

Nationale de la Recherche. A.S. was supported by BMSI YIG 2014. F.G. is supported by SigN core funding. L.Z., M.C., and I.B.G. are all sponsored by Association pour la Recherche contre le Cancer (PGA120140200851). F.C. was supported by INCA-DGOS (GOLD H78008). N.C. was supported by INCA-DGOS (GOLD study; 2012-1-RT-14-IGR-01). L'Oreal awarded a prize to M.V. We are grateful to the staff of the animal facility of Gustave Roussy and Institut Pasteur. We thank P. Gonin, B. Ryffel, T. Angelique, N. Chanthapathet, H. Li, and S. Zuberogitia for technical help. DNA sequence reads from this

study have been submitted to the NCBI under the Bioproject IDPRJNA299112 and are available from the Sequence Read Archive (SRP Study accession SRP065109; run accession numbers SRR2758006, SRR2758031, SRR2758178, SRR2758179, SRR2758180, SRR2758181, SRR2768454, and SRR2768457).

#### SUPPLEMENTARY MATERIALS

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3 April 2015; accepted 21 October 2015  
Published online 5 November 2015  
10.1126/science.aad1329

## CANCER IMMUNOTHERAPY

# Commensal *Bifidobacterium* promotes antitumor immunity and facilitates anti-PD-L1 efficacy

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T cell infiltration of solid tumors is associated with favorable patient outcomes, yet the mechanisms underlying variable immune responses between individuals are not well understood. One possible modulator could be the intestinal microbiota. We compared melanoma growth in mice harboring distinct commensal microbiota and observed differences in spontaneous antitumor immunity, which were eliminated upon cohousing or after fecal transfer. Sequencing of the 16S ribosomal RNA identified *Bifidobacterium* as associated with the antitumor effects. Oral administration of *Bifidobacterium* alone improved tumor control to the same degree as programmed cell death protein 1 ligand 1 (PD-L1)-specific antibody therapy (checkpoint blockade), and combination treatment nearly abolished tumor outgrowth. Augmented dendritic cell function leading to enhanced CD8<sup>+</sup> T cell priming and accumulation in the tumor microenvironment mediated the effect. Our data suggest that manipulating the microbiota may modulate cancer immunotherapy.

**H**arnessing the host immune system constitutes a promising cancer therapeutic because of its potential to specifically target tumor cells although limiting harm to normal tissue. Enthusiasm has been fueled by recent clinical success, particularly with antibodies that block immune inhibitory pathways, specifically CTLA-4 and the axis between programmed cell death protein 1 (PD-1) and its ligand 1 (PD-L1) (1, 2). Clinical responses to these immunotherapies are more frequent in patients who show evidence of an endogenous T cell response ongoing in the tumor microenvironment before therapy (3–6). However, the mechanisms that govern the presence or absence of this phenotype are not well understood. Theoretical sources of interpatient heterogeneity include host germline genetic differences, variability in patterns of somatic alterations in tumor cells, and environmental differences.

The gut microbiota plays an important role in shaping systemic immune responses (7–9). In the cancer context, a role for intestinal microbiota in

mediating immune activation in response to chemotherapeutic agents has been demonstrated (10, 11). However, it is not known whether commensal microbiota influence spontaneous immune responses against tumors and thereby affect the therapeutic activity of immunotherapeutic interventions, such as anti-PD-1/PD-L1 monoclonal antibodies (mAbs).

To address this question, 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 (12). We found that JAX and TAC mice exhibited significant differences in B16.SIY melanoma growth rate, with tumors growing more aggressively in TAC mice (Fig. 1A). This difference was immune-mediated: Tumor-specific T cell responses (Fig. 1, B and C) and intratumoral CD8<sup>+</sup> T cell accumulation (Fig. 1D) were significantly higher in JAX than in TAC mice. To begin to address whether this difference could be mediated by commensal microbiota, we cohoused JAX and TAC mice before tumor implantation. We found that cohousing ablated the differences in tumor growth (Fig. 1E) and immune responses (Fig. 1, F to H) between the two mouse populations, which suggested an environmental influence. Cohoused TAC

and JAX mice appeared to acquire the JAX phenotype, which suggested that JAX mice may be colonized by commensal microbes that dominantly facilitate antitumor immunity.

To directly test the role of commensal bacteria in regulating antitumor immunity, we transferred JAX or TAC fecal suspensions into TAC and JAX recipients by oral gavage before tumor implantation (fig. S1A). We found that prophylactic transfer of JAX fecal material, but not saline or TAC fecal material, into TAC recipients was sufficient to delay tumor growth (Fig. 2A) and to enhance induction and infiltration of tumor-specific CD8<sup>+</sup> T cells (Fig. 2, B and C, and fig. S1B), which supported a microbe-derived effect. Reciprocal transfer of TAC fecal material into JAX recipients had a minimal effect on tumor growth rate and antitumor T cell responses (Fig. 2, A to C, and fig. S1B), consistent with the JAX-dominant effects observed upon cohousing.

To test whether manipulation of the microbial community could be effective as a therapy, we administered JAX fecal material alone or in combination with antibodies targeting PD-L1 ( $\alpha$ PD-L1) to TAC mice bearing established tumors. Transfer of JAX fecal material alone resulted in significantly slower tumor growth (Fig. 2D), accompanied by increased tumor-specific T cell responses (Fig. 2E) and infiltration of antigen-specific T cells into the tumor (Fig. 2F), to the same degree as treatment with systemic  $\alpha$ PD-L1 mAb. Combination treatment with both JAX fecal transfer and  $\alpha$ PD-L1 mAb improved tumor control (Fig. 2D) and circulating tumor antigen-specific T cell responses (Fig. 2E), although there was little additive effect on accumulation of activated T cells within the tumor microenvironment (Fig. 2F). Consistent with these results,  $\alpha$ PD-L1 therapy alone was significantly more efficacious in JAX mice compared with TAC mice (Fig. 2G), which paralleled improved antitumor T cell responses (fig. S1C). These data indicate that the commensal microbial composition can influence spontaneous antitumor immunity, as well as a response to immunotherapy with  $\alpha$ PD-L1 mAb.

To identify specific bacteria associated with improved antitumor 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- and TAC feces-inoculated TAC mice over time ( $P = 0.001$  and  $P = 0.003$ , respectively, ANOSIM multivariate data analysis) and became similar

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to samples obtained from sham- and JAX feces-inoculated JAX mice (Fig. 3A). In contrast, 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 distance of the microbial shift was smaller (Fig. 3A).

Comparative analysis showed that 257 taxa were of significantly different relative abundance in JAX mice relative to TAC mice [false discovery rate (FDR) < 0.05, nonparametric  $t$  test] (Fig. 3B and table S1). Members belonging to several of these groups were similarly altered in JAX-fed TAC mice relative to sham- or TAC-inoculated TAC mice (Fig. 3C and tables S1 and S2). To further identify functionally relevant 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 (Fig. 2C). The only significant association was *Bifidobacterium* ( $P =$

$5.7 \times 10^{-5}$ , FDR = 0.0019, univariate regression) (table S3), which showed a positive association with antitumor T cell responses and increased in relative abundance over 400-fold in JAX-fed TAC mice (Fig. 3C). Stimulatory interactions between bifidobacteria and the host immune system, including those associated with interferon- $\gamma$  (IFN- $\gamma$ ), have been described previously (13–16). We thus hypothesized that members of this genus could represent a major component of the beneficial antitumor immune effects observed in JAX mice.

