RESEARCH ARTICLE

A Natural Polyphenol Exerts Antitumor Activity and Circumvents Anti–PD-1 Resistance through Effects on the Gut Microbiota

Meriem Messaoudene[1], Reilly Pidgeon[2], Corentin Richard[1], Mayra Ponce[1], Khoudia Diop[1], Myriam Benlaifaoui[1], Alexis Nolin-Lapalme[1], Florent Cauchois[1], Julie Malo[1], Wiam Belkaid[1], Stephane Isnard[3], Yves Fradet[4], Lharbi Dridi[2], Dominique Velin[5], Paul Oster[5], Didier Raoult[6], François Ghiringhelli[7], Romain Boidot[8,9], Sandy Chevrier[8], David T. Kysela[10], Yves V. Brun[10], Emilia Liana Falcone[11,12], Geneviève Pilon[13], Florian Plaza Oñate[14], Oscar Gitton-Quent[14], Emmanuelle Le Chatelier[14], Sylvere Durand[15,16], Guido Kroemer[15,16,17], Arielle Elkrief[1], André Marette[13], Bastien Castagner[2], and Bertrand Routy[1,18]

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ABSTRACT Several approaches to manipulate the gut microbiome for improving the activity of cancer immune-checkpoint inhibitors (ICI) are currently under evaluation. Here, we show that oral supplementation with the polyphenol-rich berry camu-camu (CC; *Myrciaria dubia*) in mice shifted gut microbial composition, which translated into antitumor activity and a stronger anti–PD-1 response. We identified castalagin, an ellagitannin, as the active compound in CC. Oral administration of castalagin enriched for bacteria associated with efficient immunotherapeutic responses (*Ruminococcaceae* and *Alistipes*) and improved the $CD8^+$/$FOXP3^+CD4^+$ ratio within the tumor microenvironment. Moreover, castalagin induced metabolic changes, resulting in an increase in taurine-conjugated bile acids. Oral supplementation of castalagin following fecal microbiota transplantation from ICI-refractory patients into mice supported anti–PD-1 activity. Finally, we found that castalagin binds to *Ruminococcus bromii* and promoted an anticancer response. Altogether, our results identify castalagin as a polyphenol that acts as a prebiotic to circumvent anti–PD-1 resistance.

SIGNIFICANCE: The polyphenol castalagin isolated from a berry has an antitumor effect through direct interactions with commensal bacteria, thus reprogramming the tumor microenvironment. In addition, in preclinical ICI-resistant models, castalagin reestablishes the efficacy of anti–PD-1. Together, these results provide a strong biological rationale to test castalagin as part of a clinical trial.

INTRODUCTION

The advent of immune-checkpoint inhibitors (ICI) has improved overall survival in multiple malignancies (1–3). However, primary therapeutic resistance remains a major hurdle. Defining mechanisms of resistance implicates several factors, including PD-L1 expression, tumor microenvironment contexture, and the tumor mutational burden determining the cancer-immune set point or threshold (4, 5). Recent metagenomic sequencing studies coupled with experiments in germ-free (GF) mice have revealed that the gut microbiome serves as another key mediator of this cancer-immune set point (5, 6). Preclinical results suggest that modulation of the gut microbiome by antibiotics (ATB) is associated with ICI resistance, and multiple reports in more than 11,000 patients have demonstrated the deleterious impact of ATB on ICI efficacy (7–9). In addition, experiments in GF or ATB-treated mice revealed that fecal microbiota transplantation (FMT) using fecal samples of patients with cancer reproduced the patients' clinical phenotype in terms of response or nonresponse upon tumor implantation and treatment with ICI (10).

Gut microbiome profiling in patients amenable to ICI revealed that high gut bacterial diversity and the presence of immunogenic bacteria, such as *Ruminococcus*, *Akkermansia muciniphila*, and *Bifidobacterium*, were associated with a stronger $CD8^+$ T-cell and $CD4^+$ Th1-dependent antitumor response and resulted in favorable clinical outcomes (10–13). FMT from ICI-responsive patients or oral supplementation with *A. muciniphila* in mice initially colonized with FMT from nonresponder (NR) patients was shown to enhance the efficacy of ICI (10, 13). Furthermore, a consortium of 11 beneficial gut bacteria induced a stronger $CD8^+$ T-cell and IFNγ immune response in the colon of mice and improved ICI activity (14). Evidence has also shown that ATB use prior

[1]University of Montreal Hospital Research Centre (CRCHUM), Montreal, Quebec, Canada. [2]Department of Pharmacology and Therapeutics, Faculty of Medicine and Health Sciences, McGill University, Montreal, Quebec, Canada. [3]Research Institute, McGill University Health Centre, Montreal, Quebec, Canada. [4]Centre de recherche du CHU de Québec, Oncology Division, CHU de Québec, Université Laval, Québec City, Quebec, Canada. [5]Service of Gastroenterology and Hepatology, Centre Hospitalier Universitaire Vaudois, University of Lausanne, Lausanne, Switzerland. [6]Aix Marseille Université, IRD, AP-HM, MEPHI, IHU-Méditerranée Infection, Marseille, France. [7]Department of Medical Oncology, Center GF Leclerc, Dijon, France. [8]Unit of Molecular Biology, Department of Biology and Pathology of Tumors, Georges-François Leclerc Cancer Center, UNICANCER, Dijon, France. [9]UMR CNRS 6302, Dijon, France. [10]Faculté de Médecine, Département de Microbiologie, Infectiologie et Immunologie, University of Montreal, Montreal, Quebec, Canada. [11]Department of Immunity and Viral Infections, Montreal Clinical Research Institute (IRCM), Montreal, Quebec, Canada. [12]Department of Medicine, University of Montreal, Montreal, Quebec, Canada. [13]Department of Medicine, Faculty of Medicine, Cardiology Axis of the Québec Heart and Lung Institute and Institute of Nutrition and Functional Foods, Laval University, Québec City, Quebec, Canada. [14]Université Paris-Saclay, INRAE, MGP, Jouy-en-Josas, France. [15]Metabolomics and Cell Biology Platforms, Gustave Roussy Cancer Campus, Villejuif, France. [16]Centre de Recherche des Cordeliers, Équipe labellisée par la Ligue contre le cancer, Université de Paris, Sorbonne Université, INSERM U1138, Institut Universitaire de France, Paris, France. [17]Pôle de Biologie, Hôpital Européen Georges Pompidou, Paris, France. [18]Hematology-Oncology Division, Department of Medicine, University of Montreal Healthcare Centre (CHUM), Montreal, Quebec, Canada.

Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

Corresponding Author: Bertrand Routy, Hemato-Oncology, University of Montreal Hospital Research Centre (CRCHUM), Montreal, Quebec H2X 3H8, Canada. Phone: 514-890-8000; E-mail: bertrand.routy@umontreal.ca

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to ICI initiation decreased gut microbiome diversity and decreased the presence of *Ruminococcaceae* in patients with advanced non–small cell lung cancer (NSCLC) and led to unfavorable outcomes (15). Taken together, these observations have initiated efforts to harness the gut microbiome as a therapeutic adjuvant to ICI (16). Recently, the first proof-of-principle FMT clinical trials in melanoma were reported, and several others evaluating FMT, probiotics, or targeted dietary modifications in combination with ICI are ongoing (17, 18). However, the use of prebiotics—compounds that favorably modify the host gut microbiome—has not yet been investigated as a microbiome-based intervention to improve ICI efficacy. Camu-camu (CC), also known as *Myrciaria dubia*, is an Amazonian berry containing several phytochemicals and has been shown to exert protective prebiotic effects against obesity and related metabolic disorders in mice through increasing the abundance of *A. muciniphila* and *Bifidobacterium* in the gut (19). Here, we evaluated whether the prebiotic action of CC could also be harnessed to shift the gut microbiome and improve antitumor activity.

RESULTS

Antitumor Activity of CC Is Microbiome-Dependent and Circumvents Anti–PD-1 Resistance

To assess the antitumor effect of CC oral supplementation, specific pathogen-free (SPF) mice received daily oral gavage of CC after MCA-205 sarcoma (ICI-sensitive) tumor inoculation (10) and were treated intraperitoneally with either anti–PD-1 (αPD-1) or an isotype control for αPD-1 (IsoPD-1) every three days. We observed a similar therapeutic efficacy of CC/IsoPD-1 and water/αPD-1 compared with water/IsoPD-1, whereas the combination of CC and αPD-1 led to further inhibition of tumor growth compared with each individual treatment (Fig. 1A). We then tested CC in an αPD-1–resistant E0771 breast cancer tumor mouse model (20) and confirmed that the αPD-1 alone did not exhibit any antitumor activity in this model (Fig. 1B). CC/IsoPD-1 demonstrated minimal antitumor activity, whereas the combination of CC/αPD-1 increased αPD-1 activity. These drug combinations were well tolerated by the animals, with no weight loss or diarrhea observed (Supplementary Fig. S1A). To further explore the therapeutic potential of CC alone or in combination with αPD-1, we recolonized ATB-treated mice by performing FMT from four responder (R) patients and four NR patients with advanced NSCLC amenable to αPD-1 (Supplementary Table S1). MCA-205 tumors were inoculated in these "avatar" mouse models, and mice were treated with CC or water, with or without αPD-1 (Fig. 1C). As previously published, FMT from NR patients conferred resistance to αPD-1, whereas FMT from R patients supported the αPD-1 antitumor effect (Fig. 1D; ref. 10). In the FMT NR avatar mice, oral supplementation of CC/IsoPD-1 induced an antitumor effect. Moreover, the combination of CC/αPD-1 further improved tumor response compared with water/αPD-1 or CC/IsoPD-1. Conversely, in FMT R avatar mice, CC alone or in combination with αPD-1 did not further enhance the antitumor response compared with water/αPD-1 (Fig. 1D).

To test our hypothesis that the antitumor effect of CC was dependent on the gut microbiota, we used three different strategies. We first demonstrated that in mice reared in GF conditions, CC oral supplementation had no antitumor activity (Fig. 1E). Second, treatment of mice with broad-spectrum ATB during CC treatment compromised the antitumor activity conferred by CC (Supplementary Fig. S1B). Third, we used fecal samples from mice treated with or without CC to perform daily FMT in SPF mice over the course of 14 days after MCA-205 inoculation (10, 21). Only FMT from CC-treated mice further improved αPD-1 activity (Fig. 1F). These experiments demonstrate that the antitumor activity of CC is microbiota-dependent.

