THE UNIVERSITY OF CHICAGO

# THE ROLE OF COMMENSAL MICROBIOTA IN SHAPING SYSTEMIC ANTI-TUMOR IMMUNITY

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES
AND THE PRITZKER SCHOOL OF MEDICINE
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

COMMITTEE ON CANCER BIOLOGY

BY
AYELET SHOSHANA SIVAN

CHICAGO, ILLINOIS
DECEMBER 2015

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

**Supplementary files**

# ACKNOWLEDGMENTS

This work is a result of a truly collaborative effort made by many individuals, to whom I owe my deepest gratitude.

To Tom Gajewski, my thesis advisor: one of the Mitzvahs (principals) in Jewish culture, is making a stranger feel welcome. I joined the lab as a stranger, but I feel today as though I am family. I owe this to you Tom for being supportive, encouraging and generous throughout my time here. This was achieved while promoting scientific excellence, precision and dedication and this was inspired by your own devotion to the success of us, your mentees. It has been a tremendous opportunity to work with you at such an exciting time. For all of this and more, Thank you.

To the Gajewski lab members, who have been for me, a home away from home. I have regarded all of you as older siblings. You are all exceptional scientists from whom I have learned so much. To Leticia Corrales, without whom this work would not be possible, thank you for your friendship, endless patience and encouragement inside and outside the lab. To Stefani Spranger, you are not only a compassionate leader, but also a close friend, who has supported me through this journey, with wisdom, a hug and a Starbucks coffee. To Seng-Ryong Woo, thank you for sharing your knowledge and expertise with a smile and a joke. To Jay Williams, thank you for your generosity with your time and effort, especially long nights at the sorter. To Brendan Horton, who has been with me through thick and thin, thank you for leading the way, and teaching me not to give up. To Randy Sweis, thank you for your openness and support, we have enjoyed so many conversations together. To Michael Leung, thank you for your kindness and assistance. And finally, to Franco, you were a companion, who helped me take on every task, big or small, with enthusiasm and dedication.

To my collaborators. To Nathaniel Hubert, you have been instrumental to this work. Thank you for your dedication to this project, humor and availability at all hours of the night. To Zach Earley, thank you for jumping on board without hesitation and providing

your expertise, insight and a lot of laughs. To Kevin Lei - thank you for being one step ahead and teaching me as you go. To Daina Ringus, thank you for your time, microbiology expertise and conversations, and to Yuan Zhang and Marlies Meisel - thank you for your contributions. To the Alegre, Kline, Savage, Chang and Jabri labs - thank you for endless advice.

To my committee members. To Marisa Alegre, who welcomed me with open arms, I am fortunate to have such a dedicated and perceptive mentor, thank you for your immense contributions to the development of this work. To Gene Chang, thank you for your enthusiasm for the project, microbiology expertise and support. To Nancy Cox, thank you for your much-needed expertise, support and leadership, and for your amazing gooseberry pies. To Geoffrey Greene, a special thank you for giving me the opportunity to pursue a PhD, believing in me and supporting me from the very beginning.

To Bana Jabri, thank you for your valuable and insightful contributions to this work.

To Kay Macleod, thank you for personally making sure that I succeed, I am so grateful for all that you have done on my behalf.

My thanks to the Cancer Biology program faculty, students and administrators for making the program an amazing one to be in. Thanks to the GCIS Animal facility, Flow Cytometry core, Functional Genomics core and to Argonne National Laboratory. I would like to thank my funding sources the Bernice Goldblatt foundation, the Cancer Biology Training grant and the Melanoma Research Alliance grant.

A special thank you to my previous mentor Dorothy Sipkins, who showed me what it is to dedicate oneself to medicine and scientific research.

I would like to acknowledge past mentors Yoram Groner and Niv Pencovich who started me on the scientific path, and Peter Savage for guiding me throughout.

I thank Amichai Amit, for believing in me and encouraging me to pursue a PhD.

To my incredible parents, whom I love, have been inspired by and to whom I owe ev-

erything past, present and future and to my sisters, grandparents, family and friends, thank you.

To Keston, whom I love with all of my heart, thank you for filling every day with warmth and sunlight.

To all of the animals in my life - Audrey, Efe, Jackson and Taconic  thank you.

# ABSTRACT

T cell infiltration of solid tumors is associated with favorable patient outcomes, yet the mechanisms underlying variable endogenous immune responses between individuals are not well understood. Distinct members of the commensal microbiota affect host immunity in various ways, suggesting that differences in the composition of this community may contribute to individual differences in anti-tumor immune responses. In Chapter 1, we examine potential effects of microbial composition on spontaneous anti-tumor immunity, by comparing B16 melanoma growth in C57BL/6 mice having distinct commensal microbiota. The two populations of mice showed robust versus weak spontaneous anti-tumor immunity. Protective responses could be transferred between one mouse population to the other upon cohousing or following fecal transfer, arguing for a microbiota-dependent effect. In Chapter 2 we examine microbial and immunologic mechanisms underlying the observed effects. 16S rRNA sequencing identified *Bifidobacterium* as associated with the anti-tumor effects. Oral administration of *Bifidobacterium* alone markedly improved tumor control to the same degree as treatment with systemic $\alpha$PD-L1, in a $CD8^+$ T cell-dependent manner, and combination treatment nearly abolished tumor outgrowth. Mechanistically, the effect was mediated by augmented dendritic cell function leading to more robust antigen-specific $CD8^+$ T cell priming and markedly increased accumulation of activated T cells in the tumor microenvironment. These data support the idea that one source of inter-subject heterogeneity with regard to spontaneous anti-tumor immunity and therapeutic effects of antibodies targeting the PD-1/PD-L1 axis may be the specific composition of gut microbes, which could be manipulated for therapeutic benefit. (Data tables are provided in a supplementary file.)

# CHAPTER 1

# INTRODUCTION

## 1.1 The clinical success of immunotherapy

The goal of curative treatment for the majority of patients with metastatic cancers has eluded clinicians and researchers alike for many years. This is due, in part, to the genetic diversity within tumors and across individuals, and the ability of tumors to undergo selection and adaptation, both locally and within distant tissues. For these reasons, conventional systemic therapies, which largely rely on non-specific targeting of cell replication or induction of apoptosis, are frequently unsuccessful, as tumors can show either primary resistance or can be selected for secondary resistance over time. In contrast, the clinical success of immunotherapy has transformed the way we think about cancer treatment. The unique genetic abnormalities that endow cancer cells with the ability to resist conventional treatment may also constitute antigens that render them susceptible to immune attack [12, 105, 11]. Anti-tumor immunity can be highly specific and adaptive, does not require tumor cells to be actively replicating or localized in one place, and can be maintained life-long through immunologic memory. In the last two decades, these concepts have gone from theory to clinical application, with a subset of advanced-stage patients experiencing powerful and lasting tumor regression with contemporary immunotherapy approaches [65, 107, 136, 47, 52].

## 1.2 The endogenous anti-tumor immune response and current immunotherapies

Pioneering studies dissecting various aspects of initiation and maintenance of protective immune responses have led to the development of immune-based therapies for cancer, that intervene at various steps of the priming and effector phases of anti-tumor immunity. The

anti-tumor response is largely initiated by dendritic cells (DCs), which upon activation in the tumor microenvironment deliver antigen and co-stimulatory signals to T cells in the tumor-draining lymph node, resulting in priming and activation of effector T cell responses against cancer-specific antigens. Activated T cells then migrate to the tumor to execute cancer cell killing. The precise molecular requirements for each step in this response are still under investigation but have become better understood over the past several years.

### *1.2.1 Tumor sensing by antigen-presenting cells (APCs)*

Pattern recognition receptors (PRRs) expressed on APCs are known to mediate recognition of pathogen-associated molecular patterns (PAMPs) and lead to initiation of immune responses in the context of infection. More recently it has been recognized that these receptors are capable of sensing endogenous signals known as damage-associated molecular patterns (DAMPs), which can be released by tumor cells undergoing cell stress or cell death, and possibly through additional mechanisms that may be revealed upon further study. DAMPs include intracellular metabolites, e.g. ATP, ER-associated proteins, e.g. Calreticulin (CRT), intracellular proteins, e.g. High-mobility group protein B1 (HMGB1) and F-actin, and nucleic acids, e.g. DNA. Recognition of these molecules by PRRs on APCs can stimulate recruitment and maturation of DCs (ATP-P2RX7) [29, 43], engulfment of tumor antigens by DCs (CRT-CD91) [41, 40] and optimal antigen presentation to T cells (HMGB1-TLR4; F-actin-DNGR1) [112, 3, 2, 111]. While chemotherapy and radiotherapy-induced cell death has been reported to release ATP or/and HMGB1 to activate DCs via the inflammasome or Toll-like receptor 4 (TLR4) pathways, respectively [3, 43], our laboratory has determined using gene-targeted mice and single cell imaging that spontaneous DC activation in vivo is mediated by tumor-derived DNA, leading to stimulator of interferon genes (STING) activation and production of type I interferon (IFN) [137]. Host type I IFN production plays a critical role in bridging innate immune sensing of tumors and mounting adaptive T cell

responses, which are ultimately required for immune-mediated tumor control, discussed in the following paragraphs.

### *1.2.2 APC-T cell interactions leading to effector T cell priming*

Productive T cell priming depends on signals provided by APCs. These include antigen presentation in the form of peptides bound to histocompatibility molecules on the surface of APCs, and engagement of surface molecules on APCs with costimulatory receptors on naive T cells. In order to activate naive T cells in the context of a tumor, APCs must acquire exogenous antigens derived from the tumor and present them to $CD8^+$ T cells in a process known as cross-presentation [62, 49]. Thus, cross-presentation plays an important role in eliciting anti-tumor T cell responses.

We, and others have demonstrated a requirement for type I IFN signaling by host DCs [21, 27], which facilitates cross-presentation of antigen to T cells by the $CD8\alpha^+$ subset of DCs [33]. Consistent with these reports, spontaneous priming of $CD8^+$ T cells was eliminated in $Batf3^{-/-}$ mice, which lack the $CD8\alpha^+$ DC lineage, in both syngeneic transplantable [49] and genetically engineered [122] mouse models of cancer. In addition to antigen-presentation, delivery of CD80 or CD86 co-stimulatory signals by DCs is necessary to lead to productive priming of effector T cells [73]. These molecules bind to CD28 on the surface of naive T cells, which provides intracellular signals that synergize with signaling downstream of T cell receptor-antigen ligation, promoting full T cell activation and initiating differentiation into effector T cells [76].

### *1.2.3 Tumor-associated antigens*

Many immunogenic human tumor antigens have now been identified [10, 106, 12, 105, 11]. These include differentiation antigens, e.g. melanocyte differentiation antigens, Melan-A/MART-1, tyrosinase, gp-100; over-expressed antigens, e.g. HER-2/neu; viral-derived anti-

gens, e.g. EBV and HPV; cancer-testis antigens whose expression is normally restricted to gametogenic and placental tissues; and mutated antigens  epitopes that arise from cancer-specific mutations. It is the existence of such antigens that enables the possibility of an adaptive response against tumors, discussed below.

### *1.2.4 Effector phase mediated by CD8$^+$ T cells*

Naive T cells are provided with antigenic and costimulatory signals upon tumor recognition by APCs. Subsequently, migration of activated CD8$^+$ T cells into the tumor site requires the CXCR3-binding chemokines CXCL9 or CXCL10 [93]. The effector phase requires IFN-$\gamma$ and either the perforin/granzyme pathway or the FasL pathway of lysis for tumor elimination [73]. Specifically, release of specialized lytic granules upon recognition of MHC Class I-bound antigen on the surface of a tumor cell leads to tumor cell-specific killing. IFN-$\gamma$ may function to upregulate components of the MHC Class I antigen processing and presentation pathway in tumor cells [119], to facilitate tumor recognition and elimination by CD8$^+$ T cells. In addition IFN-$\gamma$ has been reported to induce expression of pro-apoptotic molecules that may contribute directly to tumor cell apoptosis [16, 139], and elicit production of CXCR3 binding chemokines that have both important chemotactic and anti-angiogenic properties [127, 118]. Finally, IFN-$\gamma$ signaling in host immune cells is critical for the cytolytic activity of CD8$^+$ T cells [30]. Thus, IFN-$\gamma$ appears to target both host and tumor cells, as evidenced by the requirement for IFN-$\gamma$ responsiveness in both tumor cells [22] and host cells [30].

### *1.2.5 Immune-inhibitory mechanisms*

Although host-protective immune responses can be initiated, they are often blunted by establishment of an immune-suppressive environment within the tumor. Defined inhibitory mechanisms include suppression of CD8$^+$ effector cells by CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Tregs) [72], recruitment of myeloid-derived suppressor cells (MDSCs) [36], metabolic

deregulation via tryptophan catabolism by indoleamine-2,3-dioxygenase (IDO) [131], up-regulation of cytotoxic lymphocyte antigen 4 (CTLA-4) on T cells [77] and engagement of the inhibitory receptor PD-1 by the ligand PD-L1/B7-H1 [23, 8].

Tregs inhibit the function of $CD8^+$ T cells by producing the immunosuppressive cytokines IL-10, IL-35 and TGF-$\beta$, expressing negative co-stimulatory molecules such as CTLA-4, PD-1, and PD-L1, and by competing for IL-2, a cytokine that supports cytotoxic T cell function. MDSCs can promote Treg induction and TGF-$\beta$ production, but also deplete or sequester amino acids necessary for T cell function, such as tryptophan, through expression of IDO. CTLA-4 and PD-1 are inhibitory receptors that can be induced upon T cell activation and limit the responses of activated T cells through modulation of intracellular signaling [74, 32].

### *1.2.6 Current immunotherapies*

Knowledge of the steps involved in anti-tumor immunity provides a framework in which to develop and test therapeutic interventions aimed at improving each of these stages of anti-tumor immunity. Early phases of the response can be enhanced through vaccine approaches, with the goal of increasing the frequency of tumor antigen-specific T cells in the host [116, 66]. Improved activation of DCs that are acquiring and presenting tumor antigens can be achieved by stimulating innate immune sensing pathways, including the STING pathway via specific agonists [19].

An alternative strategy for increasing the frequency of anti-tumor T cells is ex vivo expansion of specific T cell populations for adoptive transfer into the tumor-bearing host. One such approach involves isolation and ex-vivo expansion of tumor-infiltrating T cells, followed by adoptive transfer back into the patient [104]. This strategy has generated response rates of approximately 50% in metastatic melanoma patients [25, 26]. In situations in which T cell responses have been initiated but have been blunted by an immune-suppressive tumor microenvironment, strategies for blockade of specific immune-suppressive pathways have been

pursued. Two key targets are inhibitory receptors expressed on activated T cells− CTLA-4 and PD-1 [74, 32]. Antibodies against these receptors have shown impressive clinical activity and have been FDA-approved for the treatment of patients with melanoma [47, 52] and in the case of $\alpha$PD-1 agents, also for non-small cell lung cancer [128].

## 1.3 Beyond existing therapies: patient heterogeneity and tumor immune-phenotypes

As described above, the efficacy of many of the existing immune-based therapies for cancer relies on modulating an underlying endogenous immune response initiated in the patient. Indeed, early data have indicated that clinical responses to these immunotherapies are more frequent in patients who show evidence of an endogenous T cell response ongoing in the tumor microenvironment at baseline [37, 61, 124, 130]. Despite the functional and clinical importance of this T cell-inflamed tumor microenvironment, the mechanisms that govern the presence or absence of this phenotype are not well understood. Elucidating the mechanisms that regulate spontaneous T cell infiltration in tumors is a key step in the rational development of novel approaches to improve the efficacy of immunotherapeutic strategies and expand the subset of patients who benefit clinically from these interventions.

Theoretical sources of inter-patient heterogeneity include germline genetic differences at the level of the host, variability in patterns of somatic alterations in tumor cells, and environmental differences with the potential to impact on immunity. Our laboratory has recently shown that tumor-intrinsic $\beta$-catenin pathway activation constitutes one mechanism of tumor resistance to immune infiltration [122]. In the current work, we address a second hypothesis − whether an environmental difference, specifically the composition of the gut microbiota, influences spontaneous anti-tumor immunity, thereby impacting efficacy of immunotherapeutic interventions.

## 1.4 Studying commensal microbial communities

Humans are colonized with a diverse and abundant indigenous microbiota, coating the mucosal and external surfaces of the body. The greatest density and diversity of microorganisms is found in the gut, particularly the colon, with bacteria being highly abundant. Among the phyla represented in the gastrointestinal tract are Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria, as well as Verrucomicrobia and Tenericutes. Efforts to identify commensal bacteria in the intestine were aided by technical advances in microbial ecology, which utilized 16S ribosomal RNA (rRNA) gene sequencing to classify bacteria found in various environments. Specifically, deep sequencing of short variable (V) regions of 16S rRNA, the V4, V5-V6, and V6-V7 regions, resulted in species richness estimates that were comparable to those generated during analysis of full-length sequences [113], enabling easy assimilation of these techniques to microbial analyses in the context of health and disease. Complementary experimental approaches include studies in germ-free animals, treatment with selected antibiotics, and colonization with specific bacteria by oral or rectal gavage. Using these approaches, many associations between commensal bacteria and diverse phenotypes have been identified, as discussed below.

## 1.5 Intestinal commensal microbes as systemic immune modulators

Gut bacteria provide humans with additional traits, beyond those encoded by their inherited genome, influencing diverse aspects of biological function including digestion, drug metabolism, gut epithelial integrity, and even cardiac size and behavior. One of the most studied aspects of gut bacterial function is their essential role in development and regulation of innate and adaptive immunity, with most early studies focusing on effects within intestinal tissues (reviewed in [50, 58, 78, 53]). However, a growing body of data is indicating

an important role for gut microbiota in shaping systemic immune responses, summarized in **Table S0**.

### *1.5.1 Specification of T cell subsets reaching beyond the gut*

Commensal bacteria have been shown to direct differentiation of T cells leading to expansion of specific molecular subsets, thereby impacting on systemic inflammatory processes that involve these T cell differentiation states. Early studies reported that mono-colonization of germ-free animals with *Bacteroides fragilis* resulted in $CD4^+$ T cell expansion in the spleen, as well as restoration of T helper 1/T helper 2 (Th1/Th2) balance in germ-free animals by inducing production of the Th1 cytokine IFN-$\gamma$ in splenic $CD4^+$ T cells [87]. Further studies showed that *B. fragilis* also affects T cell responses by promoting Treg function in both the intestine (Round and Mazmanian, 2010) and distant lymph nodes [97, 98]. In the latter case, oral administration of *B. Fragilis*-derived Polysaccharide A (PSA) decreased the severity of experimentally-induced encephalomyelitis (EAE), which was accompanied by accumulation of $CD103^+$ DCs and IL-10 producing Tregs in the cervical lymph nodes of myelin oligodendrocyte protein (MOG)-challenged mice [97, 98].

While *B. Fragilis* provides protective immunity, commensal bacteria known as segmented filamentous bacteria (SFB) have been shown to promote systemic inflammatory conditions such as EAE and rheumatoid arthritis, through the induction of IL-17 in spinal cords of MOG-challenged mice [79], and T helper 17 (Th17) cell expansion in the spleens of K/BxN mice [138], a murine model of rheumatoid arthritis. These systemic effects parallel the induction of Th17 cell differentiation by SFB in the small intestine [57]. These data highlight the opposing roles of specific commensals in directing T cell differentiation, supporting the idea that the balance between specific components of the gut microbial community may contribute not only to intestinal T cell homeostasis, but also to modulation of systemic T cell responses.

A third group of commensal bacteria, *Clostridium spp.*, which have been tied to accumulation of Tregs in the colon [5], have also emerged as systemic immune-modulators. Oral administration of *Clostridium spp.* to conventionally-reared [5] or germ-free mice [125] reduced serum IgE responses in allergy models, and neonatal depletion of clostridia via vancomycin treatment increased asthma severity [109]. The first of these studies demonstrated that clostridial-feeding was associated with increased numbers of $IL10^{+}CD4^{+}$ cells at extra-intestinal sites, suggesting that systemic alterations in T cell function may underlie the observed suppression of allergic immune responses [5]. An increase in peripheral Treg cells was also observed upon addition of butyrate, a short-chain fatty acid produced by commensal microorganisms such as clostridia, to the drinking water of antibiotic-treated animals [4].

Systemic abundance of a particular subset of T cells, iNKT cells, is also regulated by commensal microbiota. Absence of gut bacteria early in life leads to CXCL16-dependent accumulation of iNKT cells in both the colon and lungs, resulting in increased morbidity in experimental models of colitis and allergic asthma, respectively [100]. Taken together, these seminal studies demonstrate that commensals shape the landscape of T cell specification in the host, in both a time- and bacteria-specific manner, with implications reaching beyond the gut.

### *1.5.2 Calibration of innate immune function at non-mucosal sites*

Optimal expansion and acquisition of effector function in adaptive immune cells hinges on signals from innate immune cells. That innate immune cells may be more or less equipped to provide such signals, depending on interactions with commensal bacteria, is an emerging concept of great interest. Two studies showed that splenic DCs and peritoneal macrophages require signals from gut commensal bacteria in the steady state in order to optimally prime NK cell- [39] and $CD8^{+}$ T cell- [1] mediated anti-viral responses, respectively. Optimal

immune priming depended on robust type I IFN signaling in peripheral mononuclear cells, which was defective in germ-free or antibiotic-treated mice.

Consistent with these reports, numerous studies have demonstrated a role for commensal bacteria in facilitating viral clearance from the lungs. Specifically, oral administration of lactic acid bacteria (LAB) induced type I IFN production in splenic DCs in a TLR3-dependent manner [70], and has been shown to enhance NK cell activity, leading to suppression of influenza virus (IFV) proliferation in the lungs [69]. Oral inoculation of *Bifidobacterium longum* also enhanced NK cell activity in response to IFV infection [68]. Others have shown that commensal bacteria lead to inflammasome activation, induction of proIL-1, proIL-18, and NLRP3, in broncho-alveolar lavage, eliciting DC migration from the lung to the draining lymph nodes and improved T and B cell-mediated anti-IFV responses [55]. Intestinal bacteria have been shown to augment serum antibody responses upon administration of trivalent inactivated influenza vaccine, mediated through peripheral lymph node macrophage production of plasma cell growth factors IL-6 and TNF-$\alpha$ [99].