At the sequence level, *Bifidobacterium* operational taxonomic unit OTU\_681370 showed the largest increase in relative abundance in JAX-fed TAC mice (table S1) and the strongest association with antitumor T cell responses across all permutations (Fig. 3D and table S3). We further identified this bacterium as 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

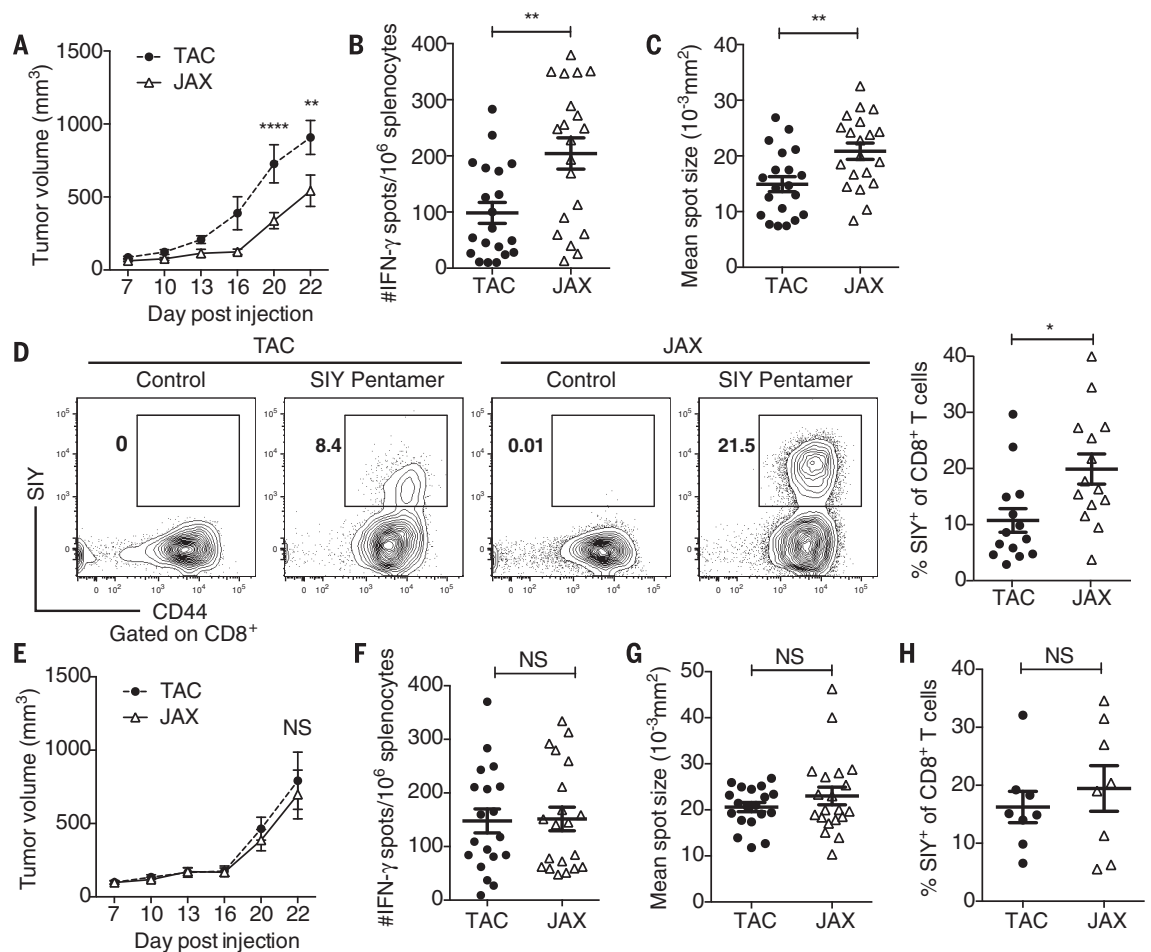
cocktail of *Bifidobacterium* species, which included *B. breve* and *B. longum* and administered this by oral gavage, alone or in combination with  $\alpha$ PD-L1, to TAC recipients bearing established tumors. Analysis of fecal bacterial content revealed that the most significant change in response to *Bifidobacterium* inoculation occurred in the *Bifidobacterium* genus ( $P = 0.0009$ , FDR = 0.015, nonparametric  $t$  test), with a 120-fold increase in OTU\_681370 (fig. S2A and table S4), which suggested that the commercial inoculum contained bacteria that were at least 97% identical to the taxon identified in JAX and JAX-fed TAC mice. An increase in *Bifidobacterium* could also be detected by quantitative polymerase chain reaction (PCR) (fig. S2B).

*Bifidobacterium*-treated mice displayed significantly improved tumor control in comparison with their non-*Bifidobacterium* treated counterparts (Fig. 3E), which was accompanied by robust induction of tumor-specific T cells in the periphery (Fig. 3F) and increased accumulation of antigen-specific CD8<sup>+</sup> T cells within the tumor

**Fig. 1. Differences in melanoma outgrowth and tumor-specific immune responses between C57BL/6 JAX and TAC mice are eliminated when mice are cohoused.** (A) B16.SIY tumor growth kinetics in newly arrived JAX and TAC mice.

(B) IFN- $\gamma$  enzyme-linked immunospot assay (ELISPOT) in tumor-bearing JAX and TAC mice 7 days after tumor inoculation. (C) Mean size of IFN- $\gamma$  spots ( $10^{-3}$  mm<sup>2</sup>). (D) Percentage of SIY<sup>+</sup> T cells of total CD8<sup>+</sup> T cells within the tumor of JAX and TAC mice as determined by flow cytometry 21 days after tumor inoculation. Representative plots (left), quantification (right).

(E) B16.SIY tumor growth kinetics in JAX and TAC mice cohoused for 3 weeks before tumor inoculation. (F) Number of IFN- $\gamma$  spots/ $10^6$  splenocytes in tumor-bearing JAX and TAC mice cohoused for 3 weeks before tumor inoculation. (G) Mean size of IFN- $\gamma$  spots ( $10^{-3}$  mm<sup>2</sup>). (H) Percentage of SIY<sup>+</sup> T cells of total CD8<sup>+</sup> T cells within the tumor of JAX and TAC mice cohoused for 3 weeks before tumor inoculation. Means  $\pm$  SEM combined from six independent experiments, analyzed by two-way analysis of variance (ANOVA) with Sidak's correction for multiple comparisons (A) and (E), or individual mice with means  $\pm$  SEM combined from four (B), (C), (F), (G) or three (D) and (H) independent experiments, analyzed by Student's  $t$  test; five mice per group per experiment; \* $P < 0.005$ , \*\* $P < 0.01$ ; NS, not significant.



(Fig. 3G and fig. S2C). These effects lasted several weeks (fig. S2, D and E).

The therapeutic effect of *Bifidobacterium* feeding was abrogated in CD8-depleted mice (fig. S3A), which indicated that the mechanism was not direct but rather through host antitumor T cell responses. Heat inactivation of the bacteria before oral administration also abrogated the therapeutic effect on tumor growth and reduced tumor-specific T cell responses to baseline (fig. S3, B to D), which suggested that the antitumor effect requires live bacteria. As an alternative strategy, we tested the therapeutic effect of *B. breve* and *B. longum* strains obtained from the American Type Culture Collection, which also showed significantly improved tumor control (fig. S4A). Administration of *Bifidobacterium* to TAC mice inoculated

with B16 parental tumor cells or MB49 bladder cancer cells also resulted in delayed tumor outgrowth (fig. S4, B and C, respectively). 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 (fig. S4D) or on tumor-specific T cell responses (fig. S4E), which suggested that modulation of antitumor immunity depends on the specific bacteria administered. Collectively, these data point to *Bifidobacterium* as a positive regulator of antitumor immunity in vivo.

Upon inoculation with *Bifidobacterium*, a small set of species were altered in parallel with *Bifidobacterium* (ANOSIM,  $P = 0.003$ ) (fig. S5A and table S4), however, for the most part, they did not resemble the changes observed with JAX feces

administration. Although we observed reductions (~2- to 10-fold) in members of the order Clostridiales, as well as in butyrate-producing species, upon *Bifidobacterium* inoculation, which could point to an inhibitory effect on the regulatory T cell compartment (17–19), we did not observe any difference in the frequency of CD4<sup>+</sup> Foxp3<sup>+</sup> T cells in tumors isolated from JAX and TAC mice (fig. S5B). Thus, although we cannot definitively rule out an indirect effect, it is unlikely that *Bifidobacterium* is acting primarily through modulation of the abundance of other bacteria.