CC Increases Microbiome Diversity and Beneficially Shifts the Composition of the Microbiome

We first sought to define the differences in microbiome composition in our avatar murine models between those receiving FMT from R or NR prior to any CC ± αPD-1 treatment. Microbiome profiling based on 16S rRNA revealed that FMT from R, which translated into αPD-1 activity, was associated with greater alpha diversity and different objective clusters when compared with mice that received FMT from NR (resistant to αPD-1; Supplementary Fig. S2A and S2B). Engraftment of *Ruminococcaceae UBA1819*, *Christensenellaceae R-7* group, *Paraprevotella*, *Bifidobacterium*, and *Oscillospiraceae UCG-003* was enriched in mice that underwent FMT from R and had tumors that were sensitive to water/αPD-1, whereas *Lachnospiraceae UCG-006*, *NK4B4*, and *FCS020* were more abundant in FMT from NR (Supplementary Fig. S2C). Altogether, these results confirm the importance of specific bacteria associated with the patient-level αPD-1 response in our murine experiments.

To elucidate the impact of CC on the gut microbiome composition, we characterized the microbiota composition in the MCA-205 murine model treated with CC without patient FMT (Fig. 1A). CC treatment was associated with increased alpha diversity compared with water/IsoPD-1, independent of αPD-1 therapy (Fig. 2A; Supplementary Fig. S3A). qRT-PCR with universal 16S rRNA gene primers confirmed an increased bacterial abundance in the CC group compared with water (Supplementary Fig. S3B). The Bray–Curtis index revealed that oral gavage with CC led to distinct bacterial clusters compared with pre-CC treatment ($P = 0.006$; Supplementary Fig. S3C), whereas oral gavage with water/αPD-1 showed no separation of the microbiome into distinct clusters ($P = 0.16$; Supplementary Fig. S3D). Differential abundance analysis showed that *Ruminococcus* bacteria at the genus level were overrepresented in the CC groups compared with the water groups (adjusted $P < 0.05$; Fig. 2B). Moreover, *Ruminococcus* and *Turicibacter* were the only genera that were consistently more abundant in both CC/IsoPD-1 and CC/αPD-1 groups compared with the water/IsoPD-1 group (Supplementary Fig. S3E and S3F). In parallel, profiling of the gut microbiome using 16S rRNA sequencing in the E0771 tumor model showed that *Ruminococcaceae UBA1819*, *Turicibacter*, *Parasutterella*, *Ruminococcus*, *A. muciniphila* (adjusted $P < 0.05$) as well as *Christensenellaceae R-7* group and *Oscillospiraceae UCG-005* ($P < 0.05$) were increased in mice treated with CC/αPD-1 compared with water/αPD-1 (Fig. 2C).

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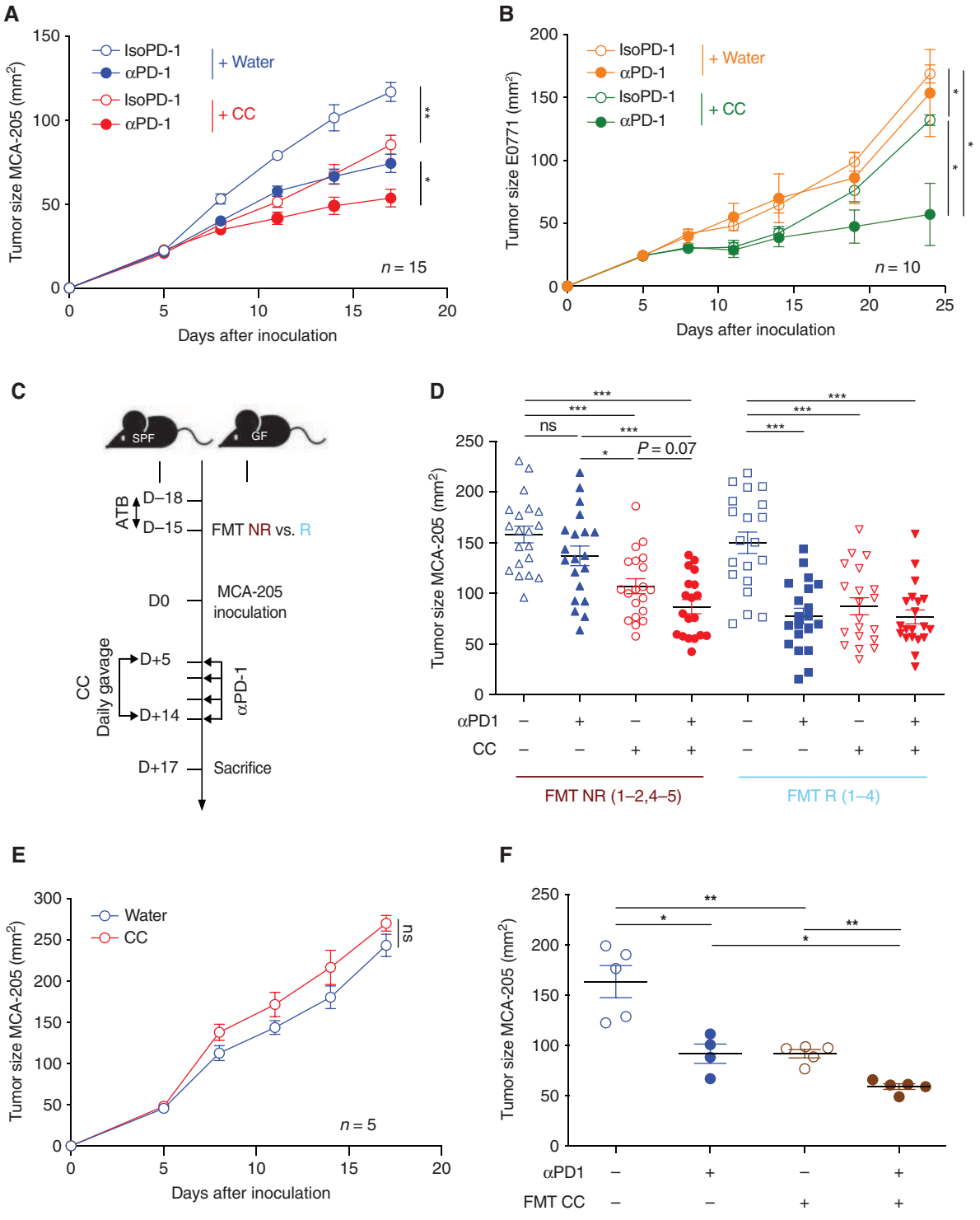

Figure 1. CC supplementation is associated with antitumor activity that is microbiome dependent and circumvents the αPD-1 resistance in mice conferred by FMT from NR patients with NSCLC. **A** and **B,** Tumor growth kinetics in SPF C57BL/6 mice after sequential injections of αPD-1 or IsoPD-1 and daily oral gavage with CC or water are depicted for (**A**) MCA-205 sarcoma ($n = 15$ mice/group) and (**B**) E0771 breast cancer ($n = 10$ mice/group). **C,** Experimental design of avatar mice experiments. FMT from feces samples from NR and R patients with NSCLC were individually performed in GF C57BL/6 mice or after 3 days of ATB in SPF C57BL/6 mice. Two weeks later, MCA-205 tumors were inoculated, and daily gavage with CC or water was performed in combination with sequential injections of αPD-1 or IsoPD-1. **D,** Pooled analysis of the mean tumor size ± SEM at sacrifice post-FMT from four NR (NR1–2, 4–5) and four R (R1–4) groups for each CC and water group. Each symbol represents one mouse. **E,** MCA-205 tumor kinetics in mice reared in GF conditions and receiving daily oral gavage with CC or water ($n = 5$ mice/group). **F,** Tumor size at sacrifice of mice bearing MCA-205 treated with αPD-1 or IsoPD-1 in combination with daily FMT with mouse feces previously supplemented with CC. Each circle represents one mouse. Means±SEM are represented in all experiments. ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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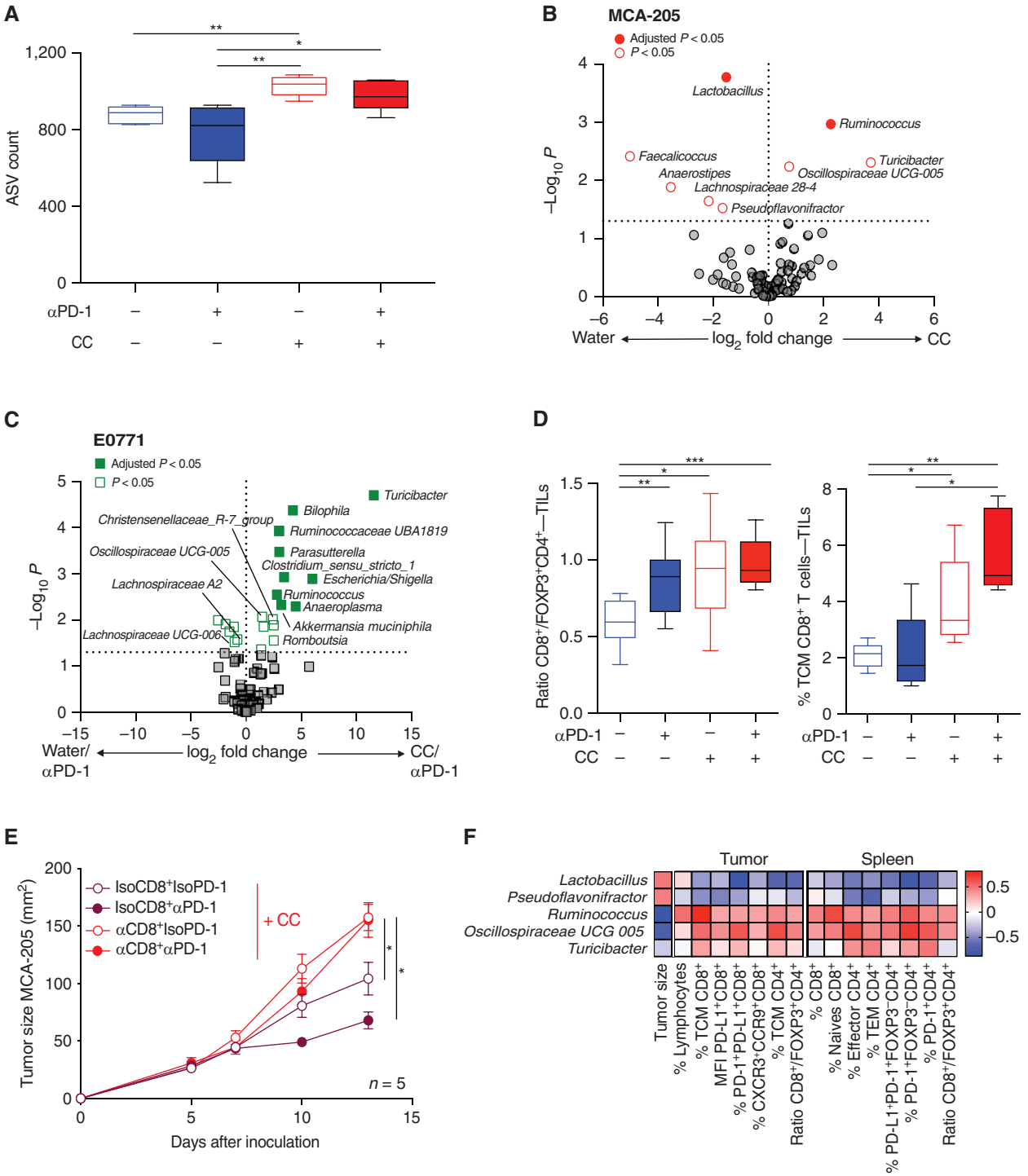

