T cell responses to secondary infection is inconclusive, leading to the consensus that TLR2 signaling exerts its protective effects solely through innate immunity.^{372,634,795,797,798,800} In the broader context of antibacterial adaptive immunity, it has often been argued that TLR signaling is minimally important, and can be bypassed, for the establishment of adaptive immune responses because patients with TLR deficiencies typically have reduced susceptibility to bacterial infection over time.^{802,803} However, here we found that TLR2 was required during both IV (primary) and IT (secondary) *Lm* administration to support CD8⁺ T cell-mediated tumor control and *Lm* bacterial burden within tumors. The requirement for TLR2 signaling to mediate control of secondary *Lm* infection in our study, but not in previous reports, likely stems from our studying *Lm* in the context of cancer, where *Lm* can colonize tumors and interact with a unique set of immune cells recruited to and shaped by the TME.⁸⁰⁷ The TME is highly immunosuppressive, which may render individual anti-*Lm* CD8⁺ T cells less effective.^{864,865} Similarly, the frequency of *Lm*-specific CD8⁺ T cells in tumor tissue may be limited by the scarcity of nutrients or signals that promote proliferation and survival, such as IL-2.⁸⁶⁶ In either case, the failure of adaptive immunity to effectively control *Lm* in the absence of TLR2 signaling during IT *Lm* administration demonstrates more generally that innate immune signals not only support the priming of adaptive immune cells, but also support their function upon reactivation in response to secondary infection in immunosuppressive environments. Therefore, the immune response to *Lm* in tumors highlights mechanisms by which TLR signaling can contribute at both early and late stages of adaptive immune responses.

Specifically, TLR2 signaling in tumors upon IT *Lm* injection was required to mediate the destabilization and depletion of Tregs, enabling CD8⁺ T cell-mediated tumor control by IV+IT *Lm*. This likely underlies the specific requirement for TLR2 during IT *Lm* administration, while other TLRs are dispensable, and may explain the requirement for TLR2 in adaptive anti-*Lm* immune responses in tumors but not in other tissues. The ability of IT *Lm* to deplete Tregs is consistent with a recent study that showed that systemic *Lm* infection transiently reduced Treg frequency, number, and suppressive function in the spleen at 3 days post-infection before Tregs rebounded at 7 days post-infection.¹⁸² While the authors did not focus on the signal that mediated the disruption of Tregs at the 3-day timepoint, they did identify PRR signaling as being significantly reduced at 7 days post-infection compared to 1 day-post-infection; our results would suggest that TLR2 signaling likely plays a role in their system. Indeed, others have shown that TLR2 signaling by small-molecule ligands negatively regulates Treg function, but did not test whether TLR2 stimulation by live bacteria or bacterial products have the same result.^{824,845,847,848,867} In contrast, we found no evidence of prior reports that tied TLR5 stimulation by *Lm* to Treg frequency or function, while the sole study to investigate a link between TLR9, Tregs, and *Lm* found that TLR9 signaling supported the generation of peripherally-induced Tregs from naive CD4⁺ T cells.⁸¹⁸ Our work therefore bridges a gap between these findings by demonstrating that bacterial TLR2 ligands can disrupt Treg suppression in the TME, an effect that is specific to TLR2 and no other TLRs in the systems that we employed. However, it is noteworthy that Treg depletion did not lead to reduced bacterial burden in our system, suggesting that TLR2 contributes to anti-*Lm* immunity by multiple mechanisms, and not solely by negatively regulating Tregs.^{797,798,800,828,829}

Prior reports which found that TLR2 mediated the disruption of Treg suppression proposed that this effect was driven by T cell-intrinsic TLR2 signaling within Tregs or effector T

cells.^{845–848,868,869} However, we did not observe evidence of TLR2 expression in Tregs, CD4⁺ conventional T cells, or CD8⁺ T cells, nor did we find evidence that T cell-intrinsic TLR2 signaling contributed to tumor control. A possible reason for this inconsistency is that the preceding reports utilized CD25 expression as the primary criterion for sorted Tregs, which may contain multiple contaminating CD25⁺ cell types including CD25⁺ dendritic cells.^{870,871} CD25⁺ cDCs could affect the results of *in vitro* T cell assays even at low frequencies, given the extreme potency of cDCs in regulating T cell responses.^{871–873} In contrast, our Treg sorting strategy was to isolate CD4⁺CD8[−]CD62L⁺RFP⁺GFP⁺ cells from *Foxp3*^{GFP-Cre};*Rosa26*^{lsL-RFP} Treg lineage-trace mice, which should facilitate the purification of *bona fide* Tregs from the broader population of CD25⁺ cells given that *Foxp3* is a more definitive marker for Tregs than CD25.⁸⁷⁴ Furthermore, even if Tregs express TLR2 at a level below the threshold of detection for the *Tlr2*^{GFP} reporter, cells with very low TLR expression may be capable of responding to high concentrations of TLR ligands *in vitro* that are normally preferentially sensed by cells with higher TLR expression, as in the case of cDC2s *in vivo* and BMDCs *in vitro* in the systems we employed.^{875,876} However, both our *in vivo* results using mixed *Tlr2*^{+/+} and *Tlr2*^{−/−} bone marrow chimera mice and *in vitro* using BMDC-Treg cocultures strongly suggest that Treg-intrinsic TLR2 signaling did not mediate the destabilization of *Foxp3* expression in our systems. Our findings therefore underscore the diversity of methods by which TLR2 signaling can regulate T cell immunity,⁷⁵ which in other circumstances may be mediated by Treg-intrinsic signaling^{845,869} but in this case occur via a T cell-extrinsic mechanism that is translated by other immune cells into an instructive cue.

Indeed, our studies using various APC-depleting mouse strains established that cDCs were vital for Treg destabilization by TLR2, while our use of cDC1- and cDC2-deficient mice confirmed that cDC2s are responsible for this effect in MC38 tumors. This finding was particularly interesting for two reasons. First, the reduced frequency of Tregs in cDC2-deficient mice and the inability of these mice to mediate Treg depletion upon IT TLR2 stimulation suggests that cDC2s exert both pro- and anti-Treg functions in the context of cancer, depending on the presence or absence of inflammatory signaling from TLR2. This is in line with recent findings that together support the existence of an important, bidirectional cDC2-Treg regulatory axis that is functional in a variety of tissues, including both mouse and human tumors.^{269,877–882} In general, cDC2s have been found to support Treg function, but in certain cases cDC2s and Tregs can provide mutually inhibitory signals that depend on tissue-specific environmental factors and the presence or absence of inflammatory signals, analogous to our results with TLR2.^{269,877–882} Second, the dependence of Treg destabilization upon cDC2s intersects with results from this and our previous work which showed that cDC1-mediated, TLR2-dependent cross-presentation of tumor antigens is required for the full efficacy of IV+IT *Im* therapy.⁸⁰⁷ Taken together, these results demonstrate how multiple distinct APC types (cDC1s and cDC2s) regulate different aspects of the adaptive immune response (CD8⁺ T cells and Tregs, respectively) in response to shared innate immune signals (TLR2) to coordinate an appropriate adaptive immune response. While this phenomenon has been widely reported in the context of primary infection and the initial priming of adaptive immunity, to our knowledge ours is the first report to establish a similar role for TLR signaling in coordinating adaptive immune responses to secondary infection.^{75,399} As alluded to above, this difference may be due to the high degree of immune suppression in the TME, which could necessitate the coordinated activity of individual APC subsets that are specialized for the regulation of Tregs and CD8⁺ T cells to overcome this barrier to effector immune responses.^{465,883,884}

In the case of tumor-specific CD8⁺ T cells, TLR2 signaling promoted the responsiveness of these cells by enhancing tumor antigen uptake and cross-presentation by cDCs. This finding is consistent with previous reports that small-molecule TLR2 ligands covalently linked to tumor antigens can enhance cross-presentation to boost antitumor immunity.^{885–889} Similarly, the activation of tumor-nonspecific bystander CD8⁺ T cells in the TME can provide immunostimulatory signals to APCs that boost cross-presentation of tumor antigens and subsequent epitope spreading.^{778,779} Indeed, T cell-dependent cross-presentation of tumor antigens and/or epitope spreading has as has been reported to occur in other therapies that activate tumor-nonspecific bystander CD8⁺ T cells, leading to the production of cytokines such as IFN- γ which can potentially upregulate cross-presentation.^{778,779,890,891} Our results suggest that IV+IT *Lm* operates by a combination of these mechanisms and Treg depletion because both IV *Lm* and IT *Lm* were necessary but insufficient to increase cross-presentation and tumor-specific T cell priming. In our combined model, IV *Lm* potentiates cross-presentation by generating *Lm*-specific T cells. In turn, IT *Lm* provides both bacterial TLR2 ligands (to drive cDC-intrinsic TLR2 signaling to enable antigen uptake and deplete tumor Tregs) and *Lm* antigens (to reactivate *Lm*-specific CD8⁺ T cells generated by IV *Lm*) which synergize to enhance tumor antigen cross-presentation by cDCs. This model is consistent with previous work from our lab which showed that IFN- γ is strongly induced in CD8⁺ T cells by IV+IT *Lm* and that IFN- γ produced by *Lm*-specific CD8⁺ T cells is required for tumor control.⁸⁰⁷ Furthermore, we have shown that blockade of IFN- γ or depletion of CD8⁺ T cells abrogated enhanced cross-presentation mediated by the depletion of CXCR3⁺ Tregs from MC38 tumors.²⁶⁶ This model is particularly intriguing because it would reveal a novel mechanism by which the innate (TLR2) and adaptive (CD8⁺ T cell) immune response against *Lm* within tumor tissue can together enhance tumor-specific adaptive immunity without additional immunotherapeutic treatments, the co-expression of tumor antigens by *Lm*, or the direct infection of tumor cells by *Lm*.^{623,634,753}

In summary, our findings have important implications for the development of immunotherapies that act on innate immune cells to promote the T cell response, including bacterial immunotherapies. First, tumor-specific CD8⁺ T cell immunity can be increased in the absence of genetically engineering the expression of tumor antigens into bacteria, instead relying upon the ability of bacteria-specific CD8⁺ T cells and bacterial TLR ligands to synergistically mediate tumor antigen cross-presentation. This finding has tremendous potential in light of many recent discoveries that several types of cancer may naturally harbor numerous microbes.^{864,892,893} Our findings suggest that eliciting T cell responses against such microbes within tumors can cause epitope spreading to tumor antigens, yielding therapeutically efficacious antitumor immunity. Second, TLR2 signaling increases antigen uptake by APCs and inhibits Treg suppression, raising the importance of targeting TLR2 therapeutically. While several TLR agonists have been tested in clinical trials, TLR2 has not been the focus of these efforts.^{655,669,682,685,686,786} However, our results, along with several other recent studies,^{705,809,894–897} indicate that TLR2 stimulation may be a more potent agonist of T cell-mediated antitumor immunity than formerly realized, likely due to its unique inhibition of Treg-mediated immunosuppression within tumors. Thus, our work supports the development of cancer immunotherapies that stimulate TLR2. Third, enhanced tumor antigen presentation in tdLN can enhance the systemic immune response against tumor antigens, providing a mechanism for intratumoral immunotherapies to ignite systemic adaptive immune responses that could be effective against disseminated metastatic cancers. In conclusion, by characterizing the molecular

and cellular mechanisms underlying the TLR2 dependency of IV+IT *Lm* therapy, we highlight how this receptor can be leveraged to elicit improved antitumor immunity.

Limitations of the study

All experiments were conducted in preclinical mouse models of disease, which can fail to recapitulate responses to therapies in humans. Additional studies using humanized mice may provide insight into the translatability of the specific mechanisms identified in this work to the human immune system. The primary strain used in this study, *Listeria monocytogenes* $\Delta actA$, has a tolerable safety profile in human clinical trials, but has been observed to form biofilms on medical implants in some patients during clinical trials. Mutations that reduce biofilm formation and the extracellular viability of *L. monocytogenes* may be considered for clinical development based upon the principles outlined in this work.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michel DuPage (dupage{at}berkeley.edu).

Materials availability

Listeria monocytogenes strains used in the study will be made available from the lead contact upon request.

Data and code availability

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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Author Contributions

Conceptualization and methodology, M.D., T.C, and D.A.P.; investigation, T.C., J.G.C., S.F., D.G.V., S.H., J.V., N.F.H., and J.Y.; writing – original draft, M.D. and T.C.; writing – review & editing, M.D., T.C., and D.A.P.; supervision, M.D., T.C., and D.A.P.; funding acquisition, M.D. and D.A.P.

3.5 Methods

Experimental models and subject details

Animal studies

C57BL/6J wild-type (JAX:000664) and CD45.1+ (JAX:002014) mice were obtained from Jackson laboratories and bred in house. OT-1 transgenic mice were obtained from Taconic (Catalog#: 2334) and bred in house. *Tlr2*^{-/-} (JAX:004650) and *Tlr2*^{GFP} (JAX:031822) mice were a gift from the Barton lab at the University of California, Berkeley. Foxp3 lineage trace mice were generated by crossing *Foxp3*^{eGFP-hCre} mice (JAX:023161) with *Rosa26*^{LSL-RFP} mice⁸⁹⁸ and were a gift from the Bluestone lab at the University of California, San Francisco. For tumor studies, syngeneic C57BL/6J mice of the above genotypes were inoculated with 5.0×10^5 MC38, 5.0×10^5 MC38^{mCherry}, 1.0×10^6 MC38- $\beta 2m^{KO}$, 1×10^6 MC38- $\beta 2m^{KO}$ -OVA, or 2.0×10^5 B16F10 tumor cells in PBS subcutaneously on the right flank. For KP sarcoma experiments, 2.0×10^5 KP sarcoma cells were implanted intramuscularly in the right hind leg.⁸⁰⁷ Tumor measurements were performed blindly across the entire experiment by a single operator measuring three dimensions of the tumor with calipers every second day beginning when tumors were palpable. All experiments were conducted according to the Institutional Animal Care and Use Committee guidelines of the University of California, Berkeley.

Cell lines

MC38 and MC38-OVA cell lines were kindly provided by Dr. Jeff Bluestone's lab. The MC38- $\beta 2m^{KO}$ and MC38- $\beta 2m^{KO}$ -OVA cell lines were generated by our lab by CRISPR-Cas9-mediated knockout of *B2m* followed by FACS to purify H2-K(b)-H2-D(b) cells using a FACS AriaTM Fusion cell sorter (BD).²⁶⁶ The MC38^{mCherry} cell line was generated by our lab as detailed below. All cell lines were maintained in DMEM (GIBCO) supplemented with 10% FBS, sodium pyruvate (GIBCO), L-Glutamine (GIBCO), and penicillin-streptomycin (GIBCO). Tumor cells were grown at 37°C with 5% CO₂.

Method Details

Generation of mCherry-expressing MC38

The MC38^{mCherry} cell line was generated by transduction of MC38 cells with an mCherry-encoding lentiviral construct as described previously.⁸⁹⁹ Briefly, VSV-G, psPax2, and mCherry-encoding vector targeting plasmid were transfected into LentiX-293T cells (Takara Bio) at a molecular ratio of 1:3:4 using LipofectamineTM Transfection Reagent (Thermo Fisher) on day 0. The following day (day 1), LentiX-293T media was swapped for fresh media. On day 4, lentiviral media was collected and filtered through a 0.45 μ m syringe-driven filter. MC38 cells were suspended in lentiviral media in the presence of 4 μ g/mL polybrene (Sigma-Aldrich) and incubated at 37°C with rotation for 1 hr. Following incubation, cells were resuspended in fresh media and expanded through serial passaging. mCherry⁺ MC38 cells were isolated to >95% purity by multiple rounds of FACS using a FACS AriaTM Fusion cell sorter (BD).

L.monocytogenes strains

All strains of *L. monocytogenes* were derived from the wild-type 10403S strain. The *Lm* constructs were based on an attenuating deletion of the *actA* gene ($\Delta actA$). *Lm* Δlgt was generated by introduction of an in-frame deletion of *lgt*.^{799,900} *Lm-OVA* expresses an LLO-OVA fusion encompassing amino acids 1-441 of Listeriolysin O (LLO) and OVA cloned in frame of LLO1-441 under the control of the *hly* promoter in the pPL2 integration vector.^{782,901} *Lm-TagBFP* expresses a secreted TagBFP protein under the control of the *actA* promoter using the pPL2 integration vector.^{827,901} All strains were cultured in filter-sterilized nutrient-rich Brain Heart Infusion (BHI) media (BD Biosciences) containing 200 μ g/mL streptomycin (Sigma-Aldrich).

Intravenous and Intratumoral Listeria infection

Overnight cultures were grown in BHI + 200 μ g/mL streptomycin at 30°C. The following day, bacteria were grown to logarithmic phase by diluting the overnight culture in fresh BHI + 200 μ g/mL streptomycin and culturing at 37°C 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 1×10^6 CFU log-phase bacteria. For intratumoral infections, frozen stocks were diluted in PBS to infect via intratumoral injections with 5×10^7 CFU log-phase bacteria. In some instances, intratumoral bacteria was substituted with 100 μ g Pam3CSK4 (Invivogen) or supplemented with 50 μ g diphtheria toxin (EMD Millipore). Uninfected control animals were mock infected by intratumoral injection of 100 μ L PBS. Mice were euthanized 1-20 days after intratumoral injections.

Colony forming unit assays from tissues

Tumors were surgically resected following euthanasia, weighed, and transferred to 0.1% NP40 buffer diluted in PBS. Organs were homogenized with a Fisherbrand™ 150 Handheld Homogenizer (Thermo Fisher) and serially diluted on non-TC treated 96-well plates (Genesee). Serial dilutions were plated on BHI + 200 μ g/mL streptomycin plates. Plates were incubated overnight at 37°C. The following day, CFUs were counted and normalized by tumor weight.

Bone marrow dendritic cell differentiation

Primary BMDCs were generated as previously described using WT B6 mice (day 0).⁹⁰² After differentiation (day 6), BMDC media was replaced with fresh media containing PBS or 1 μ g/mL Pam3CSK4 (Invivogen) to stimulate TLR2. 24 hr later (day 7), BMDCs were harvested by transferring media containing nonadherent cells to a centrifuge tube and replacing with an equal volume of cold PBS supplemented with 10 μ M EDTA to loosen semi-adherent cells. Nonadherent cells were pelleted by centrifugation, while semi-adherent cells were incubated for 10 min on ice. After incubation, semi-adherent cells were recovered by gentle pipetting, and this suspension was used to resuspend pelleted nonadherent cells. Pooled cells were washed with PBS to remove trace Pam3CSK4. BMDCs were counted and used in Treg co-culture experiments or analyzed by flow cytometry to assess purity and phenotype.

Fluorescence-activated cell sorting of Tregs

Treg lineage-trace mice were euthanized and lymph nodes and spleens surgically resected and stored on ice in PBS supplemented with 4 mM EDTA and 2% FBS. Single-cell suspensions of splenocytes and lymphocytes were generated by crushing organs through a 40 μ m cell strainer (Fisher Scientific) and red blood cells were removed by ACK lysis using RBC lysis buffer (BioLegend). CD4 T cells were magnetically enriched using the EasySep™ Mouse CD4+ T Cell Isolation Kit (STEMCELL Technologies) per the manufacturer's instructions. Isolated CD4 T cells were stained with fluorescent antibodies in the dark at 4°C for 30 min and washed with PBS prior to resuspension in Opti-MEM (Thermo Fisher) supplemented with 2% FBS for FACS. Naive Tregs (CD4+CD8-CD62L+RFP+GFP+) were sorted with a FACS Aria™ Fusion cell sorter (BD). Sorting efficiency was determined by post-sort flow cytometric analysis and Treg purity exceeded 95%.

Treg-BMDC co-culture assay

FACS-isolated Tregs were mixed with BMDCs or Dynabeads™ Mouse T-Activator CD3/CD28 for T-Cell Expansion and Activation (GIBCO) at a ratio of 4:1::BMDCs:Tregs or 1:3::beads:Tregs. BMDC-Treg cultures were supplemented with 1 μ g/mL of Ultra-LEAF™ Purified anti-mouse CD3 Antibody (BioLegend) to mimic TCR signaling. For indirect stimulation of TLR2 on Tregs mediated by cDCs, Pam3-stimulated BMDCs were used in some BMDC-Treg cultures. For direct stimulation of TLR2 on Tregs, 1 μ g/mL Pam3 (Invivogen) was included in some bead-Treg cultures. All cells were cultured in DMEM supplemented with 10% FBS, sodium pyruvate (GIBCO), L-Glutamine (GIBCO), penicillin-streptomycin (GIBCO), HEPES (GIBCO), MEM Non-Essential Amino Acid Solution (GIBCO), 50 μ M B-mercaptoethanol (GIBCO), and 200 IU/mL teceleukin (hIL2; Hoffmann-La Roche). Cultures were incubated at 37°C for 72 hr prior to flow cytometric analysis to assess Treg phenotype and Foxp3 destabilization.

Bone marrow chimera generation

Host CD45.1+ mice were subjected to irradiation using an XRad320 (Precision X-Ray Irradiation) on day 0 (550 cGy) and 16 hours later on day 1 (500 cGy). 8 hours after the second dose of radiation, mice received 4×10^6 bone marrow cells composed of equal parts (2×10^6 cells/each) $CD45^{1/1}; Tlr2^{+/-}$ and $CD45^{2/2}; Tlr2^{-/-}$ cells via tail vein injection. Bone marrow was collected by surgically resecting femurs and tibias from euthanized mice, cleaning to remove excess tissue, and flushing bone marrow into a 15 mL conical centrifuge tube with a 27-gauge needle using ice-cold RPMI supplemented with 10% FBS, sodium pyruvate (GIBCO), L-Glutamine (GIBCO), HEPES (Gibco), MEM non-essential amino acids (Gibco), penicillin-streptomycin (GIBCO), and 55 μ M 2-mercaptoethanol. Red blood cells were removed by ACK lysis using RBC lysis buffer (BioLegend) and remaining bone marrow progenitor cells were washed extensively with ice-cold PBS prior to tail vein injection. Mice received water supplemented with sulfamethoxazole-trimethoprim oral suspension (Ani Pharmaceuticals) for 30 days with daily monitoring following adoptive transfer of bone marrow. Successful reconstitution was assessed by staining tissues with fluorophore-conjugated anti-CD45.1 and -CD45.2 antibodies and flow cytometric analysis. All mice used in experiments were found to have a ~50:50 ratio of CD45.1+:CD45.2+ cells in all tissues confirming successful reconstitution.

Sample preparation for flow cytometry

Single cell suspensions were prepared as described below (“*Tissue collection and restimulation Assays*”). Dead cells were stained with Live/Dead Fixable Blue Cell Stain kit (Molecular Probes) in PBS at 4°C. Cell surface antigens were stained at 4°C using a mixture of fluorophore-conjugated antibodies. Surface marker stains for murine T cell 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 CD25 (PC61, BioLegend), anti-mouse, CD44 (IM7, BioLegend), anti-mouse CD45 (30-F11, BioLegend), anti-mouse CD45.1 (A20, BioLegend), anti-mouse CD45.2 (104, BioLegend), anti-mouse CD62L (MEL-14, BioLegend), anti-mouse CD69 (H1.23F, BioLegend), anti-mouse CD103 (2E7, BioLegend), anti-mouse Lag3 (C9B7W, BioLegend), anti-mouse PD1 (29F.1A12, BioLegend), anti-H-2Kb MuLV p15E Tetramer-KSPWFTTL (MBL), anti-H2-Kb-A2/SIINFELK tetramer (NIH tetramer core), and anti-IAb/NEKYAQAYPNVS tetramer (NIH tetramer core) in PBS. Surface marker stains for murine myeloid cell samples were carried out with anti-mouse CD16/32 TruStain FcX (93, BioLegend), anti-mouse CD45 (30-F11, BioLegend), anti-mouse F4/80 (BM8, BioLegend), anti-mouse CD45R (RA3-6B2, BioLegend), anti-mouse CD11c (N418, BioLegend), anti-mouse I-A/I-E (M5/114.15.2, BioLegend), anti-mouse SIRPa (P84, BioLegend), anti-mouse CD11b (M1/70, BioLegend), anti-mouse XCR1 (ZET, BioLegend), anti-mouse CD103 (2E7, BioLegend), anti-mouse Ly6C (HK1.4, BioLegend), anti-mouse Ly6G (1A8, BioLegend), anti-mouse CD14 (Sa14-2, BioLegend), and anti-H2-K(b)-SIINFELK (25-D1.16, BioLegend) in PBS. Cells were fixed in 4% PFA (Electron Microscopy Sciences) in PBS at room temperature for 15 minutes, permeabilized with 0.1 % Triton X-100 (Thermo Fisher) in PBS at room temperature for 10 minutes, and washed twice with 0.5% BSA (Sigma Aldrich) in PBS prior to intracellular staining. Intracellular staining for murine T cell samples was performed using anti-mouse Foxp3 (FJK-16S, eBioscience) and anti-mouse IL-2 (Dec-16, BioLegend) at 4°C in 0.5% BSA in PBS. Cells were analyzed by flow cytometry the following day to prevent signal loss from fluorescent proteins (mCherry, GFP, tagBFP). Cells were resuspended in PBS and filtered through FlowTubes™ Cap w/ 35 µm Strainer Mesh (Genesee) prior to data acquisition. In some instances, previously published data were reanalyzed to assess the impact of IV+IT *Lm* on the cross-presentation of tumor-derived ovalbumin by APC subsets.⁸⁰⁷

Tissue collection and restimulation assays

Tumors and lymphoid organs were surgically resected following euthanasia of mice according to the Institutional Animal Care and Use Committee guidelines of the University of California, Berkeley. Resected tumors were minced to 1 mm³ fragments and digested in RPMI media supplemented with 20 mg/mL DNase I (Roche), 125 U/mL collagenase D (Roche), and 2% FBS using an orbital shaker at 37°C for 1 hr. Digested tumors were filtered through a 70 µm cell strainer (Thermo Fisher). Cells from lymphoid organs were prepared by mechanical disruption with the sterile plunger of a 1 mL syringe through a 70 µm nylon mesh cell strainer (Thermo Fisher). Cell suspensions from tumors and lymphoid organs were subjected to ACK lysis to remove red blood cells using RBC lysis buffer (BioLegend) and passed through FlowTubes™ Cap w/ 35 µm Strainer Mesh (Genesee) prior to in vitro stimulation and/or flow cytometric analysis. Stimulation was performed with 3-5×10⁶ cells in Opti-MEM media (Thermo Fisher) supplemented with Brefeldin A (eBioscience), 10 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), and 0.25 µM ionomycin (Sigma-Aldrich) at 37°C for 6 hr. Surface staining,

fixation, permeabilization, and intracellular staining were performed as described above (“*Tissue collection and preparation for flow cytometry*”).