We next assessed whether translocation of *Bifidobacterium* was occurring into the mesenteric lymph nodes, spleen, or tumor; however, no *Bifidobacterium* was detected in any of the organs

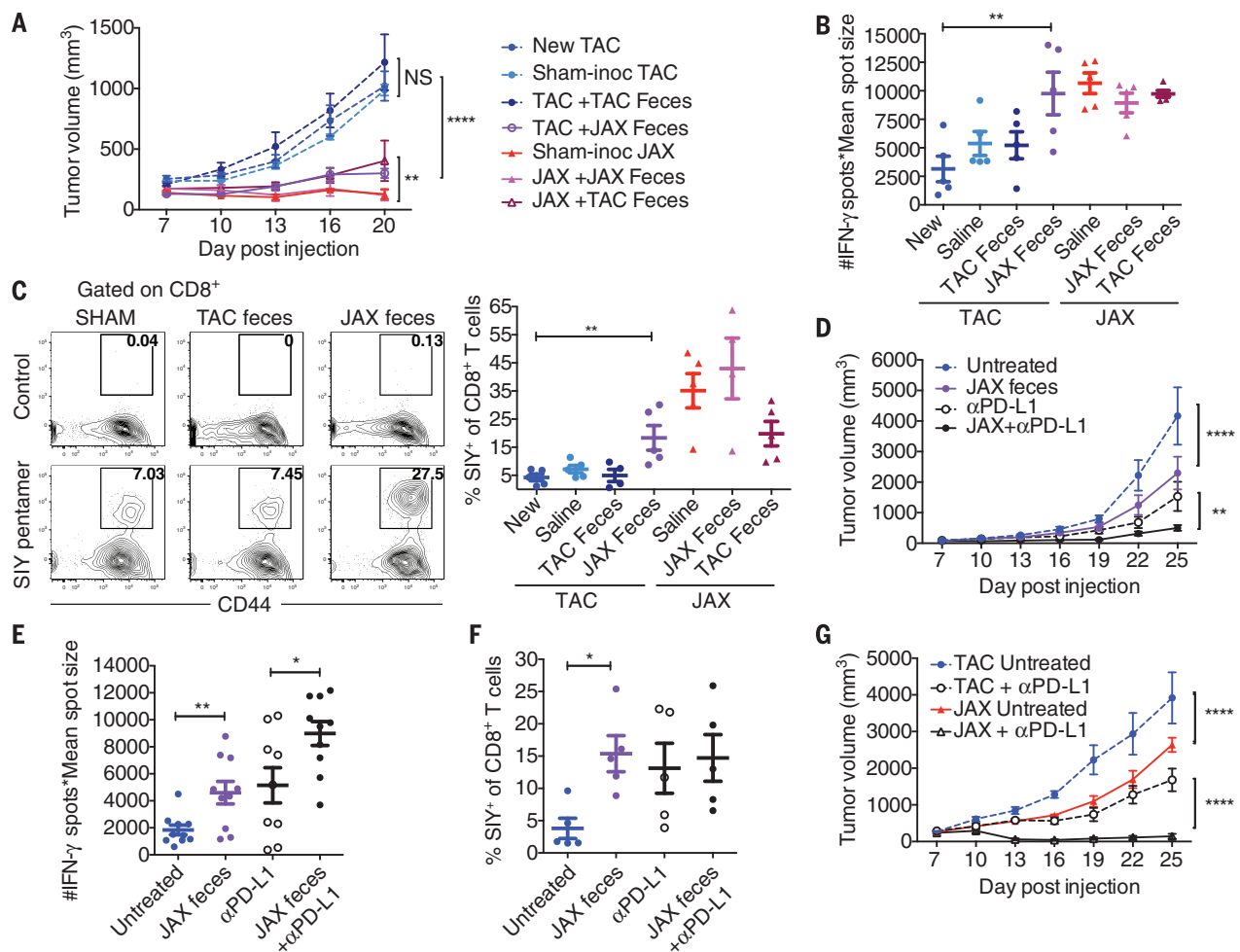
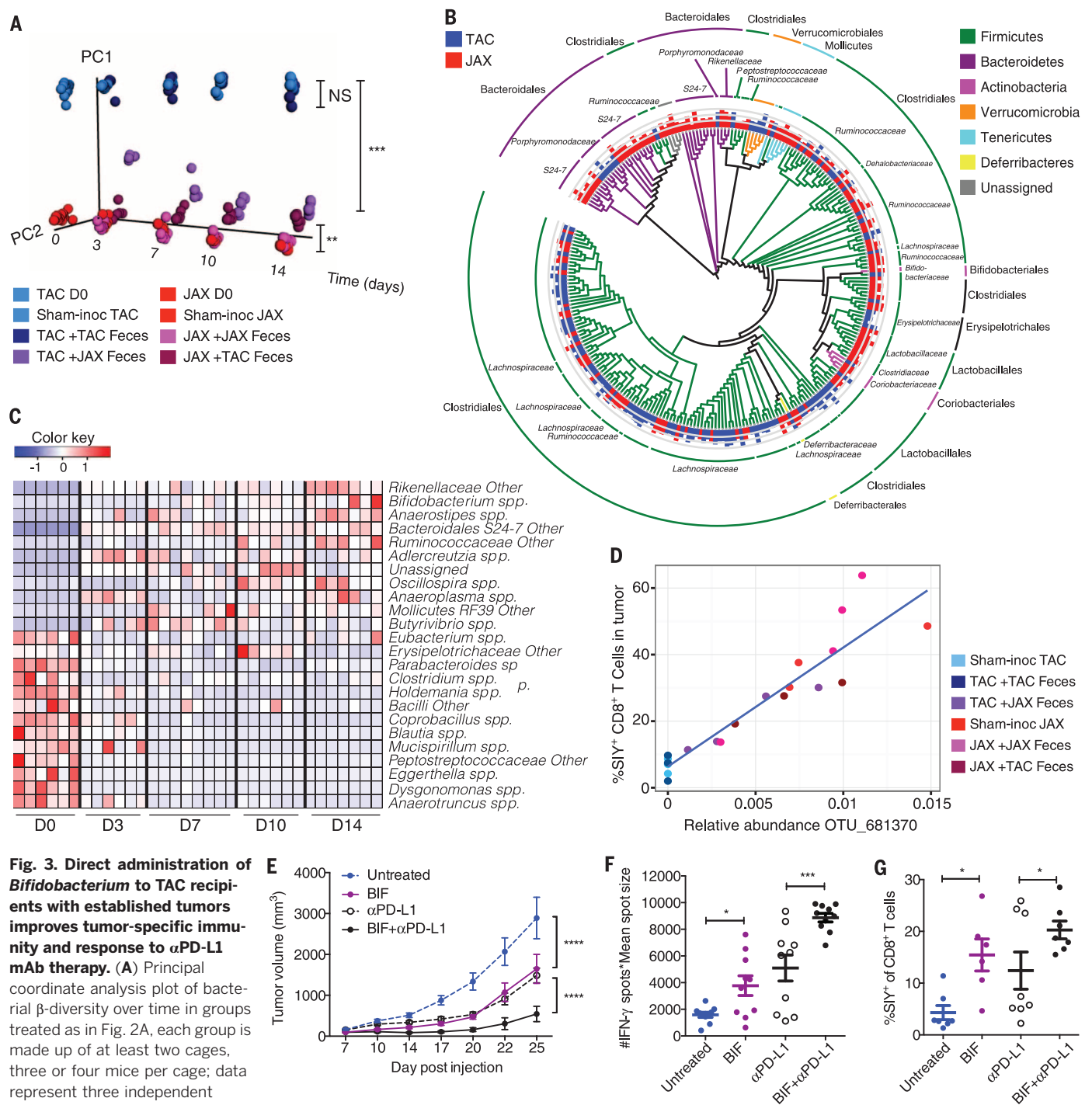


**Fig. 2. Oral administration of JAX fecal material to TAC mice enhances spontaneous antitumor immunity and response to αPD-L1 mAb therapy.**

(A) B16.SIY tumor growth in newly arrived TAC mice, TAC and JAX mice orally gavaged with phosphate-buffered saline or TAC or JAX fecal material before tumor implantation. (B) Number of IFN-γ spots × mean spot size (10<sup>-3</sup> mm<sup>2</sup>), determined by ELISPOT 7 days after tumor inoculation. (C) Percentage of SIY<sup>+</sup> CD8<sup>+</sup> T cells within the tumor of TAC and JAX mice treated as in (A), 21 days after tumor inoculation. Representative plots (left), quantification (right). (D) B16.SIY tumor growth in TAC mice, untreated or treated with JAX fecal material 7 and 14 days after tumor implantation, αPD-L1 mAb 7, 10, 13, and 16 days after tumor implantation, or both regimens. (E) IFN-γ ELISPOT as-

essed 5 days after start of treatment. (F) Percentage of tumor-infiltrating SIY<sup>+</sup> CD8<sup>+</sup> T cells, determined by flow cytometry 14 days after start of treatment. (G) B16.SIY tumor growth kinetics in TAC and JAX mice, untreated or treated with αPD-L1 mAb 7, 10, 13, and 16 days after tumor implantation. Means ± SEM analyzed by two-way analysis of variance (ANOVA) with Dunnett's (A) or Tukey's (D) and (G) correction for multiple comparisons; or individual mice with means ± SEM analyzed by one-way ANOVA with Holm-Sidak correction for multiple comparisons (B), (C), (E), and (F); data are representative of (A) to (C), (F), and (G) or combined from (D) and (E) two to four independent experiments; five mice per group per experiment; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ ; NS, not significant.



isolated from *Bifidobacterium*-gavaged tumor-bearing mice (fig. S5C). We thus concluded that the observed systemic immunological effects are likely occurring independently of bacterial translocation.