Intestinal bacterial communities can also regulate direct anti-bacterial functions of distant innate immune cells. A recent study showed that bacterial peptidoglycans are recognized by NOD1 receptors on bone marrow neutrophils, leading to enhanced killing function and protective immunity to *Streptococcus pneumoniae* and *Staphylococcus aureus* infections [17]. This study provides an important link between gut bacterial products and distant immune cells, and will be discussed further in 1.6.2.

### *1.5.3 Commensal bacteria and systemic anti-tumor immunity*

The recognition that tumor growth is profoundly influenced by host immune responses has gained traction in the last decade. However, a potential impact of gut commensal microbes on systemic anti-tumor immune responses has only begun to be investigated.

Two seminal studies showed that the therapeutic effect of chemotherapy was facilitated

by the presence of commensal microbes. In one model, treatment with oral antibiotics reduced the therapeutic effect of oxaliplatin or the response to anti-IL-10R+CpG-ODN as an immunotherapy. Mechanistically, the effect was mapped to the level of inflammatory cytokine production by myeloid cells [56]. In a second model, bacterial translocation from the gut into peripheral organs was implicated in the therapeutic effect of cyclophosphamide, as treatment with antibiotics reduced the generation of Th17 cells that were involved with anti-tumor efficacy in this system [134].

In cancer models associated with inflammation, commensal bacteria also have been reported to promote tumor growth (reviewed in [42]). In a recent study, TLR5-mediated sensing of gut commensals was shown to increase systemic levels of IL-6, leading to recruitment of MDSCs and $\gamma\delta$ suppressor T cells into the tumor microenvironment, in a p53-kras driven tumor model [110]. This study highlights the importance of dissecting the functional roles of specific components of the gut microbiota in specific inflammatory contexts. Further study should aim to reveal positive and negative commensal regulators of the endogenous anti-tumor response as well as those that may modulate response to contemporary cancer immunotherapies, which have significant therapeutic potential in patients.

## 1.6 Mechanisms underlying commensal-mediated modulation of distant host immunity

Gaining an in-depth understanding of the multitude of ways in which intestinal bacteria interact with the host is a crucial step in elucidating the mechanisms by which commensal bacteria influence distant host immunity. Below are discussed some of the emerging concepts in this field.

### *1.6.1 Physical and molecular interactions between commensal bacteria and host cells within the gut*

PRRs play a critical role in the detection of commensal bacteria. PRRs are expressed by innate immune cells as well as intestinal epithelial cells (IECs), and include the membrane- and endosome-associated TLRs, cytosolic DNA sensors (CDSs), cytoplasmic Nod-like receptors (NLRs), retinoic acid inducible gene I-like helicase receptors (RLRs) and G-protein coupled receptors (GPCRs). Commensal bacterial components, such as PSA (TLR2), LPS (TLR4), Flagellin (TLR5), dsRNA (TLR3), CpG DNA (TRL9) and short-chain fatty acids (GPR109a and GPR43) are recognized by mucosal PRRs [50]. Given that bacteria reside in the lumen or associate with the outermost layer of the intestinal mucosa, how their components physically interact with host immune cells, particularly DCs, which play a critical role in directing immune responses, is only beginning to become understood.

DCs are crucial orchestrators of immune responses, bridging between the internal organism and the outward environment, as well as between the innate and adaptive components of the immune system. Intestinal $CD11c^+$ DCs are present throughout the lamina propria (LP) and gut-associated lymphoid tissues (GALT) and are highly adept at carrying out antigen-presenting functions and cytokine production both within the LP itself [45, 60] and upon migration to GALT [9, 115, 54] (see also **Table S0**). Crosstalk between DCs and intestinal bacteria or their components can be mediated in several ways, which convey the complexity of the intestinal milieu:

- Recruitment of $CD103^+$ DCs from the LP to the intestinal epithelium, followed by direct sampling of whole bacteria from the lumen. $CD103^+$ DCs were observed sending dendrites into the intestinal lumen and capturing bacteria, which could then be processed and presented in GALT [31].

- Luminal sampling of soluble antigens by $CX3CR1^+$ mononuclear phagocytes [88, 103,

95] or specialized intestinal epithelial cells known as goblet cells [89], and subsequent transfer to LP CD103$^+$ DCs. A recent study showed that CX3CR1$^+$ mononuclear phagocytes capture soluble antigens present in the gut lumen, and transfer them to CD103$^+$ DCs via gap junctions, channels that span between adjacent cells and allow intercellular communication [88]. Similarly, goblet cells were shown to deliver low molecular weight soluble antigens from the intestinal lumen to underlying CD103$^+$ LP DCs, a process they coined goblet-cell-associated antigen passages (GAPs) [89].

- A subset of intestinal DCs can carry bacteria located within infected apoptotic IECs, and migrate to T cell areas in GALT [129].

- Another route of bacterial delivery to DCs is through M cell-mediated transcytosis of luminal or epithelial-associated commensal bacteria. M cells are specialized epithelial cells that actively phagocytose molecules and microbes, and transcytose the internalized antigens [94]. One group observed DCs capturing GFP-tagged commensal E. coli in the M-cell pocket, and this mode of interaction contributed to the initiation of antigen-specific immune response in GALT [48].

- Outer membrane vesicles (OMVs) are bilayered lipid membrane vesicles that are naturally produced by Gram-negative bacteria, including commensals (reviewed in [67]). One group recently expanded on previous studies and revealed that *B. fragilis* releases PSA in OMVs, which can be recognized directly and internalized by DCs via TLR2 to promote Treg expansion and function [120].

- DCs can also be modulated indirectly through interactions with IECs, which come in close contact with luminal and epithelial-associated bacteria. IECs can detect bacterial products through PRRs, including TLRs, NLRs, RLRs and GPCRs (reviewed in [44, 50]). For example, serum amyloid A (SAA) production by IECs in response to epithelium-associated SFB induces IL-6 and IL-23 cytokine production in DCs iso-

lated from the LP [45]. Similarly, clostridia, which reside close to the epithelium, induce TGF-$\beta$ production in IECs to drive DC-mediated differentiation of Tregs in the colon [5].

- Bacterial metabolites, such as butyrate and proprionate which may be capable of diffusing through the epithelium [20], can be recognized via GPCRs on DCs and macrophages in the intestine [121]. They are also potent histone deacetylase (HDAC) inhibitors and have been shown to direct epigenetic modifications in DCs [4], as well as in T cells [35, 121]. An emerging theme from numerous studies is that of commensal-driven epigenetic modifications in innate (and adaptive) immune cells. Some reports point to decreased hypermethylation locally in the colon upon early exposure to commensals [100], others demonstrate changes in cells isolated from extra-intestinal sites, such as impaired binding of NF-$\kappa$B and IRF3 to their respective promoters in splenic DCs of germ-free mice, accompanied by absence of activating histone marks [39], genome-wide alterations in peritoneal macrophages from antibiotic-treated mice [1] and increased hypermethylation in lungs of germ-free mice [100]. Additional studies have investigated the effects of bacterial products in vitro. Butyrate has been shown to enhance acetylation at the *Foxp3* locus directly in T cells, as well as lead to hypo-acetylation of the *Relb* locus in DCs, skewing them toward a regulatory response.

How gut interactions impact on cells at extra-intestinal sites is a new area of intense investigation. Below are discussed potential mechanisms that may bridge between interactions that occur in the gut and systemic immune effects.

### *1.6.2 Linking host-commensal interactions in the gut to systemic effects on immunity*

Evidence for precise mechanisms that link commensal-host crosstalk in the gut and long-range regulation of extra-intestinal immune responses is lacking. However, some examples exist and it is possible to speculate about potential mechanistic connections.

- Direct dissemination of naked bacteria from the gut into peripheral organs. Given that intact barrier function of the gut should normally prevent this, this possibility seems to apply to situations in which this barrier has been compromised, such as in inflammatory bowel disease or drug-induced increases in intestinal permeability. As an example, cyclophosphamide has been shown to lead to bacterial dissemination with implications on distant anti-tumor immunity [134].

- Hematogenous or lymphatic spread of bacterial products from the gut. For example, peptidoglycans (PGs), a component of bacterial cell walls, were detected in the serum and bone marrow (BM) of E. coli-colonized germ free mice. In this case, PGs were recognized by NOD1 expressed on BM neutrophils, leading to enhanced killing function and increased protective immunity to *Streptococcus pneumoniae* and *Staphylococcus aureus* [17]. While this has yet to be shown, it is possible that components such as PSA, either naked or within OMVs, also spread to other organs, given that PSA-directed changes in DCs and T cells were observed in cervical lymph nodes [97, 98].

- Dissemination of bacteria contained within a host cell. Early studies showed that CD18-expressing cells of monocyte-macrophage lineage can transport bacteria from the gastrointestinal tract to the bloodstream, spleen and liver, consistent with the idea that innate immune cells can initiate systemic immune responses to intestinal microbes [133].

- Systemic dissemination of host-derived products elicited by host-commensal interactions in the gut. For example, TLR5-mediated sensing of gut commensals led to increases in IL-6 in the serum, accompanied by distant accumulation of MDSCs [110].

- Trafficking of primed T cells from GALT to peripheral lymph nodes and spleen. Commensal-driven specification of T cell subsets within the gut is well characterized. Given that T cells are capable of trafficking out of GALT to systemic lymphoid tissues [75], it is possible that systemic changes in T cell subsets is due to migration of these subsets from the gut, where they were imprinted, to other lymphatic sites. However, this possible mechanism is not likely to account for peripheral changes observed in innate immune cells.

- Trafficking of DCs from GALT to peripheral lymph nodes and spleen. As discussed above, DCs undergo many changes in the intestine in response to commensal bacteria. These functionally altered DCs could exit the intestine and circulate to distant sites, where they exert effects on adaptive immunity. While this possibility is highly controversial [86, 75], there is evidence to support DC trafficking between tissues. For example, subcutaneous administration of adjuvant led to substantial numbers of macrophages and DCs entering the efferent lymph [46] and subcutaneous injection of DCs resulted in T cell priming in the spleen [15]. These reports suggest that innate immune cells can traffic out of secondary lymphoid tissues and enter into the bloodstream to carry out distant functions.

## 1.7 Summary

The existence of multiple members of the microbiota that affect host immunity in different ways suggests that differences in the composition of this community may contribute to individual differences in immune responses during infection, autoimmunity or other immuno-

logical conditions, and importantly, cancer. Unveiling the cellular and molecular mechanisms of these interactions has the potential to lead to exciting new therapeutic interventions based on rational modulation of mucosal-driven immunity.

Our collective data from multiple experimental perspectives have indicated that transplantable tumor models are a very good system for representing the T cell-inflamed tumor microenvironment seen in a large subset of advanced human cancer patients [38]. They show a degree of innate immune activation [137] and spontaneous T cell priming [33], with T cells becoming dysfunctional over time [124], similar to T cell-inflamed human cancers. The therapeutic benefit with interventions blocking immune-inhibitory pathways that act within the tumor microenvironment, such as $\alpha$PD-1 antibodies, is largely seen in this subset of patients [123]. This working model provides a solid rationale for testing the effects of commensal microbial composition on anti-tumor immune responses. In the following chapters, data supporting a profound role of commensal microbes in shaping anti-tumor immunity in mice will be presented.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Animals and tumor model

C57BL/6 mice were obtained from Jackson laboratory or Taconic farms. For all experiments, 68-week-old female mice were used. Mice were fed Harlan Teklan 2018 diet and housed in the SPF University of Chicago animal facility. The C57BL/6-derived melanoma cell line B16.F10.SIY (henceforth referred to as B16.SIY) was generated as described [8]. The MB49 bladder cancer cell line was originally a generous gift from Timothy L. Ratliff, Purdue University. For tumor growth experiments, mice were injected subcutaneously with $1x10^6$ B16.SIY tumor cells or parental B16.F10 tumor cells. For bladder cancer model experiments, mice were injected subcutaneously with $2x10^6$ MB49 cells. Tumor size was measured twice a week until endpoint and tumor volume was determined as length x width$^2$ x 0.5. All experimental animal procedures were approved by the University of Chicago Animal Care and Use Committee (IACUC).

## 2.2 IFN-$\gamma$ ELISPOT and SIY Pentamer analyses

ELISPOT plates (Millipore, MAIP S4510) were coated with purified $\alpha$IFN-$\gamma$ (BD) overnight at 4 degrees. Plates were blocked with 10% FBS in DMEM for 2 hours at room temperature. Whole splenocytes were plated at $10^6$ cells per well and stimulated with SIY peptide overnight at 37$^o$C. Spots were developed using the BD mouse IFN-$\gamma$ kit (Cat. No. 552569), and the number of spots was measured using an Immunospot Series 3 Analyzer and analyzed using ImmunoSpot software (Cellular Technology). For pentamer staining, cells were labeled with PE-MHC class I pentamer (Proimmune) consisting of murine H-2Kb complexed to SIYRYYGL (SIY) peptide or to control SIINFEKL peptide, and stained with CD3-AX700 (Ebioscience, 17A2), CD8-PacBlue (Biolegend, 53-6.7), CD4-APC (Pharmin-

gen, RM4-5), CD62L-PECy7 (Ebioscience, MEL-14), CD44-FITC (BD, IM7) and Fixable Viability-ef780 (Ebioscience). Stained cells were analyzed using an LSR II cytometer with FACSDiva software (BD). Data analysis was conducted with FlowJo software (Tree Star).

## 2.3 Fecal transfers and $\alpha$PD-L1 mAb immunotherapy

Fecal pellets from JAX and TAC-derived mice were collected upon arrival in our facility and each fecal pellet was resuspended in 1 ml of phosphate-buffered saline (PBS). The suspension from each fecal pellet was used for oral gavage of two recipient mice, 100$\mu$l per gavage. For prophylactic fecal transfer experiments mice were gavaged with JAX or TAC fecal suspensions once a week for two weeks prior to tumor inoculation. For therapeutic fecal transfer experiments, mice were gavaged on days 7 and 14 post tumor implantation. For combination therapy experiments, mice were additionally injected intraperitoneally with 100$\mu$g $\alpha$PD-L1 mAb (clone 10f.9g2, BioXCell) in 100$\mu$l PBS on days 7, 10, 13 and 16 post-tumor implantation.

## 2.4 Microbial DNA analysis

Bacterial DNA was extracted from murine fecal pellets using PowerSoil-htp 96 Well Soil DNA Isolation Kit (MoBio cat.# 12955-4). The V4-V5 region of the 16S rRNA encoding gene was amplified (http://www.earthmicrobiome.org/emp-standard-protocols/; Earth Microbiome Project, 2011) and sequenced at the High-Throughput Genome Analysis Core at Argonne National Laboratory. Quantitative Insights Into Microbial Ecology (QIIME) was used to trim and classify sequences [14]; specifically, the open reference OTU picking protocol was used at 97% sequence identity against the Greengenes database (05/13 release) [90]. PYNAST was used to align sequences [13] and RDP Classifier was used for taxonomic assignment [135]. Community structure was compared using weighted and un-

weighted UniFrac distances [83]. All beta-diversity analyses were performed on data rarefied to 9662 reads and after filtering out OTUs occurring in less than 35% of samples per comparison. Non-parametric t-tests were performed to determine differences in bacterial taxa occurrence between fecal communities. Principal Coordinate Analysis (PCoA) ordination was generated to visually compare beta diversity and Analysis of Similarity (ANOSIM) test statistics were performed to statistically compare within- to between- group similarity in QIIME. Newick tree files comprised of OTUs deemed significant by t-test were constructed in iTOL [80]; bar graphs representing mean log fold differences in relative abundances were plotted for each taxon alongside the phylogeny, replacing zero values with the least non-zero value observed in the comparison. Association testing was performed via standard linear regression. We tested the association between frequency of $SIY^{+}CD8^{+}$ T cells in the tumor and taxa relative abundance. We used rarefied values and removed taxa that were missing in greater than 35% of the samples. The first two principal components of the relative abundance matrix were used as covariates in the model. Specifically we fit

$$SIY\ relative\ abundance_k + PC1 + PC2 \tag{2.1}$$

where k is the kth taxa. The association was fit with lm, principal components were calculated using the package PEER and FDR was computed using the package qvalue. This analysis was performed in R. OTU_681370 was characterized via NCBI Microbial Genomes Blast, using the V4-V5 16s rRNA sequence:

TACGTAGGGTGCAAGCGTTATCCGGATTTATTGGGCGTAAAGGGCTCGTAGG
CGGTTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAACGGTGGATCCGCGCC
GGGTACGGGCGGGCTTGAGTGCGGTAGGGGAGACTGGAATTCCCGGT.

## 2.5 Bacterial administration and heat inactivation

A cocktail of lyophilized *Bifidobacterium* species (*B. bifidum, B. longum, B. lactis* and *B. breve*, Seeking Health) was resuspended in PBS at 5x$10^9$ CFU/ml. Each mouse was given 200$\mu$l of *Bifidobacterium* (1x$10^9$ CFU/mouse) by oral gavage 7 and 14 days following tumor inoculation. Heat inactivation was performed by boiling rehydrated bifidobacteria at 100°C for 2 hours. Heat-treated and live bifidobacteria were serially diluted in reduced PBS and plated on reduced clostridial medium (RCM) agar in anaerobic conditions. Plates were subsequently incubated in an anaerobic chamber for three days to test efficacy of killing. For gavage of ATCC-derived bifidobacterial cultures, ATCC 15700 *B. breve* and ATCC BAA-999 *B. longum* were cultured in RCM in an anaerobic chamber and monitored for log-phase growth using spectrophotometric measurements of turbidity every hour. At measurements of ≈0.5, cultures were pelleted, resuspended at 5x$10^9$ cells/ml PBS (as assessed by serial dilution and quantification of colony forming units, following plating on RCM agar plates and incubation in an anaerobic chamber for 48 hours) and gavaged into mice, 200$\mu$l/mouse, 1x$10^9$ cells/mouse. *Lactobacillus murinus* (provided by Yuan Zhang and Yang-Xin Fu at University of Chicago) was cultured in MRS broth overnight, then washed and resuspended in PBS at 5x$10^{10}$ CFU/ml. Each mouse was orally gavaged with 100$\mu$l of bacterial suspension (5x$10^9$ CFU/mouse) 7 and 14 days following tumor inoculation.

## 2.6 $CD8^+$ T Cell Depletion

For depletion of $CD8^+$ T cells, mice were injected intraperitoneally weekly with rat mAb anti-mouse CD8 (43.2) or isotype control IgG2b (BioXcell) at a dose of 250 $\mu$g per mouse. These regimens resulted in $>$ 99% depletion of $CD8^+$ T cells from the peripheral blood, as evaluated by flow cytometry.

## 2.7 Bacterial quantitation in peripheral organs and fecal samples

Mesenteric lymph nodes, tumor and spleen were sterilely removed, homogenized in PBS and strained through a 70$\mu$m filter. The cells were pelleted, resuspended in QIAGEN buffer ALT, and underwent Qiashredding (QIAGEN) to further lyse cells and reduce the viscosity. DNA was purified from the flow through using QIAGEN DNA mini purification kit. For spiking experiments, organs were isolated as described above and spiked with $10^9$, $10^6$, $10^3$, $10^2$ and 10 ATCC 15700 *B. breve* cells, as assessed by serial dilution of anaerobically cultured bacteria, incubating 48 hours on RCM agar plates in anaerobic conditions, and counting colony forming units. qPCR was used to quantify bacterial SSU (16S) rRNA gene abundance, in the feces and tissue as described previously [7, 28, 34]. Primer sets targeting SSU rRNA genes of bifidobacteria at the genus level were used and included BifidF (CGG GTG AGT AAT GCG TGA CC), and BifidR (TGA TAG GAC GCG ACC CCA) and probe (6FAM-CTC CTG GAA ACG GGT G) [34]. Primers and probe were synthesized by IDT and Invitrogen respectively. qPCR master mixes contained TaqMan Universal qPCR 1X Master Mix (Applied-Biosystems), 0.25M primers and probe, and either 4ng (feces) or 25ng (tissue) gDNA template. Purified genomic DNA from reference bacteria *Bifidobacterium breve* ATCC 15700D-5 was used as a standard. Cycling conditions were 50°C for 2min, 95°C for 10min, and 45 cycles of 95°C for 15sec, 60°C for 60sec using Roche Lightcycler 480 real time PCR machine. A melt curve was performed for quality assurance and efficiencies ranged under the accepted values, 90-110%. Data were normalized as copies of bacterial 16S per ng DNA.

## 2.8 Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled 2C CD8$^+$ T cell adoptive transfer

CD8$^+$ T cells were isolated from the spleen and lymph node of naive CD45.1/.2$^+$ 2C TCR Tg mice using the MACS CD8 T cell Isolation Kit (Miltenyi, Cat No. 130-095-236), labeled with 2.5 mM CFSE and injected i.v. into CD45.2$^+$ C57BL/6 mice derived from either JAX or TAC and into TAC mice gavaged with *Bifidobacterium* once a week for two weeks. 24 hours later, mice were inoculated with 1x10$^6$ B16.SIY melanoma cells s.c. Seven days post-adoptive T cell transfer, spleen and tumor-draining lymph node were harvested and restimulated ex-vivo with SIY peptide in the presence of brefeldin A. Samples were stained with Fixable Viability-ef780 (Ebioscience), CD45.1-PerCPCy5.5 (Ebioscience, E20), CD45.2-APC (Ebioscience, 104), CD3-AX700 (Ebioscience, 17A2), CD8-BV711 (Biolegend, 53-6.7), CD4-BV605 (Biolegend, RM4-5) and IFN-$\gamma$-PE (BD, XMG1.2). Intracellular IFN-$\gamma$ production and CFSE dilution were assessed in gated CD45.1/.2$^+$ 2C CD8$^+$ T cells by flow cytometry.