Adoptive transfer experiments

For tumor growth experiments, 2×10^6 *in vitro* expanded OT-I T cells were transferred IV into *Tlr2*^{+/-} and *Tlr2*^{-/-} littermate control mice 2 weeks prior to MC38 inoculations. For *in vitro* OT-I expansion, spleens and lymph nodes were collected from OT-I transgenic mice. OT I CD8⁺ T cells were activated with 1 µg/mL SIINFEKL peptide in DMEM supplemented with 10% FBS, sodium pyruvate (GIBCO), L-Glutamine (GIBCO), penicillin-streptomycin (GIBCO), HEPES (GIBCO), MEM Non-Essential Amino Acid Solution (GIBCO), 55 µM B-mercaptoethanol (GIBCO), and 200 IU/mL teceleukin (hIL2; Hoffmann-La Roche provided by NCI repository, Frederick National Laboratory for Cancer Research). In some experiments where indicated, splenocytes from WT mice immunized 1-2 weeks prior with 10^6 CFU of *Lm-OVA* IV were expanded identically to OT-Is. In these cases, SIINFEKL/K(b) tetramer staining was performed prior to adoptive transfer to validate that a pure population of T cells (>85% tetramer+) was used for all experiments. For priming experiments, 5×10^5 naive, CFSE-labeled OT-I T cells were transferred IV into MC38 tumor-bearing WT B6 mice 12 days post tumor inoculation. Naive OT-I T cells were isolated from spleens and lymph nodes using the MagniSort™ Mouse CD8⁺ T cell Enrichment Kit (Invitrogen) per the manufacturer’s instructions. Isolated naive OT-I T cells were labeled with the CFSE Cell Division Tracker Kit (BioLegend) per the manufacturer’s instructions. CFSE-labeled OT-Is were transferred IV into B6.SJL-*Ptpca Pepcb*/BoyJ (CD45.1) mice 1 day following IT injections of *Lm* or PBS.

In vivo antibody-mediated cell depletion

For tumor progression studies, IL-2 depletion was achieved by intraperitoneal injection of 200 µg per mouse of the anti-IL-2 monoclonal antibody clone JES6-1A12 (Leinco, Catalog # I-1042) one day prior IT *Lm* treatment, followed by additional doses twice weekly thereafter.

Statistical Methods

p values were obtained from unpaired two-tailed Student’s t tests for statistical comparisons between two groups of values derived from separate mice, or from paired two-tailed Student’s t tests for comparisons between tissues within individual mice, 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.

3.6 Supplementary Materials

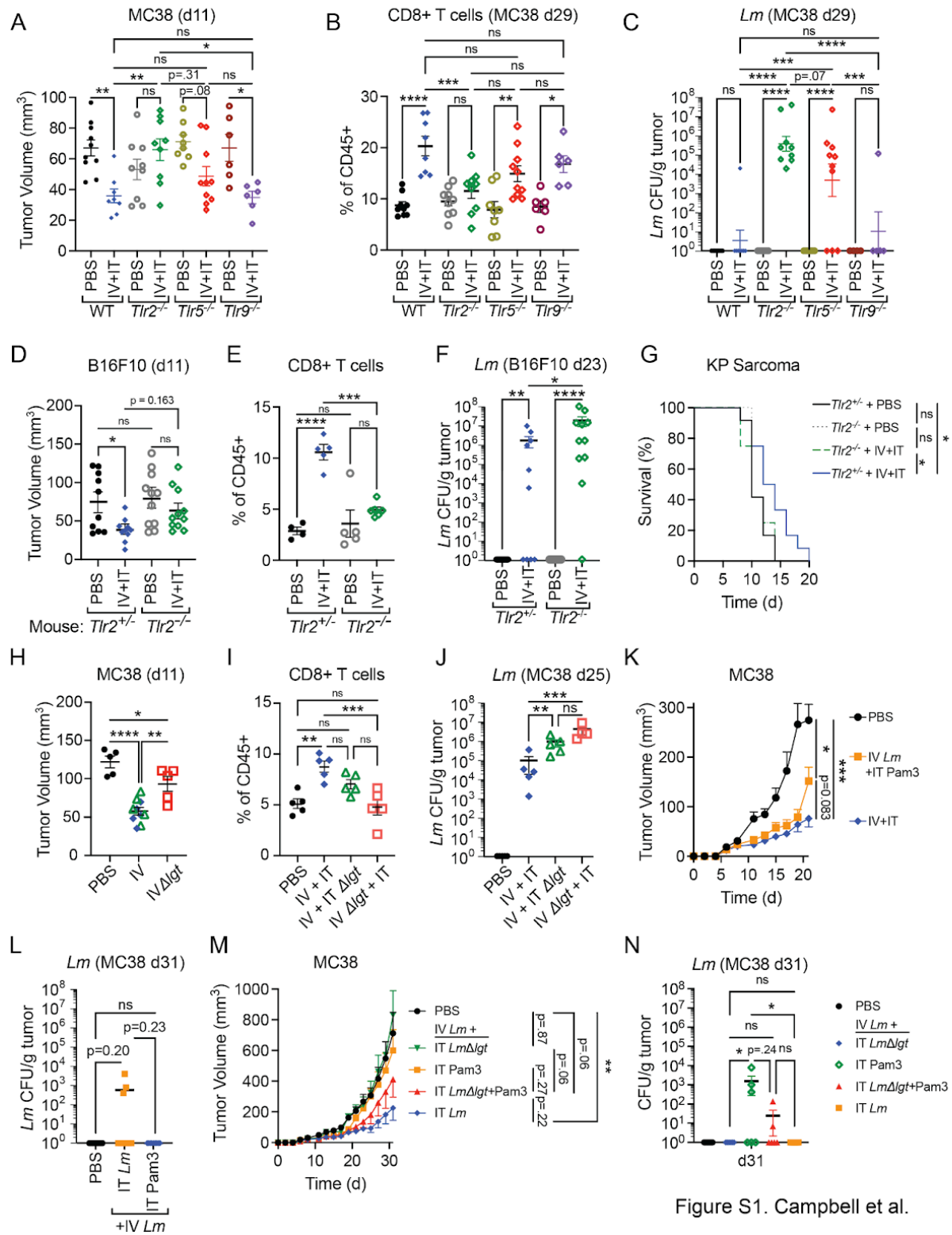


Figure S1. Campbell et al.

Figure S3.1 (related to Fig. 3.1):

- (A) MC38 tumor volume on day 11 of tumor growth from experiment in Figure 1B.
- (B) CD8⁺ T cell frequencies in MC38 tumors from experiment in Figure 1B on day 29 of tumor growth.
- (C) *Lm* CFUs recovered from MC38 tumors on day 29 of tumor growth in IV+IT *Lm*-treated WT (*Tlr2*^{+/-}), *Tlr2*^{-/-}, *Tlr5*^{-/-}, and *Tlr9*^{-/-} mice from experiment in Figure 1B.
- (D) B16F10 tumor volume on day 11 of tumor growth from experiment in panel C.
- (E) CD8⁺ T cell frequencies in B16F10 tumors from experiment in panel C on day 23 of tumor growth.
- (F) *Lm* CFUs recovered from B16F10 tumors on day 23 of tumor growth in IV+IT *Lm*-treated *Tlr2*^{+/-} or *Tlr2*^{-/-} littermate mice from experiment in Figure 1C.
- (G) Percent survival of KP sarcoma-bearing *Tlr2*^{+/-} and *Tlr2*^{-/-} mice receiving IV+IT *Lm* or PBS.
- (H) MC38 tumor volumes on day 11 of tumor growth from experiment in Figure 1H.
- (I) CD8⁺ T cell frequencies in MC38 tumors from experiment in Figure 1H on day 25 of tumor growth.
- (J) *Lm* CFUs recovered from MC38 tumors on day 25 of tumor growth in IV+IT *Lm*-treated mice that received *LmΔlgt* at the IV stage, IT stage, or *Lm* at both stages of treatment from experiment in Figure 1F.
- (K) MC38 tumor growth in IV+IT *Lm*-treated or IV *Lm*+IT Pam3-treated mice up to day 21 from experiment in Figure 1J.
- (L) *Tlr2*^{+/-} and *Tlr2*^{-/-} littermate mice were implanted subcutaneously with 5x10⁵ MC38 tumor cells (day 0). Mice were treated with IT PBS or by injection with 1x10⁶ CFU IV *Lm* on d4 followed by IT injection on d11 of 5x10⁷ CFU *Lm*, 5x10⁷ CFU *LmΔlgt*, 100 ug Pam3CSK4, or 5x10⁷ CFU *LmΔlgt* plus 100 ug Pam3CSK4.
- (M) *Lm* CFUs recovered from MC38 tumors on day 31 of tumor growth from experiment in Figure S1L. Mean ± s.e.m. and one-way ANOVA (A, B, C, D, E, F, H, I, J, M), log-rank test (G), or two-way ANOVA (K, L).

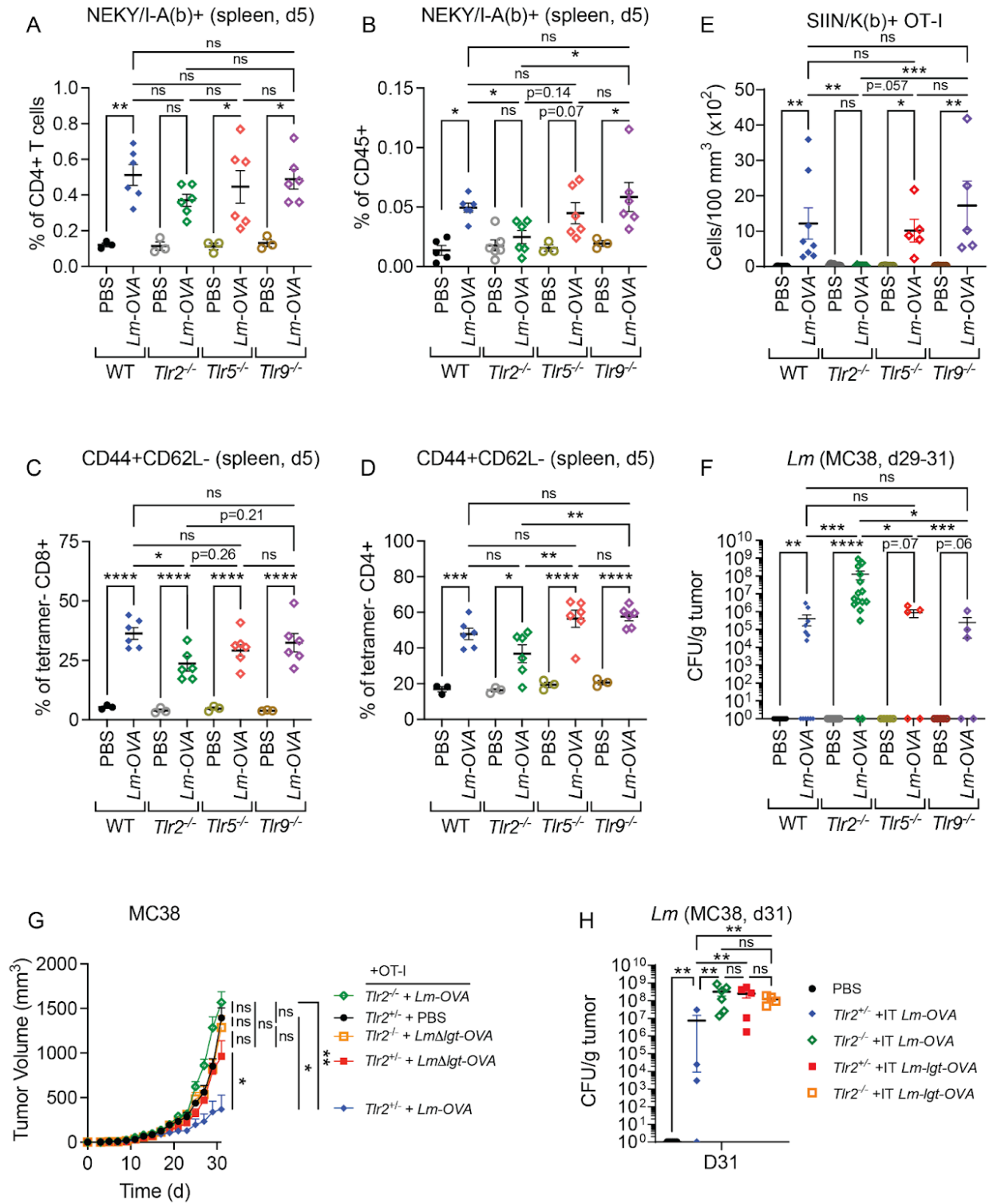
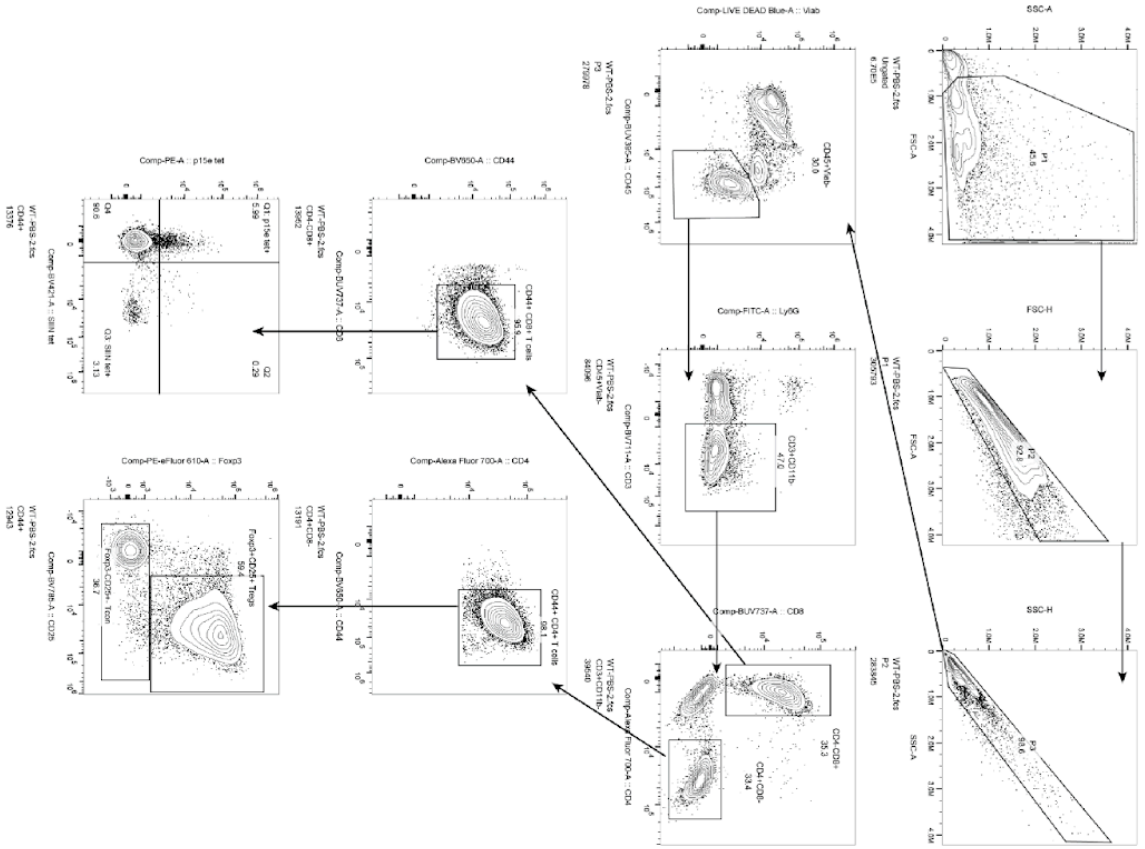


Figure S3.2 (Related to Fig. 3.2):

- (A, B) Frequency of NEKY/I-A(b) tetramer⁺ CD4⁺ T cells in the spleens of TLR2^{-/-} and TLR2^{+/-} littermates 5 days post-IV *Lm-OVA* as a percentage of CD4⁺ T cells (A) or of total CD45⁺ splenocytes (B).
- (C) Frequency of activated CD8⁺ T cells in the spleen 5 days post-IV *Lm-OVA* in *Tlr2*^{-/-} or *Tlr2*^{+/-} littermate mice stratified by SIIN/K(b) tetramer[±].
- (D) Frequency of activated CD4⁺ T cells in the spleen 5 days post-IV *Lm-OVA* in *Tlr2*^{-/-} or *Tlr2*^{+/-} littermate mice stratified by NEKY/I-A(b)+/-.
- (E) Absolute number of SIIN/K(b) tetramer⁺ OT-I T cells per 100 mm³ tumor tissue on day 31.
- (F) *Lm-OVA* CFUs recovered on day 31 from the tumors of WT (*Tlr2*^{+/-}), *Tlr2*^{-/-}, *Tlr5*^{-/-}, or *Tlr9*^{-/-} mice
- (G) MC38 tumor growth in *Tlr2*^{+/-} or *Tlr2*^{-/-} littermate mice harboring 2x10⁶ pre-activated OT-I T cells and treated IT on d11 with 5x10⁷ CFU *Lm-OVA*, *LmΔlgt-OVA*, or PBS.
- (H) *Lm-OVA* and *LmΔlgt-OVA* CFUs recovered on day 31 from the tumors of *Tlr2*^{-/-} or *Tlr2*^{+/-} littermate mice. Mean ± s.e.m. and one-way ANOVA (A, B, C, D, E, F, H) or two-way ANOVA (G).

A

T cell panel



3

Myeloid cell panel

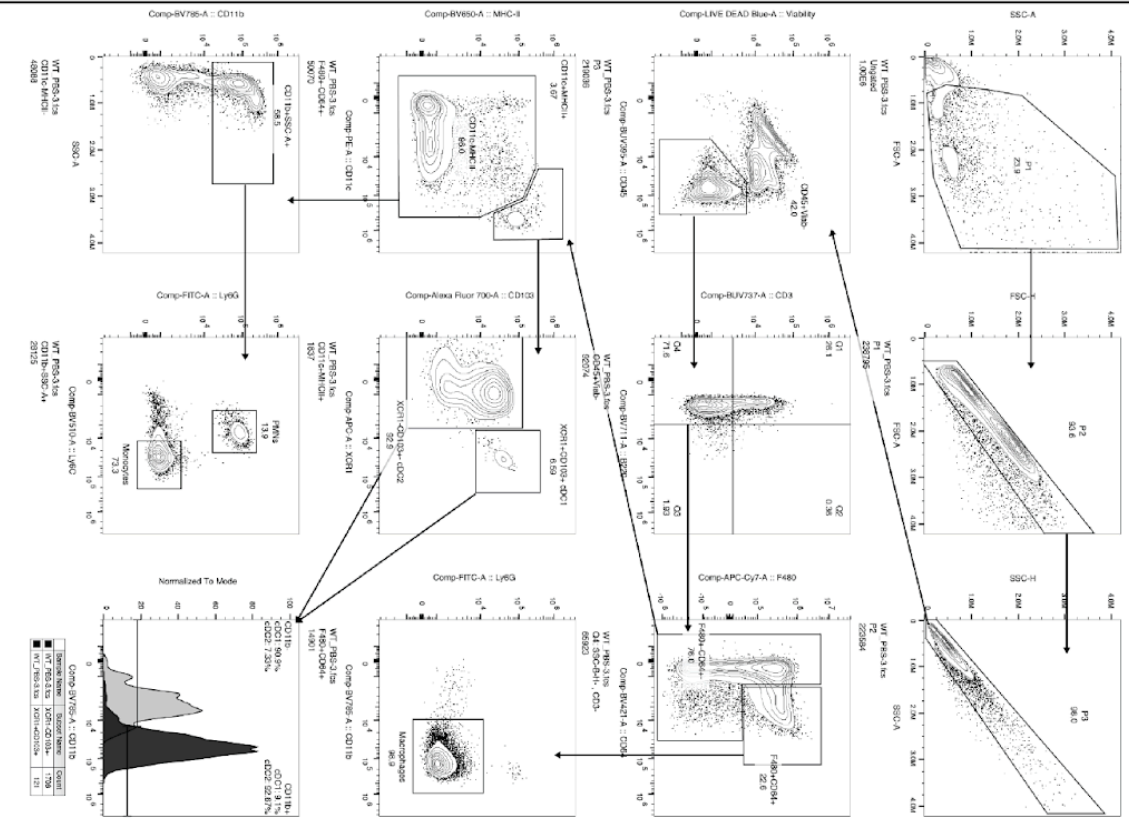


Figure S3.3 (related to Fig. 3.3):

- (A) General T cell panel gating strategy
- (B) General myeloid cell panel gating strategy

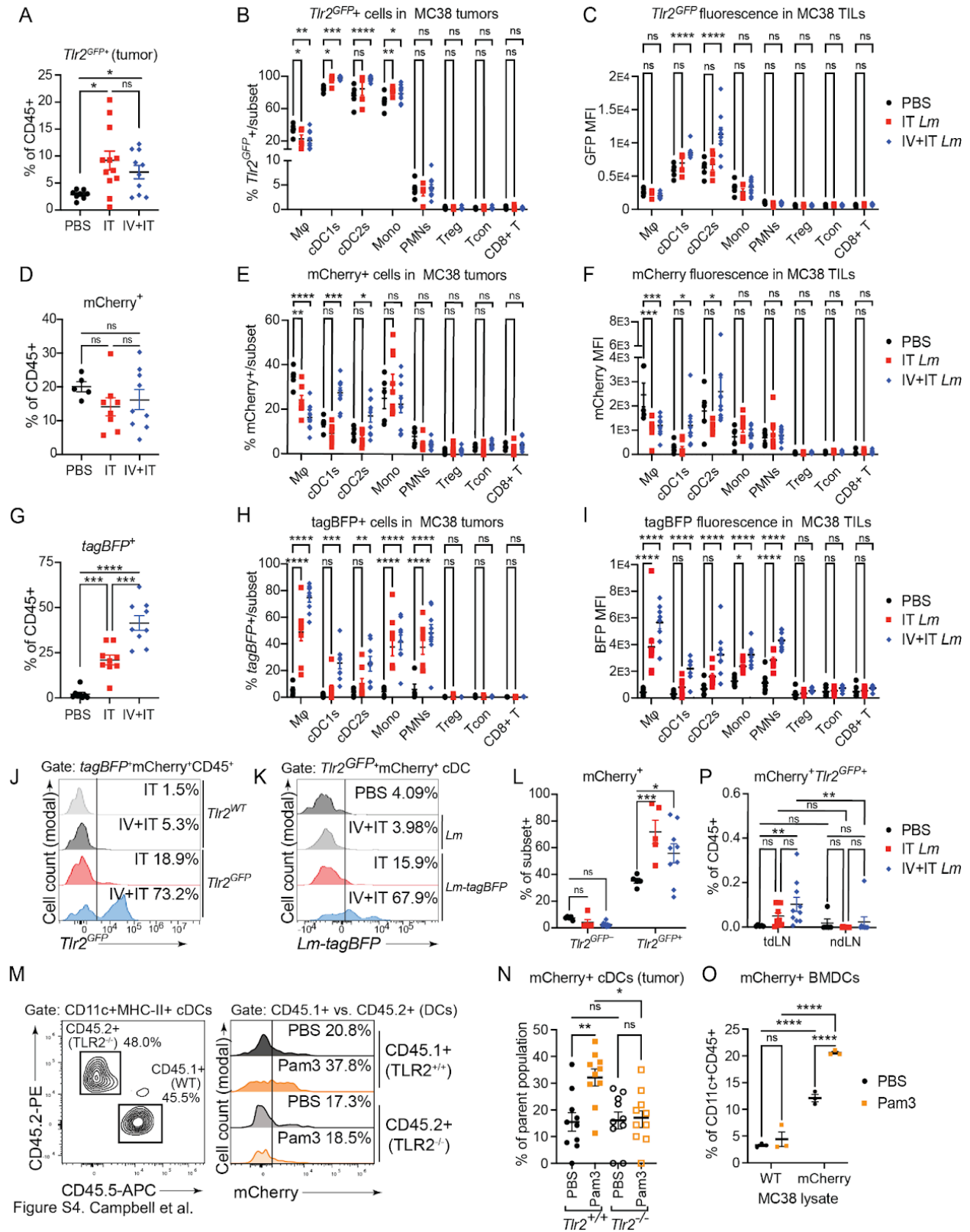


Figure S4. Campbell et al.

Figure S3.4 (related to Fig. 3.3): All data from MC38 tumors 24 hr post-IT *Lm-tagBFP* or PBS (day 12).