We subsequently interrogated the immunologic mechanisms underlying the observed differences in T cell responses between TAC, JAX, and *Bifidobacterium*-treated TAC mice (fig. S6A). CD8<sup>+</sup> SIY-specific 2C T cell receptor (TCR) Tg T cells exposed to tumors in JAX and *Bifidobacterium*-treated TAC mice exhibited greater expansion in the tumor-draining lymph node, as compared with their counterparts in TAC mice (fig. S6B).

However, they produced markedly greater IFN- $\gamma$  in both the tumor-draining lymph node and the spleen of JAX and *Bifidobacterium*-fed TAC tumor-bearing mice (Fig. 4A), consistent with our analyses of the endogenous T cell response (Figs. 1C, 2E, and 3F). These data pointed to an improvement in immune responses upstream of T cells, at the level of host dendritic cells (DCs). Consistent with this hypothesis, we found an increased percentage of major histocompatibility complex (MHC) Class II<sup>hi</sup> DCs in the tumors of JAX and *Bifidobacterium*-treated TAC mice (Fig. 4B).

We therefore used genome-wide transcriptional profiling of early tumor-infiltrating DCs isolated

from TAC, JAX, and *Bifidobacterium*-treated TAC mice (fig. S7A and table S5). Pathway analysis of 760 gene transcripts up-regulated in both JAX and *Bifidobacterium*-treated TAC-derived DCs relative to DCs from untreated TAC mice identified cytokine-cytokine receptor interaction, T cell activation, and positive regulation of mononuclear cell proliferation as significantly enriched pathways (Fig. 4C and fig. S7B). Many of these genes have been shown to be critical for antitumor responses, including those involved in CD8<sup>+</sup> T cell activation and costimulation [H2-m2 (MHC-I), *Cd40*, *Cd70*, and *Icam1*] (20–22); DC maturation (*Relb* and *Ifngr2*) (23, 24); antigen processing and

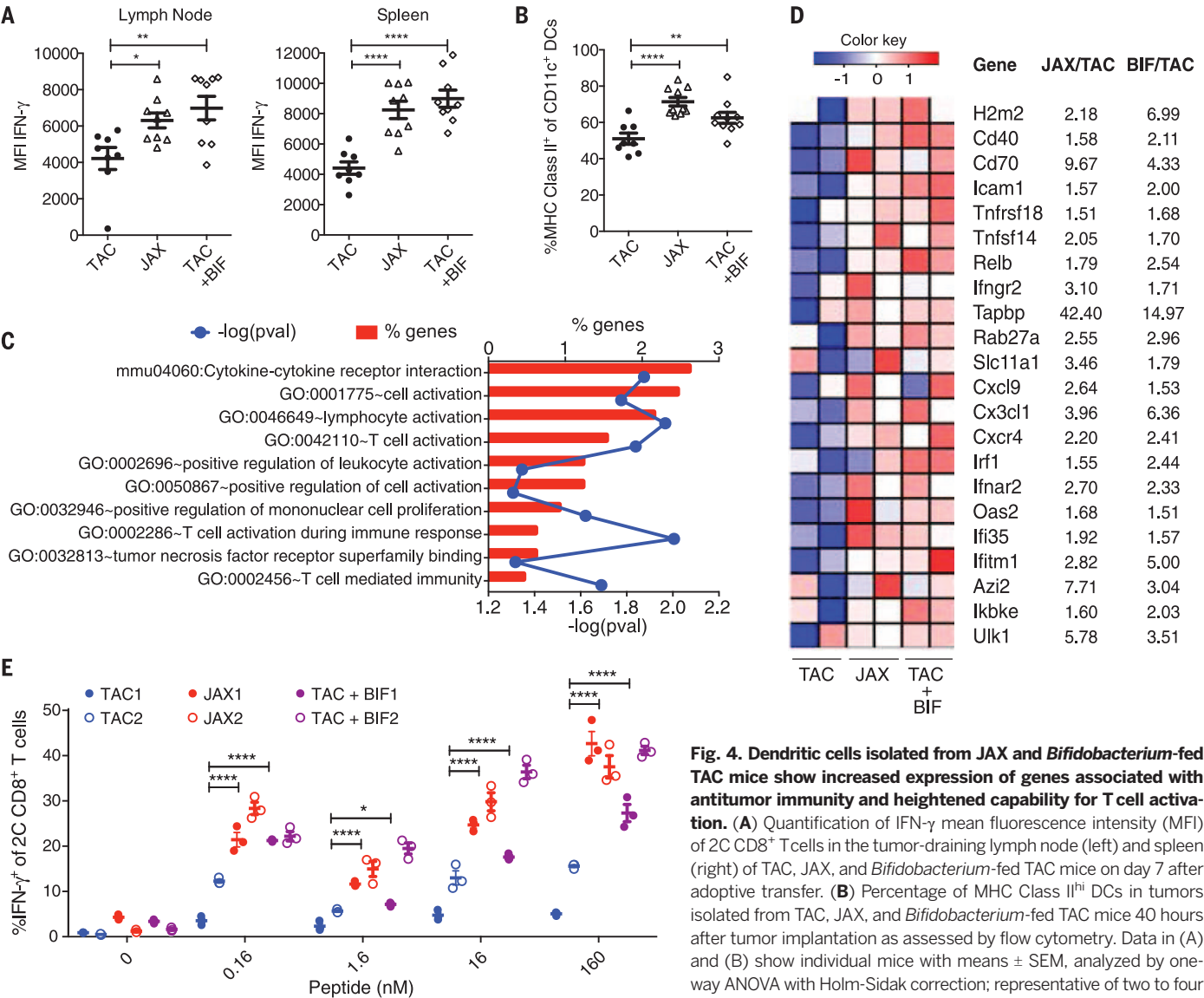


**Fig. 4. Dendritic cells isolated from JAX and *Bifidobacterium*-fed TAC mice show increased expression of genes associated with antitumor immunity and heightened capability for T cell activation.** (A) Quantification of IFN- $\gamma$  mean fluorescence intensity (MFI) of 2C CD8<sup>+</sup> T cells in the tumor-draining lymph node (left) and spleen (right) of TAC, JAX, and *Bifidobacterium*-fed TAC mice on day 7 after adoptive transfer. (B) Percentage of MHC Class II<sup>hi</sup> DCs in tumors isolated from TAC, JAX, and *Bifidobacterium*-fed TAC mice 40 hours after tumor implantation as assessed by flow cytometry. Data in (A) and (B) show individual mice with means  $\pm$  SEM, analyzed by one-way ANOVA with Holm-Sidak correction; representative of two to four independent experiments, eight or nine mice per group per experiment. (C) 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 40 hours after tumor inoculation, as assessed by DAVID pathway analysis. Red bars indicate the percentage of genes in a pathway up-regulated in DCs isolated from JAX and *Bifidobacterium*-fed TAC mice. Blue line indicates *P* values calculated by Fisher's exact test. (D) 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. (E) Quantification of IFN- $\gamma$ <sup>+</sup> 2C TCR Tg CD8<sup>+</sup> T cells stimulated in vitro with DCs purified from peripheral lymphoid tissues of naïve TAC, JAX, and *Bifidobacterium*-treated TAC mice in the presence of different concentrations of SIY peptide. Analyses in (C) to (E) were performed on data combined from two independent experiments, five mice pooled per group per experiment. (E) Technical replicates of pooled samples from each experiment separately and were analyzed by fitting a linear mixed model, with Bonferroni correction for multiple comparisons: \**P* < 0.05, \*\*\*\**P* < 0.0001.

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cross presentation (*Tapbp*, *Rab27a*, and *Slc11a1*) (25–27); chemokine-mediated recruitment of immune cells to the tumor microenvironment (*Cxcl9*, *Cxcl10*, and *Cxcr4*) (28–30); and type I interferon signaling (*Irfl*, *Ifnar2*, *Oas2*, *Ifi35*, and *Ifitm1*) (31, 32) (Fig. 4D and fig. S7C). 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 (13).