Figure 2. Impact of CC on microbiome composition and association with CD8+ T-cell antitumor activity. **A,** 16S rRNA analysis of the fecal samples from mice from the four groups in the MCA-205 experiments, and representation of the alpha diversity measured by the amplicon sequence variant (ASV) count in each group. **B** and **C,** Volcano plot representation of differential abundance analysis comparing (**B**) pooled CC vs. water (αPD-1 and IsoPD-1) groups in the MCA-205 model and (**C**) the water/αPD-1 vs. CC/αPD-1 groups in the E0771 model ($n = 10$ and 5 mice/group, respectively). Bacteria enriched in each group are represented using adjusted P value (fill shape symbol) and P value (no fill shape symbol; false discovery rate: 0.05). **D,** Flow cytometry analysis of the ratio of CD8+/FOXP3+CD4+ T cells and CD8+ T central memory (TCM) cell (CD45RB−CD62L+) subpopulations in MCA-205 TILs in the four experimental groups ($n = 10$ mice/group). **E,** Effects of anti-CD8 (αCD8) depletion or its isotype control (IsoCD8) on MCA-205 tumor growth kinetics with daily oral gavage of CC ($n = 5$ mice/group) treated or not with αPD-1 or its isotype control. **F,** Pairwise Spearman rank correlation heat map between significantly different bacteria enriched in CC/IsoPD-1 vs. water/IsoPD-1 groups with positively correlated TILs or splenocyte cytometry and matching tumor size in the MCA-205 experiment. MFI, mean fluorescence intensity; TEM, T effector memory. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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To further define how CC modifies the microbiome, we analyzed the fecal samples of "avatar" mice that received FMT followed by treatment with CC ± IsoPD-1. At the genus level, addition of CC/IsoPD-1 to FMT NR avatar mice was associated with an increase in the relative abundance of *Bifidobacterium* ($P < 0.001$) as well as *Ruminococcaceae* and *Ruminococcaceae UCG-009* ($P = 0.054$ and $P = 0.056$, respectively; Supplementary Table S2).

Altogether, these results demonstrate that CC induced a shift in the gut microbiota composition, irrespective of tumor model and αPD-1 administration. These results highlighted commonalities between the bacteria enriched in FMT from R patients with those of mice who received CC.

Antitumor Activity of CC Is CD8$^+$ T-cell Dependent and Correlates with Immunogenic Bacterial Species

We next characterized the immune-potentiating effect of CC by assessing flow cytometry surrogate markers within tumor-infiltrating lymphocytes (TIL). In the MCA-205 model without FMT (Fig. 1A), analyses revealed that the ratio of CD8$^+$/FOXP3$^+$CD4$^+$ regulatory T cells (Treg) in the three groups that showed antitumor activity (CC/IsoPD-1, CC/αPD-1, or water/αPD-1) was increased compared with water/IsoPD-1 (Fig. 2D). We further examined the subpopulations of TILs and found that CC/IsoPD-1 increased central memory (TCM) CD8$^+$ T cells (CD45RB$^-$CD62L$^+$) compared with water/IsoPD-1. To verify that the antitumor activity associated with CC was mediated by CD8$^+$ T cells, we administered an anti-CD8$^+$ monoclonal antibody in combination with CC in MCA-205–bearing mice to deplete the CD8$^+$ T-cell subpopulation. Results showed increased tumor growth compared with CC/IsoCD8$^+$ (control), indicating that the microbiome-mediated effect of CC was CD8$^+$ T-cell dependent (Fig. 2E).

We further explored the interactions between the microbiome and the systemic immune response on tumor size related to CC. In the MCA-205 murine model, pairwise comparisons using nonparametric Spearman correlations between bacteria enriched in CC/IsoPD-1 versus water/IsoPD-1 groups were performed and characterized by intratumoral cytometry immune markers and tumor size. Increase in the proportion of PD-L1$^+$CD8$^+$ T cells, TCM CD8$^+$ cells, and ratio of CD8$^+$/FOXP3$^+$CD4$^+$ T cells in MCA-205 TILs were associated with bacteria enriched by CC, such as *Ruminococcus* and *Oscillospiraceae*, and downregulation of *Lactobacillus* and *Pseudoflavonifractor* (Fig. 2F). Similarly, in splenocytes of the CC/IsoPD-1 group, CD8$^+$ T cells and CD8$^+$/FOXP3$^+$CD4$^+$ T-cell ratio correlated with *Ruminoccocus* and *Oscillospiraceae UCG-005* compared with water/IsoPD-1.

In parallel, we analyzed the TILs in the E0771 tumor model posttreatment. CC combined with αPD-1 induced activation of intratumoral CD8$^+$ T cells, which was represented by an increase in the mean fluorescent intensity of ICOS$^+$CD8$^+$ T cells when compared with water/αPD-1 (Supplementary Fig. S4A). Spearman rank correlations were performed between bacteria upregulated in water/αPD-1 and CC/αPD-1 groups with regard to immune markers and tumor size. The results further demonstrated a positive correlation between the CD8$^+$/FOXP3$^+$CD4$^+$ T-cell ratio and upregulation of ICOS$^+$FOXP3$^-$CD4$^+$ T-cell infiltration. This was associated with the abundance of *Ruminococcus*, *Christensenellaceae R-7* group, *Bilophila* and *A. muciniphila*, which correlated with a reduced tumor size (Supplementary Fig. S4B).

Castalagin Is the Bioactive Polyphenol Molecule from CC That Is Responsible for Its Antitumor Effect

CC is composed of more than 50 compounds, including multiple polyphenols that can all contribute to its prebiotic properties (22). We aimed to identify the bioactive molecule responsible for the observed microbiome-dependent antitumor activity of CC. Using high-performance liquid chromatography (HPLC), we separated the raw extract from CC according to its molecular polarity: polar (P), medium polarity (MP), and nonpolar (NP) as well as an insoluble fraction (INS; Fig. 3A; Supplementary Fig. S5A and S5B). These four fractions were independently delivered by oral supplementation in the MCA-205 tumor model as previously described and compared with CC and water. Among the four different fractions, only the P fraction was associated with an antitumor effect similar to CC (Fig. 3B; Supplementary Fig. S6). We further separated the P fraction into four subfractions according to retention times (P1–P4). The P1 fraction was mostly composed of ascorbic acid; P2 was composed of the polyphenol vescalagin and gallic acid; P3 was composed of the polyphenol castalagin; and P4 was composed of different complex polyphenols (Supplementary Fig. S7A and S7B). We tested these four subfractions in the MCA-205 model and found that P3, which is 95% castalagin, was the only fraction that was associated with an antitumor effect similar to CC (Supplementary Fig. S7C).

To validate that castalagin was responsible for the activity of subfraction P3, we obtained pure castalagin from different sources: (i) an analytical standard manufacturer and (ii) oak wood extracts. Analysis of the various sources of castalagin by liquid chromatography–mass spectrometry (LC-MS) and 2-D nuclear magnetic resonance spectroscopy confirmed that subfraction P3 and castalagin isolated from oak were identical to the analytical standard (Supplementary Fig. S8A–S8F). Using the analytical standard or castalagin from oak at similar concentrations present in fraction P (0.85 mg/kg per mouse), we obtained similar tumor inhibition in the MCA-205 and E0711 models when used alone or in combination with αPD-1, respectively (Fig. 3C and D). Next, we performed a proof-of-principle experiment to confirm the microbiome-dependent effect of castalagin whereby oral gavage of castalagin in GF mice resulted in no antitumor effect even in combination with αPD-1 (Fig. 3E).