- (A) Total *Tlr2*^{GFP+}CD45⁺ cell frequency.
- (B) *Tlr2*^{GFP+} cell subset frequency.
- (C) *Tlr2*^{GFP} MFI in CD45⁺ cell subsets.
- (D) Total mCherry⁺CD45⁺ cell frequency.
- (E) mCherry⁺ cell subset frequency.
- (F) MFI of mCherry in CD45⁺ cell subsets.
- (G) Total *tagBFP*⁺CD45⁺ cell frequency.
- (H) *tagBFP*⁺ cell subset frequency.
- (I) MFI of *Lm-tagBFP* in CD45⁺ cell subsets.
- (J) Representative flow cytometry histograms showing *Tlr2*^{GFP} fluorescence within *tagBFP*⁺mCherry⁺ CD45⁺ cells in MC38 tumors of *Tlr2*^{GFP} or WT control mice treated with IT only or IV+IT *Lm-tagBFP*.
- (K) Representative flow cytometry histograms showing *tagBFP* fluorescence within *Tlr2*^{GFP+}mCherry⁺ cDCs in MC38 tumors treated with PBS, IV+IT *Lm*, IT *Lm-tagBFP*, or IV+IT *Lm-tagBFP*.
- (L) mCherry⁺ cell frequency among *Tlr2*^{GFP-} and *Tlr2*^{GFP+} cells in MC38 tumors.
- (M) Gating of CD45.1⁺ (*Tlr2*^{+/+}) and CD45.2⁺ (*Tlr2*^{-/-}) cDCs (left) and representative mCherry fluorescence in CD45.1/.2⁺ cDCs (right) of mixed bone marrow chimera mice treated with IT Pam3 or PBS.
- (N) Quantification of mCherry⁺ frequency among *Tlr2*^{+/+} and *Tlr2*^{-/-} DCs in mixed bone marrow chimera mice treated with IT Pam3 or PBS.
- (O) Quantification of mCherry⁺ frequency among BMDCs cultured with MC38 or MC38-mCherry⁺ tumor lysate for 24 hr with or without Pam3 stimulation.
- (P) mCherry⁺*Tlr2*^{GFP+} cell frequency in lymph nodes 24 hr post-IT *Lm*. Mean ± s.e.m. and one-way ANOVA (A, D, G, N) or two-way ANOVA (B, C, E, F, H, I, L, O, P).

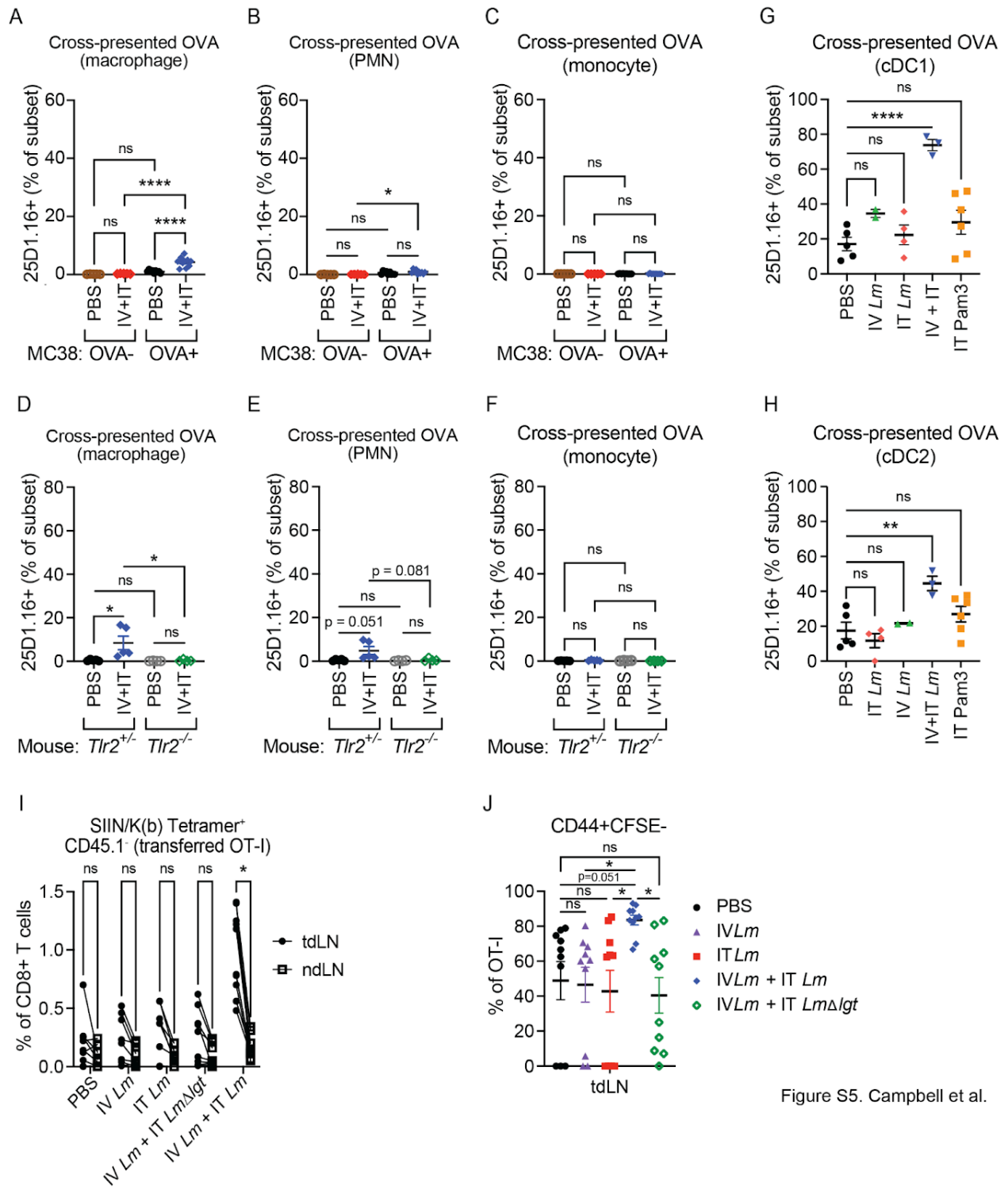


Figure S5. Campbell et al.

Figure S3.5 (related to Fig. 3.4):

(A, B, C) Frequency of SIIN/K(b)⁺ cells among macrophages (A), PMNs (B), and monocytes (C) detected by 25D1.16 staining in tumors of MC38-β2m^{KO} control or MC38-β2m^{KO}-OVA⁺ tumor-bearing mice.

(D, E, F) Frequency of SIIN/K(b)⁺ cells among macrophages (D), PMNs (E), and monocytes (F) detected by 25D1.16 staining in tumors of MC38-β2m^{KO}-OVA⁺ tumor-bearing *Tlr2*^{+/-} and *Tlr2*^{-/-} littermate mice.

(G, H) Frequency of SIIN/K(b)⁺ cells among cDC1s (G) and cDC2s (H) detected by 25D1.16 staining in tumors of MC38-β2m^{KO}-OVA⁺ tumor-bearing mice treated with PBS, IV *Lm*, IT *Lm*, IV+IT *Lm*, or IT Pam3.

(I) Paired analysis of the frequency of transferred OT-I among total CD8⁺T cells in the tdLN and ndLN.

(J) Frequency of primed, proliferating OT-I among total OT-I in the tdLN. Mean +/- s.e.m. and one-way ANOVA (A, B, C, D, E, F, G, H, J) or paired Student's t-test (I).

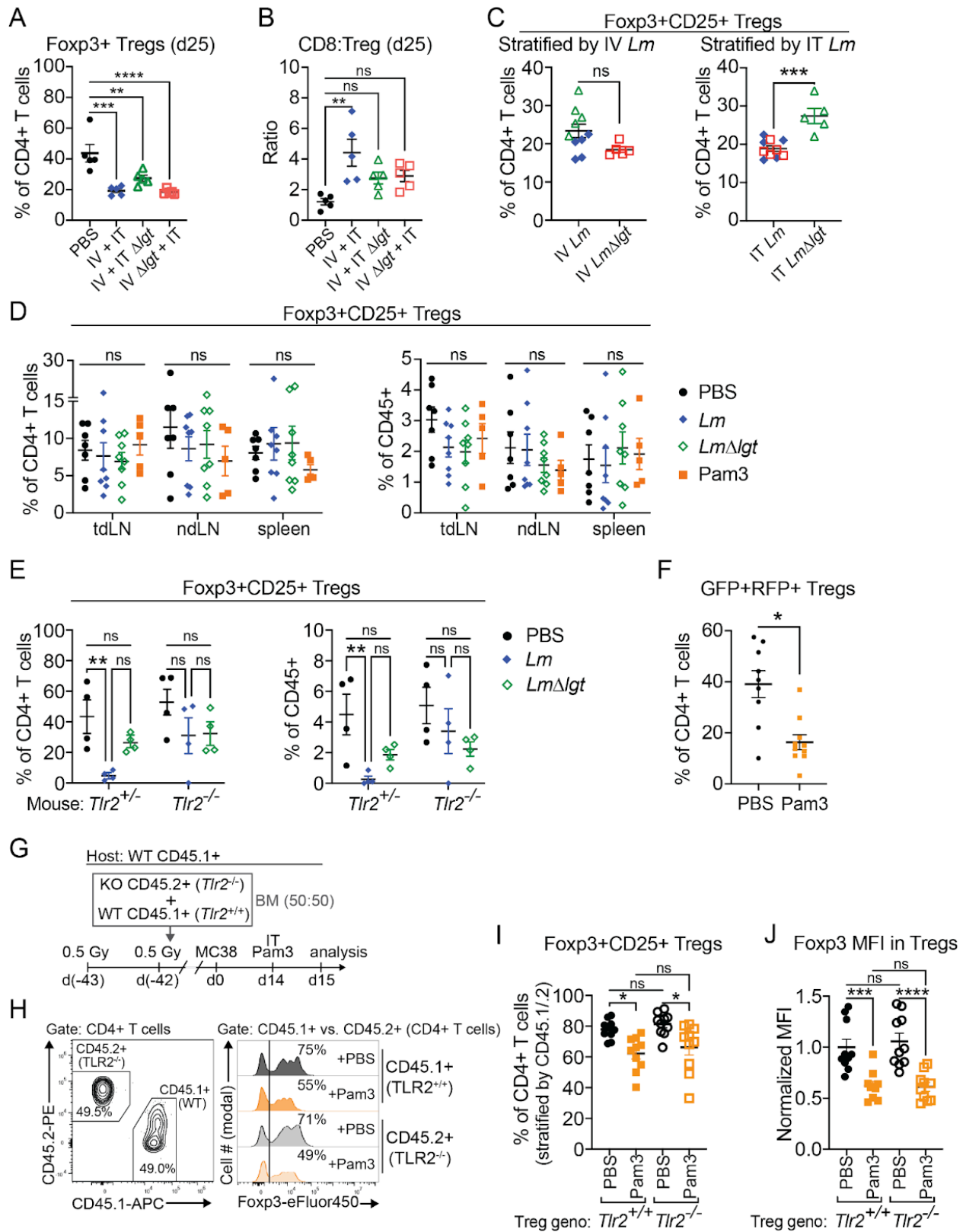


Figure S6. Campbell et al.

Figure S3.6 (related to Figures 3.5 and 3.6):

- (A) Frequency of Tregs among CD4⁺ T cells on day 25 of tumor growth in mice treated with IV+IT *Lm* using *LmΔlgt* at the IV or IT stage of treatment.
- (B) Ratio of CD8⁺ T cells:Tregs on day 25 of tumor growth in mice treated with PBS, IV+IT *Lm*, or IV+IT *Lm* using *LmΔlgt* at the IV or IT stage of treatment.
- (C) Frequency of Tregs among CD4⁺ T cells on day 25 of tumor growth in mice treated with IV+IT *Lm* using *LmΔlgt* at the IV or IT stage of treatment stratified by IV (left) or IT (right).
- (D) Frequency of Tregs as a % of CD4⁺ T cells (left) or of total CD45⁺ lymphocytes/splenocytes (right) in the tdLN, ndLN, and spleen 24 hr post-IT treatment.
- (E) Frequency of Tregs as a % of CD4⁺ T cells (left) or of total CD45⁺ TILs (right) in the tumors of *Tlr2*^{+/-} or *Tlr2*^{-/-} littermates 24 hr post-IT *Lm*.
- (F) Frequency of GFP⁺RFP⁺ Tregs among total RFP⁺ cells 24 hr post-IT PBS or Pam3.
- (G) Experimental outline for generating *Tlr2*^{+/-};CD45^{1/1}/*Tlr2*^{-/-};CD45^{2/2} mixed bone marrow chimera mice and testing whether Treg-intrinsic TLR2 signaling mediates the reduction in tumor-infiltrating Treg frequency following IT Pam3.
- (H) Representative flow cytometry histograms showing the gating of CD45.1⁺ (*Tlr2*^{+/-}) and CD45.2⁺ (*Tlr2*^{-/-}) CD4⁺ T cells in MC38 tumors and the decrease in both the frequency Foxp3⁺ cells and intensity of Foxp3 expression in both compartments 24 hr post-IT Pam3 or PBS.
- (I). Quantification of Foxp3⁺CD25⁺ Tregs as a % CD45.1⁺ or CD45.2⁺ CD4⁺ T cells in MC38 tumors.
- (J) Quantification of Foxp3 MFI within CD45.1⁺ or CD45.2⁺ Tregs. Mean +/- s.e.m. and one-way ANOVA (A, B, I, J)=, two-way ANOVA (D, E), or Student's t-test (C, F).

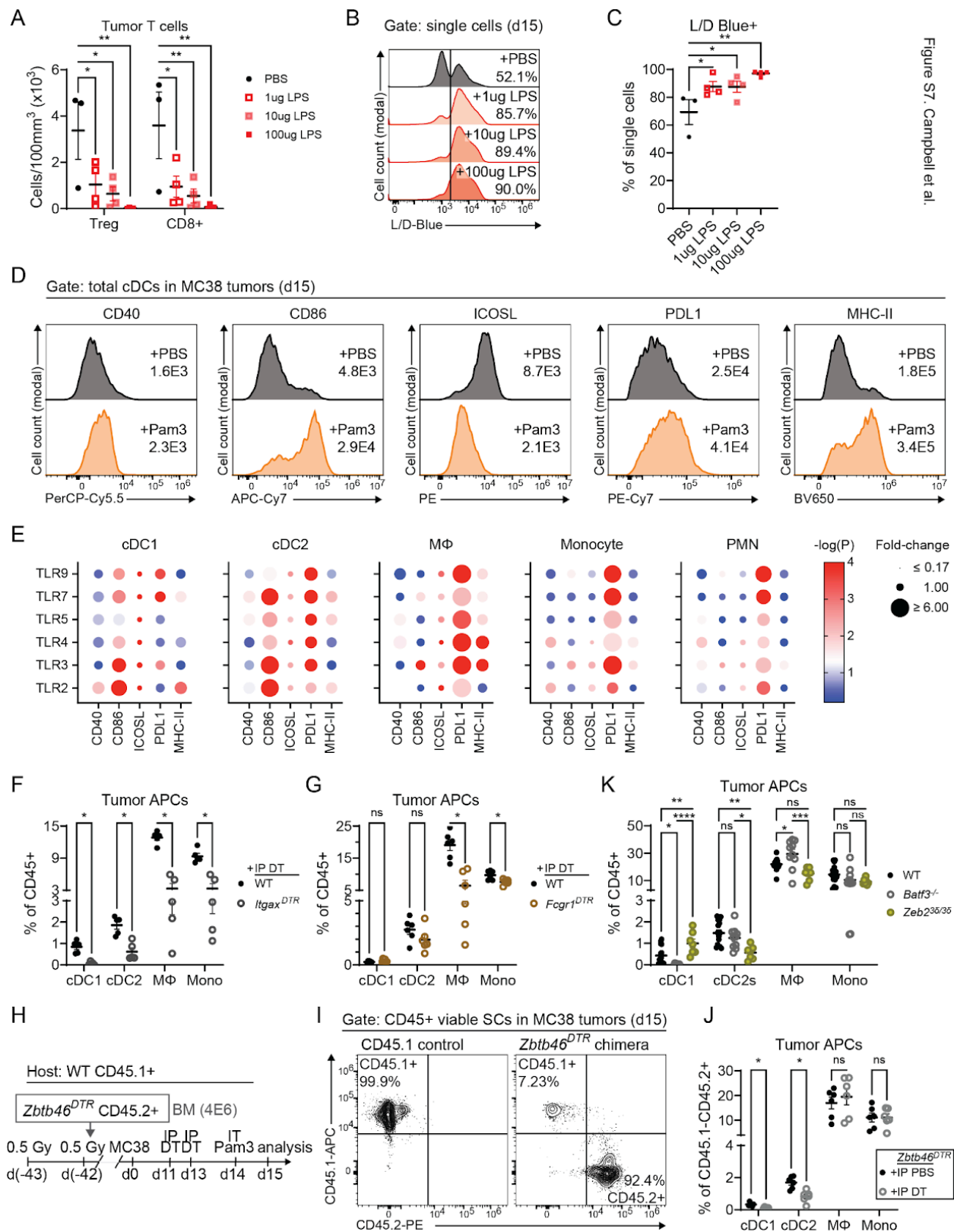
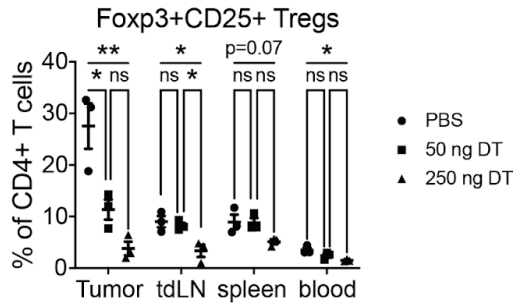


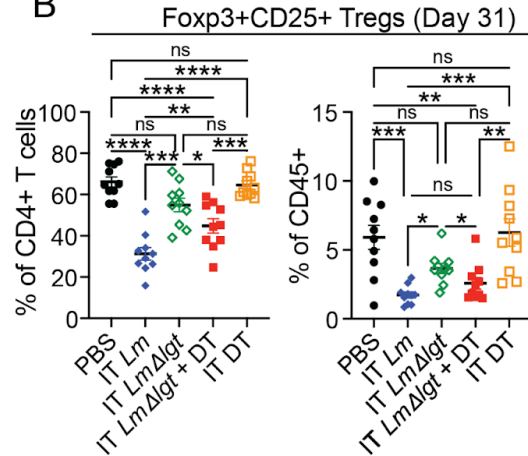
Figure S3.7 (related to Fig. 3.6):

- (A) Number of Tregs and CD8⁺ T cells per 100mm³ tumor tissue 24 hr post-IT PBS or 1, 10, or 100 ug LPS.
- (B, C) Representative flow plots (B) and quantification (C) of viability dye staining among single cells (FSC-A x SSC-A; FSC-A x FSC-H; SSC-A x SSC-H) 24 hr post-IT PBS or 1, 10, or 100 ug LPS.
- (D, E) Representative flow plots (D) and quantification of MFI fold-change relative to PBS controls (E) of CD40, CD86, ICOSL, PDL1, and MHC-II expression on tumor APCs and PMNs 24 hr post-IT PBS or TLRL.
- (F) Representative flow plots of CTV dilution in Tregs on d3 of co-culture with TLR-stimulated BMDCs.
- (G) Frequency of tumor APCs in IP DT-treated WT and *Itgax*^{DTR} mice 24 hr post-IT PBS.
- (H) Frequency of tumor APCs in IP DT-treated WT and *Fcgr1*^{Cre};*Rosa26*^{Isl-DTR} ("*Fcgr1*^{DTR}") mice 24 hr post-IT PBS.
- (I) Experimental outline for generating *Zbtb46*^{DTR} bone marrow chimera to enable cDC depletion.
- (J) Representative flow plots showing CD45.1⁺ (WT) and CD45.2⁺ (*Zbtb46*^{DTR}) staining in CD45⁺ cells of control or *Zbtb46*^{DTR} bone marrow chimera mice, showing engraftment of *Zbtb46*^{DTR} bone marrow.
- (K) Frequency of tumor APCs in PBS- or DT-treated *Zbtb46*^{DTR} bone marrow chimera mice 24 hr post-IT PBS.
- (L) Frequency of tumor APCs in WT, *Batf3*^{-/-}, and *Zeb2*^{Δ1+2+3} mice 24 hr post-IT PBS. Mean +/- s.e.m. and two-way ANOVA (A, L), one-way ANOVA (C, E), or Student's t-test (G, H, K).

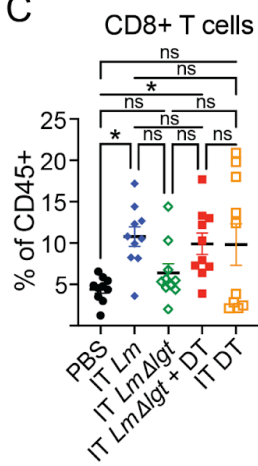
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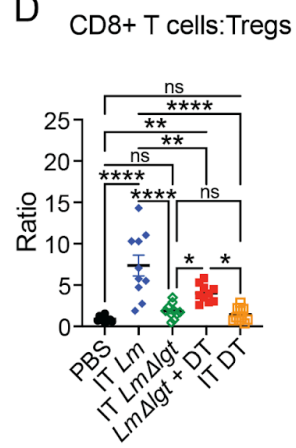
B



C



D



E

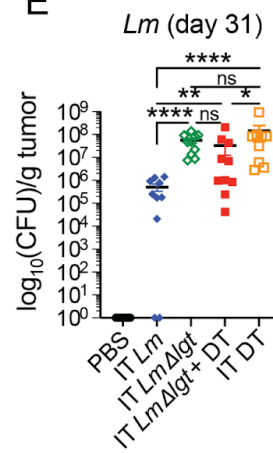
Campbell *et al.*, Figure S8

Figure S3.8 (related to Fig. 3.7A-B):

- (A) Frequency of Tregs as % of CD4⁺ T cells in tumors, tdLN, spleen, or blood 24 hr post-IT PBS or DT.
- (B) Frequency of Tregs as % of CD4⁺ T cells (left) or of total CD45⁺ TILs (right) on day 31 of tumor growth following IV *Lm* + IT treatment with indicated modifications.
- (C) Frequency of CD8⁺ T cells on day 31 of tumor growth.
- (D) Ratio of CD8⁺ T cells:Tregs on day 31 of tumor growth.
- (E) *Lm* CFUs recovered from tumors treated with *Lm* IV+IT in the indicated conditions on day 31 of tumor growth. Mean +/- s.e.m. and one-way ANOVA (B, C, D, E) or two-way ANOVA (A).

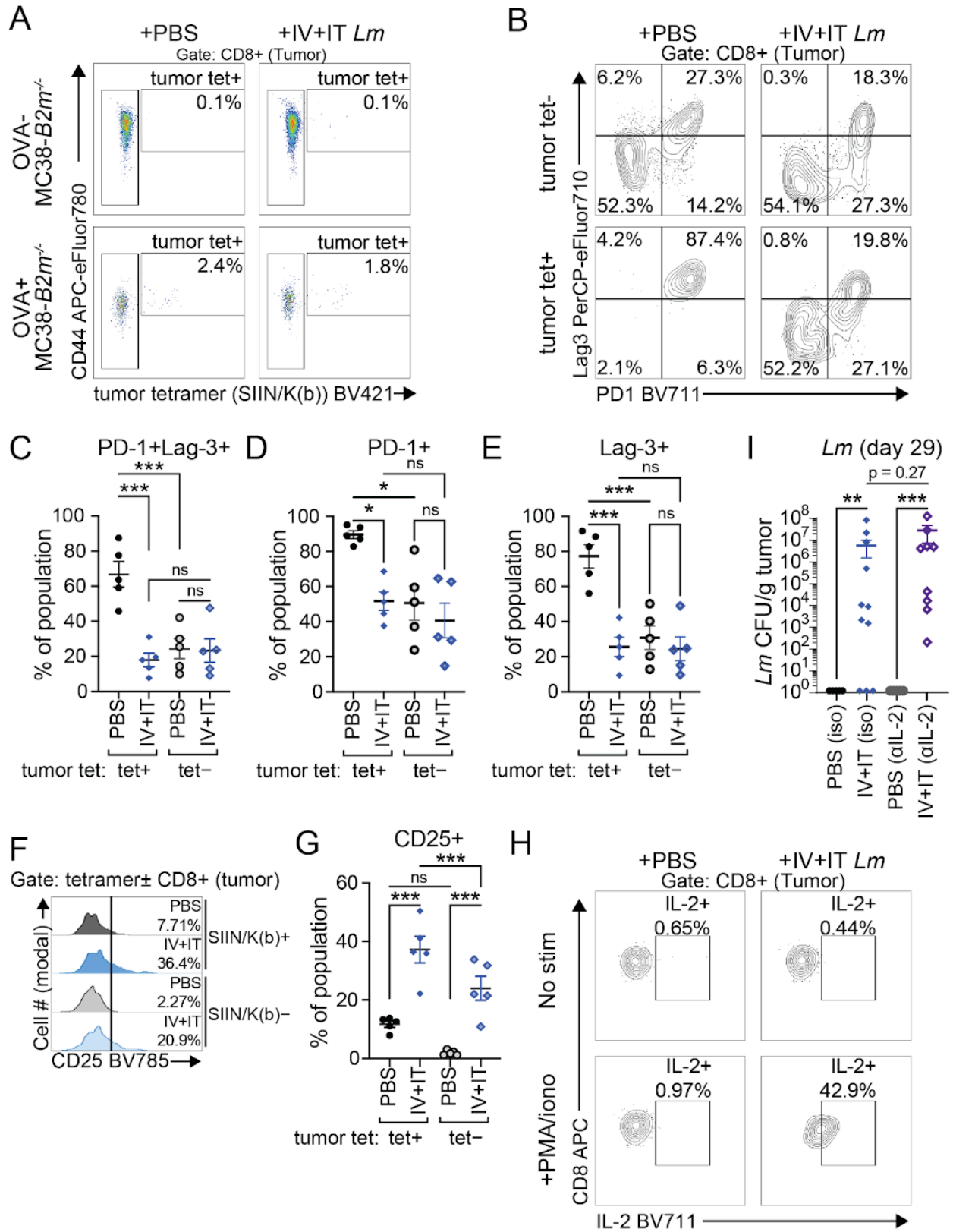


Figure S3.9 (related to Fig. 3.7C-G):

- (A) Representative flow cytometry plots showing the expansion of tumor-specific SIIN/K(b) tetramer⁺ CD8⁺ T cells in MC38- β 2m^{KO}-OVA $\pm$ tumors 1 day post-IT *Lm* or PBS (see Figure 4A).
- (B) Representative flow cytometry plots showing Lag3 and PD1 staining in tumor-specific SIIN/K(b) tetramer $\pm$ CD8⁺T cells in PBS or IV+IT *Lm*-treated OVA⁺ tumors 24 hr post-IT.
- (C) Co-expression of PD-1 and Lag3 on tumor-specific and bulk CD8⁺ T cells.
- (D) Expression of PD-1 on tumor-specific and bulk CD8⁺ T cells.
- (E) Expression of Lag3 on tumor-specific and bulk CD8⁺ T cells.
- (F) Representative flow cytometry histograms showing CD25 staining in SIIN/K(b) tetramer $\pm$ CD8⁺T cells in PBS or IV+IT *Lm*-treated OVA⁺ tumors 24 hr post-IT.
- (G) Expression of CD25 on tumor-specific and bulk CD8⁺ T cells
- (H) Representative flow cytometry plots showing IL-2 staining in CD8⁺ T cells from PBS or IV+IT *Lm*-treated tumors 7 days post-IT after 6 hr restimulation with media or PMA+ionomycin.
- (I) *Lm* CFUs recovered from IL-2-depleted or control tumors on day 29 of tumor growth. Mean $\pm$ s.e.m. and one-way ANOVA (C, D, E, G, I).