To test whether functional differences in DCs isolated from TAC, JAX, and *Bifidobacterium*-treated TAC mice could be sufficient to explain the differences in T cell priming observed in vivo, we purified DCs from lymphoid tissues of naïve TAC, JAX, and *Bifidobacterium*-treated TAC mice and tested their ability to induce carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled CD8<sup>+</sup> SIY-specific 2C TCR Tg T cell proliferation and acquisition of IFN- $\gamma$  production in vitro. DCs purified from JAX and *Bifidobacterium*-treated TAC mice induced 2C T cell proliferation at lower antigen concentration than did DCs purified from naïve TAC mice (fig. S8, A and B). Furthermore, at all antigen concentrations, JAX-derived DCs elicited elevated levels of T cell IFN- $\gamma$  production (Fig. 4E and fig. S8A). We observed similar effects upon oral administration of *Bifidobacterium* to TAC mice before DC isolation (Fig. 4E and fig. S8A). Taken together, these data suggest that commensal *Bifidobacterium*-derived signals modulate the activation of DCs in the steady state, which in turn supports improved effector function of tumor-specific CD8<sup>+</sup> T cells.

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 antitumor immunity could be 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 or to release soluble factors that disseminate systemically and lead to improved DC function.

Our results do not rule out a contribution of other commensal bacteria species in having the capability to regulate antitumor immunity, either positively or negatively. Our data support the idea that one source of intersubject heterogeneity with regard to spontaneous antitumor immunity and therapeutic effects of antibodies targeting the PD-1/PD-L1 axis may be the 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. Similar analyses can be performed in humans, by using 16S rRNA sequencing of stool samples from patients receiving checkpoint blockade or other immunotherapies, to identify commensals associated with clinical benefit.

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## ACKNOWLEDGMENTS

R code for gene expression analyses can be found at [https://github.com/kipkeston/TG\\_Microbiota](https://github.com/kipkeston/TG_Microbiota); microarray data can be found at [www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73475](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73475) and [www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73476](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73476); 16S rRNA sequencing data can be found at [www.ncbi.nlm.nih.gov/bioproject/297465](http://www.ncbi.nlm.nih.gov/bioproject/297465). The data reported in this manuscript are presented in the main paper and in the supplementary materials. We thank D. Ringus for anaerobic culture of bifidobacteria and colony quantification, Y. Zhang for providing *Lactobacillus murinus* cultures, and R. Sweis for providing the MB49 bladder cancer cell line. This work was supported by a Team Science Award from the Melanoma Research Alliance, and National Institute of Diabetes and Digestive and Kidney Diseases, NIH, P30 Digestive Disease Research Core Center Grant (DK42086), the University of Chicago Cancer Biology training program (5T32CA009594-25), and the Cancer Research Institute fellowship program. The University of Chicago has filed a patent application that relates to microbial community manipulation in cancer therapy.

## SUPPLEMENTARY MATERIALS

[www.sciencemag.org/content/350/6264/1084/suppl/DC1](http://www.sciencemag.org/content/350/6264/1084/suppl/DC1)  
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25 April 2015; accepted 9 October 2015  
Published online 5 November 2015  
10.1126/science.aac2455

## MALARIA

# Malaria parasites target the hepatocyte receptor EphA2 for successful host infection

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The invasion of a suitable host hepatocyte by mosquito-transmitted *Plasmodium* sporozoites is an essential early step in successful malaria parasite infection. Yet precisely how sporozoites target their host cell and facilitate productive infection remains largely unknown. We found that the hepatocyte EphA2 receptor was critical for establishing a permissive intracellular replication compartment, the parasitophorous vacuole. Sporozoites productively infected hepatocytes with high EphA2 expression, and the deletion of EphA2 protected mice from liver infection. Lack of host EphA2 phenocopied the lack of the sporozoite proteins P52 and P36. Our data suggest that P36 engages EphA2, which is likely to be a key step in establishing the permissive replication compartment.

Malaria infections place a tremendous burden on global health (1). Their causative agents, *Plasmodium* parasites, are transmitted to mammals as sporozoites by the bite of *Anopheles* mosquitoes. After entry into a capillary, sporozoites are carried to the liver, where they pass through multiple cells before recognizing and invading hepatocytes. During invasion, the sporozoite forms a protec-

tive parasitophorous vacuole made of hepatocyte plasma membrane, which ensconces the parasite, establishes the intrahepatocytic replication

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## Commensal *Bifidobacterium* promotes antitumor immunity and facilitates anti–PD-L1 efficacy

Ayelet Sivan, Leticia Corrales, Nathaniel Hubert, Jason B. Williams, Keston Aquino-Michaels, Zachary M. Earley, Franco W. Benyamin, Yuk Man Lei, Bana Jabri, Maria-Luisa Alegre, Eugene B. Chang, and Thomas F. Gajewski

*Science*, **350** (6264), .  
DOI: 10.1126/science.aac4255

### Gut microbes affect immunotherapy

The unleashing of antitumor T cell responses has ushered in a new era of cancer treatment. Although these therapies can cause dramatic tumor regressions in some patients, many patients inexplicably see no benefit. Mice have been used in two studies to investigate what might be happening. Specific members of the gut microbiota influence the efficacy of this type of immunotherapy (see the Perspective by Snyder *et al.*). Vétizou *et al.* found that optimal responses to anticytotoxic T lymphocyte antigen blockade required specific *Bacteroides* spp. Similarly, Sivan *et al.* discovered that *Bifidobacterium* spp. enhanced the efficacy of antiprogrammed cell death ligand 1 therapy.

*Science*, this issue, p. 1079 and p. 1084; see also p. 1031

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Supplementary Materials for  
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Published 5 November 2015 on *Science Express*  
DOI: 10.1126/science.aac4255

**This PDF file includes**

Materials and Methods  
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**Other Supplementary Material for this manuscript includes the following:**  
(available at [www.sciencemag.org/cgi/content/full/science.aac4255/DC1](http://www.sciencemag.org/cgi/content/full/science.aac4255/DC1))

Tables S1 to S7

## Materials and Methods

### Animals and tumor model

C57BL/6 mice were obtained from Jackson laboratory or Taconic farms. For all experiments 6–8-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 (33). 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  $1 \times 10^6$  B16.SIY tumor cells or parental B16.F10 tumor cells. For bladder cancer model experiments, mice were injected subcutaneously with  $2 \times 10^6$  MB49 cells. Tumor size was measured twice a week until endpoint and tumor volume was determined as  $\text{length} \times \text{width}^2 \times 0.5$ . All experimental animal procedures were approved by the University of Chicago Animal Care and Use Committee (IACUC).

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

ELISPOT plates (*Millipore, MAIP S4510*) were coated with purified  $\alpha\text{IFN-}\gamma$  (*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°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-2K<sup>b</sup> complexed to SIYRYYYGL (SIY) peptide or to control SIINFEKL peptide, and stained with CD3-AX700 (*Ebioscience, 17A2*), CD8-PacBlue (*Biolegend, 53-6.7*), CD4-APC (*Pharmingen, 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).

### Fecal transfers and $\alpha\text{PD-L1}$ 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 $\mu\text{l}$  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 $\mu\text{g}$   $\alpha\text{PD-L1}$  mAb (clone 10f.9g2, *BioXCell*) in 100 $\mu\text{l}$  PBS on days 7, 10, 13 and 16 post-tumor implantation.

### 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 (34); specifically, the open reference OTU picking protocol was used at 97% sequence identity against the Greengenes database (05/13 release) (35). PYNAST was used to align sequences (36) and RDP Classifier was used for taxonomic assignment (37). Community structure was compared using weighted and unweighted UniFrac distances (38). 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 (39); 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<sup>+</sup>CD8<sup>+</sup> 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

$$\text{SIY} \sim \text{relative\_abundance}_k + \text{PC1} + \text{PC2}$$

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  
CGGTTTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAACGGTGGATCCGCGCC  
GGGTACGGGCGGGCTTGAGTGCGGTAGGGGAGACTGGAATCCCCGGT.