## 2.9 Dendritic cell sorting and gene expression profiling

Upon arrival in our facility, TAC mice were gavaged with *Bifidobacterium* once a week for two weeks. *Bifidobacterium*-fed mice, newly arrived JAX mice, and newly arrived TAC mice were inoculated subcutaneously in both flanks with 5x10$^6$ DRAQ5-labeled B16.SIY tumor cells. 40hrs following tumor implantation, whole tumors including infiltrating immune cells were digested in collagenase (Worthington) and filtered into single cell suspensions. Samples from 5 mice in each group were pooled and subsequently stained with Fixable Viability-ef506 (Ebioscience), CD45-AF488 (Bioloegend, 30-F11), CD3-ef450 (Ebioscience, 145-2C11), CD19-PB (Ebioscience, 1D3), I-A/I-E-PECy7 (Biolegend, M5/114.15.2), CD11c-PE (Ebioscience, N418) and CD11b-PerCpCy5.5 (BD, M1/70). Live CD45$^+$CD3$^-$CD19$^-$MHCII$^{hi}$CD11c$^+$ den-

dritic cells were sorted directly into RLT Buffer (Qiagen) using FACSAriaIII (BD) and stored immediately on dry ice. Total RNA was isolated using RNeasy Micro kit (Qiagen). RNA was submitted to the Functional Genomics Facility at the University of Chicago for gene expression profiling. RNA integrity and concentration were assessed using an Agilent Bioanalyzer 2100, and all RNA samples used for microarray analysis had an RNA Integrity Number $> 9.0$. Total RNA was processed into biotinylated cRNA using the Epicentre TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0 (TAB2R71024). The cRNA was hybridized to Illumina MouseRef8v2 arrays using Illumina provided protocols and scanned using an Illumina HiScan. Quantile normalized and background subtracted values were subsequently analyzed using R. Genes whose expression value was under 10 were removed from the analysis. Mean fold-change in gene transcript levels between JAX samples relative to TAC, and BIF samples relative to TAC were calculated, and genes whose fold-change was over 1.5 in both comparisons (760 gene transcripts) were inputted into The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 for pathway analysis. Genes found to be significantly enriched ($p<0.05$) for immune function were then plotted in a heatmap using R software.

## 2.10 qRT-PCR validation of gene-expression profiling

DCs were sorted and RNA extracted as described above. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was measured by real-time qRT-PCR using specific primers/probes (Roche Universal Probe library, **Table S7**). PCR was performed using 7300 Real Time PCR machine (Applied Biosystems). The results are expressed as fold-change over TAC, normalized to 18S expression.

## 2.11 BMDC stimulation with *Bifidobacterium* in vitro

Cells isolated from the tibiae and femurs of WT C57BL/6 mice were cultured in DMEM medium containing 10% FBS and 1% penicillin/streptomycin, in the presence of rmGM-CSF (20 ng/ml; BioLegend) for 8 days at 37C with 5% CO2. BMDCs were then stimulated for 4 hours with medium alone or with rehydrated *Bifidobacterium*-containing medium at a ratio of 1:10 BMDCs to bacterial cells. Total RNA was isolated using RNeasy Micro kit (Qiagen) and submitted to the Functional Genomics Facility at the University of Chicago for gene expression profiling. RNA integrity and concentration were assessed using an Agilent-Bioanalyzer 2100, and all RNA samples used for microarray analysis had an RNA Integrity Number = 10.0.

## 2.12 Dendritic cell stimulation of CFSE-labeled 2C CD8$^+$ T cells in vitro

Upon arrival in our facility, TAC mice were gavaged with *Bifidobacterium* once a week for two weeks. DCs were purified from peripheral lymphoid tissues of CD45.2$^+$ C57BL/6 naive *Bifidobacterium*-fed mice, newly arrived JAX mice, and newly arrived TAC using Pan Dendritic cell Isolation Kit (Miltenyi, Cat No. 130-100-875). 2x10$^5$ DCs were plated 1:1 with CD45.1/.2$^+$ CFSE- labeled 2C CD8$^+$ T cells (MACS CD8$^+$ T cell Isolation Kit (Miltenyi, Cat No. 130-095-236) and incubated at 37°C for 72 hours in the presence of varying concentrations of SIY peptide. Brefeldin A was added for 6 additional hours. Samples were stained with Fixable Viability-ef780 (Ebioscience), CD45.1-PerCPCy5.5 (Ebioscience, E20), CD45.2-APC (Ebioscience, 104), CD3-AX700 (Ebioscience, 17A2), CD8-BV711 (Biolegend, 53-6.7), CD4-BV605 (Biolegend, RM4-5) and IFN-$\gamma$-PE (BD, XMG1.2). Intracellular IFN-$\gamma$ production and CFSE dilution were assessed in gated CD45.1/.2$^+$ 2C CD8$^+$ T cells by flow cytometry.

## 2.13 Statistical analysis

Tumor growth curves were analyzed using two-way ANOVA, with either Sidak's multiple comparisons post test for comparison of two groups, Dunnett's multiple comparisons post test for comparison between multiple groups and a control group, or Tukey's multiple comparisons post test for comparison of more than two groups to each other. For other comparisons, unpaired Student's $t$-test was used when comparing two groups and one-way ANOVA with Holm Sidak correction for multiple testing was used when comparing more than two groups. Microbial composition comparisons were performed using non-parametric $t$-tests. If samples were not independent (technical replicates of pooled samples), linear mixed model regression was used with Bonferroni correction for multiple testing. $p<0.05$ was considered statistically significant and denoted as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Statistical analysis was performed using GraphPad PRISM and R.

# CHAPTER 3

# THE COMMENSAL MICROBIOTA SHAPE ANTI-TUMOR IMMUNITY

## 3.1 Introduction

Harnessing the host immune system constitutes a promising approach for the treatment of cancer because of its potential to specifically target tumor cells while limiting harm to normal tissue, with durability of benefit associated with immunologic memory. Enthusiasm has been fueled by recent clinical success, particularly with antibodies that block immune inhibitory pathways, specifically CTLA-4 and the PD-1/PD-L1 axis [47, 52]. Early data have indicated that clinical responses to these immunotherapies are more frequent in patients who show evidence of an endogenous T cell response ongoing in the tumor microenvironment at baseline [37, 61, 124, 130]. Despite the functional and clinical importance of this T cell-inflamed tumor microenvironment, the mechanisms that govern the presence or absence of this phenotype are not well understood. Theoretical sources of inter-patient heterogeneity include germline genetic differences at the level of the host, variability in patterns of somatic alterations in tumor cells, and environmental differences with the potential to impact on systemic immunity.

A growing body of data has indicated an important role for gut microbiota in shaping systemic immune responses. Commensal bacteria have been shown to influence the differentiation of T cells leading to expansion of specific molecular subsets with phenotypic implications [57, 87, 108, 138]. For example, segmented filamentous bacteria have been shown to promote preferential Th17 differentiation in mice [45, 57]. Commensal bacteria also have been reported to modulate the activation threshold of innate immune cells, leading to altered systemic immunity to viral infections [1, 39]. In the cancer context, a role for intestinal microbiota in mediating immune activation in response to chemotherapeutic

agents has been demonstrated [56, 134]. However, whether commensal microbiota influence spontaneous immune responses against tumors, thereby impacting therapeutic activity of immunotherapeutic interventions such as $\alpha$PD-1/PD-L1 mAbs, is not known.

## 3.2 Differences in melanoma outgrowth and tumor-specific immune responses between C57BL/6 JAX and TAC mice are eliminated upon cohousing

To test whether differences in the specific composition of the normal microbiota influence the immune response to a growing tumor in vivo, we compared subcutaneous B16.SIY melanoma growth in genetically similar C57BL/6 mice derived from two different mouse facilities, Jackson Laboratory (JAX) and Taconic Farms (TAC), which have been shown to differ in their commensal microbes [57]. We found that JAX and TAC mice exhibited significant differences in B16.SIY melanoma growth rate, with tumors growing more aggressively in TAC mice **(Figure 3.1A)**. To evaluate whether this difference was immune-mediated, tumor antigen-specific T cell responses, as well as T cell accumulation in the tumor microenvironment, were assessed. In fact, tumor-specific T cell responses were significantly higher in JAX mice **(Figure 3.1B and Figure 3.1C)**, and markedly increased numbers of tumor-infiltrating T cells were observed **(Figure 3.1D)**.

To begin to address whether this difference could be mediated by commensal microbiota, JAX and TAC mice were co-housed for 3 weeks prior to tumor implantation. We found that co-housing ablated the differences in tumor growth **(Figure 3.1E)** and immune responses **(Figure 3.1F-H)** between the two mouse populations, arguing for an environmental influence. Notably, TAC mice appeared to acquire the JAX phenotype upon cohousing, suggesting that JAX mice might be colonized by commensal microbes that dominantly facilitate improved anti-tumor immunity.

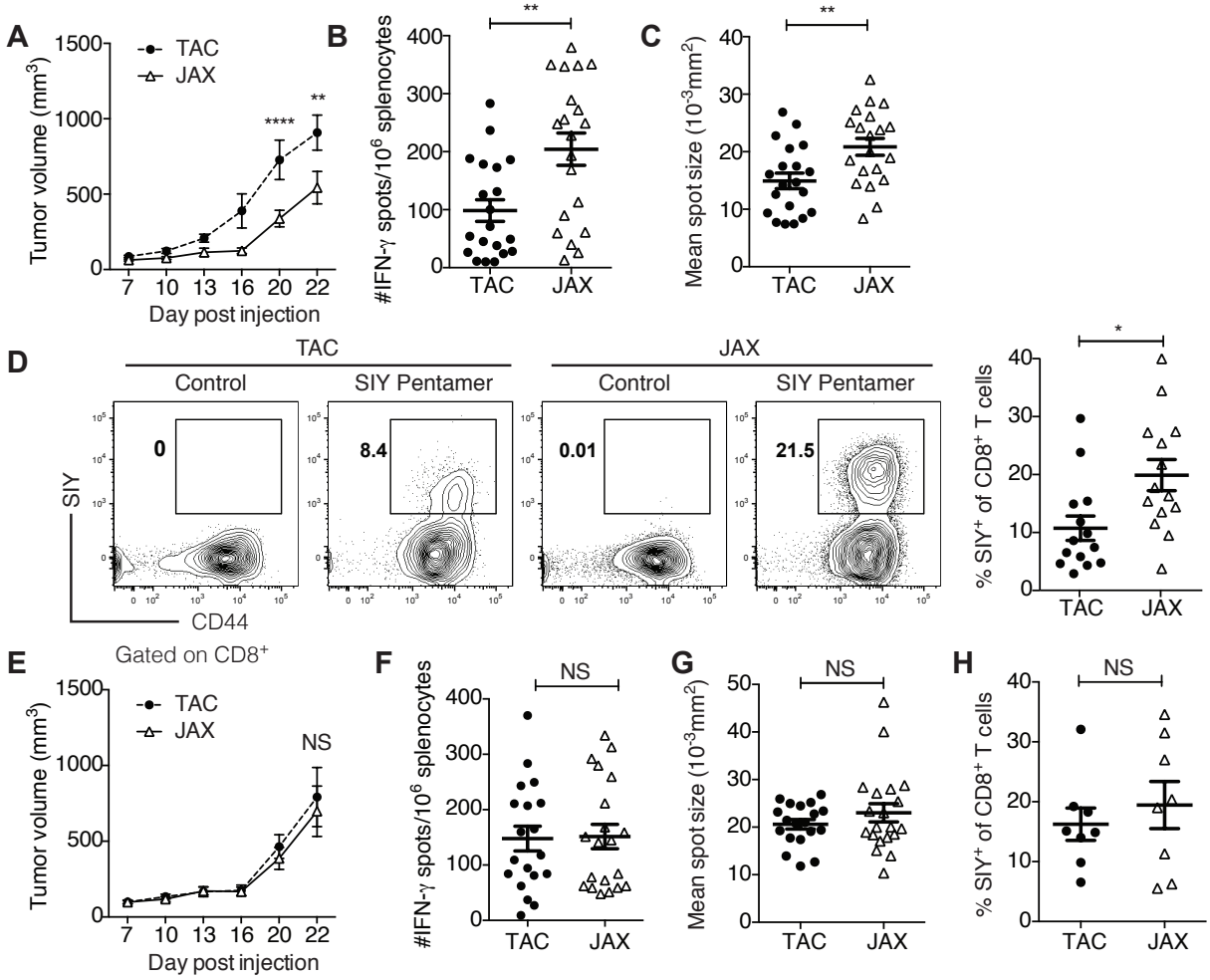


Figure 3.1: **C57BL/6 JAX and TAC mice exhibit significant differences in melanoma outgrowth and tumor-specific immune responses.** **(A)** B16.SIY tumor growth kinetics in newly arrived JAX and TAC mice. **(B)** Number of IFN-$\gamma$ spots/$10^6$ splenocytes in tumor-bearing JAX and TAC mice 7 days following tumor inoculation. **(C)** Mean IFN-$\gamma$ spot size ($10^{-3}$ mm$^2$) **(D)** Percentage of SIY$^+$ T cells of total CD8$^+$ T cells within the tumor of JAX and TAC mice as determined by flow cytometry 21 days post-tumor inoculation. Representative plots (left), quantification (right). **(E)** B16.SIY tumor growth kinetics in JAX and TAC mice cohoused for 3 weeks prior to tumor inoculation. **(F)** Number of IFN-$\gamma$ spots/$10^6$ splenocytes in tumor-bearing JAX and TAC mice cohoused for 3 weeks prior to tumor inoculation. **(G)** Mean IFN-$\gamma$ spot size ($10^{-3}$ mm$^2$). **(H)** Percentage of SIY$^+$ T cells of total CD8$^+$ T cells within the tumor of JAX and TAC mice cohoused for 3 weeks prior to tumor inoculation. Data show mean +/- SEM combined from six independent experiments, analyzed by two-way analysis of variance (ANOVA) with Sidaks correction for multiple comparisons [(A) and (E)], or individual mice with mean +/- SEM combined from four [(B), (C), (F), (G)] or three [(D) and (H)] independent experiments, analyzed by Students $t$ test; 5 mice per group per experiment; **P $< 0.01$, ****P $< 0.0001$, NS, not significant.

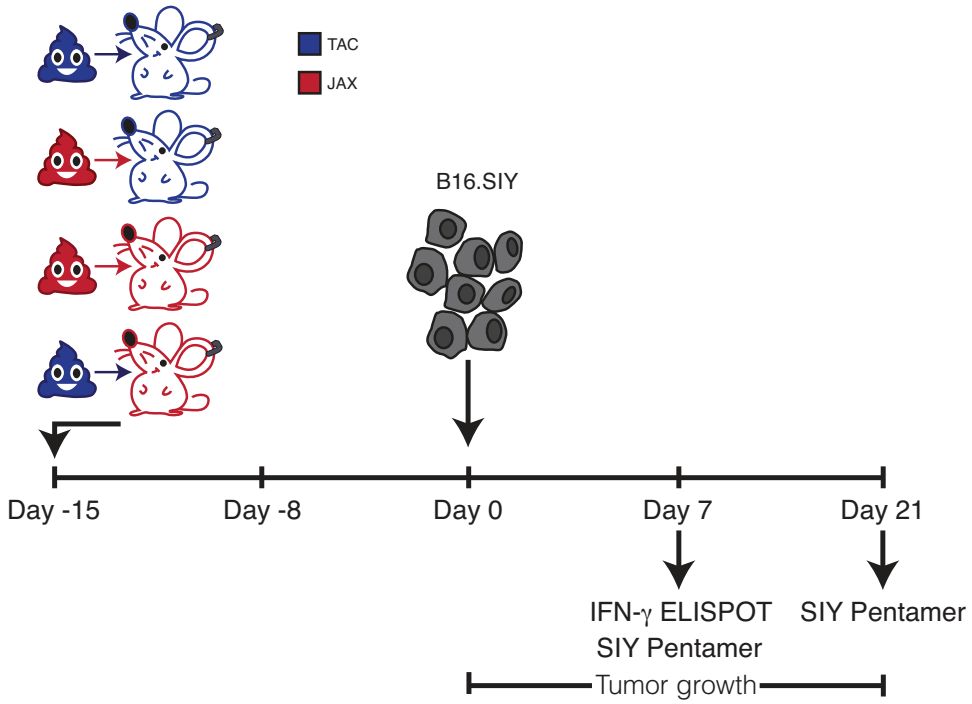


Figure 3.2: **Schematic of prophylactic fecal transfer.** Fecal pellets collected from JAX and TAC mice upon arrival in our facility were resuspended in PBS, homogenized and the suspension was introduced by oral gavage into either JAX or TAC recipients as shown, once a week for two weeks prior to B16.SIY tumor inoculation.

## 3.3 Oral administration of JAX fecal material to TAC mice enhances spontaneous anti-tumor immunity and response to $\alpha$PD-L1 mAb therapy

To directly test the role of commensal bacteria in regulating anti-tumor immunity, we transferred JAX fecal suspensions or TAC fecal suspensions into TAC and JAX recipients by oral gavage prior to tumor implantation **(Figure 3.2)**. Strikingly, we found that prophylactic transfer of JAX fecal material into TAC recipients was sufficient to delay tumor growth **(Figure 3.3A)** and to enhance induction and infiltration of tumor-specific $CD8^+$ T cells **(Figure 3.3B-D)**, supporting a microbe- or microbial product-derived effect. Importantly,

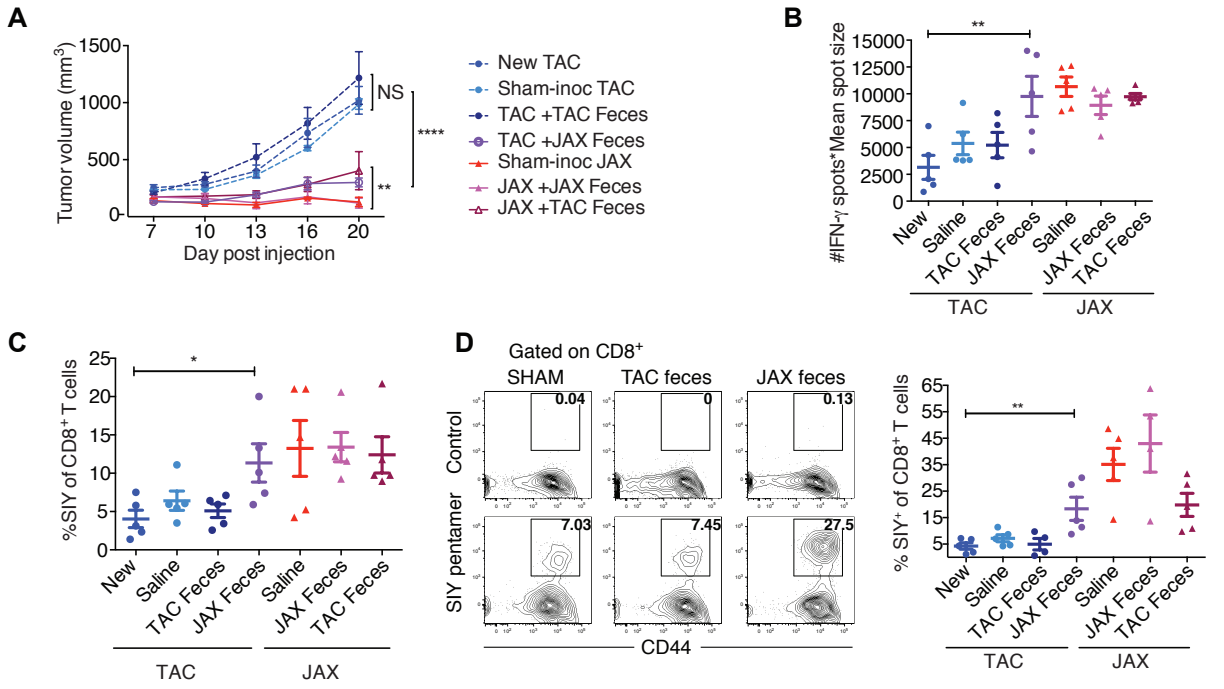


Figure 3.3: **Oral administration of JAX fecal material to TAC mice enhances spontaneous anti-tumor immunity.** **(A)** B16.SIY tumor growth in newly arrived TAC mice, TAC and JAX mice orally gavaged with PBS, TAC or JAX fecal material prior to tumor implantation. **(B)** Number of IFN-$\gamma$ spots x mean spot size ($10^{-3}$ $mm^2$), determined by ELISPOT 7 days following tumor inoculation. **(C)** Percentage of $SIY^+$ $CD8^+$ T cells within the tumor of TAC and JAX mice treated as in (A), 7 days post-tumor inoculation. **(D)** Percentage of $SIY^+$ $CD8^+$ T cells within the tumor of TAC and JAX mice treated as in (A), 21 days post-tumor inoculation. Representative plots (left), quantification (right). Data show mean +/- SEM analyzed by two-way ANOVA with Dunnetts correction for multiple comparisons (A); or individual mice with mean +/- SEM analyzed by one-way ANOVA with Holm-Sidak correction for multiple comparisons [(B) - (D)]; data are representative of two to four independent experiments; 5 mice per group per experiment; *P $<$ 0.05, **P $<$ 0.01, ****P $<$ 0.0001, NS, not significant.

the effects of housing the mice in the University of Chicago vivarium alone did not result in altered tumor growth or tumor-specific immune responses **(Figure 3.3A-D)**. Reciprocal transfer of TAC fecal material into JAX recipients had a minimal effect on tumor growth rate **(Figure 3.3A)**, and anti-tumor T cell responses **(Figure 3.3B-D)**, consistent with the JAX-dominant effects observed upon co-housing.