Castalagin Demonstrates Antitumor Activity through Shift in Microbiome Composition and Metabolite Production

We next addressed the impact of castalagin on the microbiome in SPF mice by 16S rRNA microbiome profiling in the MCA-205 model. Castalagin increased alpha diversity after oral supplementation (Supplementary Fig. S9A). At the taxa level, castalagin led to the enrichment of *Ruminococcaceae UBA1819, A. muciniphila, Ruminococcus, Blautia*, and *Alistipes*, whereas feces from the water group were enriched with

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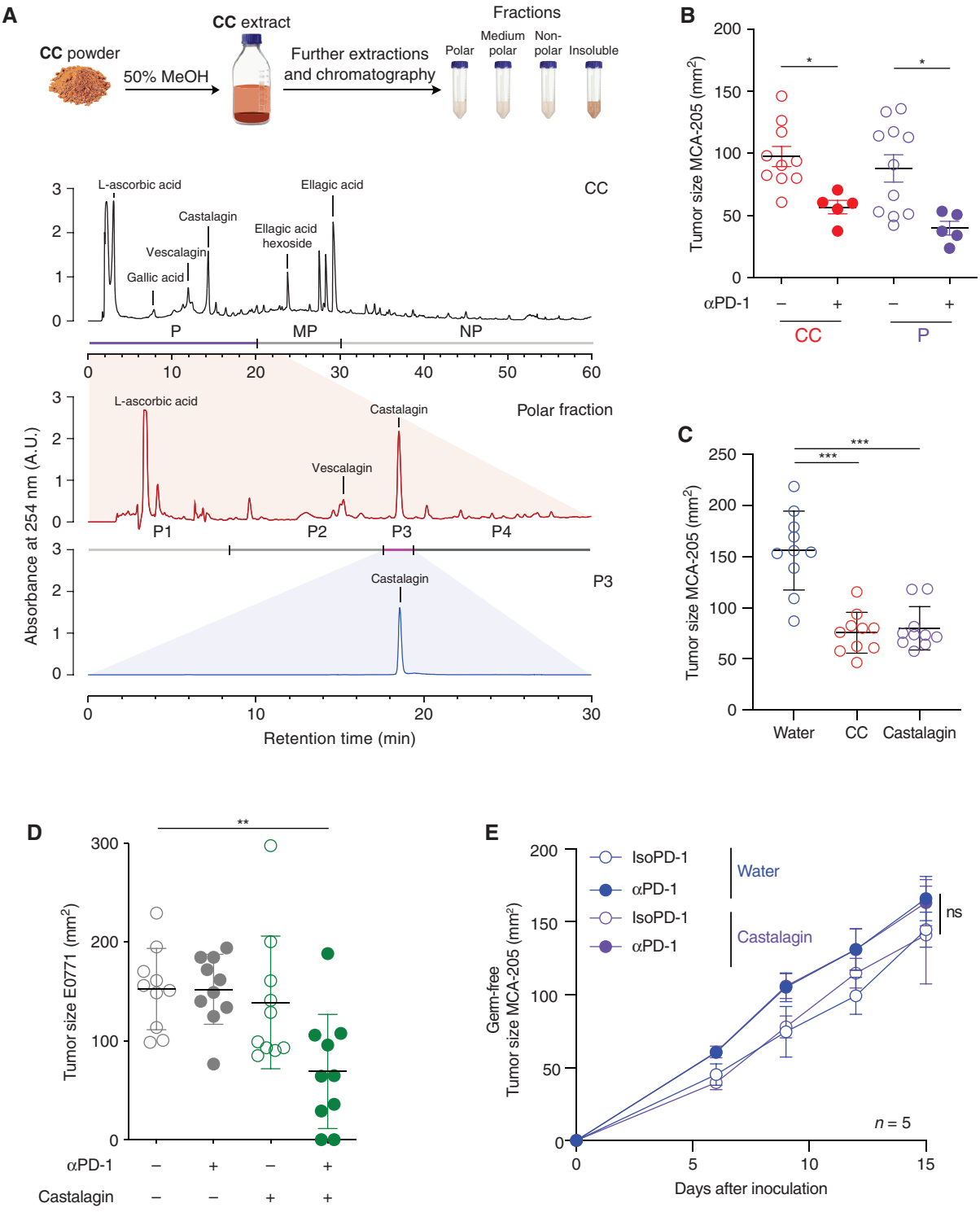

Figure 3. Identification of castalagin as the bioactive compound of CC with similar antitumor activity. **A,** Schematic of the CC fractionation procedure and summary of chromatograms (λ = 254 nm) showing the isolation of bioactive fractions from CC: CC extract (top chromatogram) containing fractions polar (P), medium polar (MP), and nonpolar (NP). Representation of the polar fraction (middle chromatogram) containing subfractions P1–4 and polar subfraction 3 (P3; bottom chromatogram). **B,** Tumor size at sacrifice of mice bearing MCA-205 treated with daily oral gavage with CC or P fraction ($n = 10$ mice/group). **C,** Tumor size at sacrifice in the MCA-205 model after daily oral supplementation with CC, castalagin at the standard concentration (0.85 mg/kg per mouse), or water ($n = 10$ mice/group). **D,** Tumor size at sacrifice of E0771-bearing SPF mice after sequential injections of αPD-1 or IsoPD-1 and a daily oral gavage with water or castalagin at the standard concentration (0.85 mg/kg per mouse; $n = 10$ mice/group). **E,** MCA-205 tumor kinetics in mice reared under GF conditions treated with sequential injections of αPD-1 or IsoPD-1 and a daily oral gavage with water or castalagin ($n = 5$ mice/group). Means ± SEM are represented in **B**–**E**. ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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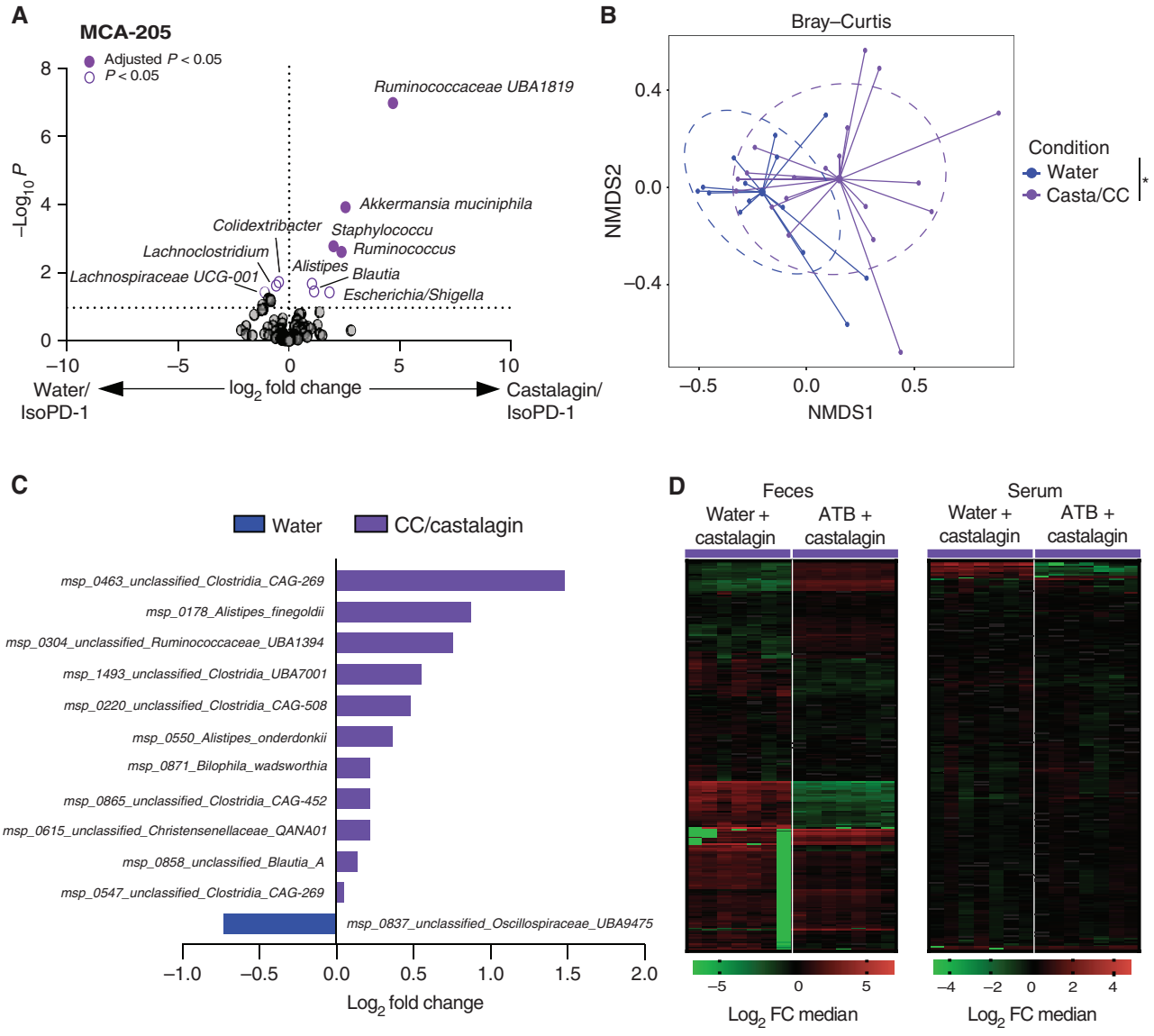

Figure 4. Castalagin influences the microbiome composition and metabolite production. **A,** Volcano plot representation of differential abundance analysis results after 16S rRNA sequencing analysis of water/IsoPD-1 vs. castalagin/IsoPD-1 groups of the MCA-205 model ($n = 10$ mice/group). Bacteria enriched in each group are represented using adjusted *P* value (fill shape symbol) and *P* value (no fill shape symbol; false discovery rate: 0.05). **B,** Metagenomics sequencing with representation of species richness in fecal samples from MCA-205 experiment in water (IsoPD-1 and αPD-1) vs. castalagin/CC (IsoPD-1 and αPD-1) groups. Bray–Curtis representation for beta diversity. **C,** Bar plot representation of differential abundance analysis results after metagenomics sequencing analysis between water (IsoPD-1 and αPD-1) and castalagin/CC (IsoPD-1 and αPD-1) groups. **D,** Heat map of change in metabolite relative abundance in the feces and serum. Hierarchical clustering (Euclidean distance, Ward linkage method) of the metabolite abundance is shown ($n = 7$ mice/group). (*continued on next page*)

Lachnospiraceae UCG-001 and *Lachnoclostridium* (Fig. 4A). Combining 16S rRNA results from all SPF experiments regardless of the tumor model, we showed *Ruminococcus*, *Alistipes*, *Parasutterella*, and *Oscillospiraceae UCG 005* post–CC and castalagin supplementation (Supplementary Fig. S9B).