#### 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 5x10<sup>9</sup> CFU/ml. Each mouse was given 200ul of *Bifidobacterium* (1x10<sup>9</sup> 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 5x10<sup>9</sup> 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, 200ul/mouse, 1x10<sup>9</sup>

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  $5 \times 10^{10}$  CFU/ml. Each mouse was orally gavaged with 100  $\mu$ l of bacterial suspension ( $5 \times 10^9$  CFU/mouse) 7 and 14 days following tumor inoculation.

#### CD8<sup>+</sup> T Cell Depletion

For depletion of CD8<sup>+</sup> 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<sup>+</sup> T cells from the peripheral blood, as evaluated by flow cytometry.

#### 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 Qias shredding (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. Real time quantitative PCR (qPCR) was used to quantify bacterial SSU (16S) rRNA gene abundance, in the feces and tissue as described previously (40-42). 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) (42). Primers and probe were synthesized by IDT and Invitrogen respectively. qPCR master mixes contained TaqMan Universal qPCR 1X Master Mix (Applied-Biosystems), 0.25  $\mu$ M 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.

#### CFSE-labeled 2C CD8<sup>+</sup> T cell adoptive transfer

CD8<sup>+</sup> T cells were isolated from the spleen and lymph node of naïve CD45.1/.2<sup>+</sup> 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<sup>+</sup> 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  $1 \times 10^6$  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,

*XMGI.2*). Intracellular IFN- $\gamma$  production and CFSE dilution were assessed in gated CD45.1/.2<sup>+</sup> 2C CD8<sup>+</sup> T cells by flow cytometry.

#### 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<sup>6</sup> 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 (*Biolegend*, 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<sup>+</sup>CD3<sup>-</sup>CD19<sup>-</sup>MHCII<sup>hi</sup>CD11c<sup>+</sup> dendritic 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.

#### Quantitative 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.

#### 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 37°C with 5% CO<sub>2</sub>. 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 AgilentBioanalyzer 2100, and all RNA samples used for microarray analysis had an RNA Integrity Number = 10.0.

#### Dendritic cell stimulation of CFSE-labeled 2C CD8<sup>+</sup> 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<sup>+</sup> 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<sup>5</sup> DCs were plated 1:1 with CD45.1/.2<sup>+</sup> CFSE- labeled 2C CD8<sup>+</sup> T cells (MACS CD8<sup>+</sup> 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-γ-PE (*BD*, XMG1.2). Intracellular IFN-γ production and CFSE dilution were assessed in gated CD45.1/.2<sup>+</sup> 2C CD8<sup>+</sup> T cells by flow cytometry.

#### 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.

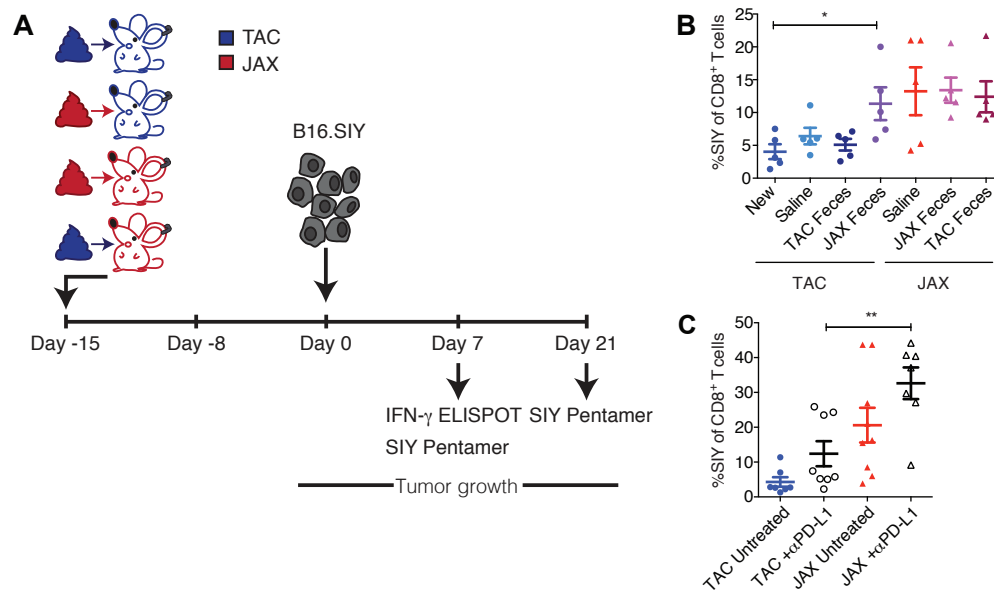


**Fig. S1.**

**(A)** 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 supernatant 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. **(B)** Percentage of SIY<sup>+</sup> T cells of total CD8<sup>+</sup> T cells within the tumor of groups as in Figure 2A, determined by flow cytometry 7 days post-tumor inoculation. **(C)** Percentage of SIY<sup>+</sup> T cells of total CD8<sup>+</sup> 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 individual mice with mean  $\pm$  SEM analyzed by one-way ANOVA with Holm-Sidak correction for multiple comparisons; data are representative (B) or combined (C) from at least two independent experiments; 5 mice per group per experiment; \* $P < 0.05$ , \*\* $P < 0.01$ .

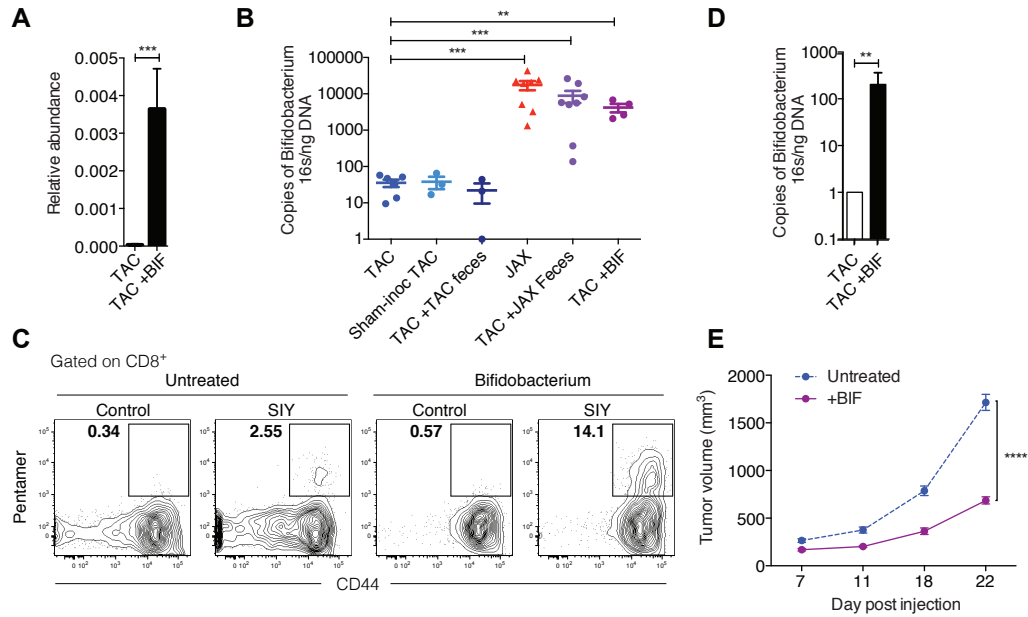


**Fig. S2**

(A) Relative abundance of *Bifidobacterium* OTU\_681370 in fecal material obtained from TAC mice 7 days following inoculation with commercial *Bifidobacterium* species. (B) *Bifidobacterium* levels in fecal material obtained from groups as shown, assessed by qPCR using genus-specific primers. (C) Representative plots showing percentage of SIY<sup>+</sup> T cells of total CD8<sup>+</sup> T cells within the tumor of untreated and *Bifidobacterium*-treated TAC mice, as assessed by flow cytometry 14 days following start of treatment. (D) *Bifidobacterium* levels in TAC mice 3 weeks post *Bifidobacterium* administration, assessed by qPCR. (E) B16.SIY tumor growth in TAC mice, untreated or inoculated with *Bifidobacterium* 6 weeks prior to tumor implantation. Data show mean  $\pm$  SD (A) or SEM [(D), (E)], or individual mice with mean  $\pm$  SEM (B), analyzed by non parametric *t*-tests [(A), (B), (D)] or two-way ANOVA with Sidak's correction (E); *n* = 5-10 mice per group; \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001.