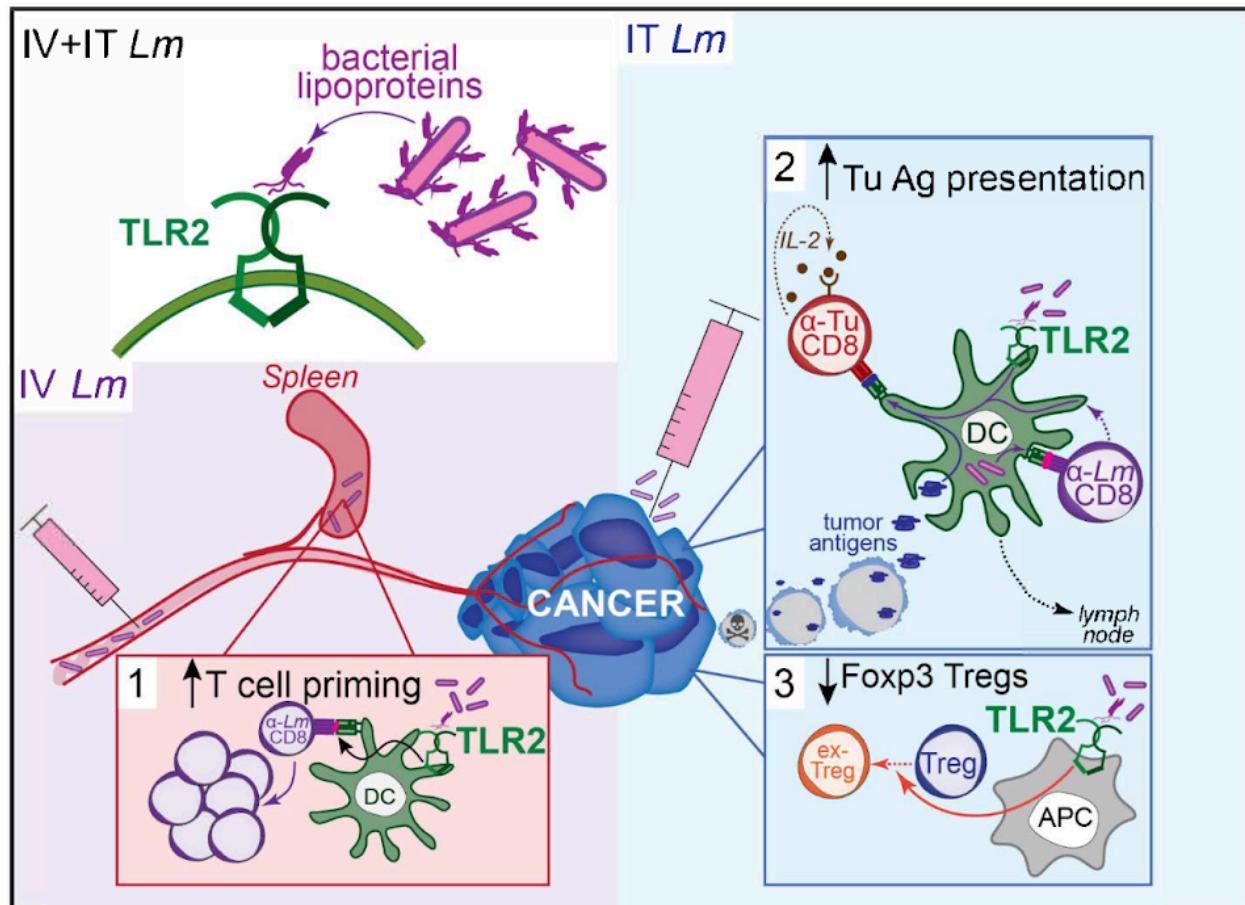


Figure S3.10 (Graphical Abstract)

(A) TLR2 contributes to optimal priming of anti-Lm T cells.

(B) APCs stimulated by TLR2 drive Foxp3 downregulation in tumor-infiltrating Tregs to generate ex-Tregs.

(C) Reduced Treg immunosuppression and direct TLR2 stimulation of DCs drive tumor antigen uptake and cross-presentation to promote tumor-specific CD8 T cell function and IL-2 signaling.

Chapter 4

Serial Toll-like receptor stimulation elicits tumor control
by modulation of regulatory and CD8⁺ T cells

4.0 Authors

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4.1 Abstract

Ligands for endosomal Toll-like receptors (TLRs; TLR3/7/9) have been heavily tested as immunotherapeutic agents, but less attention has been paid to ligands for surface TLRs (TLRs1/2/4/5/6). The lack of effort to develop surface TLR ligands (TLRLs) as cancer immunotherapeutics is in part due to assumptions based primarily on findings in non-cancer models which have shown that endosomal TLRLs are more critical drivers of CD8⁺ T cell responses to infectious disease. However, this framework fails to account for the effects that TLRLs have on other T cell subsets in tumors, including Foxp3⁺ regulatory T cells (Tregs). We therefore performed a comparative study to assess how different TLRL affected these T cell subsets within tumors. We found that ligands for both the cell-surface receptor TLR2 and the endosomal receptor TLR9 elicited tumor control without concomitant toxicity. However, these ligands functioned via distinct mechanisms. While TLR9L elicited higher frequencies and reduced exhaustion of CD8⁺ T cells in tumors, it did not strongly affect tumor Tregs. In contrast, TLR2/1L did not directly increase CD8⁺ T cell frequency, but instead reduced the frequency of tumor Tregs, enabling CD8⁺ T cell mediated tumor control without directly affecting their frequency. Dual administration of TLR2L and TLR9L elicited synergistic effects that caused a trend towards greater tumor control than with TLR2 or TLR9L

4.2 Introduction

TLR agonists are among the most-researched potential immunotherapies.^{668–670} The justification for these attempts is multifaceted, with purported benefits ranging from enhancing tumor cell phagocytosis by TLR-activated macrophages to direct tumor cell killing by TLR-induced programmed cell death.^{785,786,903,904} However, the most commonly-cited benefit of TLRs in cancer immunotherapy is to boost T cell-mediated immunity to cancer.^{785,786} In some instances, this has been ascribed to T cell-intrinsic TLR signaling, resulting in effects such as promoting anabolic metabolism to support proliferation and lowering the threshold of TCR signaling required to elicit activation.^{825,861,905–908} More frequently, the effects of TLRs on Tregs have been found to increase the T cell-activating effects of APCs, which has been a major factor in driving interest in the development of TLR ligands and agonists for cancer immunotherapy.^{785,786} This is accomplished by a network of transcription factors that are engaged downstream of TLR signaling, including NF- κ B, AP-1, and, for endosomal TLRs and TLR4, IRF3 or IRF7.³⁷⁵ Together, these transcriptional changes enhance the capacity of APCs to provide all three signals required for T cell activation, namely TCR:pMHC signaling, CD28 ligation by CD80/CD86, and cytokine receptor signaling to drive the differentiation of effector T cell subsets.^{75,77,81,91,909} This is accomplished by the expression of proinflammatory cytokines like IL-6 and IL-12, costimulatory ligands like CD80 and CD86, and components of the MHC presentation pathways by NF- κ B and AP-1.^{344,375,910,911} In the case of endosomal nucleic acid-sensing TLRs (TLRs 3/7/8/9) and TLR4, these genes are accompanied by expression of T1 IFNs by IRF-3 and IRF-7.^{375,387,911}

This final TLR-dependent signal—T1 IFNs—is one of the factors driving a major trend in the field of TLR agonists for cancer therapy, namely the preferential targeting of endosomal TLRs rather than TLRs for immunotherapy development.^{686,687,912} Several conclusions drawn from preclinical studies in mice have supported this conclusion. T1 IFNs have been shown in several preclinical models to improve CD8⁺ T cell responses to cancer.⁹¹³ Endosomal TLRs have also been found to preferentially deliver antigens within TLR-containing endosomes to the MHC-I pathway to promote tumor antigen cross-presentation, which is a critical step in generating CD8⁺ T cell responses against tumors.^{330,340,677} These benefits are compounded by the fact that cDC1s—the cell type most capable of cross-presenting antigens to initiate CD8⁺ T cell responses—respond strongly to several endosomal TLR agonists.^{503,504,914} These factors have led endosomal TLRs to be increasingly favored for clinical development over the last several years, shown by a 2023 survey that reported 22 recently-initiated clinical trials of TLR3/7/8/9 agonists but no new trials of TLR2/4/5 agonists.^{682–688} However, results to these trials have been disappointing, with no new TLR agonists approved for cancer immunotherapy since Imiquimod (a TLR7 agonist) was approved for use in the treatment of cutaneous cancers in 2004.⁶⁵⁴

This suggests that a new approach to developing TLR agonists for cancer immunotherapy is needed. An important step in this process is to more thoroughly interrogate the strengths and weaknesses of endosomal and cell-surface TLRs as targets for immunotherapy. This was the primary goal underlying the work presented in this chapter. In order to address this topic, we compared the effects of various ligands for cell-surface (TLR2 and TLR4) and endosomal (TLR3 and TLR9) TLRs in several model systems. In line with our earlier observations (**Chapter 3**), we found that intratumoral injection of a single dose of TLR2 and TLR4 ligands led to the depletion of regulatory T cells from tumors and their concomitant downregulation of Foxp3. However, serial injection of ligands for TLR2, TLR4, and TLR9 all led to tumor control, although TLR4 elicited lethal weight loss in the process. In line with published findings, *in vitro* and *in vivo* analyses showed that TLR9 elicited heightened CD8⁺ T cell responses. In agreement with our

earlier work (**Chapter 3**), TLR2 functioned in large part by abrogating the immunosuppressive activity of Tregs. Dual injection of TLR2 and TLR9 ligands elicited synergistic tumor control, suggesting that the mechanisms underlying tumor control by these compounds are at least partially distinct. Ultimately, our work shows that better antitumor immunity can be achieved by activating both cell-surface and endosomal TLRs than either type alone, likely by the simultaneous enhancement of CD8⁺ T cells and inhibition of Tregs.

4.3 Results

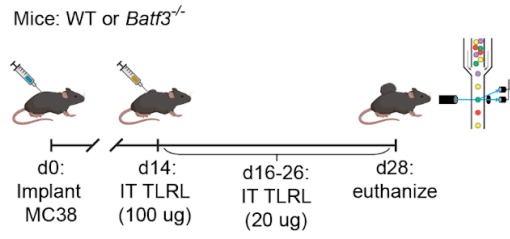
4.3.1 Serial TLR stimulation with a subset of TLRL elicits tumor control

We had previously observed that intravenous (IV) followed by intratumoral (IT) injection of *Listeria monocytogenes* lacking the *actA* virulence factor ($\Delta actA$, hereafter referred to as IV+IT *Lm*) enabled control of tumors in part by stimulating TLR2 and to a lesser extent TLR5, but not TLR9 (**Figure 3.1**). A single dose of IT TLR2L (Pam3) after IV *Lm* was insufficient to phenocopy tumor control by IV+IT *Lm*, although it did provide a degree of tumor control for ~10 d after injection (**Figures 3.1J and S3.1K**). However, other TLRL were not tested in this system, and the contribution of other TLRs to tumor control was only assessed in the context of IV+IT *Lm*, which stimulates multiple TLRs and other PRRs but which has primarily been found to stimulate TLR2.^{634,794,796,798,800} Thus, it was impossible to infer from the IV+IT *Lm* system whether TLR2 stimulation was uniquely capable of inhibiting tumor growth, or whether other TLRs could have the same effect. To answer this question, we developed a new treatment protocol of serial IT TLRL injection (**Figure 4.1A**). We implanted MC38 tumor cells subcutaneously into WT B6 mice (d0) before injecting a high dose (100 ug) of TLRL IT two weeks later (d14). This initial high dose was followed by repeated low doses (20 ug) of TLRL every second day thereafter (d16-d26) before mice were euthanized and tumors analyzed by flow cytometry (d28).

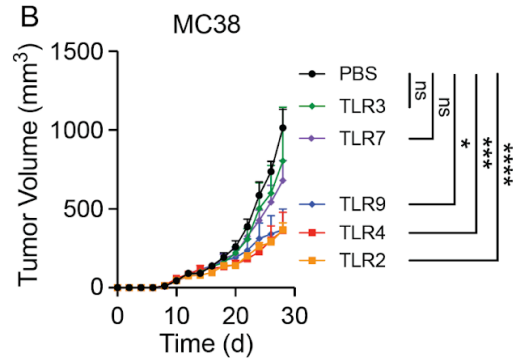
We found that tumor growth was inhibited by TLR2, TLR4, and TLR9 stimulation, but not by TLR3 or TLR7 (**Figure 4.1B**). We had previously observed that TLR2-dependent tumor control by IV+IT *Lm* required the destabilization and depletion of tumor Tregs (**Figures 3.5 and 3.7A-B**), leading us to assess the frequency and stability of tumor Tregs in TLRL-stimulated mice. We found that TLR2, TLR4, and TLR9 led to reduced Tregs as a fraction of total CD45⁺ TILs, correlating with the ability of these receptors to elicit tumor control (**Figures 4.1B-C**). However, while TLR2 and TLR4 both significantly reduced Foxp3 expression in tumor Tregs and produced a trend towards reduced Tregs as a fraction of CD4⁺ T cells, these effects were not observed with TLR3, TLR7, or TLR9 (**Figures 4.1C-D**). Rather, TLR9 stimulation significantly increased the frequency of tumor CD8⁺ T cells while producing a trend towards reduced CD4⁺ Tcons (**Figures 4.1E-F**). Consequently, both TLR2 and TLR9 stimulation led to an increase in the ratio of CD8⁺ T cells to Foxp3⁺ Tregs, which is a positive prognostic marker indicative of heightened antitumor immune responses (**Figure 4.1G**).^{839,840} In contrast, TLR4 stimulation also significantly decreased the frequency of CD4⁺ Tcon and CD8⁺ T cells in tumors (**Figures 4.1E-F**). This corresponded to a dramatic and rapid loss of body mass by mice treated with IT TLR4L, and a large increase in the frequency of dead cells and increased frequencies of monocytes and MΦs among remaining viable cells in TLR4-stimulated tumors (**Figures S3.6B-C, S4.1A-B, and data not shown**). These effects are consistent with the well-characterized role of LPS in mediating cell death via both TLR4-dependent and -independent mechanisms and suggest that serial IT TLR4L may be uniquely toxic relative to its capacity to control tumor growth.⁹¹⁵⁻⁹¹⁷ Together, these results suggest that while TLR2 is not uniquely capable of driving tumor control, only a subset of TLRs (namely TLR2, TLR4, and TLR9 among those tested) can accomplish this. Additionally, flow cytometric analysis of tumors at the end of treatment suggests that TLR2, TLR4, and TLR9 may elicit tumor control via distinct mechanisms.

Having previously observed that a single dose of IT TLR2L after IV *Lm* was insufficient to control tumors (**Figure 3.1J**), we inferred that repeated administration of TLR2 was required for this effect. To test this directly, we modified our earlier experimental protocol by treating a subset of tumors with a single high dose (100 ug, SD) of IT TLR2L while others were treated as previously (RD) (**Figure 4.1A**). We found that only mice which received RD IT TLR2L controlled their tumors and displayed the characteristic changes in Treg frequency among CD4⁺ T cells and Foxp3 expression (**Figures S4.1C-E**). While both SD and RD IT TLR2L mice had reduced frequencies of Tregs among CD45⁺ TILs, the frequency of Tregs in SD TLR2L-treated mice was intermediate between that of RD- and PBS-treated mice (**Figure S4.1F**). Ultimately, this led to only RD TLR2-stimulated mice possessing a heightened ratio of CD8⁺ T cells to Tregs (**Figure S4.1G**). Together, these results demonstrate that serial TLR2 stimulation is required for tumor control, perhaps due to a requirement for continuous TLR2 signaling to sustain Treg depletion and ablate immune suppression in the TME.

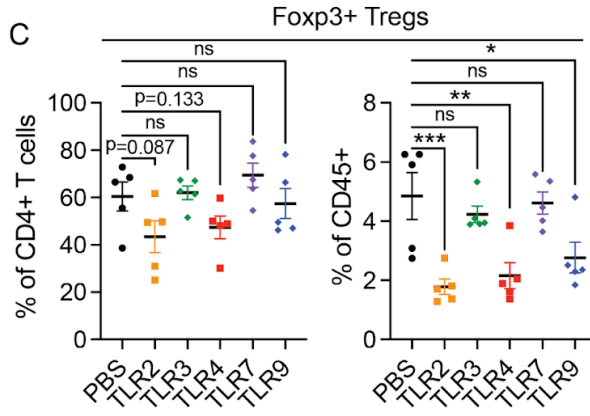
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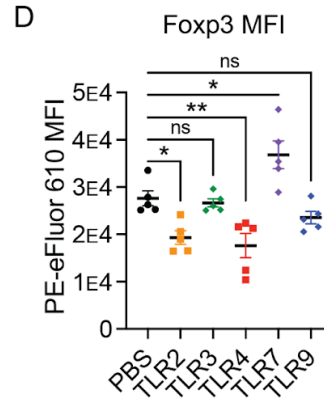
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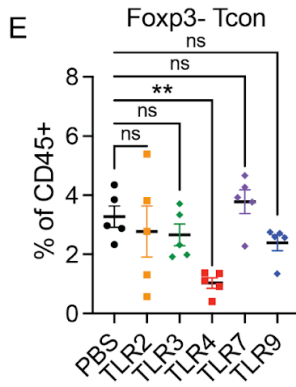
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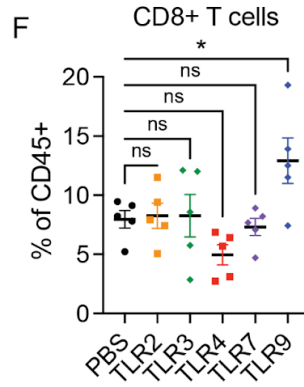
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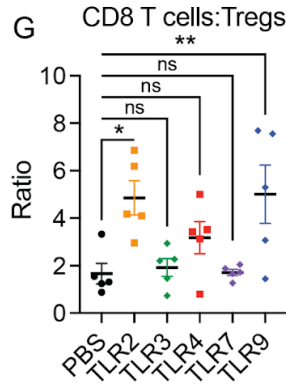
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G



Campbell, Mostafa et al. (2025) Figure 1

Figure 4.1: Repeat stimulation with TLR2/4/9 elicits control of MC38 tumors.

(A) WT B6 mice were implanted with 5E5 MC38 cells via subcutaneous injection (d0). Tumors were injected with 100 ug TLRL (d14) then with 20 ug TLRL every second day thereafter (d16-d26) and tumor growth was tracked **(B)**. Mice were then euthanized and TILs assessed by flow cytometry (d28) **(C-G)**.

(B) MC38 tumor growth in mice receiving IT TLRL for TLR2/3/4/7/9.

(C) Frequency of Tregs among CD4⁺ T cells (left) and total CD45⁺ TILs (right).

(D) Foxp3 expression in tumor Tregs.

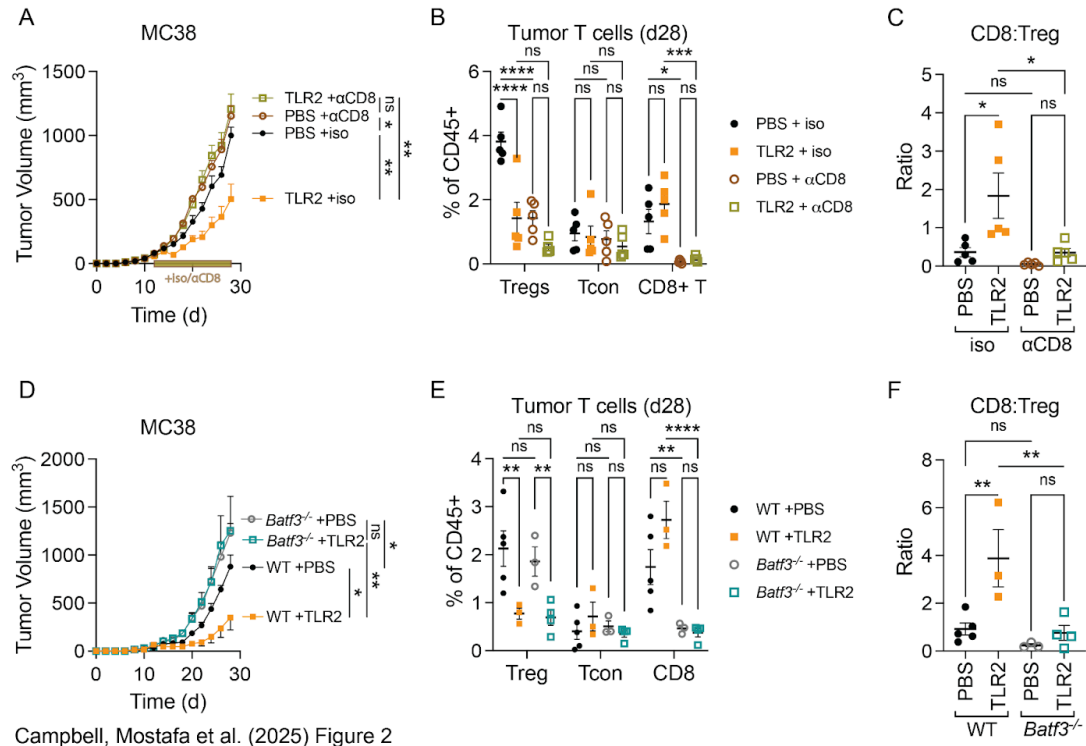
(E-F) Frequency of Foxp3⁻ Tcon (E) and CD8⁺ T cells (F) among total CD45⁺ TILs.

(G) Ratio Of CD8⁺ T cells to Foxp3⁺ Tregs. Mean $\pm$ s.e.m. and two-way ANOVA (B) or one-way ANOVA (C-G).

4.3.2 TLR2-mediated tumor control requires CD8⁺ T cells and cDC1s

Our observation that repeat IT TLR2L-mediated tumor control corresponded to an increase in the ratio of CD8⁺ T cells to Tregs without directly increasing the frequency of CD8⁺ T cells (**Figures 4.1F-G**) led us to ask whether CD8⁺ T cells played a role in the protective effect of IT TLR2L. To address this question, we repeated our experiments as in **Figure 4.1A** with the addition of anti-CD8b or isotype control depleting antibodies injected IP into tumor-bearing mice beginning 2d prior to IT treatment (d12 and d13). CD8⁺ T cell depletion completely abrogated tumor control by IT TLR2L (**Figure 4.2A**). While the frequency of Tregs among total CD45⁺ TILs was reduced relative to isotype-treated controls in anti-CD8b-treated mice, IT TLR2L nevertheless further reduced the frequency of Tregs among CD4⁺ T cells and Foxp3 expression in Tregs (**Figures 4.2B-C and S4.2A-B**). This suggests that Treg accumulation in tumors in this model is partially driven by the presence of CD8⁺ T cells, as we and other groups have reported previously.^{266,276} However, it also suggests that CD8⁺ T cells do not play a direct role in tumor Treg destabilization and depletion by IT TLR2L.

Batf3-dependent cDC1s are thought to be the most important APC driving CD8⁺ T cell responses to cancer.^{503,504} We therefore repeated our serial IT TLR2L injection experiment using *Batf3*^{-/-} or WT control mice. As with CD8⁺ T cell depletion, *Batf3* deficiency abrogated tumor control by IT TLR2L (**Figure 4.2D**). TLR2 stimulation also failed to increase the ratio of CD8⁺ T cells to Foxp3⁺ Tregs in *Batf3*^{-/-} mice, again as a result of the absence of CD8⁺ T cells (**Figure 4.2E-F**). However, IT TLR2 stimulation of *Batf3*^{-/-} mice still produced a trend towards reduced Tregs as a fraction of CD4⁺ T cells and Foxp3 expression (**Figures S4.2C-D**). This suggests that TLR2-mediated Treg depletion and destabilization occurs independently of cDC1s, as we had previously observed with a single high dose of IT TLR2L in *Batf3*^{-/-} mice (**Figure 3.6J-K**). Together, these results indicate that both cDC1s and CD8⁺ T cells are required for serial IT TLR2L-mediated tumor control, but that neither cell population plays a direct role in modulating tumor Tregs in response to TLR2 stimulation.