To test whether manipulation of the microbial community could be effective as a therapy, we utilized TAC mice bearing established tumors and administered JAX fecal material alone or in combination with $\alpha$PD-L1. Transfer of JAX fecal material alone resulted in significantly slower tumor growth **(Figure 3.4A)**, accompanied by increased tumor-specific T cell responses **(Figure 3.4B)** and infiltration of antigen-specific T cells into the tumor **(Figure 3.4C)**, to the same degree as treatment with systemic $\alpha$PD-L1 mAb. Combination treatment with both JAX fecal transfer and $\alpha$PD-L1 mAb dramatically improved tumor control **(Figure 3.4A)** and circulating tumor antigen-specific T cell responses **(Figure 3.4B)**, while there was little additive effect on accumulation of activated T cells within the tumor microenvironment, with all treatments achieving high frequencies of antigen-specific T cell infiltration by three weeks post tumor implantation **(Figure 3.4C)**. Given these results, we hypothesized that JAX mice, which harbor the protective microbes, would respond better to $\alpha$PD-L1 therapy alone as compared to TAC mice. Indeed, $\alpha$PD-L1 therapy alone was significantly more efficacious in JAX mice compared to TAC mice **(Figure 3.4D)**, which paralleled improved anti-tumor T cell responses **(Figure 3.4E)**. These data indicate that the commensal microbial composition can influence spontaneous anti-tumor immunity as well as response to immunotherapy with $\alpha$PD-L1 mAb.

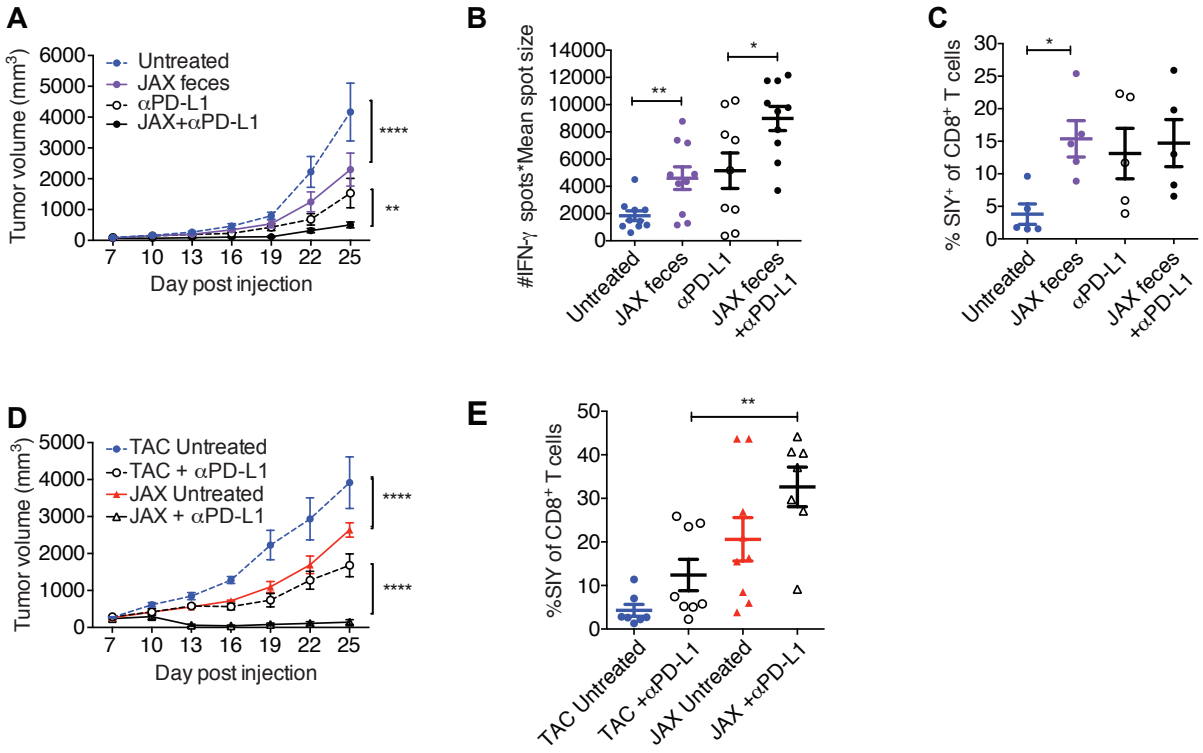


Figure 3.4: **Oral administration of JAX fecal material to tumor-bearing TAC mice enhances spontaneous anti-tumor immunity and response to $\alpha$PD-L1 mAb. (A)** B16.SIY tumor growth in TAC mice, untreated or treated with JAX fecal material 7 and 14 days post tumor implantation, $\alpha$PD-L1 mAb 7, 10, 13 and 16 days post tumor implantation, or both regimens. **(B)** IFN-$\gamma$ ELISPOT assessed 5 days after start of treatment. **(C)** Percentage of tumor-infiltrating SIY$^+$ CD8$^+$ T cells, determined by flow cytometry 14 days after start of treatment. **(D)** B16.SIY tumor growth kinetics in TAC and JAX mice, untreated or treated with $\alpha$PD-L1 mAb 7, 10, 13 and 16 days post tumor implantation. **(E)** Percentage of SIY$^+$ T cells of total CD8$^+$ T cells within the tumor of JAX and TAC mice, untreated or treated with $\alpha$PD-L1 mAb, as determined by flow cytometry 21 days post-tumor inoculation. Data show mean +/- SEM analyzed by two-way ANOVA with Tukeys correction for multiple comparisons [(A) and (D)]; or individual mice with mean +/- SEM analyzed by one-way ANOVA with Holm-Sidak correction for multiple comparisons [(B), (C), (E)]; data are representative of [(C) and (D)] or combined from [(A), (B) and (E)] two to four independent experiments; 5 mice per group per experiment; *P $<$ 0.05, **P $<$ 0.01, ****P $<$ 0.0001, NS, not significant.

# CHAPTER 4

# MECHANISMS OF COMMENSAL-REGULATED ANTI-TUMOR IMMUNITY

## 4.1 Direct administration of *Bifidobacterium* to TAC recipients with established tumors improves tumor-specific immunity and response to $\alpha$PD-L1 mAb therapy

To identify specific bacteria associated with improved anti-tumor immune responses, we monitored the fecal bacterial content over time of mice that were subjected to administration of fecal permutations, using the 16S ribosomal RNA (rRNA) miSeq Illumina platform. Principal coordinate analysis revealed that fecal samples analyzed from TAC mice that received JAX fecal material gradually separated from samples obtained from sham-inoculated TAC mice as well as TAC-inoculated TAC mice over time and became remarkably similar to samples obtained from sham-inoculated JAX mice as well as JAX-fed JAX mice **(Figure 4.1A)**. Analysis of similarity confirmed that TAC mice fed with JAX fecal material harbored significantly different microbes from those characterizing sham-inoculated or TAC-inoculated TAC mice (p=0.001 and p=0.003, ANOSIM, respectively), whereas TAC-inoculated TAC mice did not change in community diversity relative to sham-inoculated TAC mice (p=0.4, ANOSIM). Reciprocal transfer of TAC fecal material into JAX hosts resulted in a statistically significant change in community diversity (p=0.003, ANOSIM), yet the amplitude (distance) of the microbial shift was smaller **(Figure 4.1A)**.

Comparative analysis of specific bacterial taxa showed that 257 taxa were of significantly different relative abundance in newly arrived JAX mice relative to newly arrived TAC mice (FDR<0.05, non parametric *t*-test) **(Figure 4.1B)**, 284 taxa were of significantly different relative abundance in JAX-fed TAC mice relative to sham-inoculated TAC mice (FDR<0.05,

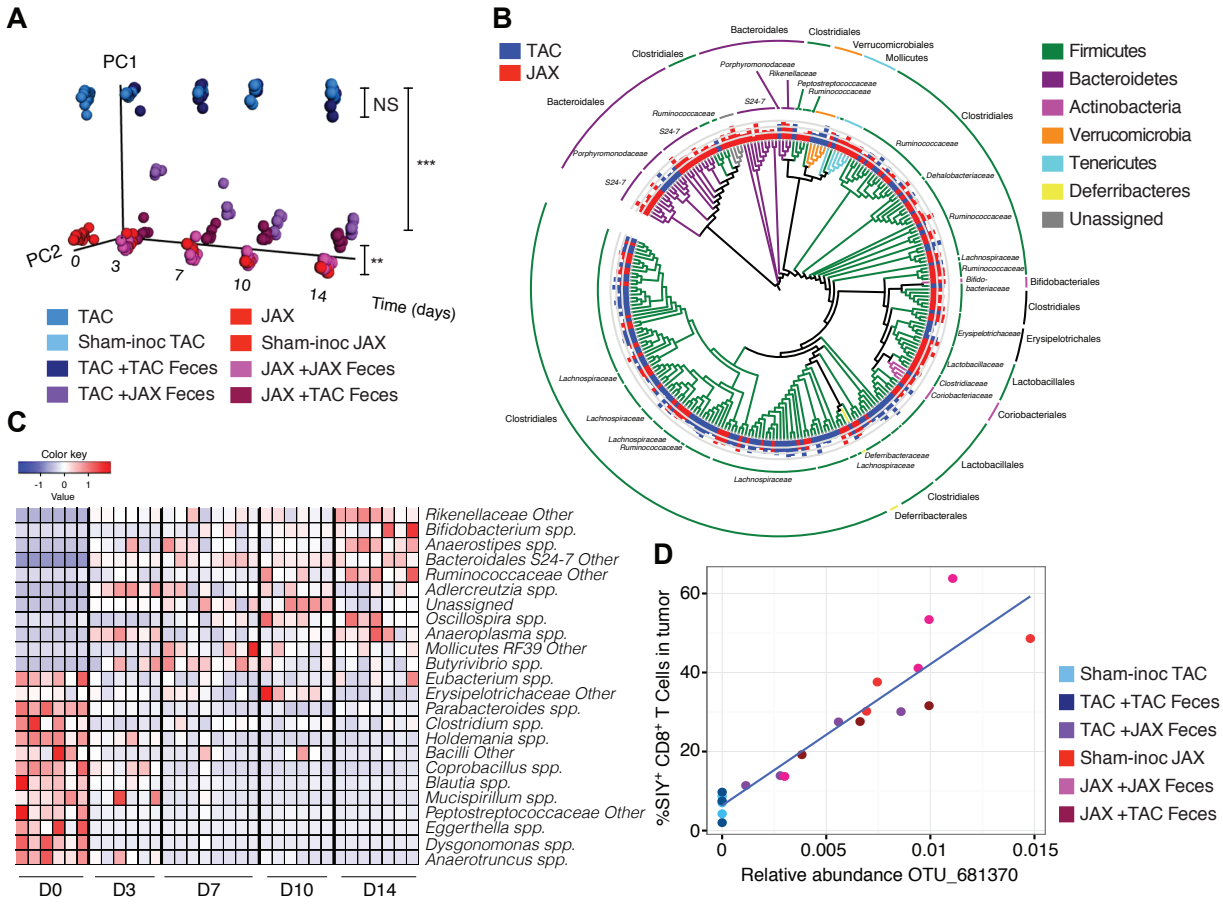


Figure 4.1: **16S rRNA sequencing reveals *Bifidobacterium* as associated with improved anti-tumor immunity.** **(A)** Principal coordinate analysis plot of bacterial $\beta$-diversity over time in groups treated as in Figure 3.3A, each group is comprised of at least two cages, 3-4 mice per cage; data represent three independent experiments; **P $<$ 0.01, ***P $<$ 0.001 (ANOSIM). **(B)** Phylogenetic analysis of taxa that are of significantly different abundance in newly arrived JAX vs TAC mice FDR$<$0.05 (non-parametric $t$ test); bars represent log-transformed fold changes, inner circle=$\log_{10}(10)$; middle circle=$\log_{10}(100)$; outer circle=$\log_{10}(1000)$. **(C)** Heatmap demonstrating relative abundance over time of significantly altered genus-level taxa in JAX-fed TAC mice FDR$<$0.05 (non-parametric $t$ test); columns depict individual mice; each timepoint shows mice from two separate cages, 3-4 mice per cage. **(D)** Correlation plot of relative abundance of *Bifidobacterium* OTU_681370 in fecal material obtained from groups as in (A) on day 14 post arrival and frequency of SIY$^+$ CD8$^+$ T cells in tumor; p=1.4x10$^{-5}$, FDR=0.0002, R2=0.86 (univariate regression).

non parametric $t$-test), and 254 taxa were of significantly different relative abundance in JAX-fed TAC mice relative to TAC-inoculated TAC mice (FDR<0.05, non parametric $t$-test) **(Table S1)**. Members belonging to several taxonomic groups overlapped between these comparisons **(Figure 4.1C** and **Table S2**). Among the significantly altered groups, *Bifidobacterium spp.*, Bacteroidales families *Rikenellaceae* and *S24-7*, Clostridiales member *Anaerostipes spp.* and unclassified taxa from the class Mollicutes showed the highest increases in abundance in JAX-fed TAC mice. Among the taxa that decreased with administration of JAX feces were *Mucispirillum spp.* of the order Deferribacterales, *Peptostreptococcace*, *Anaerotruncus spp.* and *Blautia spp.* of the order Clostridiales, *Coprobacillus spp.* of the order Erysipelotrichales and the Bacteroidales *Dysgonomonas spp.*

To glean further insight into possible functional roles among altered bacterial taxa, we asked which genus-level taxa were significantly associated with accumulation of activated antigen-specific T cells within the tumor microenvironment across all permutations (see Figure 3.3D). The only significant association was *Bifidobacterium* ($p=5.7x10^{-5}$, FDR=0.0019, univariate regression) **(Table S3)**, which showed a positive association with anti-tumor T cell responses and increased in relative abundance over 400-fold in JAX-fed TAC mice over the course of 14 days **(Figure 4.1C)**. Stimulatory interactions between bifidobacteria and the host immune system, including those supporting IFN-$\gamma$-associated immune responses, have been described previously [24, 68, 82, 91]. Given this broad statistical association and support from other studies, we hypothesized that members of this genus could represent a major component of the beneficial anti-tumor immune effects observed in JAX mice.

At the sequence level, operational taxonomic unit OTU_681370 belonging to the *Bifidobacterium* genus showed the largest increase in relative abundance in JAX-fed TAC mice **(Table S1)** and was the top significantly associated taxon with the anti-tumor T cell response across all permutations ($p=1.4x10^{-5}$, FDR=0.0002, $R^2=0.86$, univariate regression) **(Table S3)**. Relative abundance of OTU_681370 essentially mirrored the pattern of in-

filtration of activated antigen-specific T cells into the tumor microenvironment across all permutations **(Figure 4.1D)**. To further characterize this taxon we blasted the V4-V5 16S rRNA sequence of OTU_681370 using NCBI Microbial Genomes Blast and found that it was most similar to *B. breve*, *B. longum* and *B. adolescentis* (99% identity).

To test whether *Bifidobacterium spp.* may be sufficient to augment protective immunity against tumors, we obtained a commercially available cocktail of *Bifidobacterium* species, which included both *B. breve* and *B. longum* and administered this by oral gavage, alone or in combination with $\alpha$PD-L1, to TAC recipients bearing 7-day established tumors. 16S rRNA sequencing of fecal material collected from TAC mice 7 days following *Bifidobacterium* inoculation showed that the most significant increase in relative abundance occurred in the *Bifidobacterium* genus (p=0.0009, FDR=0.015, non parametric *t*-test), with a 120- fold increase in OTU_681370 **(Figure 4.2A** and **Table S4)**, suggesting that the commercial inoculum contained bacteria that were at least 97% identical to the taxon identified in JAX and JAX-fed TAC mice. A marked increase in *Bifidobacterium* could also be detected by qPCR using *Bifidobacterium* genus-specific primers **(Figure 4.2B)**.

*Bifidobacterium*-treated mice displayed significantly improved tumor control in comparison to non-*Bifidobacterium* treated counterparts **(Figure 4.2C)**, which was accompanied by robust induction of tumor-specific T cells in the periphery **(Figure 4.2D)** and markedly increased accumulation of antigen-specific $CD8^+$ T cells within the tumor **(Figure 4.2E)**. TAC mice challenged with tumor six weeks following oral administration of *Bifidobacterium* maintained increased levels of *Bifidobacterium* in their feces relative to untreated TAC mice, as well as the ability to significantly delay tumor outgrowth **(Figure 4.3A)**, suggesting that the effects of *Bifidobacterium* inoculation are durable for several weeks. The therapeutic effect of *Bifidobacterium* feeding was completely abrogated in CD8-depleted mice **(Figure 4.3B)**, arguing that the mechanism was not direct but rather through host anti-tumor T cell responses. Heat inactivation of the bacteria prior to oral administration also abrogated the

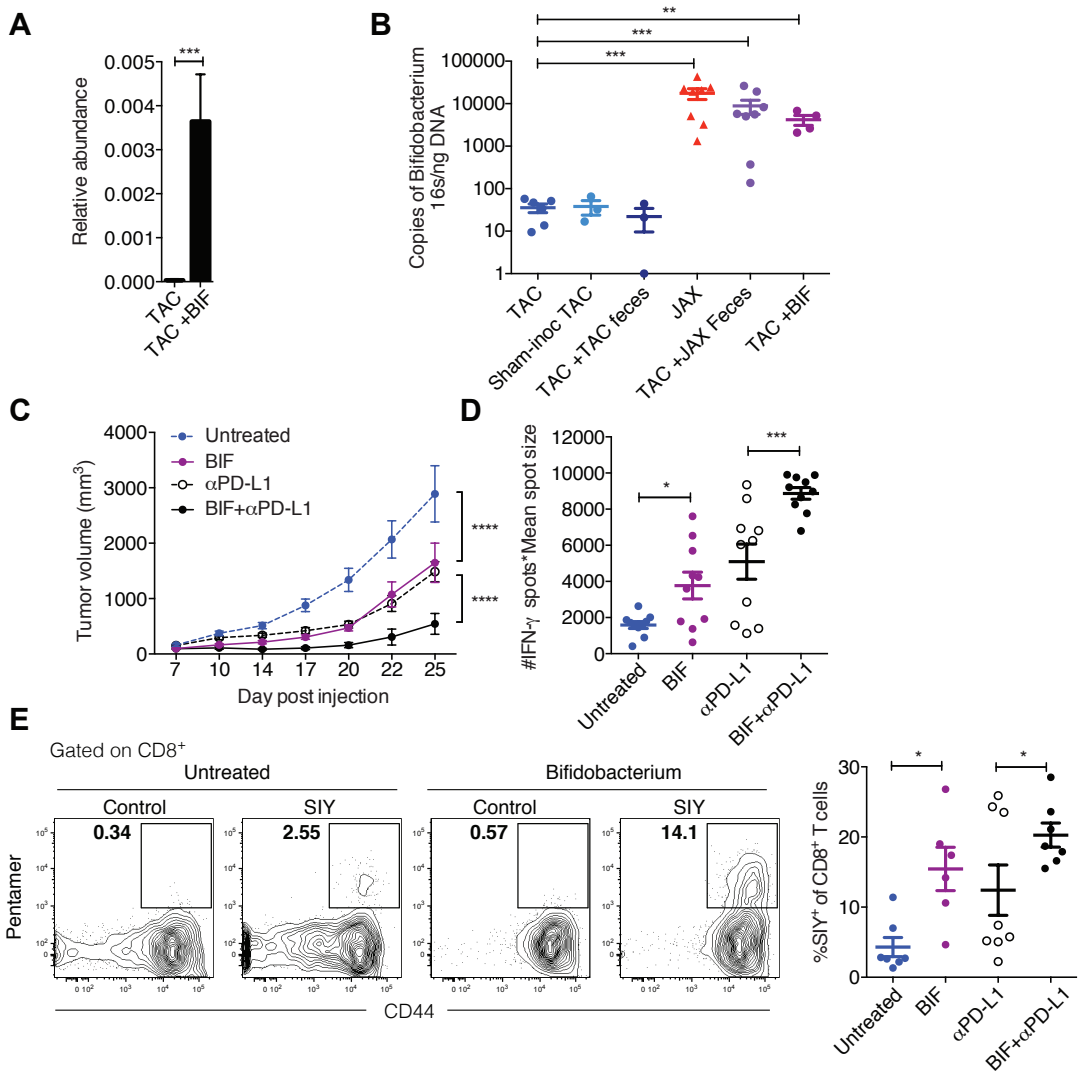


Figure 4.2: **Direct administration of *Bifidobacterium* to TAC recipients with established tumors improves tumor-specific immunity and response to αPD-L1 mAb therapy. (A)** Relative abundance of *Bifidobacterium* OTU_681370 in fecal material obtained from TAC mice inoculated with commercial *Bifidobacterium* species. **(B)** *Bifidobacterium* levels in fecal material obtained from groups as shown, assessed by qPCR. **(C)** B16.SIY tumor growth in TAC mice, untreated or treated with *Bifidobacterium* 7 and 14 days post tumor implantation, αPD-L1 mAb 7, 10, 13 and 16 days post tumor implantation, or both regimens. **(D)** IFN-γ ELISPOT, 5 days after start of treatment. **(E)** Percentage of tumor-infiltrating $SIY^+$ $CD8^+$ T cells, 14 days after start of treatment. Representative plots (left), quantification (right). Data show mean +/- SD (A) or SEM (C), analyzed by non-parametric *t*-test (A) or two-way ANOVA with Tukeys correction (C); or individual mice with mean +/- SEM analyzed by non-parametric *t*-test (B) or one-way ANOVA with Holm-Sidak correction [(D) and (E)], and are combined from two independent experiments; 5 mice per group per experiment, *P < 0.05, **P < 0.01, ***P < 0.001 ****P < 0.0001.