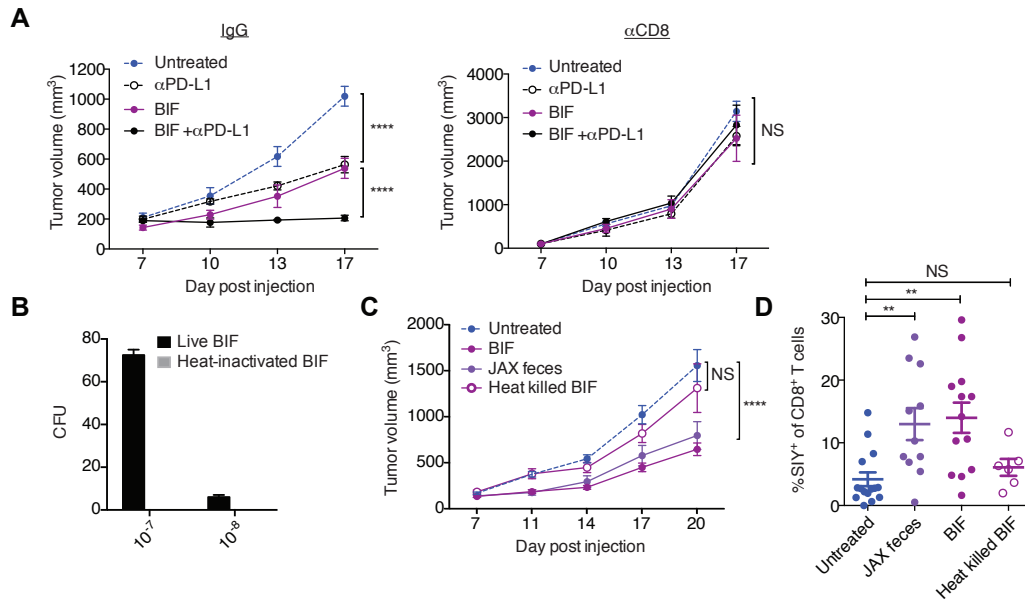


**Fig. S3**

**A)** B16.SIY tumor growth for isotype-treated (left) or CD8-depleted (right) groups as in Figure 3E. **(B)** 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. **(C)** 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. **(D)** Percentage of tumor-infiltrating SIY<sup>+</sup> T cells of total CD8<sup>+</sup> T cells for treatment groups as in (C), determined by flow cytometry 14 days after start of treatment. Data show mean  $\pm$  SEM analyzed by two-way ANOVA with Tukey's (A) or Dunnett's (C) correction for multiple comparisons; or individual mice with mean  $\pm$  SEM analyzed by one-way ANOVA with Holm-Sidak correction for multiple comparisons (D); data are representative [(A) and (B)] or combined [(C) and (D)] from at least two independent experiments; 5 mice per group per experiment; \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ , NS, not significant.

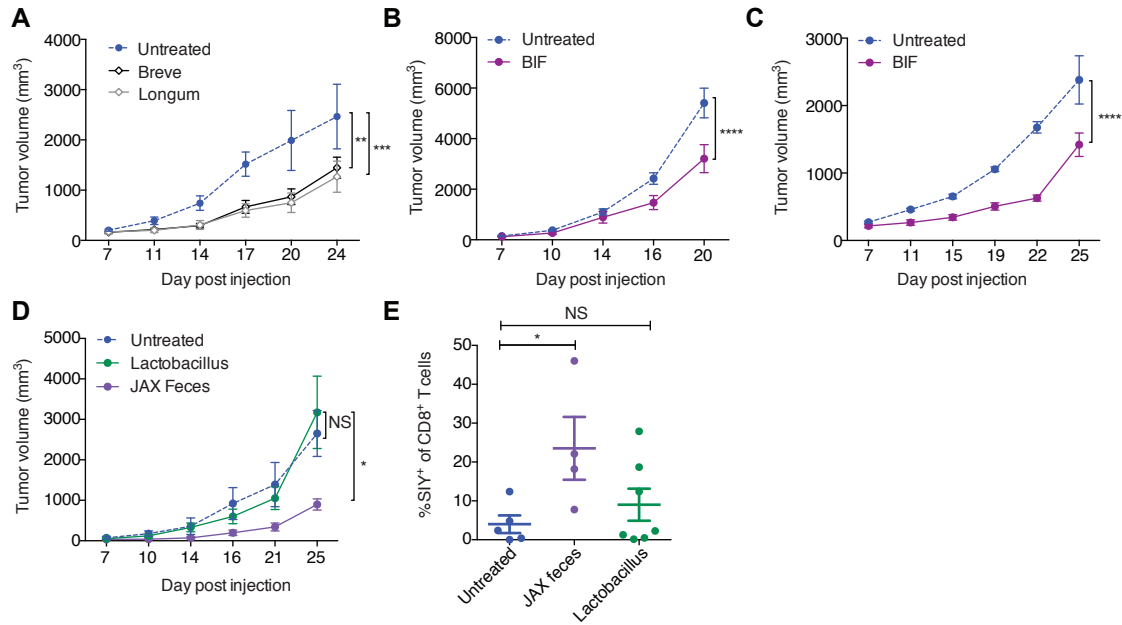


**Fig. S4**

(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<sup>+</sup> T cells of total CD8<sup>+</sup> T cells for treatment groups as in (D), determined by flow cytometry 18 days after start of treatment. Data show mean  $\pm$  SEM [(A) - (D)] or individual mice with mean  $\pm$  SEM (E), analyzed by two-way ANOVA with Dunnett's [(A), (D)] or Sidak's 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.

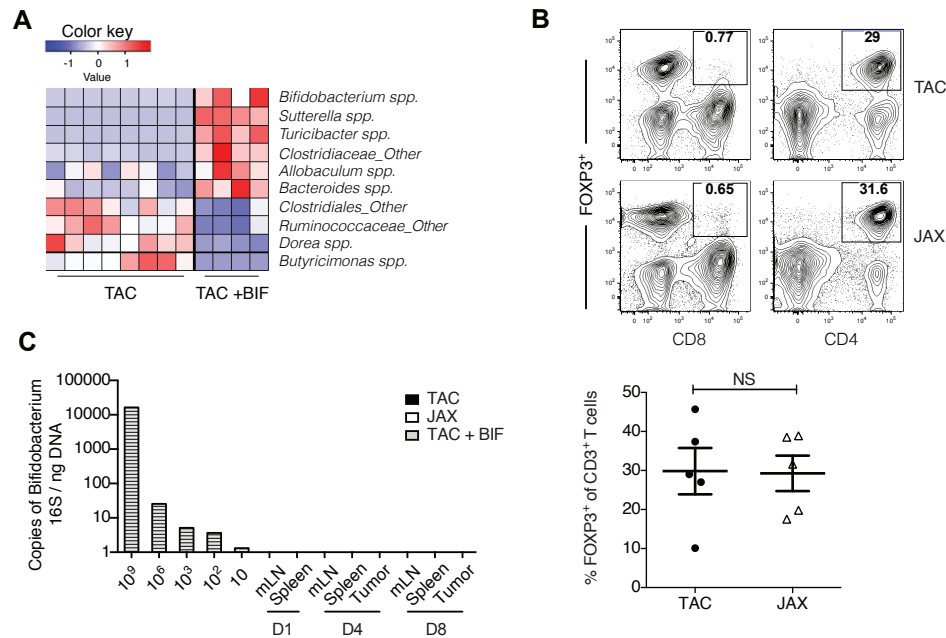


**Fig. S5**

(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<sup>+</sup> FOXP3<sup>+</sup> 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 student's *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.