Campbell, Mostafa et al. (2025) Figure 2

Figure 4.2: TLR2-mediated tumor control requires CD8⁺ T cells and cDC1s.

(A) MC38 tumor growth in WT B6 mice treated as in **Fig. 4.1A** with the addition of anti-CD8b or isotype control depleting antibody beginning two days before IT TLRL (d12).

(B) Frequency of Foxp3⁺ Tregs, Foxp3⁺CD4⁺ Tcon, and CD8⁺ T cells among total CD45⁺ TILs from **(A)**.

(C) Ratio of CD8⁺ T cells to Foxp3⁺ Tregs in tumors from **(A)**.

(D) MC38 tumor growth in *Batf3*^{-/-} or WT B6 control mice treated as in **Fig. 4.1A**.

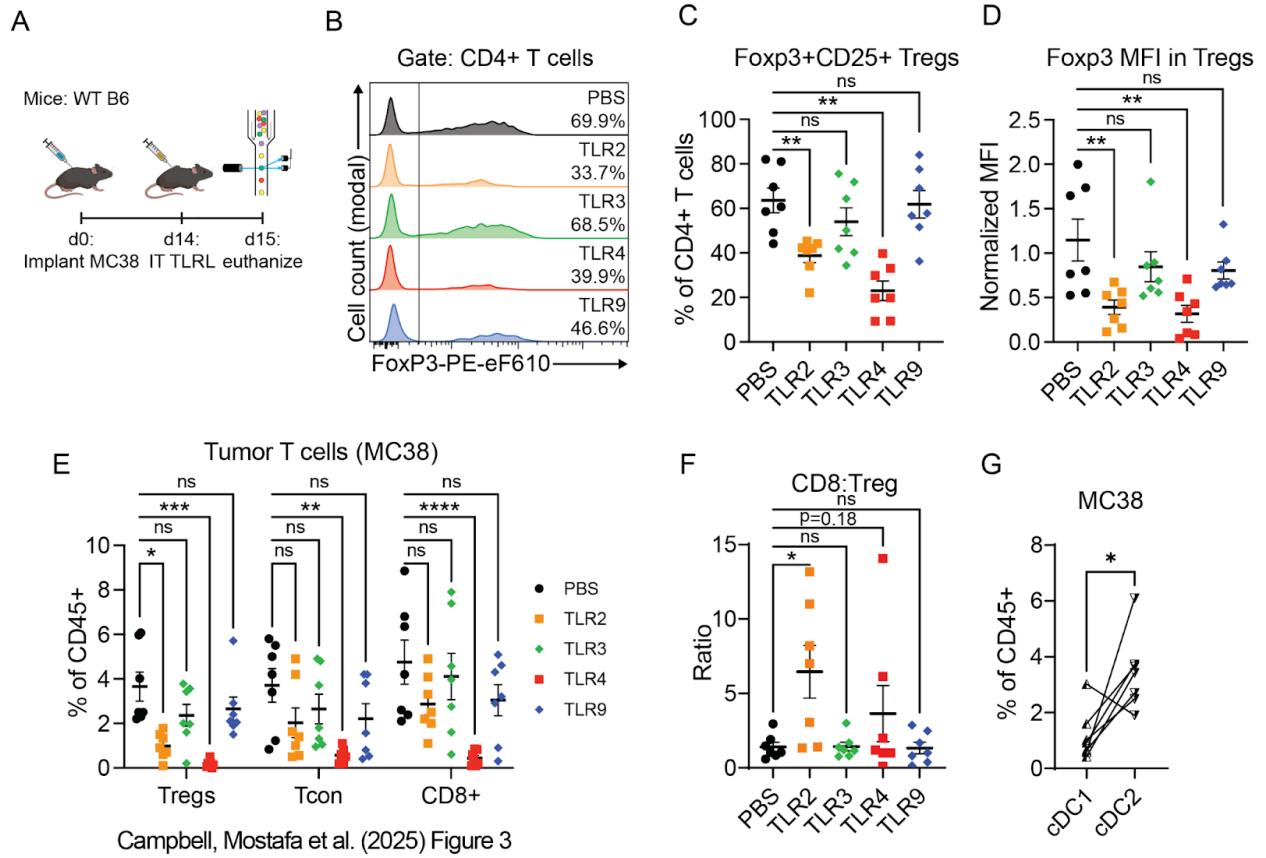
(E) Frequency of Foxp3⁺ Tregs, Foxp3⁺CD4⁺ Tcon, and CD8⁺ T cells among total CD45⁺ TILs from **(D)**.

(F) Ratio of CD8⁺ T cells to Foxp3⁺ Tregs in tumors from **(A)**. Mean ± s.e.m. and two-way ANOVA (**A, B, D, E**) or one-way ANOVA (**C, F**).

4.3.3 TLR2 mediates the rapid depletion of tumor Tregs in multiple models

Our observation of reduced Treg frequency and Foxp3 expression in Tregs at the end of tumor growth in tumors stimulated with TLR2 and TLR4 (**Figures 4.1C-D**) accorded with our finding showing similar effects of TLR2 and TLR4 but not other TLRs 24 hours after a single low dose (20 ug) of TLRL (**Figures 3.6A-C**). However, it remained possible that other TLRLs would also induce a reduction in Treg frequency or Foxp3 expression at the high dose used at the outset of treatment (100 ug). To address this, we repeated our short-term TLR stimulation experiments by implanting tumors into WT B6 mice (d0) and injecting a single high dose (100 ug) of TLRL IT (d14) 24 hours prior to euthanasia and flow cytometry analysis (**Figure 4.3A**). As with the 20 ug dose previously employed, TLR2 and TLR4 stimulation led to a reduction in Treg frequency and Foxp3 expression, while TLR3 and TLR9 stimulation did not (**Figure 4.3B-D**). Also as seen previously, TLR4 stimulation led to a reduction in the frequency of CD4⁺ Tcon and CD8⁺ T cells, such that only TLR2 stimulation significantly increased the ratio of CD8⁺ T cells to Foxp3⁺ Tregs in tumors (**Figures 4.3E-F**). This suggests that TLRL dosage is not the primary determinant of whether destabilization of Foxp3 or depletion of Tregs occurs with TLR stimulation.

We next sought to determine whether differential responses to TLRLs are consistent or whether they vary between tumor types. To test this, we adapted our experimental system shown in **Figure 4.3A** by instead implanting mice with B16F10 tumors and injecting 100 ug TLRL IT as previously described. We once again observed that TLR4 stimulation decreased Treg frequency among CD4⁺ T cells and Foxp3 expression in tumor Tregs while also depleting CD4⁺ Tcon and CD8⁺ T cells (**Figure S4.3A-D**). TLR2 stimulation of B16F10 tumors led to similar trends as observed in MC38 tumors, namely decreased Treg frequency among CD4⁺ T cells and Foxp3 expression in tumor Tregs without concurrent depletion of other T cells (**Figures S4.3A-D**). However, the effects of TLR2 stimulation in B16F10 tumors were not statistically significant and were generally milder than in MC38 tumors (**compare Figures 4.4B-E to S4.3A-D**). Also in contrast to MC38 tumors, and despite reducing CD8⁺ T cell frequency, TLR4 stimulation of B16F10 tumors increased the ratio of CD8⁺ T cells to Foxp3⁺ Tregs while TLR2 stimulation did not (**Figure S4.3E**). Notably, IT TLRL did not affect the frequency of Tregs in either local (tdLN) or distal (ndLN and spleen) SLOs in either MC38 or B16F10 tumor-bearing mice (**Figures S4.3F-G**). This contradicts the idea that differences between how IT TLRL affects systemic Tregs underlie the differences in responsiveness to TLR2 and TLR4 by MC38 and B16F10 tumors. Instead, our results suggest that while TLRL may exhibit similar effects in different models, quantitative differences in the degree of their effects within the TME itself may ultimately result in qualitatively different outcomes. However, additional experiments using other tumor models and a wider variety of TLRL are needed to answer this hypothesis.



Campbell, Mostafa et al. (2025) Figure 3

Figure 4.3: TLR2/4 but not TLR3/9 stimulation elicits rapid loss of tumor Tregs.

(A) WT B6 mice were implanted with 5E5 MC38 cells via subcutaneous injection (d0). Tumors were injected with 100 ug TLRL (d14). 24 hr later, mice were euthanized and tumors and SLOs were analyzed by flow cytometry (d15).

(B) Representative histograms showing reduced Foxp3⁺ Treg frequency and Foxp3 expression in Tregs from MC38 tumors treated with TLR2/4L compared to TLR3/9L or PBS.

(C) Frequency of Foxp3⁺ Tregs among CD4⁺ T cells in tumors.

(D) Foxp3 expression in tumor Tregs.

(E) Frequency of Foxp3⁺ Tregs, Foxp3⁻CD4⁺ Tcon, and CD8⁺ T cells among total CD45⁺ TILs.

(F) Ratio of CD8⁺ T cells to Foxp3⁺ Tregs in tumors.

(G) Frequency of XCR1⁺CD103⁺ cDC1s and SIRPα⁺CD11b⁺ cDC2s among total CD45⁺ TILs.

Mean ± s.e.m. and one-way ANOVA (**C, D, F**), two-way ANOVA (**E**), or paired Student's t-test (**G**).

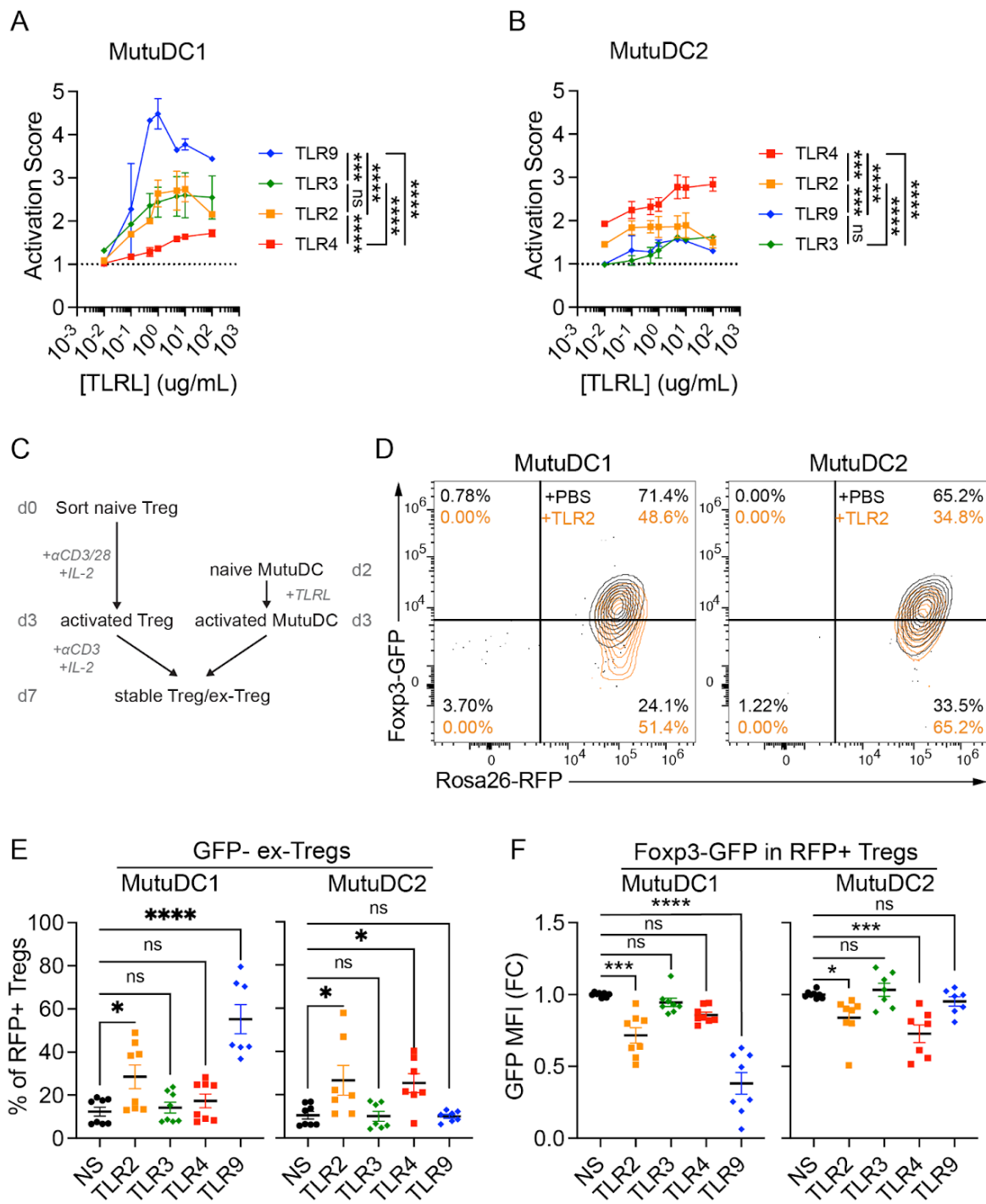
4.3.4 Different TLRs enable cDC1- and cDC2-mediated Treg destabilization

We previously found that cDC2s were required for TLR2-mediated destabilization and depletion of tumor Tregs in MC38 tumors, while cDC1s and MΦs were dispensable for these effects (**Figure 3.6J-K**). However, cDC2s outnumbered cDC1s by an approximately 2:1 ratio in both MC38 and B16F10 tumors (**Figure 4.3G and S4.3H**). This led us to ask whether cDC1s were intrinsically less capable of destabilizing Foxp3 expression in Tregs in response to TLR stimulation, or whether the relative paucity of cDC1s in tumors might explain this finding. To answer this question, we developed a variation of the *in vitro* Treg-DC co-culture assay that we used previously (**Figure 3.6E-G**). MutuDCs were used in this variation of the co-culture assay to overcome the technical challenge of isolating cDC1s and cDC2s of sufficiently high number and purity. MutuDCs are immortalized DC cell lines generated by transduction of splenic DCs with SV40 T antigens, and thus represent a more accurate simulacrum of *bona fide* cDCs than most DC models.⁹¹⁸⁻⁹²⁰ MutuDC1940 and MutuDC20956A cells (hereafter referred to as MutuDC1 and MutuDC2) were used to model cDC1s and cDC2 cells, respectively (**Figure S4A-B**).^{918,919}

Preliminary experiments testing the effect of TLR stimulation on MutuDC1 and MutuDC2 showed that each TLRL upregulated a distinct set of the surface markers CD80, CD86, ICOSL, and PDL1 on MutuDC1s vs. MutuDC2s when assessed by flow cytometry (**Figures S4.4C-D**). This led us to ask whether there were quantitative differences in the degree of MutuDC activation based on the dose of TLRL used in preliminary experiments (1 ug/mL). To address this question, we stimulated MutuDC1s and MutuDC2s with concentrations of TLRL ranging from 100 ug/mL to 10 ng/mL and assessed their activation by flow cytometry (**Figures S4.4C-D**). We found that MutuDC1 and MutuDC2 responses to TLR stimuli differed both quantitatively (e.g., TLR2L activates MutuDC2 at concentrations as low as 10 ng/mL but only activates MutuDC1 at concentrations higher than 100 ng/mL) and qualitatively (e.g., TLR2 upregulates ICOSL and PDL1 while TLR4 only upregulates ICOSL in MutuDC1s, while MutuDC2s do not upregulate ICOSL) (**S4.4C-D**). This suggested that MutuDC1 and MutuDC2 both differed intrinsically in their response to TLR stimulation and that different concentrations of TLRL are required to elicit the maximum possible degree of activation of each cell type. To determine the appropriate concentration of TLRL to activate MutuDC1 and MutuDC2, we used our flow cytometry data to compile an activation score, which was calculated as the sum of the average fold-change in MFI of each marker relative to unstimulated controls (**Figures 4.4A-B**). Using the activation score, we concluded that MutuDC1s responded most strongly to TLR9 stimulation, followed by TLR2 and TLR3 to an equal extent, and least strongly to TLR4 (**Figure 4.4A**). Conversely, MutuDC2s responded most strongly to TLR4 stimulation, then TLR2, then TLR9 and TLR3 to an equal extent (**Figure 4.4B**). In addition to the activation score, we also assessed how different concentrations of TLRL affected the viability and cell number of MutuDCs. This revealed that both viability and number of cells recovered were negatively affected by higher concentrations of all TLRL tested in both MutuDC1s and MutuDC2s (**Figures S4.4E-F**). We therefore selected TLRL concentrations that elicited the maximum degree of activation without negatively impacting viability or cell number (**Figures 4.4A-B and S4.4C-F**). A summary of these results and the concentration of TLRL used to activate MutuDC1s and MutuDC2s in subsequent experiments is listed below (**Table 4.1**).

Table 4.1: MutuDC1/2 Activation by TLRL		
Cell Line	MutuDC1	MutuDC2
Summary	TLR9>>TLR3≈TLR2>TLR4	TLR4>TLR2>TLR9≈TLR3
[TLR2L]	1.0 ug/mL	100 ng/mL
[TLR3L]	500 ng/mL	1 ug/mL
[TLR4L]	1.0 ug/mL	100 ng/mL
[TLR9L]	500 ng/mL	100 ng/mL

We then employed MutuDC1s and MutuDC2s in our APC-Treg co-culture assay. To do this, we first sorted naive Tregs from Foxp3 lineage-trace mice (see section 3.3.8 for detailed explanation of these mice) and activated these Tregs with anti-CD3/CD28 antibody-coated microbeads and IL-2 for 3 days (**Figure 4.C**).^{242,842} Activated Tregs were then mixed at a 1:1 ratio with either naive or TLR-stimulated MutuDCs in the presence of anti-CD3 antibody and IL-2 and cultured for an additional 4 days before Treg destabilization was assessed by flow cytometry (**Figure 4.4C**). We found that both MutuDC1 and MutuDC2 were capable of driving ex-Treg formation in response to TLR stimulation *in vitro* (**Figure 4.4D**). However, these cell types destabilized Tregs in response to different TLR stimuli; TLR9-stimulated MutuDC1s but not MutuDC2s induced the downregulation of Foxp3 in Tregs, while TLR4-stimulated MutuDC2s but not MutuDC1s did the same. (**Figure 4.4E-F**). However, TLR2-stimulated MutuDC1s and MutuDCs were both able to destabilize Foxp3 expression in Tregs to a similar extent (**Figure 4.4E-F**). The extent to which MutuDC1s and MutuDC2s were activated by each TLRL correlated with their destabilization of Foxp3 expression in Tregs (**Figures 4.4A-B, 4.4E-F, and Table 4.1**). A notable exception to this pattern was TLR3, which did not mediate Treg destabilization in either MutuDC1 or MutuDC2 despite its capacity to activate both cell types (**Figures 4.4A-B and 4.4E-F**). Together, these results suggested that TLR2 and cDC2s are not unique in their ability to destabilize Tregs, but that other cell types can also mediate this effect when given the appropriate TLR stimulus. However, TLR2 was the sole receptor capable of mediating Treg destabilization via both MutuDC1s and MutuDC2s.



Campbell, Mostafa et al. (2025) Figure 4

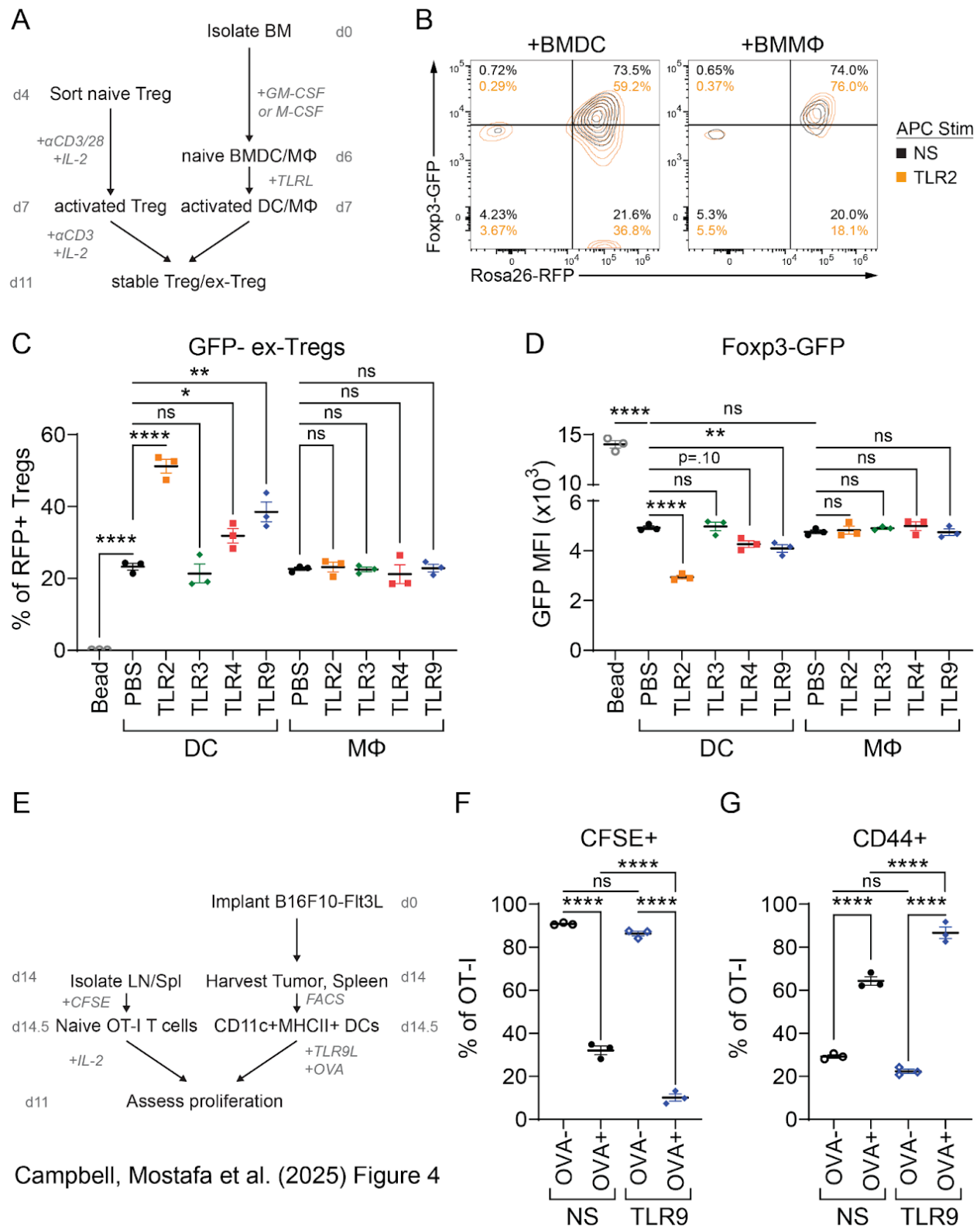
Figure 4.4: Ability of TLRs to stimulate DC1/2 correlates with Treg destabilization *in vitro*
(A-B) Activation score of MutuDC1 (A) and MutuDC2 (B) by varying concentrations of TLRL.
(C) Experimental outline for MutuDC-Treg co-culture assay.
(D) Representative flow cytometry plots showing greater ex-Treg generation in TLR2-stimulated MutuDC1 (left) and MutuDC2 (right) co-cultures.
(E) Quantification of ex-Treg frequency among total RFP⁺ Tregs/ex-Tregs upon co-culture with naive or TLR-stimulated MutuDC1 (left) or MutuDC2 (right).
(F) Quantification of normalized Foxp3-GFP expression among total RFP⁺ Tregs/ex-Tregs upon co-culture with naive or TLR-stimulated MutuDC1 (left) or MutuDC2 (right). Mean $\pm$ s.e.m. and two-way ANOVA (A, B) or one-way ANOVA (E, F).

4.3.5 TLR stimulation of DCs elicits distinct effects in Tregs and CD8⁺ T cells

We previously found that MΦs do not play a role in TLR2-mediated Treg destabilization within tumors (**Figures 3.6H-I**). However, our results with MutuDC1s and MutuDC2s suggested that APCs other than cDC2s and TLRs other than TLR2 can mediate Treg destabilization under the conditions of the APC-Treg co-culture assay. We therefore asked whether MΦs could mediate Treg destabilization by using BMDCs and BMMΦs in our APC-Treg co-culture assay (**Figure 4.5A**). To accomplish this, we generated BMDCs and BMMΦs in parallel by treating bone marrow cells with recombinant GM-CSF or conditioned media from M-CSF-overexpressing NIH 3T3 cells, respectively (**Figures 4.5A and S4.5A**).^{902,921,922} BMDCs and BMMΦs were then stimulated with TLRL or left unstimulated before being mixed at a 1:1 ratio with pre-activated lineage-trace Tregs in the presence of anti-CD3 antibody and IL-2 for 4 d before flow cytometry analysis (**Figure 4.5A**). We found that while both BMDCs and BMMΦs led Tregs to lose Foxp3 relative to anti-CD3/CD28 antibody-coated microbead controls, only BMDCs led to further Foxp3 downregulation and ex-Treg formation upon TLR stimulation (**Figures 4.5B-D**). This was not a result of TLRLs failing to stimulate BMMΦs, as BMMΦs significantly upregulated a combination of the activation markers CD80, CD86, ICOSL, and PDL1 in response to all tested TLRL (**Figure S4.5B-E**). We therefore concluded that BMMΦs are incapable of mediating Treg destabilization in response to TLR signaling, despite possessing the capacity to respond to TLRL. This suggests that the capacity to elicit the downregulation of Foxp3 in Tregs is not a general feature of TLRs and APCs but is confined to specific cell types. However, further studies are required to definitively answer the question of whether MΦs can mediate Treg destabilization in response to TLR stimulation.