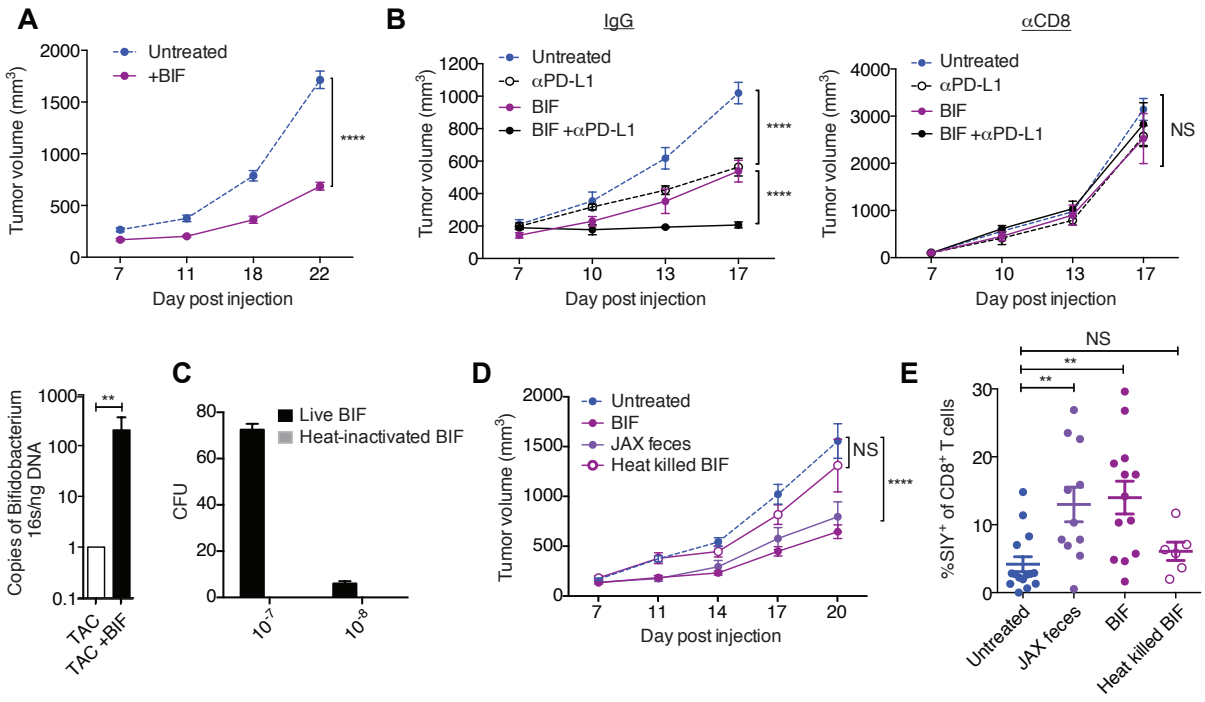


Figure 4.3: ***Bifidobacterium*-mediated effects are durable and require CD8$^+$ T cells as well as live bacteria.** **(A)** B16.SIY tumor growth in TAC mice, untreated or inoculated with *Bifidobacterium* 6 weeks prior to tumor implantation (top); *Bifidobacterium* levels in TAC mice 3 weeks post *Bifidobacterium* administration, assessed by qPCR (bottom). **(B)** B16.SIY tumor growth for isotype-treated (left) or CD8-depleted (right) groups as in Figure 4.2C. **(C)** Number of colony forming units (CFU) of live and heat inactivated bifidobacteria, plated in RCM agar following serial dilution in reduced PBS and incubated in an anaerobic chamber for 72 hours. Bars represent 2 replicate plates of each dilution. **(D)** B16.SIY tumor growth kinetics in TAC mice, untreated or treated with live *Bifidobacterium*, heat inactivated *Bifidobacterium* or JAX fecal material 7 and 14 days post tumor implantation. **(E)** Percentage of tumor-infiltrating SIY$^+$ T cells of total CD8$^+$ T cells for treatment groups as in D, determined by flow cytometry 14 days after start of treatment. Data show mean +/- SEM analyzed by two-way ANOVA with Sidak's (A), Tukeys (B) or Dunnetts (D) correction for multiple comparisons; or individual mice with mean +/- SEM analyzed by one-way ANOVA with Holm-Sidak correction for multiple comparisons (E); $n$ = *5-15* mice per group; **P $<$ 0.01, ****P $<$ 0.0001, NS, not significant.

therapeutic effect on tumor growth and reduced tumor-specific T cell responses to baseline **(Figure 4.3C-E)**, suggesting that the anti-tumor effect requires live bacteria.

As an alternative strategy, we tested the therapeutic effect of *B. breve* and *B. longum* strains obtained from the ATCC, which also showed significantly improved tumor control **(Figure 4.4A)**. Administration of *Bifidobacterium* to TAC mice inoculated with B16 parental tumor cells or MB49 bladder cancer cells also resulted in delayed tumor outgrowth (**Figure 4.4B-C)**. Oral administration of *Lactobacillus murinus* to TAC mice, which was not among the overrepresented taxa in JAX-fed mice, had no effect on tumor growth (**Figure 4.4D)** nor on tumor-specific T cell responses (**Figure 4.4E)**, suggesting that modulation of commensal bacterial communities through introduction of new bacteria in itself does not induce immunity to tumors, but rather immunity depends on the specific bacteria administered. Collectively, these data point to *Bifidobacterium* as a positive regulator of anti-tumor immunity in vivo.

## 4.2 Dendritic cells isolated from JAX and *Bifidobacterium*-fed TAC mice show increased expression of genes associated with antitumor immunity and heightened capability for T cell activation

To probe whether *Bifidobacterium* could be exerting its effects through modulation of the abundance of other bacteria, we compared taxa that showed significant changes in abundance in JAX-treated and *Bifidobacterium*-treated TAC mice (**Table S2** and **Table S4**). 16S rRNA sequencing of fecal material collected from TAC mice 7 days following *Bifidobacterium* inoculation showed a significant shift in microbial diversity (ANOSIM, p=0.003). However, relatively few changes were observed beyond the increase in *Bifidobacterium* **(Figure 4.5A)**, and they largely did not parallel the changes observed with JAX-feces administration. We

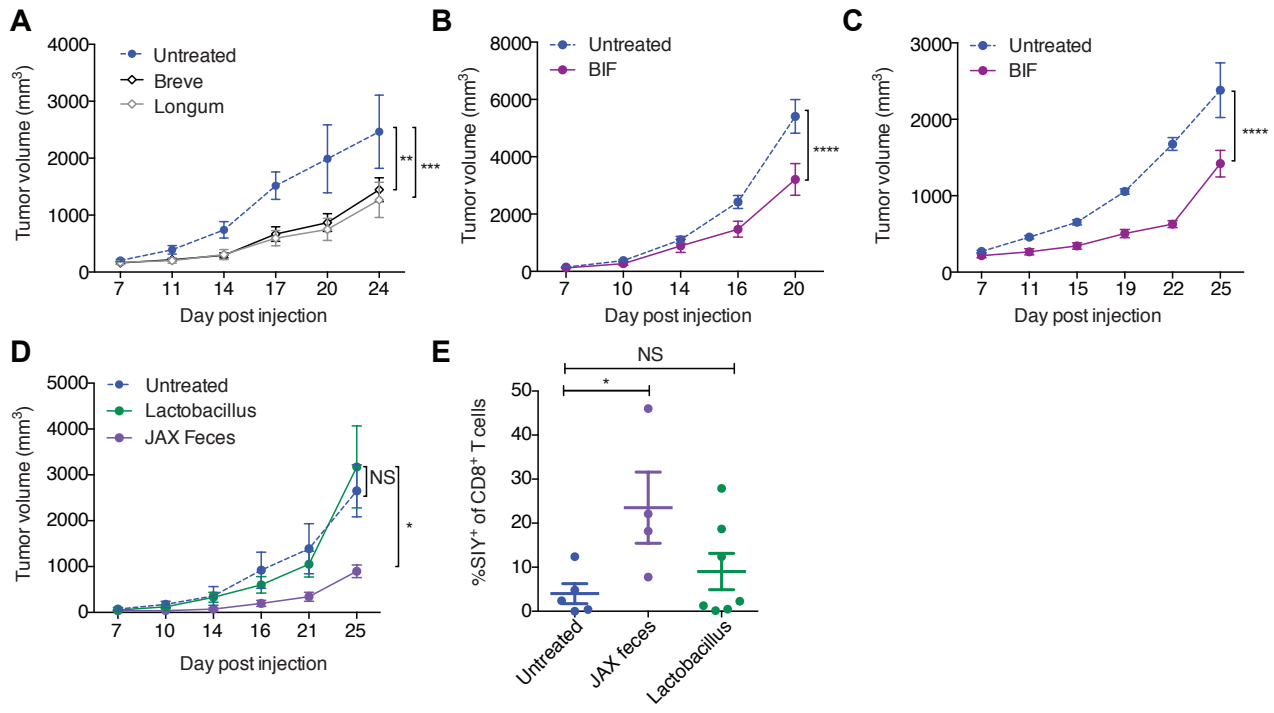


Figure 4.4: **Anti-tumor effects are specific to *Bifidobacterium* and are observed with other tumor cell types.** **(A)** B16.SIY tumor growth kinetics in TAC mice, untreated or treated with ATCC-derived *B. breve* or *B. longum*. **(B)** B16.F10 tumor growth kinetics in TAC mice, untreated or treated with *Bifidobacterium* 7 and 14 days post tumor implantation. **(C)** MB49 tumor growth kinetics in TAC mice, untreated or treated with *Bifidobacterium* 7 and 14 days post tumor implantation. **(D)** B16.SIY tumor growth kinetics in TAC mice, untreated or treated with *Lactobacillus murinus* or JAX fecal material 7 and 14 days post tumor implantation. **(E)** Percentage of tumor-infiltrating $SIY^+$ T cells of total $CD8^+$ T cells for treatment groups as in (D), determined by flow cytometry 18 days after start of treatment. Data show mean +/- SEM [(A) - (D)] or individual mice with mean +/- SEM (E), analyzed by two-way ANOVA with Dunnetts [(A), (D)] or Sidaks correction [(B), (C)], or one-way ANOVA with Holm-Sidak correction for multiple comparisons (E); n = 5-10 mice per group; *P $<$ 0.05, **P $<$ 0.01, ***P $<$ 0.001, ****P $<$ 0.0001, NS, not significant.

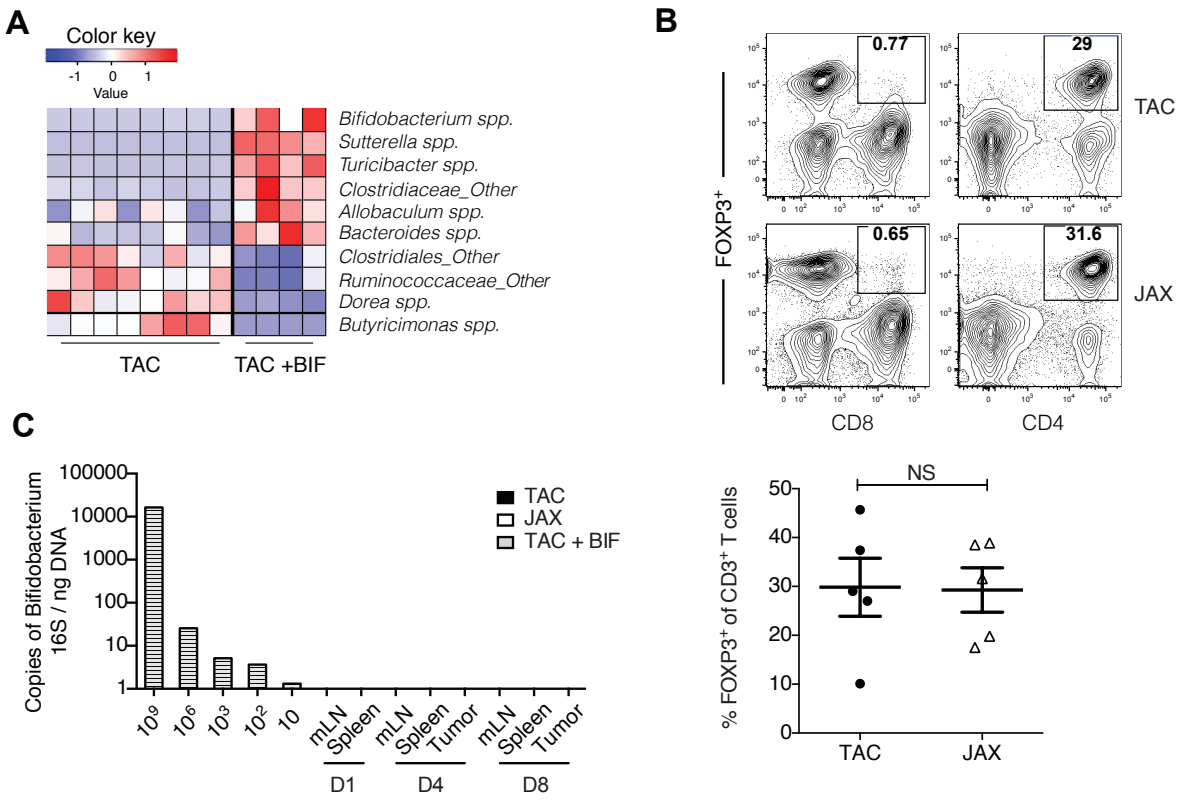


Figure 4.5: ***Bifidobacterium*-associated effects do not appear to be mediated through modulation of the abundance of other gut bacteria nor through systemic dissemination.** **(A)** Heatmap demonstrating relative abundance of significantly altered genus-level taxa in *Bifidobacterium*-fed TAC mice FDR<0.05 (non-parametric *t*-test); columns depict individual mice; n = 4-8 mice per group. **(B)** Frequency of $CD4^+$ $Foxp3^+$ T cells in tumors isolated from JAX and TAC mice 21 days post tumor inoculation, assessed by flow cytometry; representative plot (top), quantification (bottom). Data show individual mice with mean +/- SEM, analyzed by students *t*-test; n = 5 mice per group; NS, not significant. **(C)** Evaluation of translocation of *Bifidobacterium* into mesenteric lymph nodes (mLN), spleen and tumor of TAC, JAX and *Bifidobacterium*-inoculated mice, assessed by qPCR. Data show 5 mice per group over 3 time points.

observed modest reductions (≈2 to ≈10 fold) in members of the order Clostridiales as well as in butyrate-producing *Butyricimonas spp.* upon *Bifidobacterium* inoculation, which could point to an inhibitory effect on the Treg cell compartment [4, 5]. However, we did not observe any difference in the frequency of $CD4^+$ Tregs in tumors isolated from JAX and TAC mice **(Figure 4.5B)**. Furthermore, JAX feces conferred benefit even in the presence of butyrate-producing bacteria such as *Anaerostipes spp.* and *Butyrivibrio spp.*, which increased with JAX-feces inoculation **(Figure 4.1C** and **Table S2)**. Notably, *Bifidobacterium* administration did not significantly increase JAX-characteristic taxonomic groups. Thus, while we cannot definitively rule out an indirect effect of *Bifidobacterium* administration, it is unlikely that *Bifidobacterium* is exerting its effects primarily through modulation of the abundance of other bacteria.

To better understand how *Bifidobacterium* may be mediating the observed systemic effects, we assessed whether translocation of *Bifidobacterium* was occurring into the mesenteric lymph nodes, spleen or tumor. No *Bifidobacterium* was detected in any of the organs isolated from *Bifidobacterium*-gavaged tumor-bearing mice, although we could detect *Bifidobacterium* by qPCR when cell suspensions were spiked with as few as ten bacterial cells **(Figure 4.5C**). We thus concluded that the observed systemic immunological effects are likely occurring independently of bacterial translocation.

To interrogate immunologic mechanisms underlying the observed differences in T cell responses between TAC, JAX, and *Bifidobacterium*-treated TAC mice, we transferred CFSE-labeled SIY-specific 2C TCR Tg T cells into tumor-bearing mice and tested their proliferation and acquisition of IFN-$\gamma$ production ex vivo **(Figure 4.6A)**. $CD8^+$ SIY-specific 2C TCR Tg T cells exposed to tumors in JAX and *Bifidobacterium*- treated TAC mice exhibited modest but statistically significant greater expansion in the tumor-draining lymph node as compared to their counterparts in TAC mice **(Figure 4.6B)**. However, they produced markedly greater IFN-$\gamma$ in both the tumor draining lymph node and the spleen of JAX and *Bifidobacterium*-fed

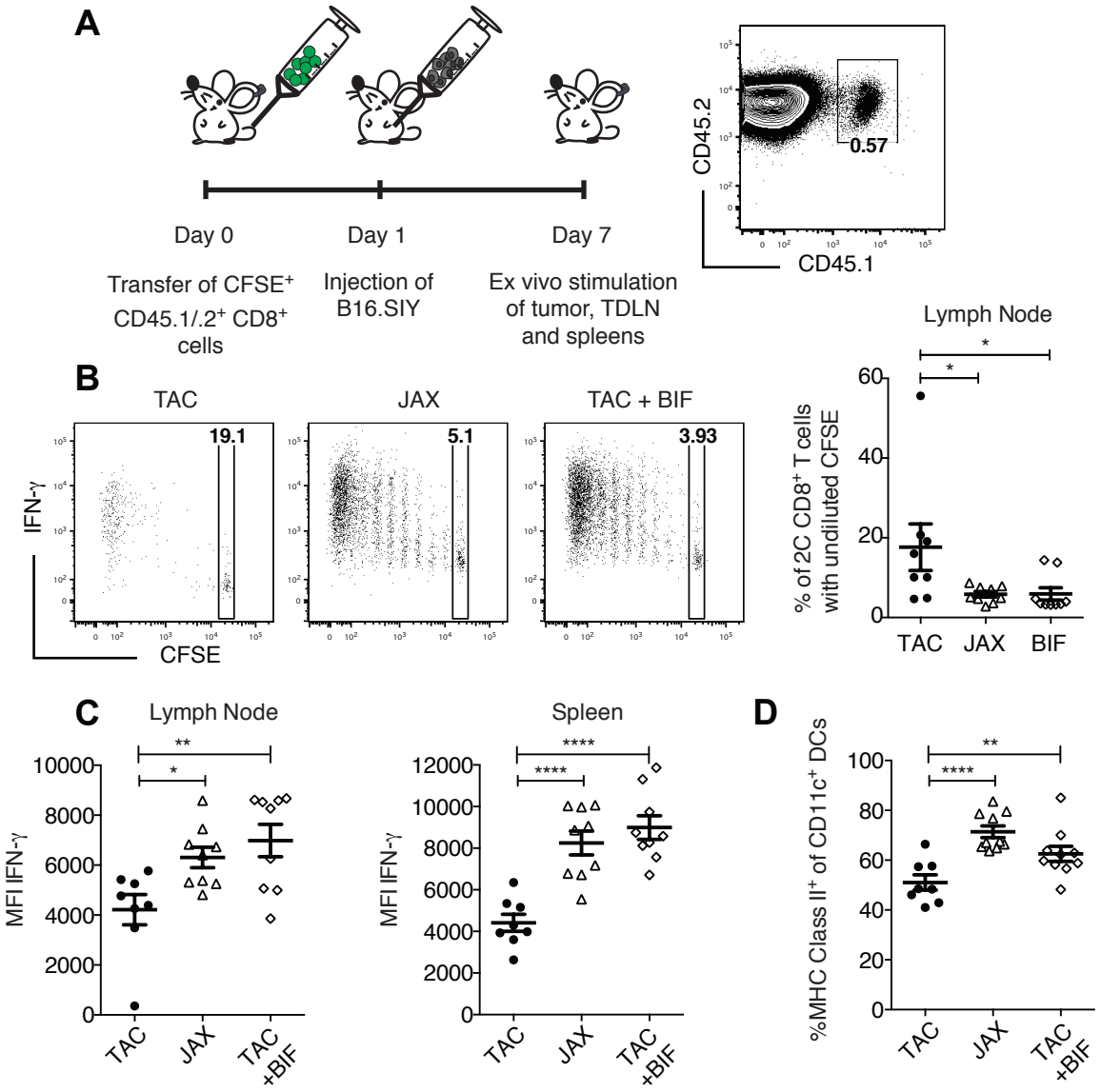


Figure 4.6: **Functional analysis of adoptively transferred antigen-specific T cells points to an effect at the level of host dendritic cells.** **(A)** Schematic of in vivo 2C proliferation assays. Intracellular IFN-$\gamma$ production and CFSE dilution were assessed in gated CD45.1$^+$/.2$^+$ CD8$^+$ 2C T cells by flow cytometry; TDLN=tumor-draining lymph node. **(B)** Representative CFSE dilution assessed in gated CD45.1$^+$/.2$^+$ 2C T cells by flow cytometry (left) and quantification (right). **(C)** IFN-$\gamma$ MFI (mean fluorescence intensity) of 2C CD8$^+$ T cells in the tumor-draining lymph node (left) and spleen (right) of TAC, JAX, *Bifidobacterium*-fed TAC mice on day 7 post adoptive transfer. **(D)** Percentage of MHC Class II$^{hi}$ DCs in tumors isolated from TAC, JAX, and *Bifidobacterium*-fed TAC mice 40 hours post tumor implantation as assessed by flow cytometry. Data show individual mice with mean +/- SEM, analyzed by one-way ANOVA with Holm-Sidak correction; representative of two to four independent experiments, 8-9 mice per group per experiment, *P $<$ 0.05, **P $<$ 0.01, ****P $<$ 0.0001.

TAC tumor-bearing mice **(Figure 4.6C)**, consistent with our analyses of the endogenous T cell response (Figures 3.1C, 3.3B, 3.4A and 4.2D). These data suggested that signals upstream of T cells in the JAX and *Bifidobacterium*-treated TAC environment enhanced acquisition of T cell effector function, pointing to an improvement in immune responses at the level of host dendritic cells (DCs). Consistent with this hypothesis, we found an increased percentage of MHC Class $II^{hi}$ DCs in the tumors of JAX and *Bifidobacterium*-treated TAC mice **(Figure 4.6D)**.