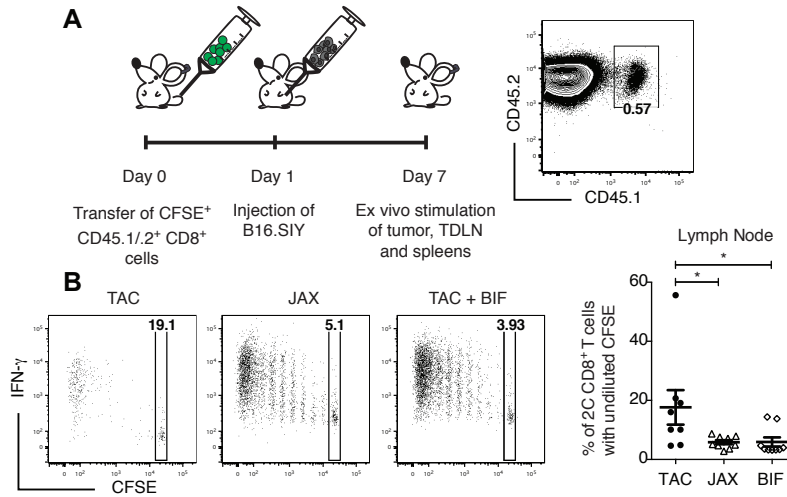


**Fig. S6**

**(A)** Schematic of *in vivo* 2C proliferation assays. CD8<sup>+</sup> T cells were isolated from the spleen and lymph node of naïve 2C TCR Tg CD45.1<sup>+</sup>.2<sup>+</sup> mice, labeled with CFSE and injected i.v. into CD45.2<sup>+</sup> C57BL/6 mice derived from either TAC, JAX or *Bifidobacterium*-treated TAC mice. 24 hours later, mice were inoculated with  $1 \times 10^6$  B16.SIY melanoma cells s.c. Spleen and tumor-draining lymph node were harvested and restimulated ex-vivo with SIY peptide. Intracellular IFN- $\gamma$  production and CFSE dilution were assessed in gated CD45.1<sup>+</sup>.2<sup>+</sup> 2C T cells by flow cytometry; TDLN=tumor-draining lymph node. **(B)** Representative CFSE dilution assessed in gated CD45.1<sup>+</sup>.2<sup>+</sup> 2C T cells by flow cytometry (**left**) and quantification (**right**). Data show individual mice  $\pm$  SEM and are representative of two independent experiments, 8 mice per group per experiment; analyzed by one-way ANOVA with Holm-Sidak correction;  $*P < 0.05$ .

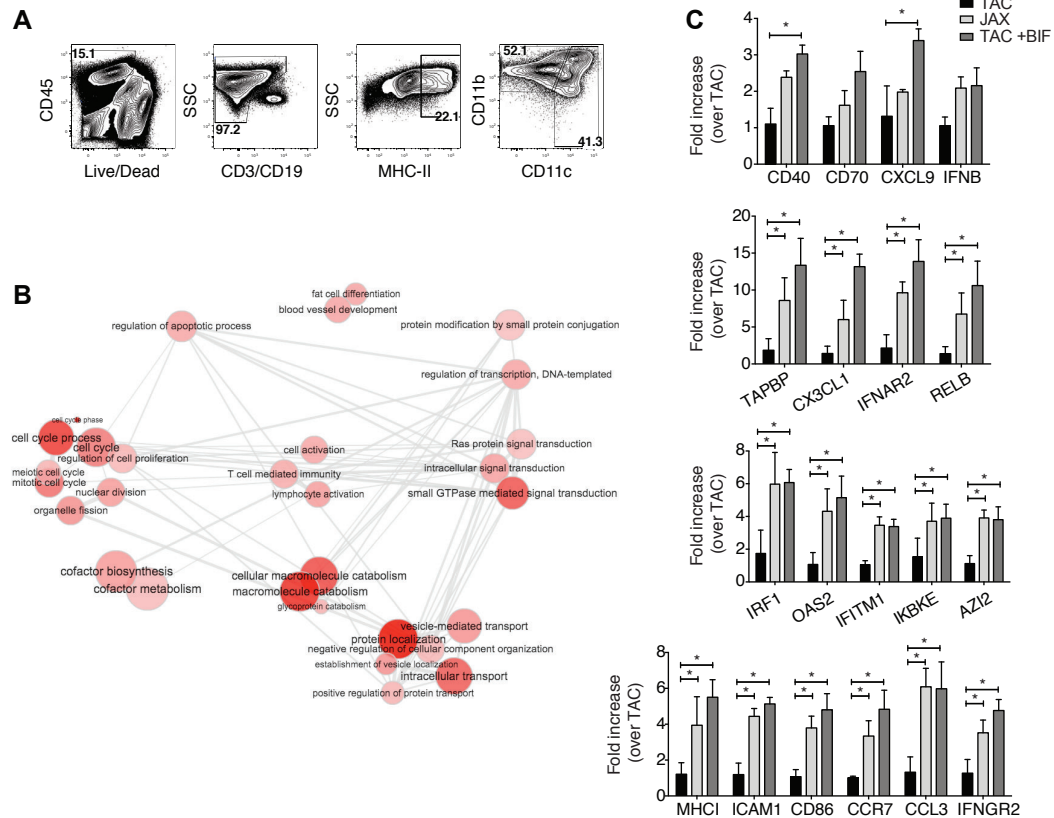


**Fig. S7**

**(A)** Representative plots depicting the strategy for isolation of DCs from tumors in JAX, TAC and *Bifidobacterium*-treated TAC mice: live CD45<sup>+</sup>CD3<sup>-</sup>CD19<sup>-</sup>MHCII<sup>hi</sup>CD11c<sup>+</sup> dendritic cells were sorted as shown. **(B)** All enriched biological pathways and functions found within the subset of elevated genes (fold change  $\geq 1.5$ ) 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)** qPCR validation of genes identified by microarray gene expression profiling as in (B). Data show mean  $\pm$  SEM, and are combined from two to three independent experiments, 5 mice pooled per sample, per experiment; analyzed by student's *t*-test \**FDR* < 0.5.

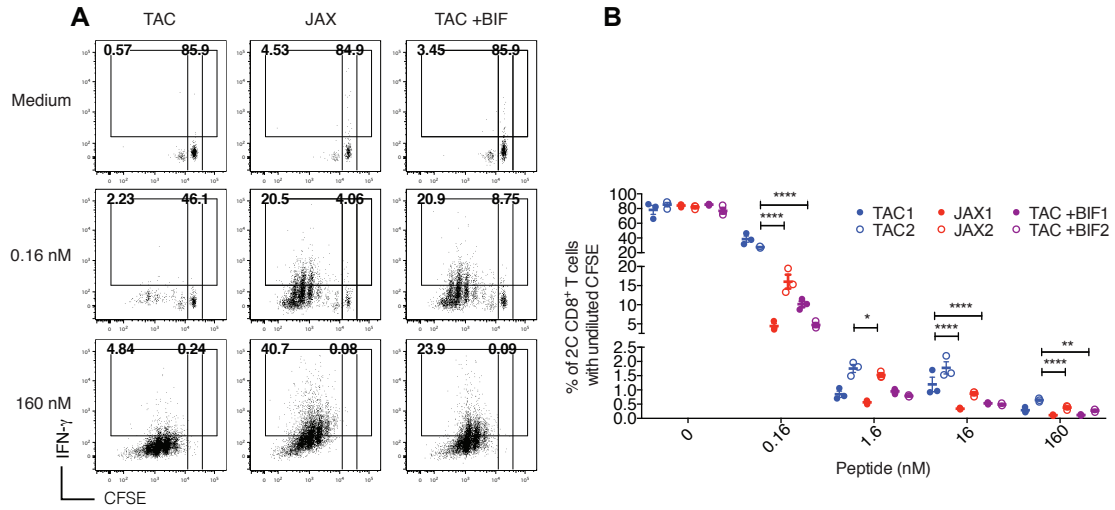


**Fig. S8**

**(A)** Representative flow plots of CFSE dilution and IFN- $\gamma$  production in 2C CD8<sup>+</sup> 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. **(B)** Percentage of 2C CD8<sup>+</sup> T cells with undiluted CFSE, 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. 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; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ .

**Table S1.** Comparison of bacterial abundance between JAX and TAC mice, sham-, TAC-, and JAX-fed TAC mice at sequence level.

**Table S2.** Comparison of bacterial abundance between JAX and TAC mice, sham-, TAC-, and JAX-fed TAC mice at genus level.

**Table S3.** Univariate association testing between bacterial abundance and antigen-specific T cell infiltration across all permutation groups at sequence and genus levels.

**Table S4.** Comparison of bacterial abundance between TAC and *Bifidobacterium*-fed TAC mice at sequence and genus levels.

**Table S5.** Gene expression profiling results (Illumina microarray) comparing gene expression in DCs isolated from tumors of TAC, JAX, and *Bifidobacterium*-fed TAC mice.

**Table S6.** Gene expression profiling results (Illumina microarray) comparing gene expression in BM-DCs untreated or stimulated with *Bifidobacterium* in vitro.

**Table S7.** Primer sequences and probes (Roche Universal Probe Library) for qPCR validation of gene transcripts up-regulated by  $\geq 1.5$ -fold in both JAX and *Bifidobacterium*-treated TAC-derived DCs relative to DCs from untreated TAC mice.

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