To confirm these changes in microbiome composition, we performed metagenomics shotgun sequencing of murine feces. Shannon and Simpson indexes were significantly more elevated posttreatment with CC and castalagin, whereas observed species were numerically increased but not significant (Supplementary Fig. S9C). Supplementation with CC/castalagin demonstrated unique bacterial cluster compared with water control and was associated with enrichment of *Ruminococcaceae UBA1394*, *Alistipes finegoldii*, *Alistipes onderdonki*, *Christensenellaceae QANA01*, and *Bilophila wadsworthia* post–CC and castalagin supplementation (Fig. 4B and C). In addition to an enrichment of *Ruminococcaceae UBA 1394*, the castalagin/IsoPD-1 group showed that *Ruminococcaceae UBA 3818, Ruthenibacterium lactatiformans*, and *A. muciniphila* were also increased post-castalagin (Supplementary Fig. S9D). Of note,

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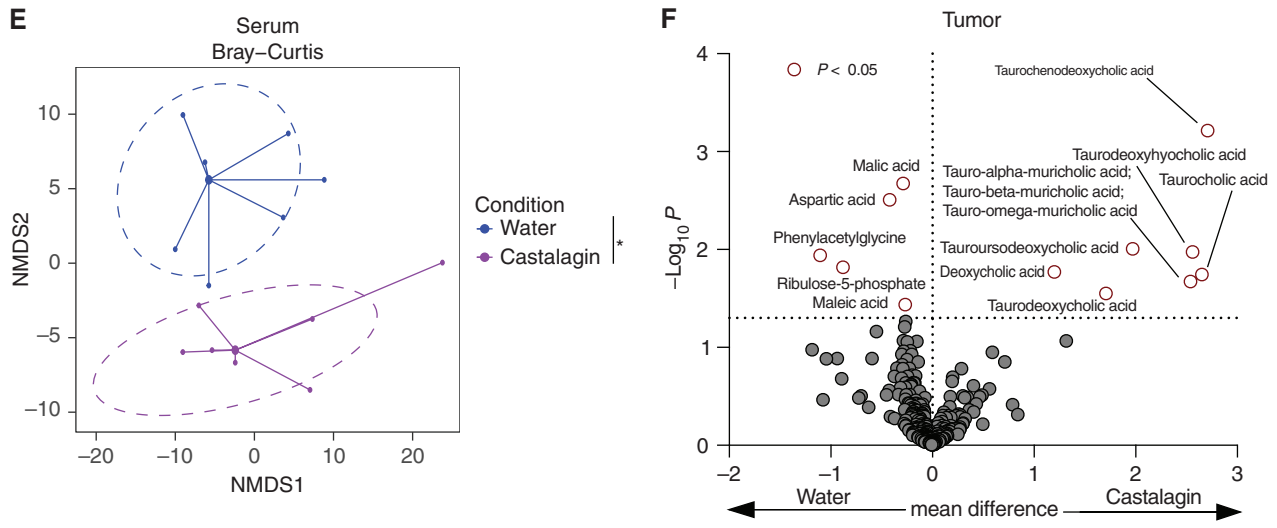

Figure 4. ***(Continued)*** **E,** Metabolites' principal coordinate analysis between water and castalagin in serum. **F,** Volcano plot representation for the differential metabolite difference from the tumor of mice receiving water or castalagin ($n = 7$ mice/group). The horizontal dotted black line shows where $P = 0.05$ with points above indicating metabolites with significantly different abundance ($P < 0.05$). *, $P < 0.05$.

Ruthenibacterium lactatiformans would correspond to the *Ruminococcaceae UBA1819* signal identified by 16S rRNA according to the SILVA rRNA database.

Based on our results showing a shift in microbiome composition post-castalagin, we investigated the impact of castalagin on metabolite production. Mice were treated with either 14 days of broad-spectrum ATB conditioning or water. MCA-205 tumor cells were then inoculated, and each group was subdivided and treated with castalagin or PBS. Metabolite extraction and measurement by a combination of chromatographic and mass spectrometric methods was performed on the serum, feces, tumor, liver, ileum, and colonic contents. Metabolite production post–CC supplementation was divergent in water compared with ATB groups in the feces and serum, supporting the microbiome-dependent mechanism of castalagin (Fig. 4D). Next, we evaluated the mice that did not receive ATB and found a significant metabolomic shift in the serum of mice treated with castalagin compared with water control (Fig. 4E). More specifically, castalagin was associated with an upregulation of taurine-conjugated bile acids in the tumor and serum (Fig. 4F; Supplementary Fig. S10A). Conversely, secondary taurinated bile acids were decreased in feces post-castalagin (Supplementary Fig. S10B and S10C). These results revealed that castalagin alone was able to shift the gut microbiome composition, translating into a distinct metabolic phenotype and providing further evidence that it represents the active microbiome-dependent component of CC.

Similar to CC, Systemic Immune Effects of Castalagin Are CD8$^+$ T-cell Dependent

We next examined whether castalagin could also reproduce the systemic immune response of CC, using five approaches. First, flow cytometry analysis in MCA-205 experiments confirmed that the polyphenol upregulated TCM CD8$^+$ T cells in TILs from SPF mice, whereas no difference in frequency was observed in GF mice (Fig. 5A). In the E0771 model, castalagin was also associated with an increase in the frequency of memory CD8$^+$ T cells (CD44^{hi}CD62L$^-$CD8$^+$ T cells) in combination with αPD-1 in both TILs and splenocytes when compared with water/αPD-1 (Supplementary Fig. S11A). Second, immunofluorescence staining of tumors further demonstrated an increase of the CD8$^+$/CD4$^+$FOXP3$^+$ T-cell ratio in the castalagin/IsoPD-1 group compared with water/IsoPD-1 (Fig. 5B and C). Third, to support our demonstration that castalagin modulates TILs, tumor transcriptome profiling was performed through RNA sequencing (RNA-seq) to define differentially expressed pathways in the MCA-205 models. We observed an upregulation of gene pathways including antigen processing and presentation, T-cell receptor signaling pathway, as well as NF-κB signaling associated with a more inflamed tumor microenvironment in the castalagin/IsoPD-1 group compared with water/IsoPD-1 control (Fig. 5D). Within these pathways, transcriptome analysis showed an overexpression of *CD3ε*, *CD8α*, *IFNγ*, and *MAPK11* in the castalagin group (Supplementary Fig. S11B). Fourth, using the CIBERSORT program to estimate immune cells from RNA-seq, castalagin was associated with a numerically higher frequency of CD8$^+$ T cells compared with water groups (Supplementary Fig. S11C). Lastly, an *in vivo* CD8$^+$ T-cell killing assay was performed after mice received an intravenous injection of carboxyfluorescein succinimidyl ester (CSFE)–labeled target cells as previously described (23). After injection of these OT-1 cells, mice were immunized with CpG/OVA. CSFE^{lo} pulsed OVA OT-I splenocytes and CFSE^{hi} no-pulsed OVA OT-I splenocytes were then transferred to the mice. We observed a lower proportion of CFSE^{lo} CD8$^+$ T cells in the draining lymph nodes in mice in the castalagin group versus the water group corresponding to higher percentage of killing, pointing to the CD8$^+$ T cell–dependent mechanism of castalagin (Fig. 5E).

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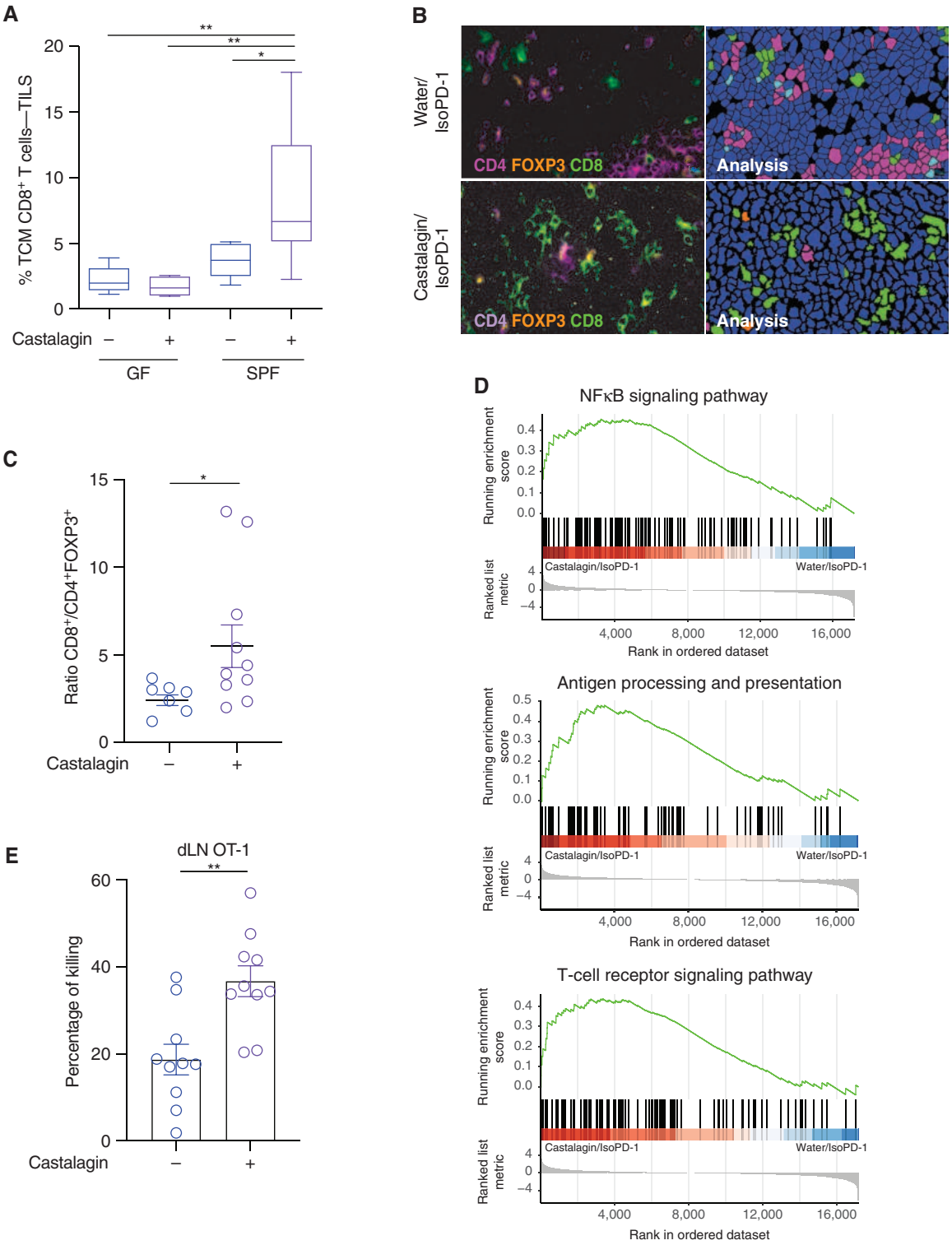

Figure 5. Immune-potentiating effect of castalagin. **A,** Flow cytometry analysis of MCA-205 TIL and CD8⁺ TCM cell (CD45RB⁻ CD62L⁺) subpopulation in the GF and SPF experiments comparing castalagin vs. water ($n = 10$ mice/group). **B,** Representative immunofluorescence images of MCA-205 tumors for CD8⁺, CD4⁺, and FOXP3⁺ cells in castalagin/IsoPD-1 and water/IsoPD-1 groups. **C,** Tumor immunofluorescence for CD8⁺/FOXP3⁺CD4⁺ cell ratio results for both groups shown in **B**. Each circle represents one tumor. **D,** Pathway classification from RNA-seq results using gene set enrichment analysis based on the KEGG database comparing castalagin/IsoPD-1 vs. water/IsoPD-1 groups of MCA-205 tumors. **E,** Percentage of killing of CD8⁺ T OT-1 cells from the draining lymph node (dLN) in mice treated with castalagin or water and immunized with CpG/OVA ($n = 10$ mice/group). *, $P < 0.05$; **, $P < 0.01$.