We next returned to the question of how TLR9 signaling mediates tumor control despite not directly affecting tumor Treg frequency or Foxp3 expression (**Figures 4.1C-G and 4.3B-F**). We had previously observed that serial IT TLR9L injection increased the frequency of CD8⁺ T cells in tumors (**Figure 4.1F**). This led us to hypothesize that TLR9 mediates tumor control by promoting either the *de novo* priming of naive CD8⁺ T cells or the proliferation of preexisting CD8⁺ T cells, both of which are consistent with reported effects of IT TLR9 agonists in various murine cancer models.^{923–926} To answer this question, we modified our APC-T cell co-culture system to study how TLR stimulation of DCs affects tumor-specific CD8⁺ T cell priming *in vitro*. We began by implanting WT B6 mice with B16 tumor cells engineered to overexpress soluble Flt3L, which leads to the drastic expansion of DCs both within tumors and in SLOs (**Figure 4.5E**).⁹²⁷ 14 days later, mice were euthanized, tumors and spleens were processed into single-cell suspensions, and CD11c⁺MHC-II⁺ DCs were isolated using FACS. DCs were then mixed with naive, CFSE-labeled OT-I T cells with or without soluble OVA and TLR9L and co-cultured for 3 days (**Figure 4.5E**). We found that OT-I T cells co-cultured with either splenic or tumor DCs proliferated and upregulated CD44 only with OVA present, confirming that our system was able to recapitulate antigen-specific CD8⁺ T cell priming (**Figures 4.5F-G and S4.5F-G**). In line with our hypothesis, OT-I T cell proliferation and CD44 upregulation were significantly increased in splenic DC co-cultures (**Figures 4.5F-G**). A similar trend was also observed in OT-Is co-cultured with tumor-derived DCs (**Figures S4.5F-G**). Together, these results suggest that TLR9 stimulation of DCs can improve the priming of naive tumor-specific CD8⁺ T cells, consistent with published reports and our own findings *in vivo* (**Figure 4.1F**).^{923–926}

Finally, we performed a 13 analyte bead-based ELISA to attempt to identify specific cytokines and chemokines that may play a role in driving the specific effects of TLR2 and TLR9 on Tregs and CD8⁺ T cells in tumors. However, we found that most of the cytokines and chemokines that were upregulated by TLR2 were also upregulated by TLR9, albeit to somewhat varying degrees (**Figures S4.5H-I**). The most notable exceptions were IFN- β , IL-27, and CCL2 which were significantly upregulated by TLR4 and TLR9 but not TLR2, and CCL3, which was significantly upregulated by TLR2 but not TLR4 or TLR9 (**Figures S4.5H-I**). IFN- β and IL-27 play roles in driving the priming and supporting the function of CD8⁺ T cells, while CCL2 has been found to play a role in generating CD8⁺ T cell memory.^{913,928,929} These roles are consistent with our putative finding that IT TLR9L drives improved CD8⁺ T cell responses. CCL3 has been shown to mediate the ubiquitin-dependent degradation of Foxp3 in Tregs from psoriasis patients, but conversely CCL3 has also been reported to support Treg migration and function in a model of skeletal muscle injury.^{930,931} It therefore possible—albeit unlikely—that differential induction of CCL3 is the primary driver of greater Treg destabilization by TLR2 relative to other TLRs, although further experiments are necessary to clarify whether this is the case.



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Figure 4.5: TLRs exhibit differential effects on Tregs and CD8⁺ T cells via DCs *in vitro*

(A) Experimental outline for BM-derived APC-Treg co-culture assay.

(B) Representative flow cytometry plots showing increased GFP-RFP⁺ ex-Treg frequency upon Treg co-culture with DC (left) but not MΦ (right) stimulated with TLR2.

(C-D) Quantification of GFP-RFP⁺ ex-Treg frequency **(C)** and Foxp3-GFP MFI **(D)** in Tregs.

(E) Experimental outline for *ex vivo* DC-OT-I co-culture assay.

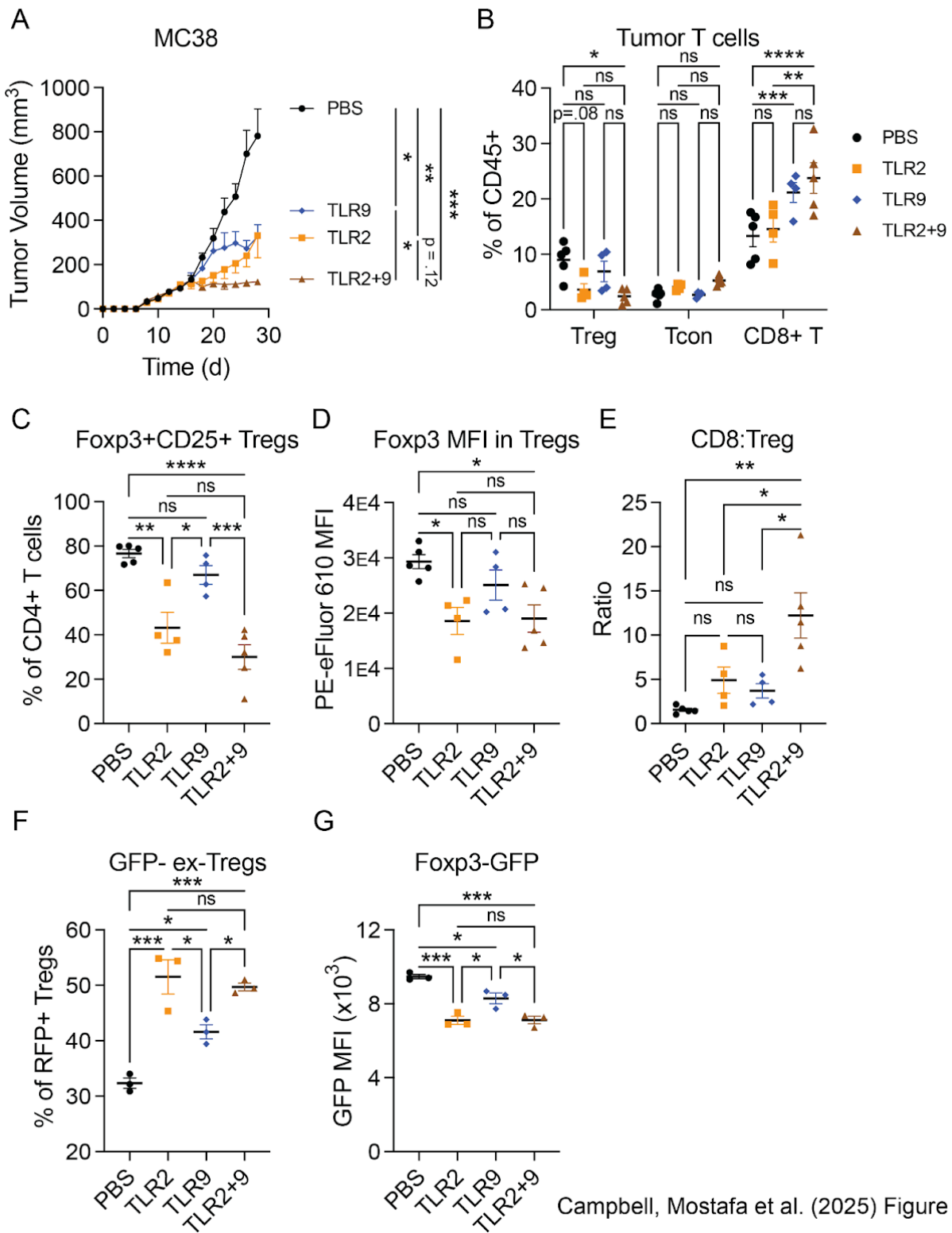
(F-G) Quantification of CFSE dilution **(F)** and CD44 upregulation **(G)** in OT-I T cells cultured with unstimulated or TLR9-stimulated DCs from spleens of B16F10-Flt3L tumor-bearing mice.

Mean ± s.e.m. and one-way ANOVA **(C, D, F, G)**.

4.3.6 Dual injection of TLR2L and TLR9L elicits synergistic tumor control

We previously noted that both IT TLR2L and IT TLR9L can slow MC38 tumor growth, but that IT TLR9L increases the frequency of CD8⁺ T cells without depleting Foxp3⁺ Tregs while IT TLR2L depletes Foxp3⁺ Tregs without increasing the frequency of CD8⁺ T cells (**Figures 4.1C and 4.1F**). This led us to hypothesize that TLR2L and TLR9L function to limit tumor growth via distinct mechanisms, which in turn led us to ask whether combining TLR2L and TLR9L might synergize to further improve tumor control. We therefore repeated our serial IT TLR experiments as in **Figure 4.1A** using TLR2L, TLR9L, or an equal mix of both compounds (TLR2+9, totaling 100 ug on d14 and 20 ug every other day thereafter). As expected, TLR2+9 stimulation elicited greater tumor control than either TLR2 or TLR9 stimulation alone (**Figure 4.6A**). Heightened tumor control in TLR2+9-treated mice corresponded to an increase in the frequency of CD8⁺ T cells and a decrease in the frequency of Tregs among total CD45⁺ TILs, while TLR2 and TLR9 altered only Treg and CD8⁺ T cell frequencies, respectively (**Figure 4.6B**). However, while TLR2+9 decreased the frequency of Tregs among CD4⁺ T cells and Foxp3 expression by Tregs in tumors, it did not do so to a greater extent than TLR2 alone (**Figures 4.6C-D**). Ultimately, the combined effect of both depleting Tregs and increasing the frequency of CD8⁺ T cells in tumors led TLR2+9 to have a much higher CD8⁺ T cell to Treg ratio than either TLR2- or TLR9-treated tumors (**Figure 4.6E**). This was accompanied by a synergistic decrease in the expression of the inhibitory receptors LAG3 and PD1 on tumor tetramer-specific CD8⁺ T cells in the tumors of TLR2+9-treated mice (**Figures S4.6A-D**). Together, these results support our hypothesis that combined TLR2 and TLR9 treatment exerts synergistic tumor control by a combination of Treg- and CD8⁺ T cell-directed mechanisms.

The failure of repeat IT TLR2+9 to deplete Tregs or downregulate Foxp3 more than IT TLR2 alone was consistent with our previous results which did not show TLR9-induced Treg depletion or Foxp3 downregulation *in vivo* (**Figures 4.1C-D and 4.3C-D**). However, we found that TLR9-stimulated BMDCs and MutuDC1s elicited Treg destabilization *in vitro* (**Figures 4.4E-F and 4.5C-D**). This led us to return to our APC-Treg co-culture assay to test whether Tregs were destabilized to a greater extent by BMDCs stimulated by TLR2+9 than either TLR2 or TLR9 alone. However, we did not observe any increase in ex-Treg formation or Foxp3 downregulation in Tregs co-cultured with TLR2+9-treated BMDCs relative to TLR2- or TLR9-treated BMDCs (**Figures 4.6F-G**). Similarly, analysis of BMDCs prior to co-culture with Tregs did not reveal synergistic activation of BMDCs by the combination of TLR2+9 (**Figures S4.6E-H**). This was consistent with our *in vivo* results, but leaves several questions about the mechanism by which TLR2+9 elicits tumor control unanswered.



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Figure 4.6: Dual treatment with TLR2/9 elicits synergistic control of MC38 tumors.

(A) MC38 tumor growth in WT B6 mice treated as in **Fig. 4.1A**, except that some mice received a combination of 50 ug each TLR2L and TLR9L (d14, 100 ug TLRL total) followed by 10 ug each TLR2L and TLR9L every other day thereafter (d16-d26, 20 ug TLRL total).

(B) Frequency of Foxp3⁺ Tregs, Foxp3⁺CD4⁺ Tcon, and CD8⁺ T cells among total CD45⁺ TILs.

(C-D) Frequency of Foxp3⁺ Tregs among CD4⁺ T cells **(C)** and Foxp3 expression in tumor Tregs **(D)**.

(E) Ratio of CD8⁺ T cells to Foxp3⁺ Tregs in tumors.

(F-G) GFP-RFP⁺ ex-Treg frequency among Tregs **(F)** and Foxp3-GFP expression in Tregs **(G)** co-cultured with BMDCs stimulated by TLR2L, TLR9L, or TLR2L+TLR9L. Mean $\pm$ s.e.m. and two-way ANOVA **(A, B)** or one-way ANOVA **(C, D, E, F, G)**.

4.4 Discussion

Our previous work showed that TLR2—but not TLR5 or TLR9—plays a critical role in tumor control by the IV+IT *Lm* immunotherapy model by stimulating cDC2s and reversing Treg-mediated tumor immune suppression (**Chapter 3**). This led us to ask whether the minimal impact of TLR5 or TLR9 on tumor growth or tumor Tregs reflected ligand availability during *Lm* infection. To address this question, we delivered IT injections of individual TLR ligands (TLRL) either as single doses (to assess immediate effects on T cells) or as repeated doses (to test whether serial stimulation could mediate tumor control). Only a subset of TLRL—those for TLR2 and TLR9—promoted tumor control upon repeat IT injection without causing significant weight loss. Flow cytometry analysis of TLR2- and TLR9-stimulated tumors at early (24 hours, single dose) and late (14 days, repeated dosing) timepoints showed that these ligands differentially affected tumor T cells. As previously observed, TLR2 stimulation reduced both the frequency and Foxp3 expression of tumor Tregs. TLR9 did not affect Tregs but instead increased tumor CD8⁺ T cell frequency. To model these effects, we used *in vitro* T-cell–DC co-cultures and found that TLR2 stimulation induced Treg destabilization via both cDC1s and cDC2s, whereas TLR9 triggered significant Treg destabilization only through cDC1s and enhanced CD8⁺ T-cell priming. Our combined *in vivo* and *in vitro* data suggested that TLR2L and TLR9L mediate tumor control through distinct mechanisms, which is supported by the synergistic tumor control by IT TLR2+9. Together, these results show that dual TLR2- and TLR9-mediated tumor control proceeds through non-overlapping mechanisms involving both Tregs and CD8⁺ T cells and likely requires contributions from both cDC1s and cDC2s.

Our observation that repeat IT TLR2 stimulation mediated tumor control was in line with our findings using IV+IT *Lm* (**Chapter 3**). However, whether Treg destabilization and depletion plays a role in tumor control by IT TLR2L alone is more difficult to interpret. In the case of IT *Lm*, we assessed the impact of TLR2-mediated Treg destabilization by using IT *Lm*Δ*Igt*—which does not stimulate TLR2, deplete Tregs, or control tumors—supplemented with IT DT, which at the correct dose depletes Tregs to a similar extent to IT TLR2L but does not control tumors following a single IT injection. However, repeat IT DT cannot be used as a direct substitute for IT TLR2L, because repeat IT DT can itself control tumors.^{127,286} This is consistent with but does not prove that TLR2-mediated Treg depletion from tumors plays a causal role in tumor control by repeated IT TLR2L. Interpretation of this result is further complicated by the fact that repeated IT DT-mediated tumor control requires either CD4⁺ Tcon (in WT tumors) or CD4⁺ Tcon and NK cells (in β2m-deficient tumors).^{127,286} In contrast, we found that IT TLR2L-mediated tumor control required CD8⁺ T cells (**Figure 4.2A**). While we did not directly test the requirement for CD4⁺ Tcon or NK cells in our system, this result suggests that the mechanism of TLR2L-mediated tumor control is at least partially distinct from outright Treg depletion.

Despite being required for tumor control by serial IT TLR2L, CD8⁺ T cells did not appear to exert any impact on Treg depletion by TLR2 (**Figure 4.2B-C**). This suggests that CD8⁺ T cells instead exert their protective effects via other mechanisms such as cytotoxicity or the production of inflammatory cytokines, which have been well described elsewhere.^{103,104} Treg depletion by TLR2 was also unaffected in cDC1-deficient *Batf3*^{-/-} mice, which nevertheless failed to control tumors following repeated IT TLR2L injection (**Figure 4.2D**). This is consistent with our earlier work showing that TLR2-dependent downregulation of Foxp3 in Tregs and depletion of Tregs in tumors still occurred in *Batf3*^{-/-} mice (**Figures 3.6J-K**). Instead, cDC1s appeared to support CD8⁺

T cell responses in order to enable tumor control by TLR2 (**Figures 4.2E-F**). Our existing results do not fully explain how this occurs, as we did not observe a significant increase in CD8⁺ T cell frequency with repeat IT TLR2L. One possibility consistent with published reports is that is that cDC1s are required to promote tumor-specific CD8⁺ T cell responses but do not play a direct role in TLR2-mediated tumor control, instead merely generating the CD8⁺ T cells which populate tumors and respond to IT TLR2L.^{503,504} A similar possibility that is consistent with our results from both CD8 depletion and *Batf3*^{-/-} mice is that TLR2 acts directly on CD8⁺ T cells to enhance their function in tumors, although this is not consistent with our results showing the absence of *Tlr2*^{GFP} expression at detectable levels in tumor CD8⁺ T cells (**Figure 3.3C**).^{825,932,933} Another is that TLR2 acts on both cDC2s (to mediate Treg depletion) and cDC1s, eliciting a cDC1-dependent signal required for CD8⁺ T cell function (e.g., IL-12).⁹³⁴ Ultimately, the resolution of these questions will likely require using models to inducibly deplete cDC1s or cDC1-specific knockout of TLR2 using a floxed *Tlr2* allele.⁹³⁵⁻⁹³⁷

Several of our findings support the conclusion that the effects of TLRs on Tregs result from qualitative differences in the effects elicited by these pathways, rather than solely from quantitative differences in the degree of stimulation elicited by each TLR. Most notably, we did not observe any effect of TLR3 on tumor Tregs *in vivo* or in any of the *in vitro* systems we used to model Treg destabilization by TLR signaling. The most obvious difference between TLR3 and other TLRs is the unique dependence of TLR3 signaling upon the TRIF and its subsequent signaling cascade, whereas the other TLRs we assessed utilize either the MyD88-dependent pathway (TLR2 and TLR9) or both TRIF and MyD88 (TLR4).³⁷⁵ This would suggest that TRIF pathway-dependent genes such as T1 IFN do not play a role in TLR-dependent Foxp3 destabilization in Tregs in the systems which we have employed here, despite potentially regulating of Treg stability in other contexts.^{938,939} However, the fact that the TRIF- and MyD88-dependent TLR signaling pathways converge on several shared transcription factors (e.g., NF-κB), and that the transcription factors utilized by MyD88-dependent TLRs differ between the cell-surface TLR2 and TLR9 (e.g., IRF-3), makes this result more difficult to interpret.⁹³⁸⁻⁹⁴¹ More thorough characterization of the transcriptional changes elicited by TLRs that do or do not destabilize Tregs in our model systems would help to clarify whether differential gene expression downstream of each TLR plays a role in this phenotype.

Another interpretation of our results is that the expression pattern of specific TLRs (e.g., TLR2 and TLR9) and the abundance of cell types expressing them (e.g., cDC1s and cDC2s) is the main factor underlying differential responses of tumor Tregs to different TLR *in vivo*. This conclusion is supported by several parallel lines of evidence from these systems. The two TLRs that depleted Tregs and led to their downregulation of Foxp3 *in vivo* were TLR2 and TLR4. This was reminiscent of Treg destabilization by TLR2- and TLR4-stimulated MutuDC2s in our co-culture assays (**Figures 4.3 and 4.4**) and our previous finding that TLR2-mediated Treg destabilization *in vivo* was cDC2-dependent (**Figures 3.6J-K**). The fact IT TLR9L does not destabilize tumor Tregs *in vivo*, despite the ability of TLR9-stimulated MutuDC1s to destabilize Tregs *in vitro*, also correlates with the fact that cDC2s outnumber cDC1s by a 2:1 margin in MC38 tumors (**Figure 4.3G**). This correlation could be further substantiated if IT TLR9L injection were found to destabilize tumor Tregs in a tumor setting wherein cDC1s predominate over cDC2s, such as in tumors of *Zeb2*^{Δ1+2+3} mice (**Figure S3.7K**) or following IT delivery of XCL1.⁹⁴²

A major gap in our study thus far is the question of how repeated IT TLR9L injection mediates tumor control. Our observation that TLR9 stimulation increases the frequency of CD8⁺ T cells and the CD8⁺ T cell to Treg ratio in tumors is consistent with a CD8⁺ T cell-dependent mechanism (**Figures 4.1B-C**). This conclusion is also supported by several studies showing that intratumoral TLR9 agonists elicited CD8⁺ T cell responses against tumors.⁹²³⁻⁹²⁶ OT-I T cells co-cultured with TLR9-stimulated DCs derived from B16-Flt3L tumor-bearing mice showed enhanced priming relative to unstimulated DCs, supporting a role for IT TLR9 in enhancing *de novo* priming of CD8⁺ T cells (**Figure 4.5F-G**). However, we did not test TLR2-stimulated DCs in this system, nor have we verified that TLR9 stimulation improves CD8⁺ T cell priming *in vivo*. Similarly, we have not tested whether TLR9 enhances the function of preexisting CD8⁺ T cells within tumors. The former model could be tested by adoptive transfer of CFSE-labeled OT-I T cells to mice following IT TLR2L or TLR9L and tracking their proliferation in the tdLN. Alternatively, the contribution of CD8⁺ T cell priming to tumor control by IT TLR9L could be tested by administering FTY720 to prevent the trafficking of newly-primed CD8⁺ T cells from migrating from the tdLN to tumors. However, the large body of evidence around the role played by TLR9 in CD8⁺ T cell-mediated makes this question less interesting to pursue in isolation.

Instead, the mechanism by which dual TLR2+9 treatment elicits synergistic control may be more interesting for further study. Thus far, we have stated that TLR2+9 likely functions via distinct effects on Tregs and CD8⁺ T cells by TLR2 and TLR9 respectively. However, it is also possible that the synergistic control of tumor growth elicited by TLR2+9 is not due to distinct mechanisms of tumor control, but rather due to the amplification of either a Treg-dependent or CD8⁺ T cell-dependent mechanism alone. With respect to the former possibility, our preliminary results from BMDC-Treg co-cultures suggest that dual TLR2- and TLR9-stimulated BMDCs do not destabilize Tregs to a greater extent than TLR2-stimulated BMDCs (**Figures 4.6F-G**). This was in line with the failure of IT TLR2+9L to elicit greater Treg depletion among CD4⁺ T cells or Foxp3 downregulation in Tregs than TLR2 alone (**Figures 4.6C-D**). Both of these results suggest that combinatorial effects on Tregs do not underlie synergistic tumor control by IT TLR2+9L. However, several recent reports may support the idea that TLR2+9 enhances CD8⁺ T cell responses irrespective of effects on Tregs. TLR2 was recently shown to enhance IL-12 and IFN- β secretion by DCs activated by endosomal TLRs relative to DCs activated by endosomal TLRs alone.⁹⁴³ Similarly, a combined TLR2/3 agonist has been shown to elicit synergistic activation of DCs to drive heightened CD8⁺ T cell responses upon IT injection.^{896,944} However, these studies did not analyze Tregs in detail, leaving open the possibility that their results are consistent with our own proposed model of synergy between Treg- and CD8⁺ T cell-directed mechanisms of TLR2 and endosomal TLRs.

Altogether, the results presented in this chapter and our previous work (**Chapter 3**) challenge the belief that endosomal TLRs are uniquely suited for the development of cancer immunotherapies.^{686,789} Instead, we find that cell-surface TLRs including TLR2 can also mediate CD8⁺ T cell-dependent immune control, albeit perhaps indirectly by reducing Treg-mediated immune suppression rather than by directly promoting CD8⁺ T cell priming or function. While we did find that *Batf3*-dependent cDC1s and CD8⁺ T cells were required for TLR2L-mediated tumor control, the results from our MutuDC-Treg co-culture system (**Figure 4.4**) and intratumoral TLR2L injection (**Figures 3.6J-K**) suggest that cCD2s are also critical to this process. While we do not challenge the conventional wisdom that cDC1s are required for CD8⁺ T cell-mediated antitumor immunity, our findings do provide additional context by suggesting

that the cDC1-CD8⁺ T cell axis intersects with and is regulated by a similar cDC2-Treg axis.^{269,503,504,877–882} This would suggest that immunotherapies seeking to drive optimal antitumor CD8⁺ T cell responses should target both of these axes, a conclusion that is supported by our findings from both the IV+IT *Lm* and IT TLR2+9L models we have employed. Our work also aligns with the current thought that CD8⁺ T cell-mediated immunity not only benefits from the expansion of CD8⁺ T cells, but also from reducing immunosuppression of these cells by Tregs within tumors.⁹⁴⁵ An additional implication of our findings is that TLR-dependent interactions with cDC1s and cDC2s locally within the TME play a continuing role in shaping CD8⁺ and Treg responses even after priming has occurred. This conclusion is supported by recent studies from our lab and others which have shown a requirement for DCs in tumor tissue to regulate T cells in tumors.^{266,326,473,508,509} Together, our and other labs' findings suggest that an update is needed to the canonical model that DC activation by inflammatory stimuli in tissues invariably leads to DC "maturation" resulting in trafficking to SLOs and *de novo* T cell priming.^{75,267,448,453,462,465,946} Instead, at least some fraction of activated DCs appear to remain within tumor tissues to regulate ongoing T cell responses. However, whether this activity is unique to tumors, and how our understanding of it might influence the development of immunotherapies in the future, will ultimately require further investigation.