We therefore employed genome-wide transcriptional profiling of early tumor-infiltrating DCs isolated from TAC, JAX and *Bifidobacterium*-treated TAC mice **(Figure 4.7A** and **Table S5)**. In total, there were 760 gene transcripts upregulated by ≈1.5-fold in both JAX and *Bifidobacterium*-treated TAC-derived DCs relative to DCs from untreated TAC mice. Pathway analysis identified cytokine-cytokine receptor interaction, T cell activation, and positive regulation of mononuclear cell proliferation as significantly enriched pathways among upregulated genes **(Figure 4.7B-C)**. Importantly, many of these genes have been shown to be critical for anti-tumor responses including those involved in $CD8^+$ T cell activation and costimulation (*H2-m2* (MHC-I), *Cd40*, *Cd70*, *Icam1*)) [6, 85, 114], DC maturation (*Relb*, *Ifngr2*) [101, 102], antigen processing and cross presentation (*Tapbp*, *Rab27a*, *Slc11a1*) [18, 59, 126], chemokine-mediated recruitment of immune cells to the tumor microenvironment (*Cxcl9*, *Cx3cl1*, *Cxcr4*) [63, 96, 140] and type I IFN signaling (*Irf1*, *Ifnar2*, *Oas2*, *Ifi35*, *Ifitm1*) [33, 137] **(Figure 4.8A)**. Up-regulation of these genes, as well as additional genes that showed an upward trend by gene expression profiling including *Cd86*, *Ccr7*, *Ccl3* and *Ifn-β*, was validated by qRT-PCR **(Figure 4.8B)**. Expression of these genes was also increased in murine bone marrow-derived DCs stimulated with *Bifidobacterium* in vitro **(Table S6)**, consistent with previous reports that these species of *Bifidobacterium* can directly elicit DC maturation and cytokine production [82].

To test whether functional differences in DCs isolated from TAC, JAX and *Bifidobac-*

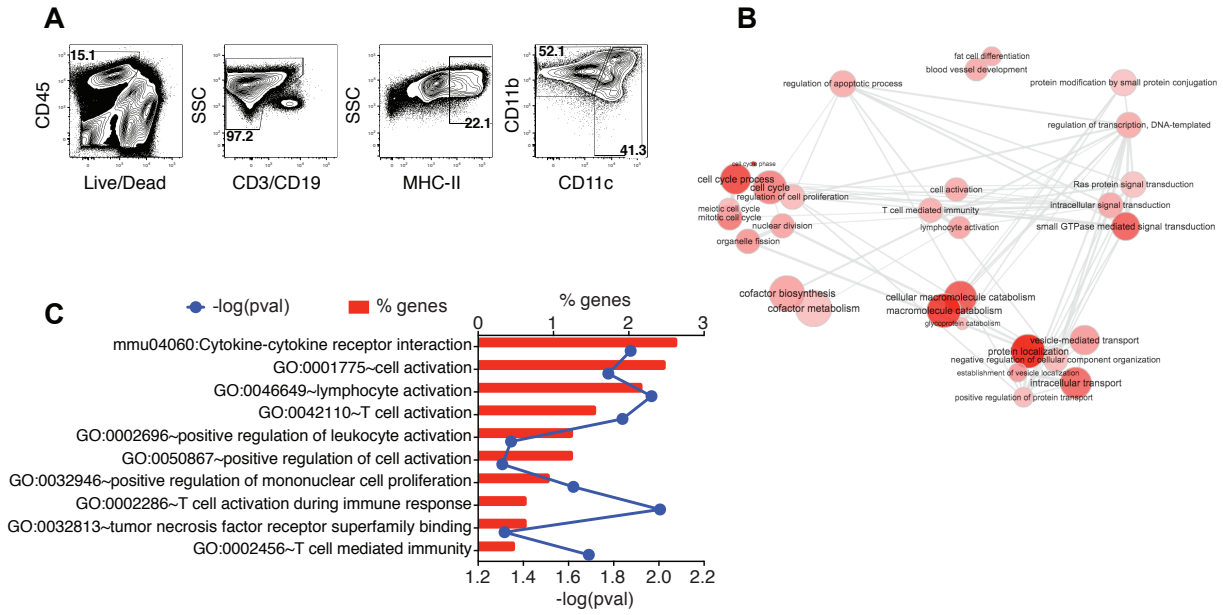


Figure 4.7: **Genome-wide transcriptional profiling of tumor-infiltrating dendritic cells isolated from JAX and *Bifidobacterium*-fed TAC mice reveals increased expression of genes associated with innate and adaptive immune activation. (A)** Representative plots depicting the strategy for isolation of DCs from tumors in JAX, TAC and *Bifidobacterium*-treated TAC mice: live $CD45^{+}CD3^{-}CD19^{-}MHCII^{hi}CD11c^{+}$ dendritic cells were sorted as shown. **(B)** Enriched biological pathways and functions found within the subset of elevated genes in JAX and *Bifidobacterium*-treated TAC-derived DCs relative to untreated TAC DCs isolated from tumors 40hrs post inoculation, as assessed by DAVID pathway analysis. **(C)** Immune-associated enriched biological pathways; Red bars indicate the percent of genes in a pathway upregulated in DCs isolated from JAX and *Bifidobacterium*-fed TAC mice. Blue line indicates p-values calculated by Fishers exact test.

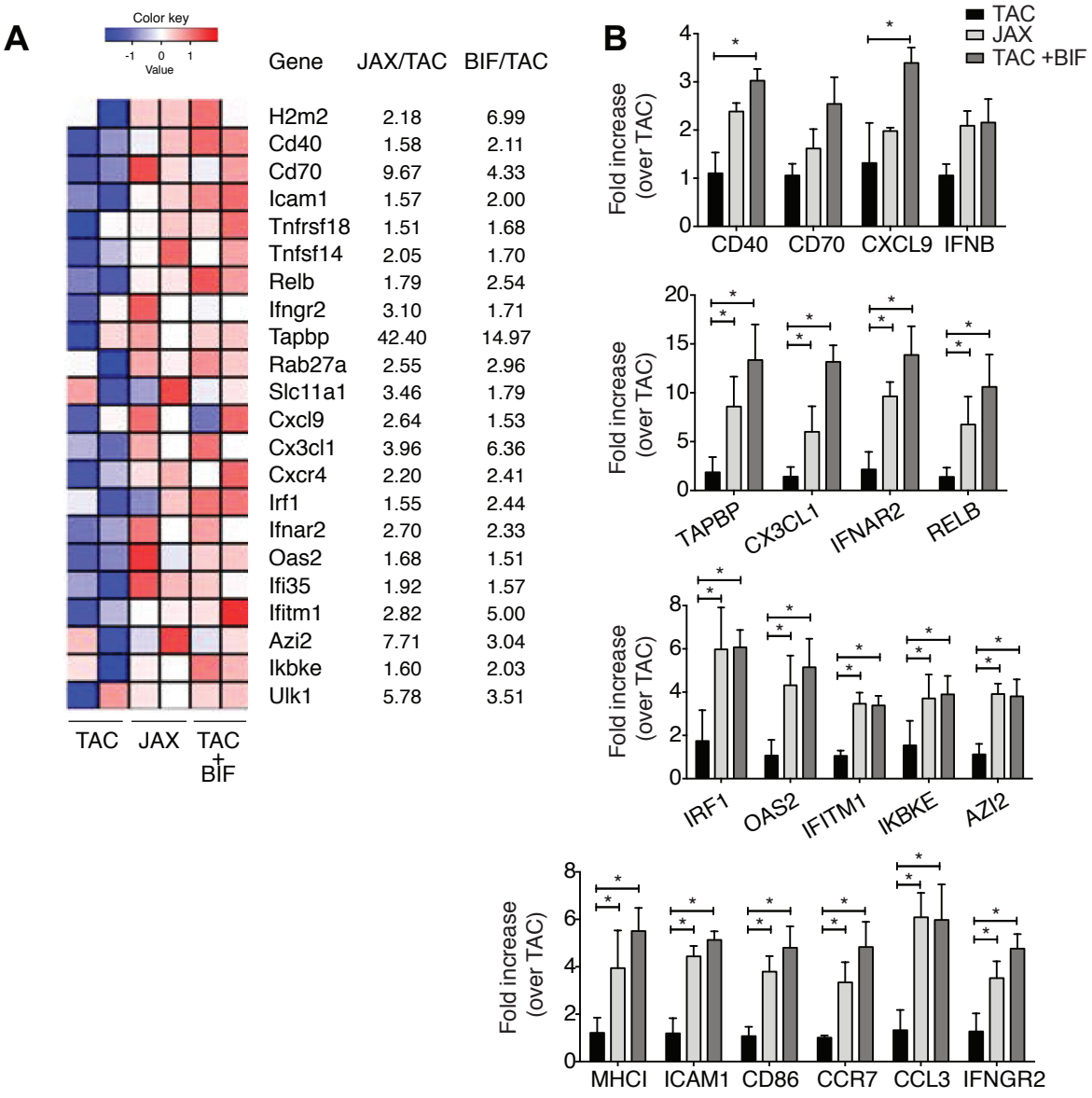


Figure 4.8: **Dendritic cells isolated from tumors of JAX and *Bifidobacterium*-fed TAC mice show increased expression of key genes associated with antitumor immunity.** **(A)** Heat map of key antitumor immunity genes in DCs isolated from JAX, *Bifidobacterium*-treated TAC or untreated TAC mice; Mean fold-change for each gene transcript is shown on the right. **(B)** qRT-PCR validation of genes identified by microarray gene expression profiling as in (A). Analyses were performed on data combined from two to three independent experiments, 5 mice pooled per group per experiment; Data in (B) show mean +/-SEM, and were analyzed by students *t*-test; *FDR<0.5.

*terium*-treated TAC mice could be sufficient to explain the differences in T cell priming observed in vivo, we purified DCs from peripheral lymphoid tissues of naive TAC, JAX, and *Bifidobacterium*-treated TAC mice and tested their ability to induce CFSE-labeled $CD8^+$ SIY-specific 2C TCR Tg T cell proliferation and acquisition of IFN-$\gamma$ production in vitro. DCs purified from naive JAX and *Bifidobacterium*-treated TAC mice induced 2C T cell proliferation at lower antigen concentration compared to DCs purified from naive TAC mice **(Figure 4.9A)**. Furthermore, at all antigen concentrations, JAX-derived DCs elicited markedly elevated levels of T cell IFN-$\gamma$ production, and this heightened APC ability could be conferred to TAC mice upon oral administration of *Bifidobacterium* prior to DC isolation **(Figure 4.9B)**. Taken together, these data suggest that commensal *Bifidobacterium*-derived signals modulate the activation of innate antigen-presenting cells in the steady state, which in turn supports improved effector function of tumor-specific $CD8^+$ T cells.

## 4.3 Discussion

Our studies demonstrate an unexpected role for commensal *Bifidobacterium* in enhancing anti-tumor immunity in vivo . Given that beneficial effects are observed in multiple tumor settings, and that alteration of innate immune function is observed, this improved anti-tumor immunity appears to occurring in an antigen-independent fashion. The necessity for live bacteria may imply that *Bifidobacterium* colonizes a specific compartment within the gut that enables it to interact with host cells that are critical for modulating DC function, that either migrate to influence DCs through cell-cell contact or act through release of soluble factors that disseminate systemically **(Figure 4.10)**. It is also possible that *Bifidobacterium* itself releases soluble factors that disseminate systemically leading to improved DC function **(Figure 4.10)**.

Our results do not rule out a potential contribution of other commensal bacteria species in having the capability to regulate anti-tumor immunity, either positively or negatively. Our

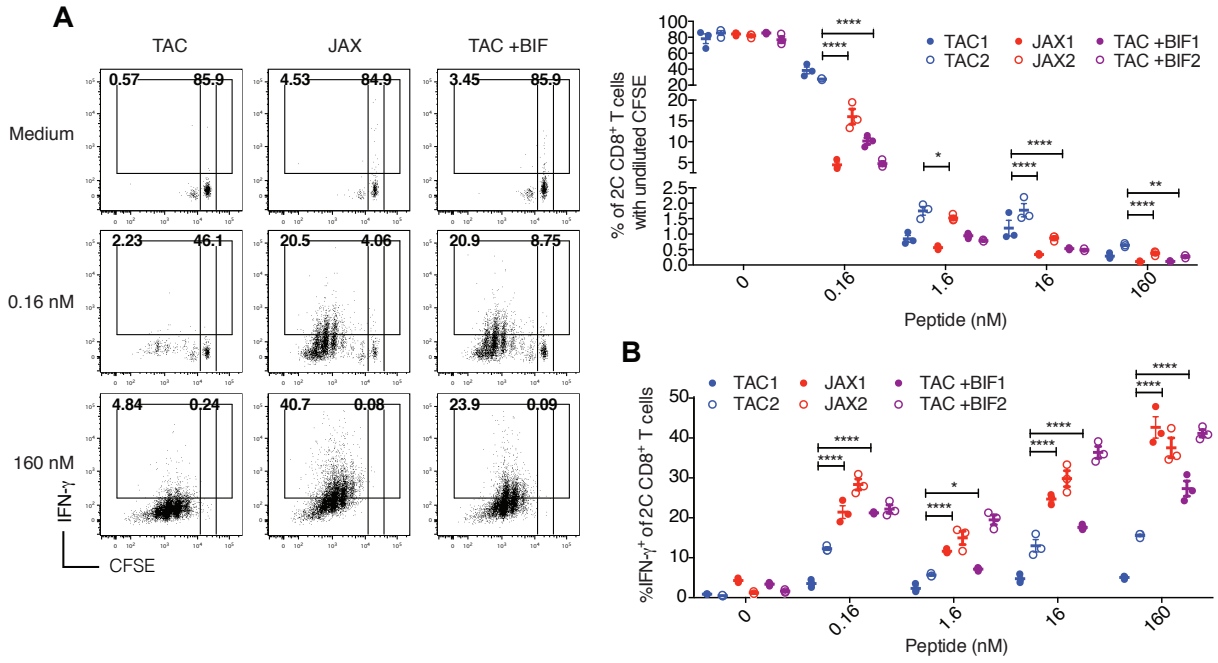


Figure 4.9: **Dendritic cells isolated from tumors of JAX and *Bifidobacterium*-fed TAC mice show heightened capability for CD8$^+$ T cell activation. (A)** Representative flow plots (left) of CFSE dilution and IFN-$\gamma$ production in 2C CD8$^+$ T cells stimulated in vitro with DCs purified from naive TAC, JAX and *Bifidobacterium*-treated TAC mice in the presence of different concentrations of SIY peptide as shown; Percentage of 2C CD8$^+$ T cells with undiluted CFSE (right). **(B)** Quantification of IFN-$\gamma^+$ 2C TCR Tg CD8$^+$ T cells stimulated in vitro with DCs as in (A). Data are combined from two independent experiments, 5 mice pooled per group per experiment, and show technical replicates of pooled samples from each experiment separately; analyzed by fitting a linear mixed model, with Bonferroni correction for multiple comparisons; *P $<$ 0.05, **P $<$ 0.01, ****P $<$ 0.0001.

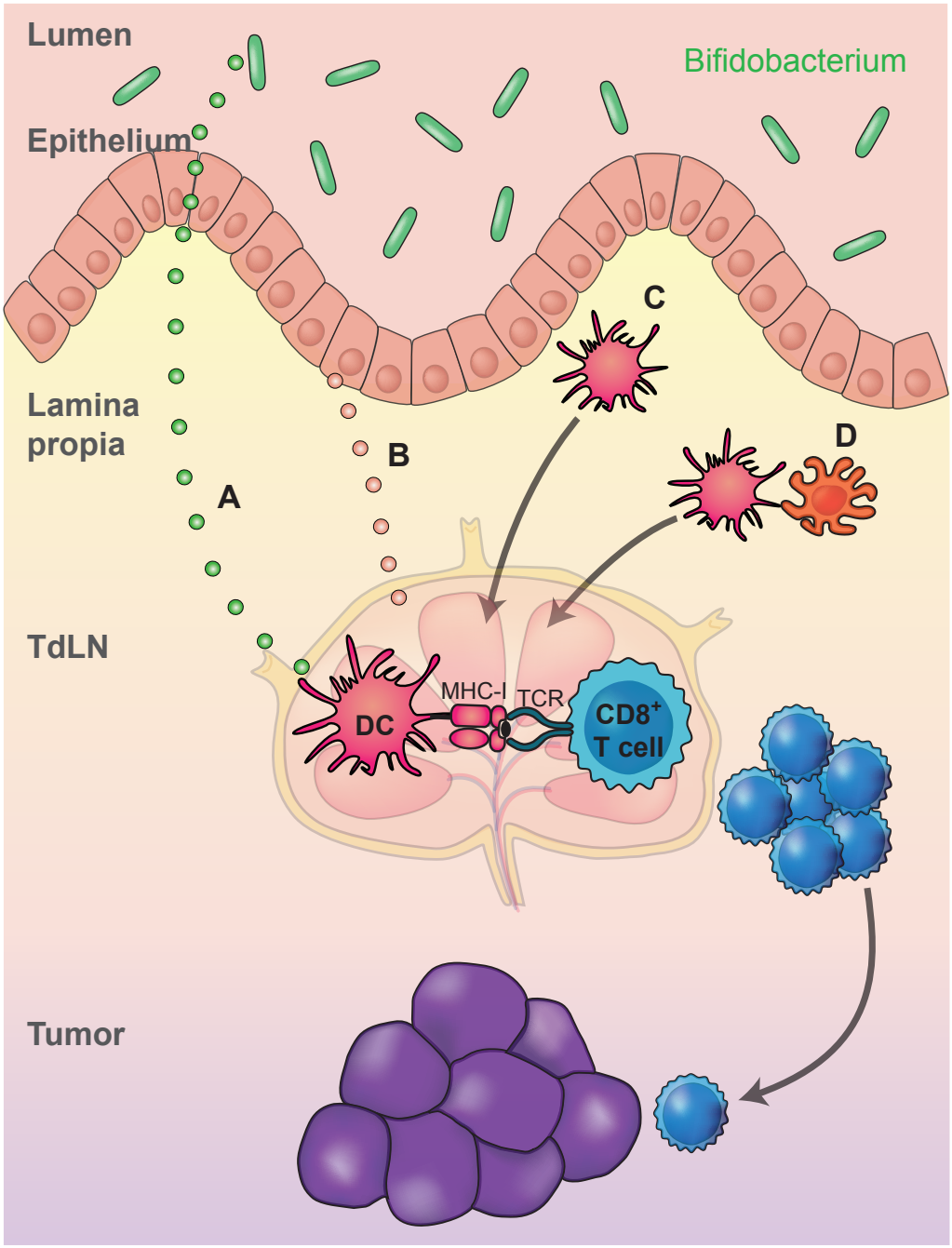


Figure 4.10: **Potential mechanisms for *Bifidobacterium*-mediated improvement in anti-tumor immune responses. (a-b)** A systemic factor might be released by *Bifidobacterium* (a) or by host cells, such as intestinal epithelial cells (b) that promotes global DC pre-activation. **(c)** Altered DCs or DC precursors might be pre-activated locally in the intestinal lamina propria and disseminate to distant sites. **(d)** A host cell, altered by crosstalk with *Bifidobacterium*, might interact with DCs to modulate DC function.

data support the idea that one source of inter-subject heterogeneity with regard to spontaneous anti-tumor immunity and therapeutic effects of antibodies targeting the PD-1/PD-L1 axis may be the specific composition of gut microbes, which could be manipulated for therapeutic benefit. These principles could apply to other immunotherapies such as antibodies targeting the CTLA-4 pathway, a subject of current investigation. Similar analyses can be performed in humans using 16S rRNA sequencing of stool samples from patients receiving checkpoint blockade or other immunotherapies, to identify commensals associated with clinical benefit. It is therefore conceivable that clinical activity of these agents might similarly be improved through rational modulation of the commensal microbiota.

# CHAPTER 5

# DISCUSSION

## 5.1 Commensal-mediated calibration of DCs

Innate immune cells have largely been perceived as ready for optimal activity upon encountering activating stimuli. DCs undergo maturation and activation leading to induction of gene expression programs required for effective priming of adaptive immunity. Our data suggest that the ability of DCs to undergo these changes and mount effector T cell responses is in fact modulated by signals derived from specific components of the commensal microbiota. These data are consistent with previous studies showing alteration of innate immune cell gene expression and function in response to commensal-derived signals, with implications for adaptive immune responses [1, 39].

The idea that maintenance of a basal state of activation in a particular cell population promotes responsiveness to secondary stimuli is supported by other studies. For example, mathematical modeling in yeast cells shows that a system with basal signaling exhibits higher efficiency, with faster response times and higher sensitivity to variations in external signals, than would systems without basal signaling [84]. In immunological systems, it has been demonstrated that induction of a basal activation level in T cells is required for responsiveness to subsequent encounters with foreign antigens. This basal activation is provided by self-recognition of self peptide-MHC complexes present on DCs and in some cases stromal cells. Following DC depletion in mice, it has been reported that T cells became impaired in TCR signaling and immune synapse formation, and consequently were hyporesponsive to antigen [51]. Collectively, data from our study and others introduce the idea that like T cells, responsiveness of DCs to stimuli, such as viral infection and tumor challenge, may also be enhanced through maintenance of a basal activation state, provided in part by commensal bacteria. This is supported by our observation that DCs isolated from naive TAC, JAX and

*Bifidobacterium*-fed mice already exhibit differences in the capability to prime T cells.

Maintenance of basal activation could be achieved through bacterial-mediated epigenetic modification in DCs, as has been reported previously in other model systems [39]. Additional study is necessary, but it is tempting to speculate that DCs isolated from naive JAX and *Bifidobacterium*-fed TAC mice exhibit changes in histone acetylation or methylation in regions of key genes that are important for mounting adaptive anti-tumor responses. These may include genes that were found to be differentially expressed in our study, such as *MHC Class I*, costimulatory markers *Cd40*, *Cd70*, *Cd80*, *Cd86*, *Relb* (a critical regulator of DC maturation), the chemokines *Cxcl9* and *Cxcl10*, antigen-processing machinery such as the *Tap* genes, and type I IFN-associated transcripts.

An important family of genes that showed increased induction in tumor-infiltrating DCs of JAX and *Bifidobacterium*-fed mice was the type I IFN-associated genes. Type I IFN signaling in $CD11c^+$ DCs has been shown to lead to accumulation of Batf3-lineage DCs within the tumor microenvironment, which then cross-present tumor-associated antigens to $CD8^+$ T cells [33]. Interestingly, we also found an increased frequency of Batf3-lineage $CD11b^-$ $CD103^+$ DCs in early tumors isolated from JAX and *Bifidobacterium*-treated mice (unpublished data), potentially contributing to the augmented $CD8^+$ T cell responses observed in these mice.