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Given that CC and castalagin induced similar shifts in the gut microbiome composition, translating into a modification in the TILs, and that castalagin is the only polyphenol enriched in fraction P3 that induced an antitumor response, we deduced that castalagin is the compound responsible for the antitumor activity of CC.

Castalagin Can Circumvent αPD-1 Resistance and Has a Dose-Dependent Antitumor Effect That Correlates with *Ruminococcaceae* Abundance

As previously performed with our avatar mice, we tested the therapeutic effects of castalagin following FMT using feces from four NR patients with NSCLC in ATB-treated and GF mice. The addition of castalagin alone was able to exert antitumor activity but also increased αPD-1 activity (Fig. 6A and B). Profiling of fecal samples from these FMT experiments using 16S rRNA further confirmed that castalagin treatment increased the relative abundance of *Ruminococcus*, *Alistipes*, *Christensenellaceae R-7* group, and *Paraprevotella* compared with the water control group (Fig. 6C; Supplementary Fig. S12A). Conversely, *Lachnosclostridium* was underrepresented after castalagin treatment.

To determine if castalagin activity was dose dependent, we performed oral gavage in mice using different concentrations of castalagin from 0 to 2.55 mg/kg. The antitumor activity became significant only at 0.85 mg/kg, plateauing at higher concentrations (Fig. 6D; Supplementary Fig. S12B). Moreover, to determine if the abundance of *Ruminococcaceae* correlated with castalagin activity, we performed qRT-PCR with *Ruminococcaceae*-specific primers on the feces from the dose-dependent experiments. *Ruminococcaceae* was not increased in the 0.21 mg/kg concentration dose of castalagin (with no antitumor effect) compared with the control (0 mg/kg; Fig. 6E). However, *Ruminococcaceae* abundance was significantly increased following supplementation with castalagin at 0.85 mg/kg (with antitumor effect), and no further incrementation of *Ruminococcaceae* abundance was observed beyond this concentration.

Castalagin Binds Preferentially to the Cellular Envelope of *Ruminococcus*

In the gut, castalagin is hydrolyzed to castalin and ellagic acid, which is further converted into urolithins by intestinal bacterial species (ref. 24; Supplementary Fig. S13). We next sought to elucidate if the metabolites of castalagin or its diastereomer, vescalagin (Supplementary Fig. S14A–S14G), were involved in its antitumor activity and detectable in fecal samples. In contrast to castalagin, no antitumor effects were observed in our MCA-205 model with vescalagin or with the downstream metabolites castalin, ellagic acid, and urolithin A (Fig. 7A). This result suggests that bypassing the metabolism by gut bacteria does not lead to an antitumor effect and that the metabolism of castalagin by gut bacteria might be required for the microbiota composition shift.

We were not able to detect castalagin using HPLC analysis on feces and serum of mice treated with up to 20× = 17 mg/kg of daily castalagin. Therefore, to assess if castalagin was metabolized differently by individual patients with cancer, we collected fecal samples from 23 patients with NSCLC treated with ICI (12 NR and 11 R; Supplementary Table S3) and performed *ex vivo* castalagin metabolism assays. Bacteria present in the feces from 91% of R patients compared with 41% in NR were capable of metabolizing castalagin to end-product metabolites such as urolithin A, isourolithin A, and urolithin B ($P < 0.05$; Fig. 7B; Supplementary Fig. S15A and S15B). We then analyzed metagenomic microbiome profiling between metabolizers and nonmetabolizers and found that the metabolizers showed a higher alpha diversity and distinct clusters (Supplementary Fig. S15C and S15D) as well increased abundance of *Ruminococcus bicirculans*, *Ruminococcus bromii, Alistipes finegoldii, Alistipes onderdonki*, and *Eubacterium* (Fig. 7C).

In both murine and human experiments, *Ruminococcus* was one of the bacteria consistently enriched post–castalagin supplementation. Therefore, we sought to demonstrate an interaction between castalagin and *Ruminococcus*, which is known to be beneficial for patients with cancer amenable to ICI (25). Although *Ruminococcus* are fastidious bacteria, *R. bromii* and *R. bicirculans* have been successfully cultivated and were enriched in metabolizer patients. We compared the growth curves of *R. bromii* and *R. bicirculans* against different control bacteria with castalagin and did not observe any beneficial effect on growth (Supplementary Fig. S16).

To test for a potential interaction between castalagin and *Ruminococcus*, we labeled castalagin with fluorescein (fluo-castalagin; Supplementary Fig. S17A), which was incubated in the presence of *R. bromii* and *R. bicirculans* as well as control bacteria *Bacteroides thetaiotaomicron* and *Escherichia coli* in anaerobic conditions. Flow cytometry analysis revealed no cell labeling with free fluorescein, whereas 79%, 47%, and 32% fluo-castalagin labeled with *R. bromii*, *R. bicirculans*, and *B. thetaiotaomicron* cells, respectively (Fig. 7D and E; Supplementary Fig. S17B). In contrast, only 7% of *E. coli* cells were labeled. Moreover, the addition of an excess (100×) of unlabeled castalagin significantly decreased *R. bromii* labeling efficiency, providing evidence of a competitive effect of free castalagin with the fluo-castalagin probe (Supplementary Fig. S17C). We used fluorescence microscopy to examine labeled bacteria and observed that fluo-castalagin was not internalized by *R. bromii* cells but remained associated with the cell envelope (Fig. 7E). Importantly, the fluorescence was weaker for *B. thetaiotaomicron* and barely detectable for *E. coli* cells compared with *R. bromii*, similar to our flow cytometry results. To further demonstrate an interaction between *R. bromii* and castalagin, we inoculated MCA-205 in GF mice. Oral supplementation of neither *R. bromii* nor castalagin had an effect, whereas the combination of both demonstrated antitumor activity (Fig. 7F). This additive antitumor effect of castalagin and *R. bromii* was then validated in SPF mice (Supplementary Fig. S18A). Importantly, qRT-PCR showed that the combination translated to a significant increase in *Ruminococcaceae* and *R. bromii* relative abundance in both GF and SPF mice feces (Fig. 7G; Supplementary Fig. S18B–S18D).

Finally, in a proof-of-principle experiment, we analyzed the feces of two non–cancer patients enrolled in a clinical trial (NCT04058392) addressing the safety of oral administration of CC (26). Preliminary fecal metagenomics revealed a positive trend in diversity and toward enrichment of *R. bromii*, consistent with the results obtained in CC-treated mice (Supplementary Fig. S18E).

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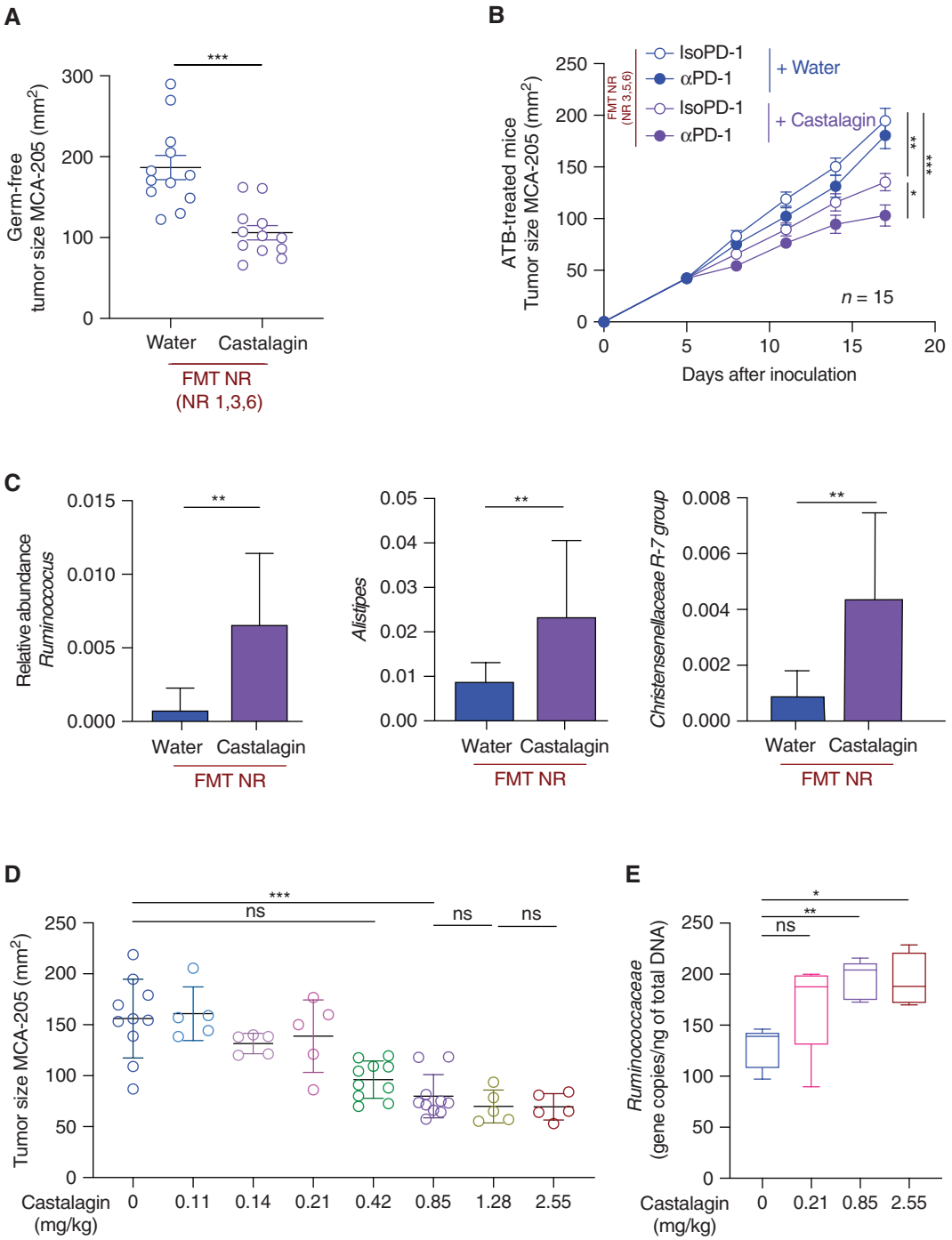