4.5 Methods

Experimental models and subject details

Animal studies

C57BL/6J wild-type (JAX:000664) and *Batf3*^{-/-} (JAX:013755) mice were obtained from Jackson laboratories and bred in house. OT-1 transgenic mice were obtained from Taconic (Catalog#: 2334) and bred in house. Foxp3 lineage trace mice were generated by crossing *Foxp3*^{eGFP-hCre} mice (JAX:023161) with *Rosa26*^{LSL-RFP} mice⁸⁹⁸ and were a gift from the Bluestone lab at the University of California, San Francisco. For tumor studies, syngeneic C57BL/6J mice of the above genotypes were inoculated with 5.0×10⁵ MC38, 2.0×10⁵ B16F10, or 2.0×10⁵ B16-F1t3L tumor cells in PBS subcutaneously on the right flank. Tumor measurements were performed blindly across the entire experiment by a single operator measuring three dimensions of the tumor with calipers every second day beginning when tumors were palpable. All experiments were conducted according to the Institutional Animal Care and Use Committee guidelines of the University of California, Berkeley.

Cell lines

The MC38 and B16F10 cell lines were kindly provided by Dr. Jeff Bluestone's lab. Cancer cell lines were maintained in DMEM (GIBCO) supplemented with 10% FBS, sodium pyruvate (GIBCO), L-Glutamine (GIBCO), and penicillin-streptomycin (GIBCO). Tumor cells were grown at 37°C with 5% CO₂. MutuDC1940 (MutuDC1) and MutuDC2 were purchased from Applied Biological Materials Inc and were maintained in IMDM (GIBCO) supplemented with 10% heat-inactivated FBS, L-Glutamine (GIBCO), penicillin-streptomycin (GIBCO), HEPES (GIBCO), NaHCO₃ (GIBCO), and B-mercaptoethanol (GIBCO).^{918,919}

Method Details

Intratumoral TLRL injection

Lyophilized TLRL were reconstituted in LAL water per the manufacturer's instructions to a final concentration of 1 mg/mL. 14 days after tumor implantation, mice received IT injections of 100uL of PBS either without additions or with 100 ug Pam3CSK4, poly(I:C), LPS from *Salmonella typhimurium*, or ODN1668 (Invivogen). For tumor growth experiments, mice were treated as above and with additional doses of 20 ug of TLRL in 100 uL PBS beginning on d16 and again every second day thereafter. Mice were euthanized 1-14 days after IT injections.

Bone marrow dendritic cell differentiation

Primary BMDCs were generated as previously described using WT B6 mice (day 0).⁹⁰² After differentiation (day 6), BMDC media was replaced with fresh media containing PBS or 1 ug/mL Pam3CSK4 (Invivogen) to stimulate TLR2. 24 hr later (day 7), BMDCs were harvested by transferring media containing nonadherent cells to a centrifuge tube and replacing with an equal volume of cold PBS supplemented with 10 uM EDTA to loosen semi-adherent cells. Nonadherent cells were pelleted by centrifugation, while semi-adherent cells were incubated for 10 min on ice. After incubation, semi-adherent cells were recovered by gentle pipetting, and this

suspension was used to resuspend pelleted nonadherent cells. Pooled cells were washed with PBS to remove trace Pam3CSK4. BMDCs were counted and used in Treg co-culture experiments or analyzed by flow cytometry to assess purity and phenotype.

Bone marrow macrophage differentiation

Primary BMMΦs were generated as previously described using WT B6 mice (day 0).⁹²¹ After differentiation (day 6), BMMΦ media was replaced with fresh media containing PBS or 1 ug/mL Pam3CSK4, 10 ug/mL poly(I:C), 100 ng/mL LPS from *S. typhimurium*, or 1 ug/mL ODN 1668 (Invivogen) to stimulate TLR2/3/4/9 respectively. 24 hr later (day 7), BMMΦs were harvested by replacing media with an equal volume of cold PBS supplemented with 10 uM EDTA and incubating for 10 min at room temperature. After incubation, cells were recovered by vigorous pipetting, centrifuged, and washed once with PBS to remove trace TLRL. BMMΦs were counted and used in Treg co-culture experiments or analyzed by flow cytometry to assess purity and phenotype.

Fluorescence-activated cell sorting of Tregs

Treg lineage-trace mice were euthanized and lymph nodes and spleens surgically resected and stored on ice in PBS supplemented with 4 mM EDTA and 2% FBS. Single-cell suspensions of splenocytes and lymphocytes were generated by crushing organs through a 40 um cell strainer (Fisher Scientific) and red blood cells were removed by ACK lysis using RBC lysis buffer (BioLegend). CD4 T cells were magnetically enriched using the EasySep™ Mouse CD4+ T Cell Isolation Kit (STEMCELL Technologies) per the manufacturer's instructions. Isolated CD4 T cells were stained with fluorescent antibodies in the dark at 4°C for 30 min and washed with PBS prior to resuspension in Opti-MEM (Thermo Fisher) supplemented with 2% FBS for FACS. Naive Tregs (CD4+CD8-CD62L+RFP+GFP+) were sorted with a FACS Aria™ Fusion cell sorter (BD). Sorting efficiency was determined by post-sort flow cytometric analysis and Treg purity exceeded 95%.

Treg-APC co-culture assay

FACS-isolated Tregs were mixed with Dynabeads™ Mouse T-Activator CD3/CD28 for T-Cell Expansion and Activation (GIBCO) at a ratio of 1:3::beads:Tregs in the presence of 2000 IU/mL teceleukin (hIL2; Hoffmann-La Roche) and activated for 4 days. Activated Tregs were reisolated by using magnetic columns to remove Dynabeads™ and washed once with PBS. Activated Tregs were then mixed with unstimulated or TLR-stimulated BMDCs, BMMΦs, MutuDC1s, MutuDC2s, or additional Dynabeads™ at a 1:1 ratio. Treg co-cultures were supplemented with 1 ug/mL of Ultra-LEAF™ Purified anti-mouse CD3 Antibody (BioLegend) to mimic TCR signaling and 2000 IU/mL teceleukin. All cells were cultured in DMEM supplemented with 10% FBS, sodium pyruvate (GIBCO), L-Glutamine (GIBCO), penicillin-streptomycin (GIBCO), HEPES (GIBCO), MEM Non-Essential Amino Acid Solution (GIBCO), and 50 uM B-mercaptoethanol (GIBCO). Cultures were incubated at 37°C for 72 hr prior to flow cytometric analysis.

OT-I-DC co-culture assay

Tumors, LNs, and spleens of B16-Flt3L tumor-bearing mice were surgically resected following euthanasia of mice according to the Institutional Animal Care and Use Committee guidelines of the University of California, Berkeley. Resected organs were crushed through 40 µm nylon mesh cell strainers (Thermo Fisher) and red blood cells were removed by ACK lysis using RBC lysis buffer (BioLegend). Dendritic cells were enriched using the EasySep™ Mouse Pan-DC Enrichment Kit II (StemCell) per the manufacturer's instructions then stained with anti-CD11c-BV605, anti-I-A/I-E-FITC, anti-CD3-PerCP-Cy5.5, and anti-CD19 PE (BioLegend) in the dark at 4°C for 30 min and washed with PBS prior to resuspension in Opti-MEM (Thermo Fisher) supplemented with 2% FBS for FACS. DCs (CD11c⁺I-A/I-E⁺CD3⁻CD19⁻) were sorted with a FACS Aria™ Fusion cell sorter (BD). Sorting efficiency was determined by post-sort flow cytometric analysis and Treg purity exceeded 95%. 10,000 DCs/well were then plated on 96-well TC-treated U-bottom plates (Thermo Scientific) +/- recombinant OVA-PE-TexasRed (Thermo Scientific) and +/- ODN1668 (InVivoGen) in 100 µL DMEM supplemented with 10% FBS, sodium pyruvate (GIBCO), L-Glutamine (GIBCO), penicillin-streptomycin (GIBCO), HEPES (GIBCO), MEM Non-Essential Amino Acid Solution (GIBCO), and 50 µM B-mercaptoethanol (GIBCO). OT-I T cells were isolated using the EasySep™ Mouse CD8⁺ T Cell Isolation Kit (StemCell) then stained with the CFSE Cell Division Tracker Kit (BioLegend). 50,000 CFSE-stained OT-I-s/well were then mixed with DCs and incubated at 37°C for 3 days before flow cytometry analysis.

Sample preparation for flow cytometry

Single cell suspensions were prepared as described below ("*Tissue collection*"). Dead cells were stained with Live/Dead Fixable Blue Cell Stain kit (Molecular Probes) in PBS at 4°C. Cell surface antigens were stained at 4°C using a mixture of fluorophore-conjugated antibodies. Surface marker stains for murine T cell 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 CD25 (PC61, BioLegend), anti-mouse CD44 (IM7, BioLegend), anti-mouse CD45 (30-F11, BioLegend), anti-mouse CD45.1 (A20, BioLegend), anti-mouse CD45.2 (104, BioLegend), anti-mouse CD62L (MEL-14, BioLegend), anti-mouse CD69 (H1.23F, BioLegend), anti-mouse CD103 (2E7, BioLegend), anti-mouse Lag3 (C9B7W, BioLegend), anti-mouse PD1 (29F.1A12, BioLegend), anti-H-2Kb MuLV p15E Tetramer-KSPWFTTL (MBL), anti-H2-Kb-A2/SIINFEKEL tetramer (NIH tetramer core), and anti-IAb/NEKYAQAYPNVS tetramer (NIH tetramer core) in PBS. Surface marker stains for murine myeloid cell samples were carried out with anti-mouse CD16/32 TruStain FcX (93, BioLegend), anti-mouse CD45 (30-F11, BioLegend), anti-mouse F4/80 (BM8, BioLegend), anti-mouse CD45R (RA3-6B2, BioLegend), anti-mouse CD11c (N418, BioLegend), anti-mouse I-A/I-E (M5/114.15.2, BioLegend), anti-mouse SIRPa (P84, BioLegend), anti-mouse CD11b (M1/70, BioLegend), anti-mouse XCR1 (ZET, BioLegend), anti-mouse CD103 (2E7, BioLegend), anti-mouse Ly6C (HK1.4, BioLegend), anti-mouse Ly6G (1A8, BioLegend), and anti-mouse CD14 (Sa14-2, BioLegend) in PBS. Cells were fixed in 4% PFA (Electron Microscopy Sciences) in PBS at room temperature for 15 minutes, permeabilized with 0.1 % Triton X-100 (Thermo Fisher) in PBS at room temperature for 10 minutes, and washed twice with 0.5% BSA (Sigma Aldrich) in PBS prior to intracellular staining. Intracellular staining for murine T cell samples was performed using anti-mouse Foxp3 (FJK-16S, eBioscience) in 0.5% BSA in PBS. Cells were analyzed by

flow cytometry between one and three days after sample preparation. Cells were resuspended in PBS and filtered through FlowTubes™ Cap w/ 35 µm Strainer Mesh (Genesee) prior to data acquisition.

Tissue collection and restimulation assays

Tumors and lymphoid organs were surgically resected following euthanasia of mice according to the Institutional Animal Care and Use Committee guidelines of the University of California, Berkeley. Resected tumors were minced to 1 mm³ fragments and digested in RPMI media supplemented with 20 mg/mL DNase I (Roche), 125 U/mL collagenase D (Roche), and 2% FBS using an orbital shaker at 37°C for 1 hr. Digested tumors were filtered through a 70 µm cell strainer (Thermo Fisher). Cells from lymphoid organs were prepared by mechanical disruption with the sterile plunger of a 1 mL syringe through a 70 µm nylon mesh cell strainer (Thermo Fisher). Cell suspensions from tumors and lymphoid organs were subjected to ACK lysis to remove red blood cells using RBC lysis buffer (BioLegend) and passed through FlowTubes™ Cap w/ 35 µm Strainer Mesh (Genesee) prior to in vitro stimulation and/or flow cytometric analysis. Stimulation was performed with 3-5×10⁶ cells in Opti-MEM media (Thermo Fisher) supplemented with Brefeldin A (eBioscience), 10 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), and 0.25 µM ionomycin (Sigma-Aldrich) at 37°C for 6 hr. Surface staining, fixation, permeabilization, and intracellular staining were performed as described above (“*Tissue collection and preparation for flow cytometry*”).

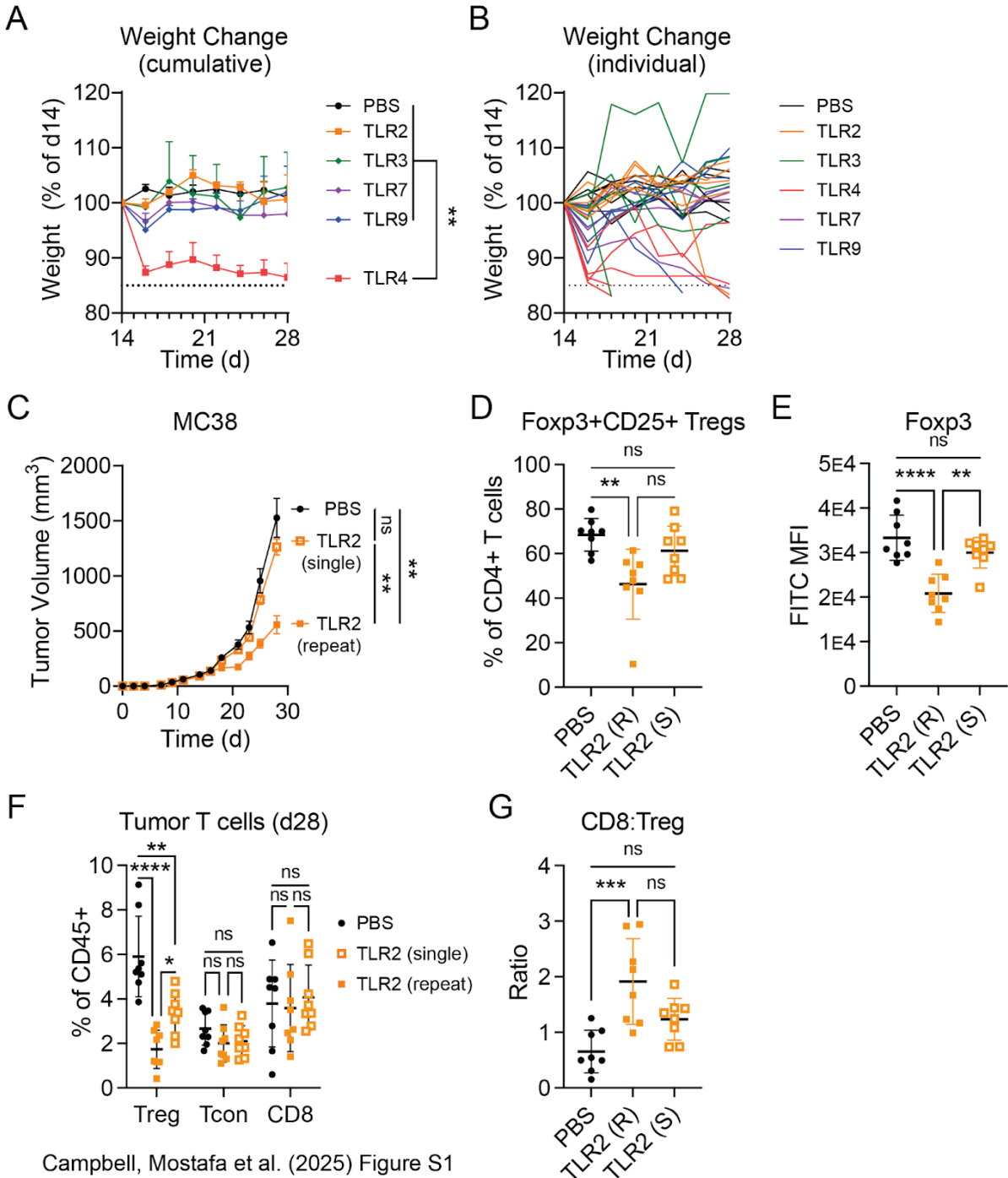
In vivo antibody-mediated cell depletion

For tumor progression studies, CD8⁺ T cell depletion was achieved by intraperitoneal injection of 200 µg per mouse of the anti-CD8b2 antibody clone 53-5.8 (Leinco, Catalog #C2382) two days and one day prior to IT *Lm* treatment, followed by additional doses twice weekly thereafter.

Statistical Methods

p values were obtained from unpaired two-tailed Student's t tests for statistical comparisons between two groups of values derived from separate mice, or from paired two-tailed Student's t tests for comparisons between tissues within individual mice, 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.

4.6 Supplementary Materials



Campbell, Mostafa et al. (2025) Figure S1

Figure S4.1 (related to Figure 4.1):

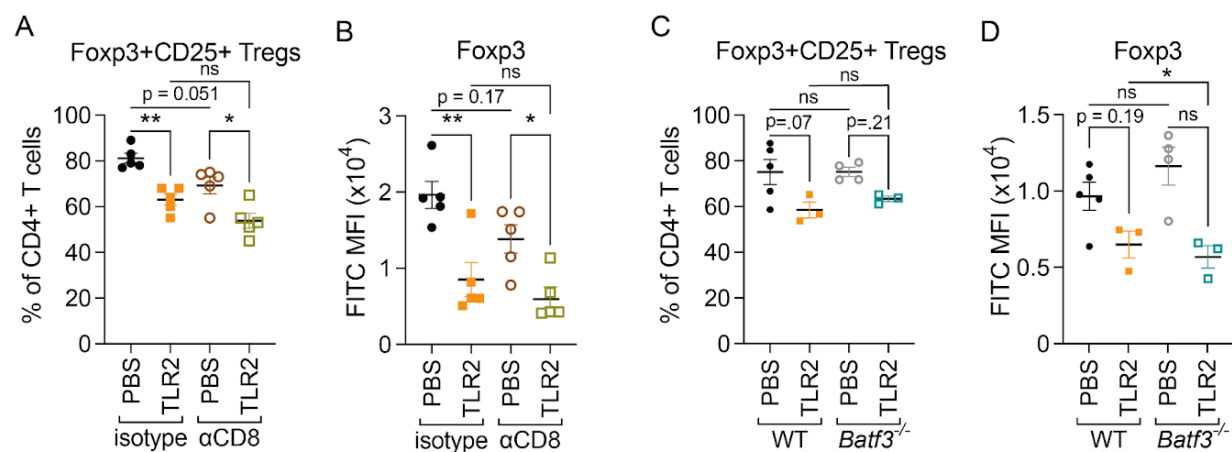
(A-B) Cumulative weight loss within TLRL treatment groups including values mice euthanized at humane endpoints **(A)** and noncumulative weight loss showing values for individual mice **(B)** for experiment shown in **Fig. 4.1A-G**.

(C) MC38 tumor growth in mice treated as in **Fig. 4.1A** or receiving only a single 100 ug dose of IT TLR2L (d14) without additional 20 ug doses thereafter.

(D-E) Frequency of Foxp3⁺ Tregs among CD4⁺ T cells **(D)** and Foxp3 expression in tumor Tregs **(E)**.

(F) Frequency of Foxp3⁺ Tregs, Foxp3⁻CD4⁺ Tcon, and CD8⁺ T cells among total CD45⁺ TILs.

(G) Ratio of CD8⁺ T cells to Foxp3⁺ Tregs in tumors. Mean $\pm$ s.e.m. and two-way ANOVA **(A, C, F)** or one-way ANOVA **(D, E, G)**.

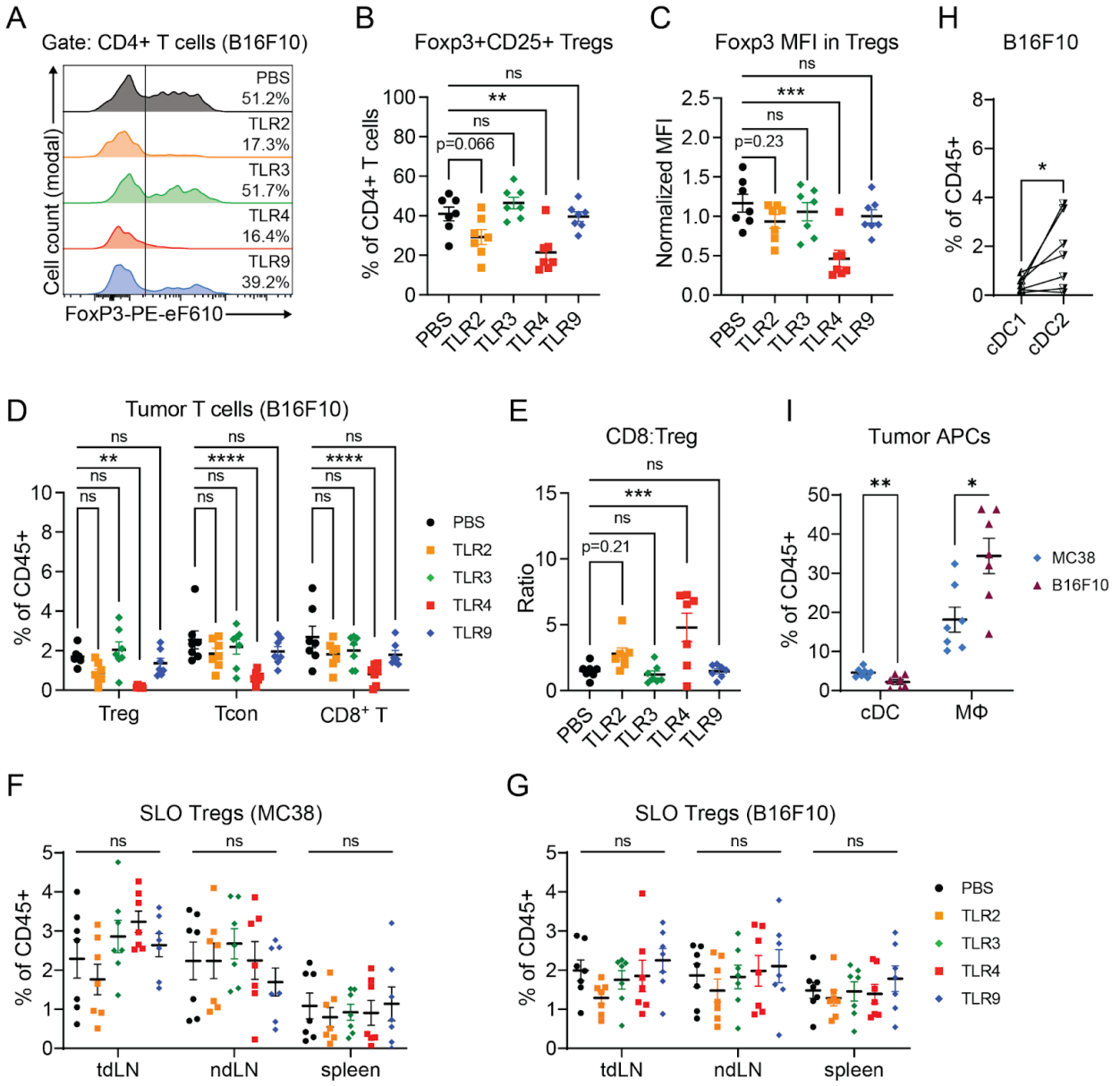


Campbell, Mostafa et al. (2025) Figure S2

Figure S4.2 (related to Figure 4.2):

(A-B) Frequency of Foxp3⁺ Tregs among CD4⁺ T cells **(A)** and Foxp3⁺ MFI in tumor Tregs **(B)** for experiment shown in **Fig. 4.2A-C**.

(C-D) Frequency of Foxp3⁺ Tregs among CD4⁺ T cells **(C)** and Foxp3⁺ MFI in tumor Tregs **(D)** for experiment shown in **Fig. 4.2D-F**. Mean $\pm$ s.e.m. and one-way ANOVA **(A, B, C, D)**.



Campbell, Mostafa et al. (2025) Figure S3

Figure S4.3 (related to Figure 4.3):

(A) Representative histograms showing reduced Foxp3⁺ Treg frequency and Foxp3 expression in Tregs from B16F10 tumors treated with TLR2/4L compared to TLR3/9L or PBS.