## 5.2 Linking the gut to systemic immunity

It was unexpected that a specific member of the commensal microbiota had such a robust effect on the function of immune cells residing in extra-intestinal lymphoid organs. Evidence for precise mechanisms that link commensal-host crosstalk in the gut and long-range regulation of extra-intestinal immune responses is lacking. However, some examples exist and it is possible to speculate about potential mechanistic connections. Our results have ruled out bacterial dissemination to peripheral organs, including the tumor, whether through

hematogenous or lymphatic spread or within host cells. Adoptive T cell transfer experiments and analyses of DCs within the tumor and peripheral lymphoid organs point to a functional effect at the level of host DCs. These results suggest that T cell trafficking from the gut cannot explain the observed effects in innate immune cells at extra-intestinal sites. Thus, the necessity for live bacteria may imply that *Bifidobacterium* colonizes a specific compartment within the gut that enables it to interact with host cells that are critical for modulating DC function, that either migrate to influence DCs through cell-cell contact or act through release of soluble factors that disseminate systemically. It is also possible that DCs (or DC precursors) are altered within the intestinal mucosa and migrate to peripheral sites, or that *Bifidobacterium* itself releases soluble factors that disseminate systemically leading to improved DC function (see [17]). Future studies will be required to unveil the precise interactions between gut commensals, including *Bifidobacterium*, and host cells that lead to poising of DCs for optimal T cell priming in extra-intestinal lymphoid organs.

## 5.3 *Bifidobacterium*, molecular cues, and effects on immunity

*Bifidobacterium spp.*, belonging to the phylum Actinobacteria, form a dominant fraction of the human gastrointestinal microbiota, particularly in infants. They comprise approximately 30 distinct species, and are Gram-positive, anaerobic or microaerophilic bacteria that can break down oligosaccharides in the large intestine to produce acetate and lactate. Bifidobacteria have been associated with health benefits, and are commercially available as probiotics. Members belonging to the *Bifidobacterium* genus have widely been associated with correcting Th1/Th2 imbalances. For example, they have been shown to favor the development of Th1 responses by increasing the local and systemic expression of IFN-$\gamma$ in vivo [24, 91], and decreases in abundance of bifidobacteria are associated with development of atopy in humans [64]. Consistent with our results, oral administration of *B. longum* resulted in increased levels of the immune-stimulatory cytokines IFN-$\gamma$, IL-2, IL-12 and IL-18 in the

lungs of influenza-infected mice, leading to enhanced NK priming and function [68]. Furthermore, *Bifidobacterium* species, including *B. longum* and *B. breve*, are capable of inducing DC maturation directly, as evidenced by upregulation of CD40, CD80 and CD86, as well as production of Th1-associated cytokines [82].

The precise molecular interactions between *Bifidobacterium* and host cells remain an open question. Given that beneficial effects in our study are observed in multiple tumor settings, and that alteration of innate immune function is observed, the effects appear to occur in an antigen-independent fashion. Therefore, *Bifidobacterium* likely acts as an adjuvant for DCs, priming them for enhanced function. Candidate molecules serving as stimulatory adjuvants include CpG DNA, which can activate TLR9 in host cells, and as yet undefined cell-wall components. Bifidobacterial DNA is characterized by a high frequency of CpG motifs [92], which has also been associated with stimulation of macrophages in vitro [81]. In addition, intraperitoneal administration of cell wall preparations derived from *Bifidobacterium* to sarcoma-bearing mice resulted in delayed tumor outgrowth through an immune-mediated mechanism [117], pointing to a potential adjuvant effect of *Bifidobacterium* cell wall components. Future studies will need to address the molecular crosstalk between commensal *Bifidobacterium* and host cells, leading to immunologic modulation.

## 5.4 Other commensal bacteria and anti-tumor immunity

Our results do not rule out a potential contribution of other commensal bacteria species in having the capability to regulate anti-tumor immunity, either positively or negatively. Our results indicate that other bacterial groups such as members of the *Rikenellaceae* and *Ruminococcaceae* families may also play a role. Interestingly, both of these taxonomic groups were found to be associated with TNF production and efficacious tumor responses to CpG-ODN treatment [56].

The use of genetically similar mice derived from different facilities, initiated by seminal

work from the Littman group [57], is a unique and rich source of information on how specific components of commensal bacteria may influence host immunity. This model allows for the study of inter-individual heterogeneity in microbial composition, as observed in humans, with the advantage of utilizing normally developed immune-competent hosts, where germ-free and antibiotic-treated animal models often fall short. The possibility to compare gut composition in animals derived from additional vendors and expand on our findings is compelling. In fact, we have observed that mice obtained from Harlan laboratories show dramatically weaker anti-tumor responses when compared to several other vendors (unpublished data), pointing to potential inhibitory bacteria present in these mice. Further characterization and identification of commensal bacteria that impact on anti-tumor immunity will be an important goal of future studies.

## 5.5 Therapeutic use of intestinal microbes or their products

Alteration of the gut microbiota in humans via fecal transplants of stool from healthy donors has been effective in case studies [71] and clinical trials [132] of refractory C. difficile infection, and is becoming the standard of care for this condition. While fecal transplantation has established a proof of principle for the feasibility of manipulating human microbiota as a therapeutic strategy, treatment with specific microorganisms is more desirable for medical purposes. Therefore, future studies should aim to unveil specific bacteria and bacterial products associated with anti-tumor responses and immunotherapeutic efficacy, with the goal of applying these findings to the clinic.

Our data support the idea that one source of inter-subject heterogeneity with regard to spontaneous anti-tumor immunity and therapeutic effects of immunotherapies, such as antibodies targeting the PD-1/PD-L1 axis, may be the specific composition of gut microbes, which could be manipulated for clinical benefit. Similar analyses can be performed in humans using 16S rRNA sequencing of stool samples from patients receiving checkpoint blockade or

other immunotherapies, to identify commensals associated with clinical benefit. It is therefore conceivable that clinical activity of these agents might similarly be improved through rational modulation of the commensal microbiota.

# CHAPTER 6

# PUBLICATIONS

- Commensal *Bifidobacterium* promotes anti-tumor immunity and facilitates anti-PD-L1 efficacy. **Sivan A**, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, Benyamin F, Lei YM, Jabri B, Alegre MA, Chang EB and Gajewski TF. *Science*. 2015 Nov 5; DOI: 10.1126. PMID: 26541606.

- E-selectin and SDF-1 regulate breast cancer cell occupancy of pro-dormancy bone marrow vascular niches. Price TT, Burness ML, **Sivan A**, Cheng R, Lee CH, Olivere L, Comatas K, Magnani, Lyerly HK, Cheng Q, McCall C and Sipkins DA. *Science Translational Medicine, in revision*. November 2015.

- The Microbiota Modulates Skin Allograft Rejection. Lei YM, Chen L, Wang Y, Stefka A, Nair L, Molinero L, Theriault B, Aquino-Michaels K, **Sivan A**, Nagler C, Gajewski TF, Chong AS, Bartman C, Alegre MA. *Journal of Clinical Investigation, submitted*. November 2015.

- Aire-dependent thymic development of tumor-associated regulatory T cells. Malchow S, Leventhal DS, Nishi S, Fischer BI, Shen L, Paner GP, **Amit AS**, Kang C, Geddes JE, Allison JP, Socci ND, Savage PA. *Science*. 2013 Mar 8;339(6124):1219-24. PMID: 23471412

- Cell-autonomous function of Runx1 transcriptionally regulates mouse megakaryocytic maturation. Pencovich N, Jaschek R, Dicken J, **Amit A**, Lotem J, Tanay A, Groner Y. *PLoS One*. 2013 May 23;8(5):e64248; PMID: 23717578

# BIBLIOGRAPHY


[1] M.C. Abt, L.C. Osborne, L.A. Monticelli, T.A. Doering, T. Alenghat, G.F. Sonnenberg, M.A. Paley, M. Antenus, K.L. Williams, J. Erikson, et al. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity*, 37:158 – 170, 2012.

[2] S. Ahrens, S. Zelenay, D. Sancho, P. Hanc, S. Kjaer, C. Feest, G. Fletcher, C. Durkin, A. Postigo, M. Skehel, et al. F-actin is an evolutionarily conserved damage-associated molecular pattern recognized by DNGR-1, a receptor for dead cells. *Immunity*, 36:635 – 645, 2012.

[3] L. Apetoh, F. Ghiringhelli, A. Tesniere, M. Obeid, C. Ortiz, A. Criollo, G. Mignot, M.C. Maiuri, E. Ullrich, P. Saulnier, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nature medicine*, 13:1050 – 1059, 2007.

[4] N. Arpaia, C. Campbell, X. Fan, S. Dikiy, J. van der Veeken, P. deRoos, H. Liu, J.R. Cross, K. Pfeffer, P.J. Coffer, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature*, 504:451 – 455, 2013.

[5] K. Atarashi, T. Tanoue, T. Shima, A. Imaoka, T. Kuwahara, Y. Momose, G. Cheng, S. Yamasaki, T. Saito, Y. Ohba, et al. Induction of colonic regulatory t cells by indigenous clostridium species. *Science*, 331:337 – 341, 2011.

[6] S.P. Bak, M.S. Barnkob, A. Bai, E.M. Higham, K.D. Wittrup, and J. Chen. Differential requirement for CD70 and CD80/CD86 in dendritic cell-mediated activation of tumor tolerized CD8 T cells. *Journal of immunology*, 189:1708 – 1716, 2012. Baltimore, Md : 1950.

[7] M. Barman, D. Unold, K. Shifley, E. Amir, K. Hung, N. Bos, and N. Salzman. Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. *Infection and Immunity*, 76:907 – 915, 2008.

[8] C. Blank, I. Brown, A.C. Peterson, M. Spiotto, Y. Iwai, T. Honjo, and T.F. Gajewski. PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor TCR transgenic CD8+ T cells. *Cancer Research*, 64:1140 – 1145, 2004.

[9] M. Bogunovic, F. Ginhoux, J. Helft, L. Shang, D. Hashimoto, M. Greter, K. Liu, C. Jakubzick, M.A. Ingersoll, M. Leboeuf, et al. Origin of the lamina propria dendritic cell network. *Immunity*, 31:513 – 525, 2009.

[10] T. Boon, T.F. Gajewski, and P.G. Coulie. From defined human tumor antigens to effective immunization? *Immunology today*, 16:334 – 336, 1995.

[11] T. Boon and P. van der Bruggen. Human tumor antigens recognized by T lymphocytes. *The Journal of experimental medicine*, 183:725 – 729, 1996.

[12] V.G. Brichard and D. Lejeune. GSK's antigen-specific cancer immunotherapy programme: Pilot results leading to phase III clinical development. *Vaccine*, 25:Supplement 2, B61 – B71, 2007.

[13] J.G. Caporaso, K. Bittinger, F.D. Bushman, G.L. DeSantis, T.Z. andersen, and R. Knight. PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics*, 26:266 – 267, 2010.

[14] J.G. Caporaso, J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E.K. Costello, N. Fierer, A.G. Pena, J.K. Goodrich, J.I. Gordon, et al. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7:335 – 336, 2010.

[15] M.D. Catalina, M.C. Carroll, H. Arizpe, A. Takashima, P. Estess, and M.H. Siegelman. The route of antigen entry determines the requirement for l-selectin during immune responses. *The Journal of experimental medicine*, 184:2341 – 2351, 1996.

[16] Y.E. Chin, M. Kitagawa, K. Kuida, R.A. Flavell, and X.Y. Fu. Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. *Molecular and cellular biology*, 17:5328 – 5337, 1997.

[17] T.B. Clarke, K.M. Davis, E.S. Lysenko, A.Y. Zhou, Y. Yu, and J.N. Weiser. Recognition of peptidoglycan from the microbiota by nod1 enhances systemic innate immunity. *Nature medicine*, 16:228 – 231, 2010.

[18] E.B. Compeer, T.W.H. Flinsenberg, S.G. van der Grein, and M. Boes. Antigen processing and remodeling of the endosomal pathway: requirements for antigen cross-presentation. *Frontiers in Immunology*, 3: –, 2012.

[19] L. Corrales, Laura H. Glickman, Sarah M. McWhirter, David B. Kanne, Kelsey E. Sivick, George E. Katibah, S.-R. Woo, E. Lemmens, T. Banda, Justin J. Leong, et al. Direct activation of sting in the tumor microenvironment leads to potent and systemic tumor regression and immunity. *Cell Reports*, 11:1018 – 1030, 2015.

[20] J.H. Cummings, E.W. Pomare, W.J. Branch, C.P. Naylor, and G.T. Macfarlane. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut*, 28:1221 – 1227, 1987.

[21] M.S. Diamond, M. Kinder, H. Matsushita, M. Mashayekhi, G.P. Dunn, J.M. Archambault, H. Lee, C.D. Arthur, J.M. White, U. Kalinke, et al. Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *The Journal of experimental medicine*, 208:1989 – 2003, 2011.

[22] A.S. Dighe, E. Richards, L.J. Old, and R.D. Schreiber. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity*, 1:447 – 456, 1994.

[23] H. Dong and L. Chen. B7-H1 pathway and its role in the evasion of tumor immunity. *Molecular Medicine*, 81:281 – 287, 2003.

[24] P. Dong, Y. Yang, and W.P. Wang. The role of intestinal bifidobacteria on immune system development in young rats. *Early human development*, 86:51 – 58, 2010.

[25] M.E. Dudley, J.R. Wunderlich, P.F. Robbins, J.C. Yang, P. Hwu, D.J. Schwartzentruber, S.L. Topalian, R. Sherry, N.P. Restifo, A.M. Hubicki, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science*, 298:850 – 854, 2002.

[26] M.E. Dudley, J.R. Wunderlich, J.C. Yang, R.M. Sherry, S.L. Topalian, N.P. Restifo, R.E. Royal, U. Kammula, D.E. White, S.A. Mavroukakis, et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *Journal of clinical oncology*, 23:2346 – 2357, 2005. official journal of the American Society of Clinical Oncology.

[27] G.P. Dunn, A.T. Bruce, K.C. Sheehan, V. Shankaran, R. Uppaluri, J.D. Bui, M.S. Diamond, C.M. Koebel, C. Arthur, J.M. White, et al. A critical function for type i interferons in cancer immunoediting. *Nature immunology*, 6:722 – 729, 2005.

[28] Z.M. Earley, S. Akhtar, S.J. Green, A. Naqib, O. Khan, A.R. Cannon, A.M. Hammer, N.L. Morris, X. Li, J.M. Eberhardt, et al. Burn injury alters the intestinal microbiome and increases gut permeability and bacterial translocation. *PloS one*, 10, 2015.

[29] M.R. Elliott, F.B. Chekeni, P.C. Trampont, E.R. Lazarowski, A. Kadl, S.F. Walk, D. Park, R.I. Woodson, M. Ostankovich, P. Sharma, et al. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature*, 461:282 – 286, 2009.

[30] F. Fallarino and T.F. Gajewski. Cutting edge: differentiation of antitumor CTL in vivo requires host expression of stat1. *Journal of immunology*, 163:4109 – 4113, 1999.

[31] J. Farache, I. Koren, I. Milo, I. Gurevich, K.W. Kim, E. Zigmond, G.C. Furtado, S.A. Lira, and G. Shakhar. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. *Immunity*, 38:581 – 595, 2013.

[32] G.J. Freeman, A.J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L.J. Fitz, N. Malenkovich, T. Okazaki, M.C. Byrne, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *The Journal of experimental medicine*, 192:1027 – 1034, 2000.

[33] M.B. Fuertes, A.K. Kacha, J. Kline, S.R. Woo, D.M. Kranz, K.M. Murphy, and T.F. Gajewski. Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8$\alpha$+ dendritic cells. *The Journal of experimental medicine*, 208:2005 – 2016, 2011.

[34] F.O. Furet JP, Gourmelon M., Bridonneau C., Tap J., Mondot S., Dor J., and Corthier G. Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. *FEMS Microbiology Ecology*, 68:351 – 362, 2009.

[35] Y. Furusawa, Y. Obata, S. Fukuda, T.A. Endo, G. Nakato, D. Takahashi, Y. Nakanishi, C. Uetake, K. Kato, T. Kato, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature*, 504:446 – 450, 2013.

[36] D.I. Gabrilovich and S. Nagaraj. Myeloid-derived suppressor cells as regulators of the immune system. *Nature reviews Immunology*, 9:162 – 174, 2009.

[37] T.F. Gajewski, J. Louahed, and V.G. Brichard. Gene signature in melanoma associated with clinical activity: a potential clue to unlock cancer immunotherapy. *Cancer journal*, 16:399 – 403, 2010.

[38] T.F. Gajewski, H. Schreiber, and Y.X. Fu. Innate and adaptive immune cells in the tumor microenvironment. *Nature immunology*, 14:1014 – 1022, 2013.

[39] S.C. Ganal, S.L. Sanos, C. Kallfass, K. Oberle, C. Johner, C. Kirschning, S. Lienenklaus, S. Weiss, P. Staeheli, P. Aichele, et al. Priming of natural killer cells by nonmucosal mononuclear phagocytes requires instructive signals from commensal microbiota. *Immunity*, 37:171 – 186, 2012.

[40] S.J. Gardai, K.A. McPhillips, S.C. Frasch, W.J. Janssen, A. Starefeldt, J.E. Murphy-Ullrich, D.L. Bratton, P.A. Oldenborg, M. Michalak, and P.M. Henson. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of lrp on the phagocyte. *Cell*, 123:321 – 334, 2005.

[41] A.D. Garg, D.V. Krysko, T. Verfaillie, A. Kaczmarek, G.B. Ferreira, T. Marysael, N. Rubio, M. Firczuk, C. Mathieu, A.J. Roebroek, et al. A novel pathway combining calreticulin exposure and ATP secretion in immunogenic cancer cell death. *The EMBO journal*, 31:1062 – 1079, 2012.

[42] W.S. Garrett. Cancer and the microbiota. *Science*, 348:80 – 86, 2015.

[43] F. Ghiringhelli, L. Apetoh, A. Tesniere, L. Aymeric, Y. Ma, C. Ortiz, K. Vermaelen, T. Panaretakis, G. Mignot, E. Ullrich, et al. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nature medicine*, 15:1170 – 1178, 2009.

[44] Y. Goto and I.I. Ivanov. Intestinal epithelial cells as mediators of the commensal-host immune crosstalk. *Immunology and cell biology*, 91:204 – 214, 2013.

[45] Y. Goto, C. Panea, G. Nakato, A. Cebula, C. Lee, M.G. Diez, T.M. Laufer, L. Ignatowicz, and I.I. Ivanov. Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation. *Immunity*, 40:594 – 607, 2014.

[46] J.G. Hall. Studies on the adjuvant action of beryllium. I. effects on individual lymph nodes. *Immunology*, 53:105 – 113, 1984.

[47] O. Hamid, C. Robert, A. Daud, F.S. Hodi, W.J. Hwu, R. Kefford, J.D. Wolchok, P. Hersey, R.W. Joseph, J.S. Weber, et al. Safety and tumor responses with lambrolizumab anti-PD-1 in melanoma. *The New England journal of medicine*, 369:134 – 144, 2013.

[48] K. Hase, K. Kawano, T. Nochi, G.S. Pontes, S. Fukuda, M. Ebisawa, K. Kadokura, T. Tobe, Y. Fujimura, S. Kawano, et al. Uptake through glycoprotein 2 of $FimH^+$ bacteria by M cells initiates mucosal immune response. *Nature*, 462:226 – 230, 2009.

[49] K. Hildner, B.T. Edelson, W.E. Purtha, M. Diamond, H. Matsushita, M. Kohyama, B. Calderon, B.U. Schraml, E.R. Unanue, M.S. Diamond, et al. Batf3 deficiency reveals a critical role for CD8$\alpha$+ dendritic cells in cytotoxic T cell immunity. *Science*, 322:1097 – 1100, 2008.

[50] D.A. Hill and D. Artis. Intestinal bacteria and the regulation of immune cell homeostasis. *Annual review of immunology*, 28:623 – 667, 2010.

[51] K. Hochweller, G.H. Wabnitz, Y. Samstag, J. Suffner, G.J. Hmmerling, and N. Garbi. Dendritic cells control T cell tonic signaling required for responsiveness to foreign antigen. *Proceedings of the National Academy of Sciences*, 107:5931 – 5936, 2010.

[52] F.S. Hodi, S.J. O'Day, D.F. McDermott, R.W. Weber, J.A. Sosman, J.B. Haanen, R. Gonzalez, C. Robert, D. Schadendorf, J.C. Hassel, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *The New England journal of medicine*, 363:711 – 723, 2010.

[53] L.V. Hooper, D.R. Littman, and A.J. Macpherson. Interactions between the microbiota and the immune system. *Science*, 336:1268 – 1273, 2012.

[54] F.-P. Huang, N. Platt, M. Wykes, J.R. Major, T.J. Powell, C.D. Jenkins, and G.G. MacPherson. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *The Journal of experimental medicine*, 191:435 – 444, 2000.

[55] T. Ichinohe, I.K. Pang, Y. Kumamoto, D.R. Peaper, J.H. Ho, T.S. Murray, and A. Iwasaki. Microbiota regulates immune defense against respiratory tract influenza a virus infection. *Proceedings of the National Academy of Sciences*, 108:5354 – 5359, 2011.

[56] N. Iida, A. Dzutsev, C.A. Stewart, L. Smith, N. Bouladoux, R.A. Weingarten, D.A. Molina, R. Salcedo, T. Back, S. Cramer, et al. Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. *Science*, 342:967 – 970, 2013.

[57] I.I. Ivanov, K. Atarashi, N. Manel, E.L. Brodie, T. Shima, U. Karaoz, D. Wei, K.C. Goldfarb, C.A. Santee, S.V. Lynch, et al. Induction of intestinal th17 cells by segmented filamentous bacteria. *Cell*, 139:485 – 498, 2009.