Figure 6. Castalagin circumvents αPD-1 resistance in mice conferred by FMT from NR patients with NSCLC and influences the microbiome composition in a dose-dependent manner. **A,** FMT of stool sample from three NR patients with NSCLC (NR 1, 3, and 6) was performed in GF C57BL/6 mice ($n = 12$/group). Two weeks later, MCA-205 sarcoma cells were inoculated, and mice received a daily oral gavage with castalagin or water. **B,** MCA-205 tumor growth kinetics in the ATB-avatar model after FMT from three NR patients (NR 3, 5, and 6) with daily oral supplementation with castalagin or water in combination with αPD-1 or IsoPD-1. **C,** Relative abundance analysis results after 16S rRNA sequencing analysis of *Ruminococcus*, *Alistipes*, and *Christensenellaceae R-7* group between castalagin and water groups in the NR FMT experiments. **D,** MCA-205 tumor size at sacrifice of mice that received daily oral gavage of castalagin with increasing concentrations from 0 mg/kg to 2.55 mg/kg. **E,** Real-time PCR of DNA extracted from mouse feces after 6 days of oral gavage with water or castalagin at 0.21, 0.85, and 2.55 mg/kg, using specific primers for *Ruminococcaceae* detection in the MCA-205 experiment ($n = 5$/group). Means±SEM are represented. ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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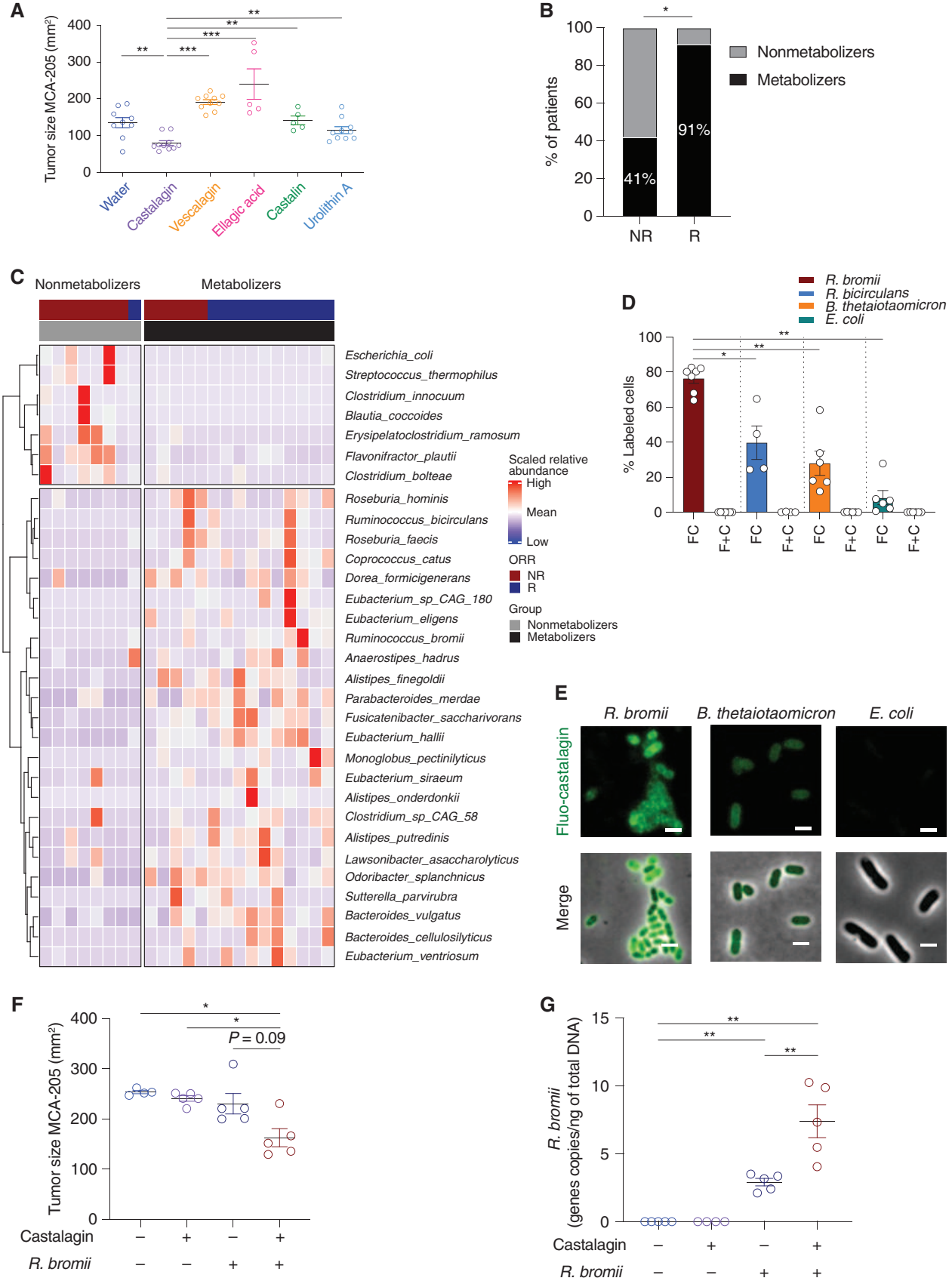

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DISCUSSION

The gut microbiome has emerged as a key player in shaping tumor immunosurveillance and potentiating ICI efficacy. Harnessing the gut microbiome is thus considered a novel and promising strategy for adjuvant therapy in cancer. CC was previously shown to exert strong microbiome-dependent activity against obesity-linked metabolic syndrome, mainly through the enrichment of *A. muciniphila* (19).

In this study, we discovered that the polyphenol castalagin, an abundant ellagitannin in CC, is the principal bioactive molecule that carries the antitumor activity of this prebiotic extract. In two tumor models, we showed that, similar to CC, the antitumor effect of castalagin induced a beneficial shift in microbiome composition, independent of αPD-1 administration. Metagenomics sequencing corroborated that castalagin treatment reproduced the effect that CC had on the abundance of *Ruminococcacceae, Alistipes*, and *Ruthenibacterium lactatiformans* (corresponds to the 16S rRNA signal of *Ruminococcaceae UBA1819*), which correlated with the recruitment of $CD8^+$, $PD\text{-}L1^+CD8^+$, and $CD8^+/FOXP3^+CD4^+$ T cells within the tumor microenvironment. In addition, we showed that these gut microbial/immune changes induced by castalagin were accompanied by an increase in taurine-conjugated bile acids. Furthermore, we have developed an efficient extraction and purification protocol using oak wood, ensuring a readily available and accessible source of castalagin for future therapeutic trials.

We also show that the action of castalagin is specific because other closely related metabolites and its diastereomer vescalagin did not exhibit antitumor activity *in vivo*. Castalagin metabolism likely gives consuming bacteria a competitive advantage that leads to their expansion, causing a global shift in composition, which in turn shifts the production of metabolites and exerts an antitumor effect, increasing αPD-1 activity and circumventing resistance. Moreover, secondary taurinated bile acids are known to inhibit colon adenoma (27) and are decreased after ATB treatment, which has also been associated with ICI resistance (28). Furthermore, using fluorescent castalagin, we demonstrated a non–energy-dependent but specific interaction between castalagin and the cell wall of certain bacteria, preferentially with *R. bromii* compared with *R. bicirculans*. This suggests that, even in the absence of direct metabolism, castalagin itself may promote the growth of *R. bromii* and possibly other beneficial bacteria in the gut. Although the castalagin–bacterial interaction did not provide a competitive advantage in nutrient-rich media *in vitro*, it may provide some advantage within a complex and nutrient-restricted environment such as the gut. Ellagitannins are known for their ability to scavenge reactive radicals (29), and the administration of grape polyphenols in a mouse model of metabolic disease was shown to decrease gut reactive oxygen species (ROS; ref. 30). Thus, bacteria that associate with castalagin may be protected from ROS present at the oxic–anoxic interface of the intestinal epithelium (31, 32). Polyphenols can also chelate ferric iron via their catechol moieties, which may provide an advantage in an iron-deprived environment (33). Importantly, our finding that NSCLC responders enriched with *Ruminococcus* were able to metabolize castalagin and our observation of an increase in *Ruminococcus* post-CC treatment further suggest that castalagin has a propensity to interact with specific and beneficial bacteria.

The present study validates the importance of specific immunogenic gut bacteria in modulating response to ICI. In patients with advanced melanoma, higher baseline diversity and overrepresentation for *Ruminococcaceae* including *R. bromii* were associated with an increased proportion of $CD8^+$ T cells in the tumor and peripheral blood and correlated with a beneficial response (12). In addition, *Ruminococcus bacterium cv2* is one of 11 beneficial bacteria present in the VE800 probiotic capsule that was linked to increased $IFN\gamma^+CD8^+$ T cells in the colon and tumors and is currently under investigation in a clinical trial (NCT04208958; ref. 14). Specific bacteria modulating ICI outcomes are further demonstrated with *A. muciniphila*, which predicted the response in 153 patients with advanced NSCLC and renal cell cancer amenable to ICI (10). This observation was recently validated in a prospective trial of 338 patients with NSCLC (25). In this study, *R. bromii* was the most abundant bacteria in patients with detectable *A. muciniphila* in their feces. Moreover, in the first clinical trials combining FMT with ICI, *Ruminococcaceae* spp. and *A. muciniphila* were both enriched post-FMT in R patients with melanoma refractory to ICI (18, 34).