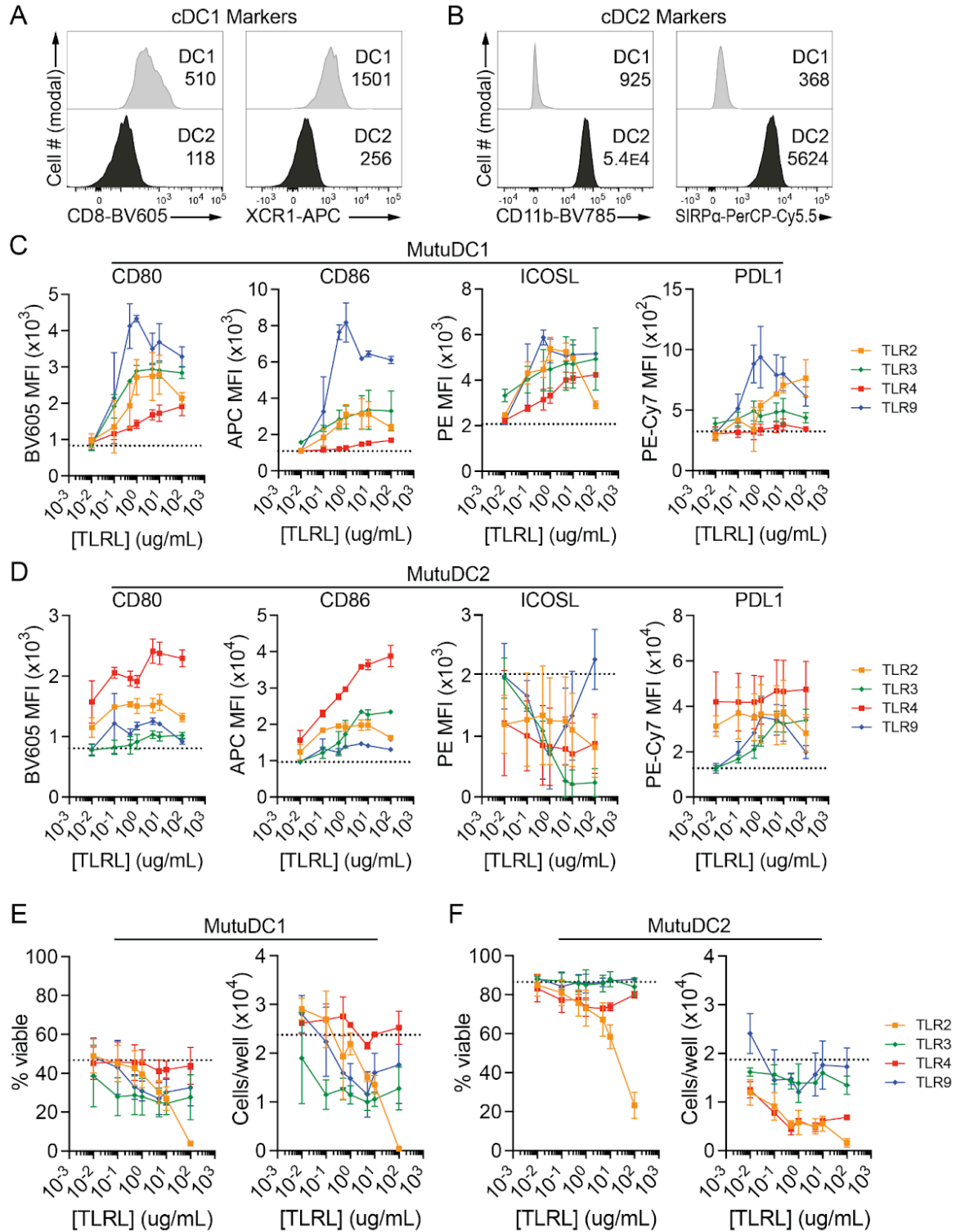
(B-C) Frequency of Foxp3⁺ Tregs among CD4⁺ T cells **(B)** and Foxp3⁺ MFI in tumor Tregs **(C)**.

(D) Frequency of Foxp3⁺ Tregs, Foxp3-CD4⁺ Tcon, and CD8⁺ T cells among total CD45⁺ TILs.

(E) Ratio of CD8⁺ T cells to Foxp3⁺ Tregs.

(F) Frequency of Foxp3⁺ Tregs among total CD45⁺ cells in SLOs of MC38 tumor-bearing mice from experiment shown in **Fig. 4.3A-F**.

(G) Frequency of Foxp3⁺ Tregs among total CD45⁺ cells in SLOs of B16F10 tumor-bearing mice from experiment shown in **(A-E)**. Mean $\pm$ s.e.m. and one-way ANOVA **(B, C, E)**, two-way ANOVA **(D, F, G)**, or paired **(H)** or unpaired **(I)** Student's t-test.



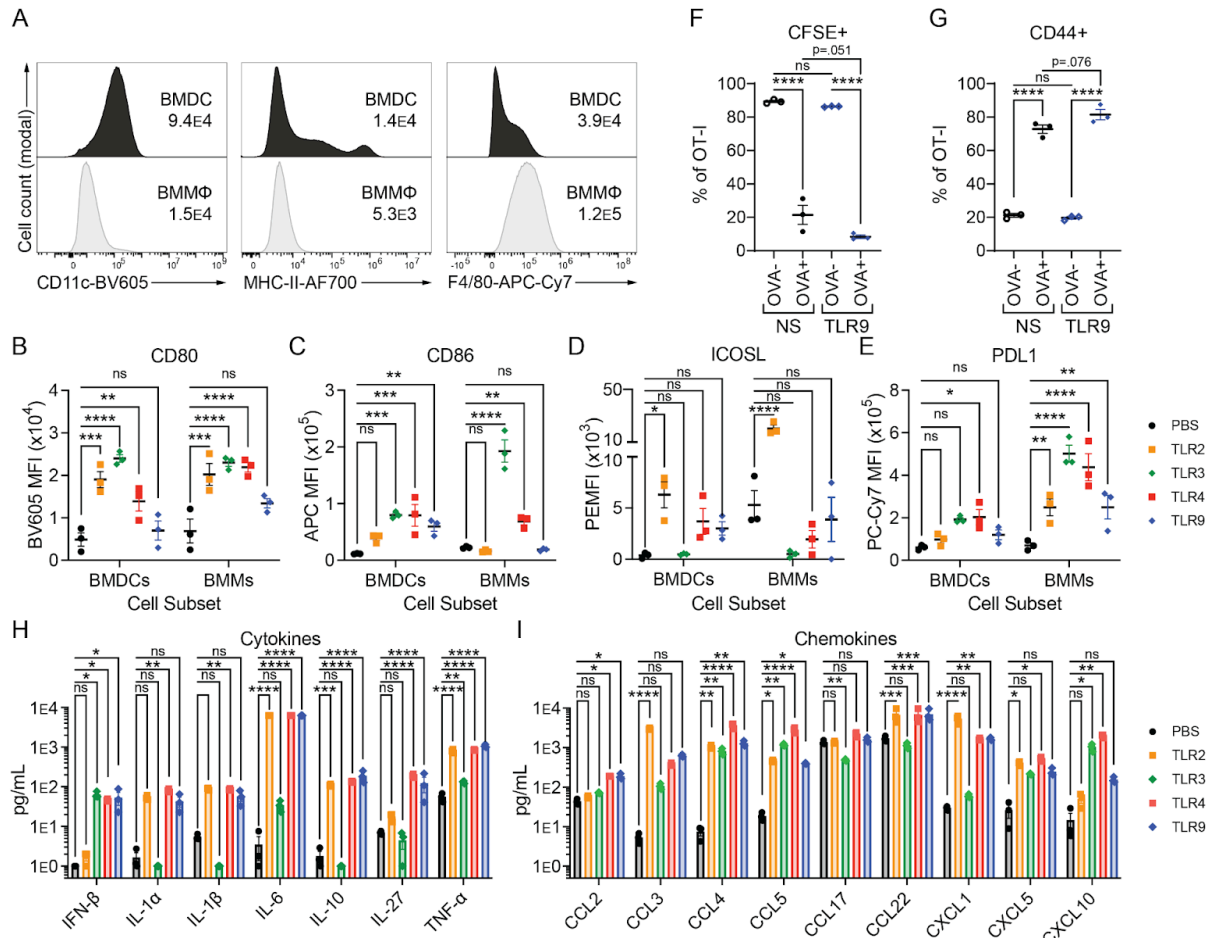
Campbell, Mostafa et al. (2025) Figure S4

Figure S4.4 (related to Figure 4.5):

(A-B) Flow cytometry histograms showing expression and MFI of cDC1-specific **(A)** and cDC2-specific **(B)** surface markers in MutuDC1 and MutuDC2 cells.

(C-D) Expression of individual activation markers used to calculate activation score on MutuDC1 **(C)** and MutuDC2 **(D)** upon stimulation with varied concentrations of TLRL.

(E-F) Viability (left) and cell number (right) of MutuDC1 **(E)** and MutuDC2 **(F)** upon stimulation with varied concentrations of TLRL. Mean $\pm$ s.e.m. with average MFI **(C, D)**, viability **(E, F left)**, or cell number **(E, F right)** of unstimulated MutuDC1 and MutuDC2 indicated by dotted line.



Campbell, Mostafa et al. (2025) Figure S5

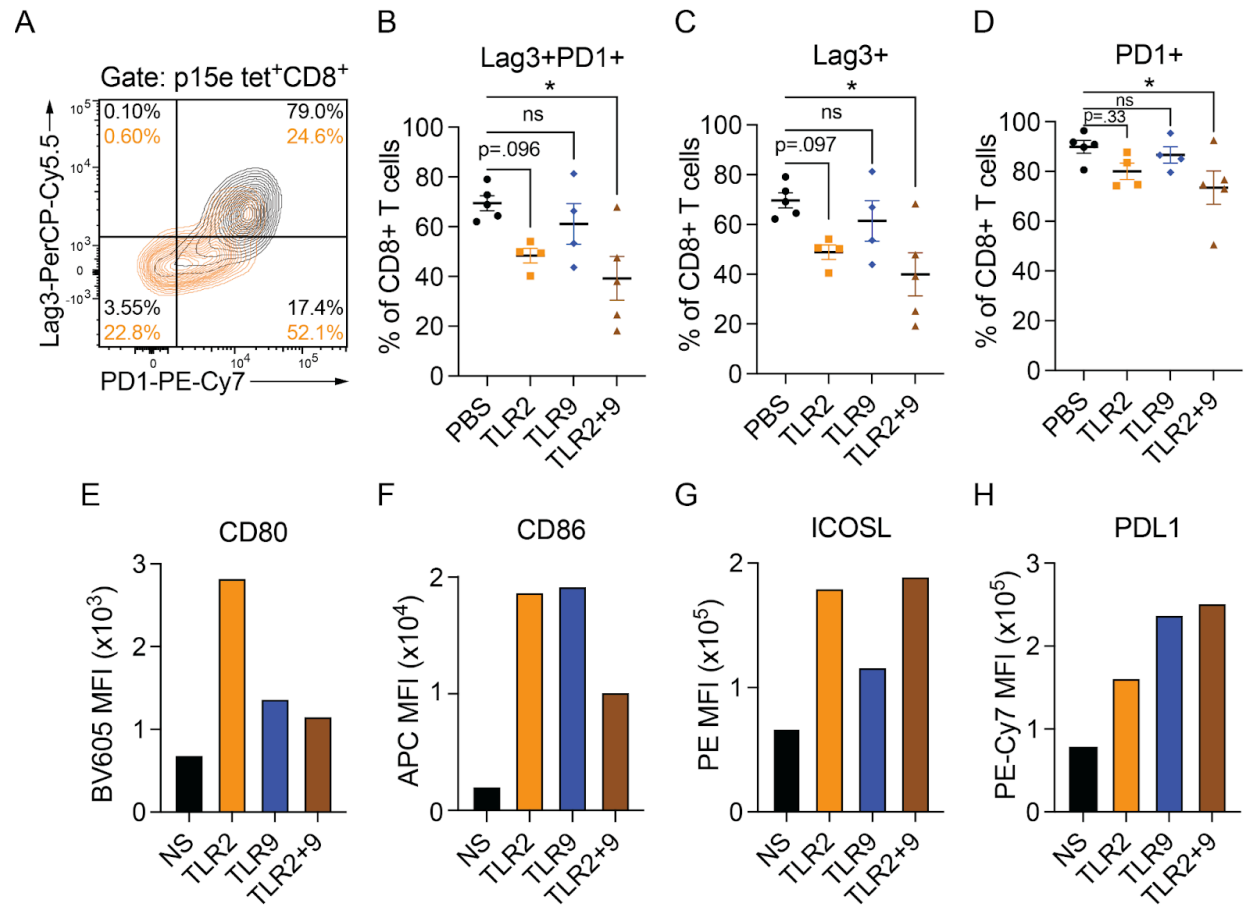
Figure S4.5 (related to Figure 4.5):

(A) Flow cytometry histograms showing expression and MFI of DC-specific (CD11c, MHC-II) and MΦ-specific (F4/80) surface markers in BMDC and BMMΦ.

(B-E) Quantified expression of CD80 **(B)**, CD86 **(C)**, ICOSL **(D)**, and PDL1 **(E)** in naive and TLR-stimulated BMDC and BMMΦ.

(F-G) Quantification of CFSE dilution **(F)** and CD44 upregulation **(G)** in OT-I T cells cultured with unstimulated or TLR9-stimulated DCs from B16F10-Flt3L tumors.

(H-I) Concentration of cytokines **(H)** and chemokines **(I)** in supernatants of naive and TLR-stimulated BMDC. Mean $\pm$ s.e.m. and two-way ANOVA **(B, C, D, E, F, G, H, I)**.



Campbell, Mostafa et al. (2025) Figure S6

Figure S4.6 (related to Figure 4.6):

(A) Representative flow cytometry plots showing Lag3 and PD1 expression in tumor-specific CD8⁺ T cells (p15e tetramer⁺) in tumors of PBS- (black) or TLR2+9-treated (orange) mice.

(B-D) Quantification of Lag3⁺PD1⁺ **(B)**, total Lag3⁺ **(C)**, and total PD1⁺ **(D)** among tumor-specific CD8⁺ T cells in PBS-, TLR2-, TLR9-, and TLR2+9-treated mice

(E-H) Expression of CD80 (E), CD86 (F), ICOSL (G), and PDL1 (H) in PBS-, TLR2-, TLR9-, and TLR2+9-stimulated BMDCs prior to Treg co-culture. Mean $\pm$ s.e.m. and one-way ANOVA **(B, C, D)** or individual values **(E, F, G, H)**.

Chapter 5

Concluding Remarks

If my thesis work is to be distilled into a single theme, it would be the role played by APCs in determining the course of T cell responses to cancer immunotherapy. This first became evident when we found that cDC1s were required for tumor control by IV+IT *Lm*, in part due to their role in re-engaging tumor antigen-specific CD8⁺ T cells (**Chapter 2**). However, using a broader definition that incorporates non-professional APCs, this theme also applies to our results showing that *Lm* could generate an inhibitory or inflammatory TME by either recruiting and persisting in or recruiting and then killing PMN-MDSCs within tumors (**Chapter 2**). The presence or absence of *Lm*-specific CD8⁺ T cells was critical in mediating the switch between the two outcomes, as MHC-I antigen presentation by host but not tumor cells. This finding is consistent with recent publications which have shown the activation of bystander T cells within tumors can drive antitumor immune responses.^{778,779,890,891} Together with these reports, our findings suggest that tumor immunity can be promoted by first generating a strong antimicrobial T cell response and then recalling these T cells to tumor tissue.

The modulation of PMNs by the TME played a major role in the immunosuppressive effects of IT *Lm* injection alone. This aligns with a substantial body of work showing that tumors can modulate innate immune cells, including APCs, to limit T cell-mediated antitumor immunity.^{324,502,520,740,741} Our findings using PMN depletion prior to IT *Lm* injection raise the question of whether tumor-mediated modulation of APC function has played a role in the failure of *Lm* cancer vaccines to earn clinical approval (**Chapter 2**).^{621–623} Conversely, both we and other groups have found that TLR2 stimulation can act on tumor APCs to abrogate immune suppression within the TME by Tregs and other suppressive cells, suggesting that the process by which *Lm* modulates the TME is neither fully suppressive nor stimulatory (**Chapter 3**).⁹⁴⁷ Instead, IT *Lm* drives a shift from one inhibitory population (Tregs) to another (PMN-MDSCs). Therefore, one approach to improve the clinical efficacy of *Lm*-based cancer vaccines may be to design *Lm* vectors that inhibit MDSC polarization either instead of, or in addition to, expressing tumor antigens without disrupting its capacity to destabilize Tregs. This might be accomplished by engineering *Lm* to secrete antagonists of receptors that drive MDSC differentiation.⁹⁴⁸ An alternative approach would be to leverage the intracellular life cycle of *Lm* and its selective growth within PMN-MDSCs to eliminate these cells by employing *Lm* strains that mediate cell death upon entry into the cytosol (**Chapter 2**).^{949,950} Ultimately, the insights from our studies suggest that using *Lm* to modulate immunosuppression within the TME may be as, if not more impactful than, the traditional goal of maximizing CD8⁺ T cell priming.

This leads to another question raised by our results, namely, what aspects of bacterial physiology and pathogenicity are necessary for tumor control by IV+IT *Lm* injection? Our IV+IT *Lm* experiments with TLR2- and MHC-I-deficient mice and CD8⁺ T cell depletion show that cytosolic localization of *Lm* and TLR2 signaling are both critical to CD8⁺ T cell-mediated tumor control but do not rule out the possibility that other mechanisms could compensate for these effects (**Chapter 2-3**). One avenue to address this question is to use *Lm* variants deficient for cytosolic entry (e.g., *Δhly*) or growth within the cytosol (e.g., *ΔpurA*).^{636,951} However, several unpublished results from our lab suggest that *Lm* in tumors often behaves differently than in other settings. First, IT injection of *LmΔhly-tagRFP* resulted in the accumulation of RFP⁺ PMNs, indicative of unexpected phagosomal escape by this strain within tumors. Second, IV *Lm* followed by IT *LmΔhly* controls tumors, which according to our model requires the presentation of cytosolic antigens on MHC-I to reactivate *Lm*-specific CD8⁺ T cells (**Chapter 2**).^{636,951,952} One strategy to overcome these limitations is to compare the ability of other bacteria to mediate

tumor control following IV+IT injection. For example, to test the requirement for cytosolic localization to mediate tumor control we could use Gram-positive, predominantly extracellular bacteria (e.g., *Staphylococcus aureus*),⁹⁵³ while to test the requirement for Treg depletion by TLR2 as opposed to other TLRs we could use intracellular Gram-negative bacteria that predominantly activate TLR4 (e.g., *Shigella flexneri*).^{954,955} Immunity to these bacteria is also more dependent upon CD4⁺ T cells and/or B cells than *Listeria* in non-tumor settings, which, according to our results thus far, would render them less effective in the setting of IV+IT.^{956,957} However, this approach also has shortcomings, not least of which is that these bacteria are unique organisms with very different mechanisms of pathogenesis.^{953,955} Moreover, *S. aureus* is not strictly extracellular in all cases, and *S. flexneri* does not exclusively activate TLR4.^{954,958} Therefore, a combination of *Lm* mutants, knockout mice, and other pathogens will likely be required to fully answer the question of which specific features of *Lm* enable tumor control by IV+IT *Lm* injection.

Despite my initial hypotheses to the contrary, my work also aligns with the model that different TLRs preferentially stimulate different APC types. This conclusion was most directly supported by *in vitro* analysis of cDC1- and cDC2-derived MutuDCs, which despite their improvements over previous DC cell lines are still an artificial system (**Chapter 4**).^{918–920} *Ex vivo* characterization of the response of tumor APCs to TLR stimulation would provide more compelling evidence in support of this model, but this approach is limited by the low frequency and number of APCs in tumors, especially cDC1s and cDC2s (**Chapters 3 and 4**). However, my work challenges the idea that CD8⁺ T cell-mediated antitumor immunity is best achieved by direct stimulation of endosomal TLRs.^{686,687,912} Instead, I found that stimulation of TLR2 in cDC2s abrogated Treg-mediated immunosuppression to provide a comparable degree of tumor control to the endosomal TLR9, and that a combination of TLR2 and TLR9 led to greater control than either alone (**Chapter 4**). I interpret these results to show that endosomal and cell-surface TLRs regulate different aspects of the CD8⁺ T cell response in the MC38 tumor model. Namely, TLR9 appeared to be more important for the generation of CD8⁺ T cells in tumors, while TLR2 sustained the activity of these cells by destabilizing and depleting Tregs. This could influence the use of TLR agonists in cancer immunotherapy, which despite underwhelming performance as monotherapies have shown greater promise when delivered alongside Treg-depleting antibodies, analogously to my own results with TLR2+9 (**Chapter 4**).^{682–685,896,944,959–961}

However, my work with different TLRs failed to address the question of what drives these differential responses to TLR ligands (TLRL). One interpretation of my findings is that the different responses to each TLRL were caused by diverging signaling cascades downstream of each TLR.^{375,385} One example of these differences that I cite repeatedly in this work is that TLR9 can drive T1 IFN expression by activating IRF-3, while TLR2 cannot in most cases.^{938–941} Alternatively, the fact that TLR2, TLR4, and TLR9 can all elicit Treg destabilization under some conditions, but TLR3 could not under any condition, might support a role for MyD88-dependent, TRIF-independent signals in reversing Treg immunosuppression (**Chapters 3–4**).^{938,941} However, the convergence of the MyD88- and TRIF-dependent TLR signaling pathways on a largely-overlapping set of transcription factors and effector genes suggests that this explanation is not sufficient to fully explain the different effects elicited by TLRL in our systems.^{938–941} This theory might be tested by employing specific inhibitors of the transcription factors downstream of TLR signaling, or by employing mutant mice that lack the signaling intermediates required to engage them.^{962,963}

An alternative explanation, which I favor, is that these effects are due to the degree of expression of individual TLRs on specific APC types, the relative abundance of those cell types within tumors, and the competency of these cell types to produce the signals required to modulate T cell responses. For example, our results using *Tlr2^{GFP}* mice showed that *Tlr2* is expressed by essentially all APCs in tumors (cDC1, cDC2, and macrophages), but that cDC2s were required for TLR2-mediated Treg destabilization (**Chapter 3**). While both MutuDC1 and MutuDC2 were able to destabilize Tregs in response to TLR2 *in vitro*, cDC2s outnumbered cDC1s by a greater than 2:1 margin in tumors (**Chapter 4**). While macrophages outnumbered cDC1s and cDC2s in tumors, TLR-stimulated BMMΦs were incapable of destabilizing Tregs (**Chapter 4**). Together, these findings may provide a rationale for the specific requirement for cDC2s to mediate Treg destabilization and depletion from tumors downstream of TLR2. This model would be supported if it was found that the degree of TLR expression by DC subsets in tumors correlated with the capacity of ligands for those TLRs to destabilize tumor Tregs. Conversely, we might expect that IT TLR9 stimulation would drive Treg destabilization in a model in which cDC1s outnumber cDC2s (e.g., MC38 tumors in *Zeb2^{Δ1+2+3}* mice) (**Chapter 3**).

An extension of this theory is that specific cell types (e.g., cDC2s) are well-positioned to mediate the effects of different TLRs (e.g., TLR2) on Tregs within tumors. Previous work from our lab showed that CXCR3⁺ Tregs closely associate with CD11c⁺ cells in the TME, which we inferred were cDC1s based on their high expression of the CXCR3 ligand CXCL9 and the failure of CXCR3⁺ Tregs to accumulate in tumors of *Batf3^{-/-}* cDC1-deficient mice.²⁶⁶ However, cDC1s are less common than cDC2s in the TME, and while MutuDC1s could destabilize Tregs in response to TLR2 *in vitro*, cDC1s were dispensable for TLR2-mediated Treg destabilization *in vivo* (**Chapter 3-4**). A possibility raised by this observation is that a specific subset of Tregs, perhaps those which are CXCR3⁻, are depleted upon IT TLR2 stimulation. While this could result simply from the closer proximity of these cells to cDC2s, it might also reflect a specific regulatory axis between this subset, TLR2, and cDC2s, analogous to the regulation of CXCR3⁺ Tregs by IFN-γ and cDC1s.²⁶⁶ Similarly, this might reflect differences in the stability of Foxp3 expression by specific Treg subsets other than cDC1-associated, CXCR3⁺ Th1-like Tregs.^{156,157,245,250,964} A possible candidate for this subset is Th17-like Tregs, which exist in a tightly-regulated equilibrium with conventional Th17 cells.¹⁴⁶ Moreover, Tregs have been shown to be susceptible to destabilization and reprogramming into a Th17-like phenotype by both Th17-promoting cytokines and by TLR2.⁹⁶⁴⁻⁹⁶⁹ Ultimately, more thorough characterization of tumor Tregs before and after IT TLR2 stimulation will be necessary to address these hypotheses. In particular, confocal microscopy analysis using cDC2-specific and Treg markers would address the contribution of spatial distribution to Treg destabilization by TLR2, while transcriptional profiling and transcription factor staining could show whether specific Treg subsets are susceptible to TLR2-mediated destabilization of Foxp3 expression.

To close, I will return to the theme which opened this section, namely, how antitumor immune responses were coordinated by multiple DC subsets. My work with *Lm* showed that cDC1s not only mediated the priming CD8⁺ T cells in SLOs, but played a role in sustaining CD8⁺ T cell function by cross-presenting tumor antigens within the TME (**Chapters 2-3**). Likewise, my work with *Lm* and specific TLRs showed that cDC2s were important not only for generating tumor-infiltrating Tregs, but also destabilized Tregs in response to TLR2 signaling (**Chapters 3-4**). While this could reduce immunosuppression in tumors, tumor-specific CD8⁺ T cell frequency and function were increased only when *Lm*-specific CD8⁺ T cell-derived

cytokines (**Chapters 2-3**) or TLR9 signaling (**Chapter 4**) were present to activate cDC1s. While my model differs from other models of TLR-induced Treg destabilization and antitumor immunity by emphasizing the interplay between cDC subsets, it is consistent with published reports that have shown both cDC1s and cDC2s to be capable of orchestrating CD8⁺ T cell responses in cancer.^{269,502–504,509} A greater distinction between this and existing models is that my work emphasizes the continued role of DCs in regulating T cells within the TME. This phenomenon has been reported elsewhere but has been less thoroughly investigated than the role played by DCs in controlling T cell responses in SLOs.^{266,326,473,508,509} However, our work thus far does not definitively prove this model, as our findings related to this hypothesis have mostly been more descriptive than functional. This could be addressed in part by inhibiting the trafficking of DCs and T cells between tumors and SLOs, or by depleting DCs from tumors but not SLOs. If correct, we might expect that both CD8⁺ T cells and Tregs would still be primed in SLOs, but would fail to accumulate or to exercise their functions effectively within the TME.

In this work, I focused on the ways in which *Lm* and TLRLs could reshape the immune landscape of tumors to promote or inhibit tumor growth. Our findings suggest several avenues for further basic studies to delineate the mechanisms by which these changes unfold. My work also has potential translational applications, namely in suggesting that the efficacy of cancer immunotherapies can be maximized by engaging multiple DC populations both within and outside of tumors to provide multimodal support of CD8⁺ T cell-mediated immunity. Together, I believe that the results presented in my thesis provide novel insights to expand upon existing concepts of the role played by APCs in driving antitumor immunity.

Chapter 6

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