[58] I.I. Ivanov and K. Honda. Intestinal commensal microbes as immune modulators. *Cell host and microbe*, 12:496 – 508, 2012.

[59] C. Jancic, A. Savina, C. Wasmeier, T. Tolmachova, J. El-Benna, P.M. Dang, S. Pascolo, M.A. Gougerot-Pocidalo, G. Raposo, M.C. Seabra, et al. Rab27a regulates phagosomal pH and NADPH oxidase recruitment to dendritic cell phagosomes. *Nature cell biology*, 9:367 – 378, 2007.

[60] M.H. Jang, M.N. Kweon, K. Iwatani, M. Yamamoto, K. Terahara, C. Sasakawa, T. Suzuki, T. Nochi, Y. Yokota, P.D. Rennert, et al. Intestinal villous M cells: an antigen entry site in the mucosal epithelium. *Proceedings of the National Academy of Sciences*, 101:6110 – 6115, 2004.

[61] R.R. Ji, S.D. Chasalow, L. Wang, O. Hamid, H. Schmidt, J. Cogswell, S. Alaparthy, D. Berman, M. Jure-Kunkel, N.O. Siemers, et al. An immune-active tumor microenvironment favors clinical response to ipilimumab. *Cancer immunology, immunotherapy*, 61:1019 – 1031, 2012. CII.

[62] O.P. Joffre, E. Segura, A. Savina, and S. Amigorena. Cross-presentation by dendritic cells. *Nature reviews Immunology*, 12:557 – 569, 2012.

[63] K. Kabashima, N. Shiraishi, K. Sugita, T. Mori, A. Onoue, M. Kobayashi, J. Sakabe, R. Yoshiki, H. Tamamura, N. Fujii, et al. CXCL12-CXCR4 engagement is required for migration of cutaneous dendritic cells. *The American Journal of Pathology*, 171:1249 – 1257, 2007.

[64] M. Kalliomaki, P. Kirjavainen, E. Eerola, P. Kero, S. Salminen, and E. Isolauri. Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *The Journal of allergy and clinical immunology*, 107:129 – 134, 2001.

[65] M. Kalos, B.L. Levine, D.L. Porter, S. Katz, S.A. Grupp, A. Bagg, and C.H. June. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Science Translational Medicine*, 3: –, 2011.

[66] P.W. Kantoff, C.S. Higano, N.D. Shore, E.R. Berger, E.J. Small, D.F. Penson, C.H. Redfern, A.C. Ferrari, R. Dreicer, R.B. Sims, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *New England Journal of Medicine*, 363:411 – 422, 2010.

[67] M. Kaparakis-Liaskos and R.L. Ferrero. Immune modulation by bacterial outer membrane vesicles. *Nature reviews Immunology*, 15:375 – 387, 2015.

[68] T. Kawahara, T. Takahashi, K. Oishi, H. Tanaka, M. Masuda, S. Takahashi, M. Takano, T. Kawakami, K. Fukushima, H. Kanazawa, et al. Consecutive oral administration of Bifidobacterium longum MM-2 improves the defense system against influenza virus infection by enhancing natural killer cell activity in a murine model. *Microbiology and immunology*, 59:1 – 12, 2015.

[69] T. Kawashima, K. Hayashi, A. Kosaka, M. Kawashima, T. Igarashi, H. Tsutsui, N.M. Tsuji, I. Nishimura, T. Hayashi, and A. Obata. Lactobacillus plantarum strain YU from fermented foods activates Th1 and protective immune responses. *International immunopharmacology*, 11:2017 – 2024, 2011.

[70] T. Kawashima, A. Kosaka, H. Yan, Z. Guo, R. Uchiyama, R. Fukui, D. Kaneko, Y. Kumagai, D.J. You, J. Carreras, et al. Double-stranded RNA of intestinal commensal but not pathogenic bacteria triggers production of protective interferon-beta. *Immunity*, 38:1187 – 1197, 2013.

[71] A. Khoruts and M.J. Sadowsky. Therapeutic transplantation of the distal gut microbiota. *Mucosal immunology*, 4:4 – 7, 2011.

[72] J. Kline, I.E. Brown, Y.Y. Zha, C. Blank, J. Strickler, H. Wouters, L. Zhang, and T.F. Gajewski. Homeostatic proliferation plus regulatory T-cell depletion promotes potent rejection of B16 melanoma. *Clinical cancer research*, 14:3156 – 3167, 2008. an official journal of the American Association for Cancer Research.

[73] J. Kline, L. Zhang, L. Battaglia, K.S. Cohen, and T.F. Gajewski. Cellular and molecular requirements for rejection of B16 melanoma in the setting of regulatory T cell depletion and homeostatic proliferation. *Journal of immunology*, 188:2630 – 2642, 2012.

[74] M.F. Krummel and J.P. Allison. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *The Journal of experimental medicine*, 182:459 – 465, 1995.

[75] J.W. Kupiec-Weglinski, J.M. Austyn, and P.J. Morris. Migration patterns of dendritic cells in the mouse. traffic from the blood, and T cell-dependent and -independent entry to lymphoid tissues. *The Journal of experimental medicine*, 167:632 – 645, 1988.

[76] A. Lanzavecchia, G. Iezzi, and A. Viola. From TCR engagement to T cell activation: a kinetic view of T cell behavior. *Cell*, 96:1 – 4, 1999.

[77] D.R. Leach, M.F. Krummel, and J.P. Allison. Enhancement of antitumor immunity by CTLA-4 blockade. *Science*, 271:1734 – 1736, 1996.

[78] Y.K. Lee and S.K. Mazmanian. Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science*, 330:1768 – 1773, 2010.

[79] Y.K. Lee, J.S. Menezes, Y. Umesaki, and S.K. Mazmanian. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proceedings of the National Academy of Sciences*, 108:4615 – 4622, 2011.

[80] I. Letunic and P. Bork. Interactive tree of life v2: online annotation and display of phylogenetic trees made easy. *Nucleic acids research*, 39:475 – 478, 2011.

[81] Y. Li, X. Qu, H. Yang, L. Kang, Y. Xu, B. Bai, and W. Song. Bifidobacteria dna induces murine macrophages activation in vitro. *Cellular and molecular immunology*, 2:473 – 478, 2005.

[82] P. Lopez, M. Gueimonde, A. Margolles, and A. Suarez. Distinct Bifidobacterium strains drive different immune responses in vitro. *International journal of food microbiology*, 138:157 – 165, 2010.

[83] C. Lozupone and R. Knight. UniFrac: a new phylogenetic method for comparing microbial communities. *Applied Environmental Microbiology*, 71:8228 – 8235, 2005.

[84] J. Macia, S. Regot, T. Peeters, N. Conde, R. Sol, and F. Posas. Dynamic signaling in the Hog1 MAPK pathway relies on high basal signal. *Transduction*, 2: –, 2009.

[85] M.F. Mackey, J.R. Gunn, C. Maliszewsky, H. Kikutani, R.J. Noelle, and R.J. Jr. Barth. Dendritic cells require maturation via CD40 to generate protective antitumor immunity. *Journal of immunology*, 161:2094 – 2098, 1998.

[86] A.J. Macpherson and T. Uhr. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science*, 303:1662 – 1665, 2004.

[87] S.K. Mazmanian, C.H. Liu, A.O. Tzianabos, and D.L. Kasper. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell*, 122:107 – 118, 2005.

[88] E. Mazzini, L. Massimiliano, G. Penna, and M. Rescigno. Oral tolerance can be established via gap junction transfer of fed antigens from $CX3CR1^+$ macrophages to $CD103^+$ dendritic cells. *Immunity*, 40:248 – 261, 2014.

[89] J.R. McDole, L.W. Wheeler, K.G. McDonald, B. Wang, V. Konjufca, K.A. Knoop, R.D. Newberry, and M.J. Miller. Goblet cells deliver luminal antigen to $CD103^+$ dendritic cells in the small intestine. *Nature*, 483:345 – 349, 2012.

[90] D. McDonald, M.N. Price, J. Goodrich, E.P. Nawrocki, T.Z. DeSantis, G.L. Probst, A. andersen, R. Knight, and P. Hugenholtz. An improved greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME journal*, 6:610 – 618, 2012.

[91] O. Menard, M.-J. Butel, V. Gaboriau-Routhiau, and A.-J. Waligora-Dupriet. Gnotobiotic mouse immune response induced by Bifidobacterium sp. strains isolated from infants. *Applied and Environmental Microbiology*, 74:660 – 666, 2008.

[92] O. Menard, V. Gafa, N. Kapel, B. Rodriguez, M.J. Butel, and A.J. Waligora-Dupriet. Characterization of immunostimulatory CpG-rich sequences from different Bifidobacterium species. *Applied and environmental microbiology*, 76:2846 – 2855, 2010.

[93] M.E. Mikucki, D.T. Fisher, J. Matsuzaki, J.J. Skitzki, N.B. Gaulin, J.B. Muhitch, A.W. Ku, J.G. Frelinger, K. Odunsi, T.F. Gajewski, et al. Non-redundant requirement for CXCR3 signalling during tumoricidal T-cell trafficking across tumour vascular checkpoints. *Nature communications*, 6:7458, 2015.

[94] M.R. Neutra, N.J. Mantis, and J.-P. Kraehenbuhl. Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nature immunology*, 2:1004 – 1009, 2001.

[95] J.H. Niess, S. Brand, X. Gu, L. Landsman, S. Jung, B.A. McCormick, J.M. Vyas, M. Boes, H.L. Ploegh, J.G. Fox, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science*, 307:254 – 258, 2005.

[96] S. Nukiwa, M. andarini, J. Zaini, H. Xin, M. Kanehira, T. Suzuki, T. Fukuhara, H. Mizuguchi, T. Hayakawa, Y. Saijo, et al. Dendritic cells modified to express fractalkine/CX3CL1 in the treatment of preexisting tumors. *European journal of immunology*, 36:1019 – 1027, 2006.

[97] J. Ochoa-Reparaz, D.W. Mielcarz, L.E. Ditrio, A.R. Burroughs, S. Begum-Haque, S. Dasgupta, D.L. Kasper, and L.H. Kasper. Central nervous system demyelinating disease protection by the human commensal bacteroides fragilis depends on polysaccharide a expression. *Journal of immunology*, 185:4101 – 4108, 2010.

[98] J. Ochoa-Reparaz, D.W. Mielcarz, Y. Wang, S. Begum-Haque, S. Dasgupta, D.L. Kasper, and L.H. Kasper. A polysaccharide from the human commensal bacteroides fragilis protects against CNS demyelinating disease. *Mucosal immunology*, 3:487 – , 2010.

[99] J.Z. Oh, R. Ravindran, B. Chassaing, F.A. Carvalho, M.S. Maddur, M. Bower, P. Hakimpour, K.P. Gill, H.I. Nakaya, F. Yarovinsky, et al. TLR5-mediated sensing of gut microbiota is necessary for antibody responses to seasonal influenza vaccination. *Immunity*, 41:478 – 495, 2014.

[100] T. Olszak, D. An, S. Zeissig, M.P. Vera, J. Richter, A. Franke, J.N. Glickman, R. Siebert, R.M. Baron, D.L. Kasper, et al. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science*, 336:489 – 493, 2012.

[101] J. Pan, M. Zhang, J. Wang, Q. Wang, D. Xia, W. Sun, L. Zhang, H. Yu, Y. Liu, and X. Cao. Interferon-gamma is an autocrine mediator for dendritic cell maturation. *Immunology letters*, 94:141 – 151, 2004.

[102] A.R. Pettit, C. Quinn, K.P. MacDonald, L.L. Cavanagh, G. Thomas, W. Townsend, M. Handel, and R. Thomas. Nuclear localization of RelB is associated with effective antigen-presenting cell function. *Journal of immunology*, 159:3681 – 3691, 1997. Baltimore, Md : 1950.

[103] M. Rescigno, M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J.-P. Kraehenbuhl, and P. Ricciardi-Castagnoli. Dendritic cells express

tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature immunology*, 2:361 – 367, 2001.

[104] N.P. Restifo, M.E. Dudley, and S.A. Rosenberg. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nature reviews Immunology*, 12:269 – 281, 2012.

[105] P.F. Robbins, Y.C. Lu, M. El-Gamil, Y.F. Li, C. Gross, J. Gartner, J.C. Lin, J.K. Teer, P. Cliften, E. Tycksen, et al. Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive t cells. *Nature medicine*, 19:747 – 752, 2013.

[106] S.A. Rosenberg. A new era for cancer immunotherapy based on the genes that encode cancer antigens. *Immunity*, 10:281 – 287, 1999.

[107] S.A. Rosenberg, J.C. Yang, R.M. Sherry, U.S. Kammula, M.S. Hughes, G.Q. Phan, D.E. Citrin, N.P. Restifo, P.F. Robbins, J.R. Wunderlich, et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using t-cell transfer immunotherapy. *Clinical cancer research*, 17:4550 – 4557, 2011. an official journal of the American Association for Cancer Research.

[108] J.L. Round and S.K. Mazmanian. Inducible Foxp3$^+$ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proceedings of the National Academy of Sciences*, 107:12204 – 12204, 2010.

[109] S.L. Russell, M.J. Gold, M. Hartmann, B.P. Willing, L. Thorson, M. Wlodarska, N. Gill, M.R. Blanchet, W.W. Mohn, K.M. McNagny, et al. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO reports*, 13:440 – 447, 2012.

[110] M.R. Rutkowski, T.L. Stephen, N. Svoronos, M.J. Allegrezza, A.J. Tesone, A. Perales-Puchalt, E. Brencicova, X. Escovar-Fadul, J.M. Nguyen, M.G. Cadungog, et al. Microbially driven TLR5-dependent signaling governs distal malignant progression through tumor-promoting inflammation. *Cancer cell*, 27:27 – 40, 2015.

[111] D. Sancho, O.P. Joffre, A.M. Keller, N.C. Rogers, D. Martinez, P. Hernanz-Falcon, I. Rosewell, and C. Reis e Sousa. Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature*, 458:899 – 903, 2009.

[112] P. Scaffidi, T. Misteli, and M.E. Bianchi. Release of chromatin protein hmgb1 by necrotic cells triggers inflammation. *Nature*, 418:191 – 195, 2002.

[113] P.D Schloss. The effects of alignment quality, distance calculation method, sequence filtering, and region on the analysis of 16s rrna gene-based studies. *PLoS computational biology*, 6, 2010.

[114] A. Scholer, S. Hugues, A. Boissonnas, L. Fetler, and S. Amigorena. Intercellular adhesion molecule-1-dependent stable interactions between t cells and dendritic cells determine cd8+ t cell memory. *Immunity*, 28:258 – 270, 2008.

[115] O. Schulz, E. Jaensson, E.K. Persson, X. Liu, T. Worbs, W.W. Agace, and O. Pabst. Intestinal CD103$^+$, but not CX3CR1$^+$, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *The Journal of experimental medicine*, 206:3101 – 3114, 2009.

[116] D.J. Schwartzentruber, D.H. Lawson, J.M. Richards, R.M. Conry, D.M. Miller, J. Treisman, F. Gailani, L. Riley, K. Conlon, B. Pockaj, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *New England Journal of Medicine*, 364:2119 – 2127, 2011.

[117] K. Sekine, E. Watanabe-Sekine, T. Toida, T. Kasashima, T. Kataoka, and Y. Hashimoto. Adjuvant activity of the cell wall of bifidobacterium infantis for in vivo immune responses in mice. *Immunopharmacology and immunotoxicology*, 16:589 – 609, 1994.

[118] C. Sgadari, A.L. Angiolillo, B.W. Cherney, S.E. Pike, J.M. Farber, L.G. Koniaris, P. Vanguri, P.R. Burd, N. Sheikh, G. Gupta, et al. Interferon-inducible protein-10 identified as a mediator of tumor necrosis in vivo. *Proceedings of the National Academy of Sciences*, 93:13791 – 13796, 1996.

[119] V. Shankaran, H. Ikeda, A.T. Bruce, J.M. White, P.E. Swanson, L.J. Old, and R.D. Schreiber. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*, 410:1107 – 1111, 2001.

[120] Y. Shen, M.L. Giardino Torchia, G.W. Lawson, C.L. Karp, J.D. Ashwell, and S.K. Mazmanian. Outer membrane vesicles of a human commensal mediate immune regulation and disease protection. *Cell host and microbe*, 12:509 – 520, 2012.

[121] N. Singh, A. Gurav, S. Sivaprakasam, E. Brady, R. Padia, H. Shi, M. Thangaraju, P.D. Prasad, S. Manicassamy, D.H. Munn, et al. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity*, 40:128 – 139, 2014.

[122] S. Spranger, R. Bao, and T.F. Gajewski. Melanoma-intrinsic beta-catenin signalling prevents anti-tumour immunity. *Nature*, 523:231 – 235, 2015.

[123] S. Spranger, H.K. Koblish, B. Horton, P.A. Scherle, R. Newton, and T.F. Gajewski. Mechanism of tumor rejection with doublets of CTLA-4, PD-1/PD-L1, or IDO blockade involves restored IL-2 production and proliferation of CD8$^+$ T cells directly within the tumor microenvironment. *Journal for immunotherapy of cancer*, 2:3, 2014.

[124] S. Spranger, R.M. Spaapen, Y. Zha, J. Williams, Y. Meng, T.T. Ha, and T.F Gajewski. Up-regulation of PD-L1, IDO, and Tregs in the melanoma tumor microenvironment is driven by CD8$^+$ T cells. *Science translational medicine*, 5:200ra116, 2013.

[125] A.T. Stefka, T. Feehley, P. Tripathi, J. Qiu, K. McCoy, S.K. Mazmanian, M.Y. Tjota, G.Y. Seo, S. Cao, B.R. Theriault, et al. Commensal bacteria protect against food

allergen sensitization. *Proceedings of the National Academy of Sciences*, 111:13145 – 13150, 2014.

[126] C.B. Stober, S. Brode, J.K. White, J. Popoff, and J.M. Blackwell. Slc11a1, formerly Nramp1, is expressed in dendritic cells and influences major histocompatibility complex class ii expression and antigen-presenting cell function. *Infection and Immunity*, 75:5059 – 5067, 2007.

[127] R.M. Strieter, S.L. Kunkel, D.A. Arenberg, M.D. Burdick, and P.J. Polverini. Interferon gamma-inducible protein 10 IP-10, a member of the C-X-C chemokine family, is an inhibitor of angiogenesis. *Biochemical and biophysical research communications*, 210:51 – 57, 1995.

[128] S.L. Topalian, F.S. Hodi, J.R. Brahmer, S.N. Gettinger, D.C. Smith, D.F. McDermott, J.D. Powderly, R.D. Carvajal, J.A. Sosman, M.B. Atkins, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *The New England journal of medicine*, 366:2443 – 2454, 2012.

[129] M.B. Torchinsky, J. Garaude, A.P. Martin, and J.M. Blander. Innate immune recognition of infected apoptotic cells directs T(H)17 cell differentiation. *Nature*, 458:78 – 82, 2009.

[130] P.C. Tumeh, C.L. Harview, J.H. Yearley, I.P. Shintaku, E.J. Taylor, L. Robert, B. Chmielowski, M. Spasic, G. Henry, V. Ciobanu, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature*, 515:568 – 571, 2014.

[131] C. Uyttenhove, L. Pilotte, I. Theate, V. Stroobant, D. Colau, N. Parmentier, T. Boon, and B.J. Van den Eynde. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nature medicine*, 9:1269 – 1274, 2003.

[132] E. van Nood, A. Vrieze, M. Nieuwdorp, S. Fuentes, E.G. Zoetendal, W.M. de Vos, C.E. Visser, E.J. Kuijper, J.F.W.M. Bartelsman, J.G.P. Tijssen, et al. Duodenal infusion of donor feces for recurrent clostridium difficile. *New England Journal of Medicine*, 368:407 – 415, 2013.

[133] A. Vazquez-Torres, J. Jones-Carson, A.J. Baumler, S. Falkow, R. Valdivia, W. Brown, M. Le, R. Berggren, W.T. Parks, and F.C. Fang. Extraintestinal dissemination of salmonella by CD18-expressing phagocytes. *Nature*, 401:804 – 808, 1999.

[134] S. Viaud, F. Saccheri, G. Mignot, T. Yamazaki, R. Daillere, D. Hannani, D.P. Enot, C. Pfirschke, C. Engblom, M.J. Pittet, et al. The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. *Science*, 342:971 – 976, 2013.

[135] Q. Wang, G.M. Garrity, J.M. Tiedje, and J.R. Cole. Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied Environmental Microbiology*, 73:5261 – 5267, 2007.

[136] J.D. Wolchok, H. Kluger, M.K. Callahan, M.A. Postow, N.A. Rizvi, A.M. Lesokhin, N.H. Segal, C.E. Ariyan, R.A. Gordon, K. Reed, et al. Nivolumab plus ipilimumab in advanced melanoma. *The New England journal of medicine*, 369:122 – 133, 2013.

[137] S.R. Woo, M.B. Fuertes, L. Corrales, S. Spranger, M.J. Furdyna, M.Y. Leung, R. Duggan, Y. Wang, G.N. Barber, K.A. Fitzgerald, et al. STING-dependent cytosolic DNA sensing mediates innate immune recognition of immunogenic tumors. *Immunity*, 41:830 – 842, 2014.

[138] H.J. Wu, I.I. Ivanov, J. Darce, K. Hattori, T. Shima, Y. Umesaki, D.R. Littman, C. Benoist, and D. Mathis. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity*, 32:815 – 827, 2010.

[139] X. Xu, X.Y. Fu, J. Plate, and A.S. Chong. IFN-gamma induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. *Cancer Research*, 58:2832 – 2837, 1998.

[140] L. Zhang, J.R. Conejo-Garcia, D. Katsaros, P.A. Gimotty, M. Massobrio, G. Regnani, A. Makrigiannakis, H. Gray, K. Schlienger, M.N. Liebman, et al. Intratumoral t cells, recurrence, and survival in epithelial ovarian cancer. *New England Journal of Medicine*, 348:203 – 213, 2003.