Altogether, this is the first study demonstrating the microbiome-dependent antitumor effect of a single polyphenol as a prebiotic, paving the way for future clinical trials using strategies with castalagin as an ICI adjuvant in patients with cancer.

METHODS

Murine Studies

All animal studies were approved by the Institutional Animal Care Committee (CIPA) and carried out in compliance with the Canadian Council on Animal Care guidelines (Ethics numbers: C18029BRs and C18025BRs). Murine experiments were conducted

Figure 7. Castalagin and not its metabolites provides antitumor activity and directly interacts with the cellular envelope of *Ruminococcus*. **A,** Tumor size at sacrifice of mice that received castalagin or its downstream metabolites or its diastereomer vescalagin. Structures of these compounds are shown in Supplementary Fig. S13. **B,** Relative proportion of castalagin metabolizers and nonmetabolizers based on *ex vivo* castalagin metabolism assays using fecal samples from NSCLC ICI-responder (R; $N = 11$) and ICI-nonresponder (NR; $N = 12$) patients. Associations between *ex vivo* castalagin metabolism and patient response status were determined using the Fisher exact test on the number of patients in each category ($P = 0.0272$). Detailed assignments of metabolic phenotypes (metabotypes) for patients are reported in Supplementary Fig. S15B. **C,** Heat map representation of metagenomic microbiome sequencing from 23 patients with NSCLC amenable to ICI and segregated between castalagin nonmetabolizer and metabolizers. ORR, objective response rate. **D,** Labeling experiments on *R. bromii*, *R. bicirculans*, *E. coli*, and *B. thetaiotaomicron*. Cells were incubated with either 2 μmol/L fluo-castalagin (FC) or 2 μmol/L free fluorescein and free castalagin (F + C) for 1 hour at 37°C. Each circle represents one independent experiment. **E,** Representative epifluorescence microscopy images of fluo-castalagin–labeled *R. bromii*, *E. coli*, and *B. thetaiotaomicron*. Merge represents superimposed images of fluorescence and phase contrast. Scale bar, 2 μm. **F,** Tumor size at sacrifice of GF mice bearing MCA-205 treated with castalagin or water in combination with oral gavage with *R. bromii* or PBS. Each circle represents one animal. **G,** Real-time qPCR of DNA extracted from mouse feces using specific primers for *R. bromii* detection in GF mice bearing MCA-205 tumors. Mean ± SEM represented. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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using seven-week-old female C57BL/6 mice, obtained from Charles River. GF female C57BL/6 mice were purchased from the International Microbiome Centre Germ-Free Facility (University of Calgary, Canada) and maintained at the Centre de recherche du Centre hospitalier de l'Université de Montréal (CRCHUM) GF Facility.

Cell Culture, Reagents, and Tumor Cell Lines

MCA-205 fibrosarcoma cells and E0771 mammary adenocarcinoma, class I MHC H-2^b syngeneic cell lines for C57BL/6 mice were used for this study. MCA-205 and E0771 cells were cultured as previously described (10, 20). All cultures were checked for *Mycoplasma* using PlasmoTest Mycoplasma Detection Kit (InvivoGen).

Subcutaneous Model of MCA-205 Sarcoma and E0771 Breast Cancer

Syngeneic C57BL/6 mice were implanted subcutaneously with 0.8×10^6 MCA-205 cells or 0.5×10^6 E0771 cells. When tumors reached 20 to 35 mm^2 in size, mice were treated four times (or two times for flow cytometry analysis; see section below) intraperitoneally every three days with αPD-1 mAb (250 μg/mouse; clone RMP1-14, Bio X Cell) or isotype control (clone 2A3, Bio X Cell). Upon treatment initiation, mice received a daily oral gavage with the following products: *Myrciaria dubia*, CC raw extract (Sunfood; 200 mg/kg per mouse); fractions from extraction round 1 (P, MP, NP, and INS; 40.18 mg/kg per mouse); fractions from extraction round 2 (P1, P2, P3, and P4; equivalent to fraction P dose of 40.18 mg/kg per mouse); vescalagin (extracted from CC; see isolation process below; 0.85 mg/kg per mouse); ellagic acid (0.85 mg/kg per mouse; Sigma-Aldrich); urolithin A (0.85 mg/kg per mouse; Sigma-Aldrich); castalin (0.5 mg/kg per mouse; PhytoProof, Sigma-Aldrich); and castalagin (PhytoProof, Sigma-Aldrich, or isolated from food grade oak; see isolation process below). In the control group, mice received daily oral gavage with water (100 μL). Tumor area was routinely monitored every three days by means of a caliper. In the $CD8^+$ depletion experiments, we used the anti-CD8 mAb (150 μg/mouse; clone 53-6.7, Bio X Cell) or isotype control (clone 2A3, Bio X Cell).

Antibiotic Treatments

For experiments with ATB, mice were treated with an ATB solution containing ampicillin (1 mg/mL), streptomycin (5 mg/mL), and colistin (1 mg/mL; Sigma-Aldrich), which was added to the sterile drinking water of mice (10). ATB activity was confirmed as previously described (10). For the FMT experiments in SPF-reared mice, mice received three days of the same combination of ATB prior to FMT. For the metabolomic experiments, mice received one week of the same combination of ATB for the ATB group, then tumor cells were injected subcutaneously. For the ATB groups, the mice continued to receive ATB after tumor cell implantation, throughout the experiment until sacrifice. When tumors reached 20 to 35 mm^2 in size, mice were treated with a daily oral gavage of castalagin or water for six days as described above. The stools and blood (for serum) were collected three days after the start of oral gavage, and the liver, tumor, ileum, and colon contents were collected at sacrifice and snap-frozen for metabolomic analysis described in Supplementary Materials and Methods.

FMT Experiments

Appropriate ethics approval was obtained at the CRCHUM in Montreal (Ethics 17.035). As previously published (10), FMT was performed by thawing fecal material from ten different patients with NSCLC amenable to ICI after patient records were retrospectively analyzed to identify their response status (Supplementary Table S1). Two weeks after FMT, tumor cells were injected subcutaneously, and mice were treated with αPD-1 or isotype control with or without CC, castalagin, or water as described above.

Flow Cytometry Analysis of Immune Cells

Tumors and spleens were harvested at 9 days after the first injection of αPD-1 in mice bearing MCA-205 tumors and at 11 days after the first injection of αPD-1 in mice bearing E0771 tumors. TILs and splenocyte suspensions were prepared as previously published (10). Two million tumor cells or splenocytes were preincubated with purified anti-mouse CD16/CD32 (clone 93; eBioscience) for 30 minutes at 4°C before membrane staining with anti-mouse antibodies listed in Supplementary Table S4. For intracellular staining, the FOXP3 staining kit (eBioscience) was used. Dead cells were excluded using the Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies). Samples were acquired on a BD Fortessa 16-color cytometer (BD), and analyses were performed with FlowJo software (BD).

Immunofluorescence Staining

Murine tumors preserved in optimal cutting temperature compound were cut (5-μm-thick sections) and adhered to microscope slides and stored at –80°C. At the start of the experiment, slides were air-dried and washed with cold acetone. To prevent nonspecific binding to biotin, the Endogenous Avidin Biotin Blocking Kit (Thermo Fisher) was used. Additionally, tissues were incubated with 10% donkey serum in order to reduce background staining. Primary antibodies used were anti-CD4, anti-CD8, and anti-FOXP3. Donkey anti-goat and donkey anti-rat conjugated to AF-488 were used as secondary antibodies, and slides were incubated with Cy3-streptavidin to detect biotinylated antibodies (Supplementary Table S5). Nuclei were visualized by counterstaining with DAPI (Thermo Fisher). Images were generated using the whole slide scanner Olympus BX61VS (20×0.75NA objective with a resolution of 0.3225 mm).

VIS software from Visiopharm was used for whole tissue immunofluorescence analysis. The protocol for the analysis is detailed in Supplementary Materials and Methods.

CC Extraction and Analysis

Polyphenols in CC were extracted in 50% and 90% MeOH and analyzed by LC-MS according to the procedure of Fracassetti and colleagues (22). The supernatants from both extracts were combined and filtered prior to analysis. We compared the relative retention times at 254 nm and negative ion mass spectra from our LC-MS analysis to those from a published characterization of CC polyphenols (22).

Identification of Castalagin as the Active Compound in CC

To discover which compounds in CC were responsible for its antitumor activity, CC was fractionated by extraction (0, 50, and then 90% MeOH) or by reverse-phase chromatography (see Supplementary Materials and Methods for detailed descriptions). Fractions were iteratively screened in the MCA-205 sarcoma mouse model to determine the active fractions.

Fluo-Castalagin Synthesis and Purification

Castalagin was monofunctionalized with fluorescein via a transesterification reaction with 5/6-carboxyfluorescein succinimidyl ester (fluorescein-NHS). Briefly, castalagin (3.2 μmol/L) was dissolved in 200 μL dimethylformamide and then reacted with fluorescein-NHS (2 eq.) in the presence of triethylamine (2 eq.) and 4-dimethyaminopyridine (Sigma). Following the workup with DOWEX 50WX8 resin, the crude mixture was analyzed by LC-MS. Peaks corresponding to monofunctionalized fluo-castalagin were isolated by HPLC.

Bacterial Strains and Culture Conditions

All bacteria were grown under anaerobic conditions at 37°C in an anaerobic chamber (Coy Laboratory Products, 5% H_2, 20% CO_2, 75% N_2). *Ruminococcus bromii* (strain ATCC 27255) and *Ruminococcus bicirculans* (strain CRCHUM12^T) were grown on anaerobe basal broth (ABB; Oxoid) agar plates. *Escherichia coli* (strain CRCHUM10)

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and *Bacteroides thetaiotaomicron* (strain CRCHUM9) were grown on tryptone soya agar with 5% sheep blood (Oxoid). *R. bicirculans*, *B. thetaiotaomicron*, and *E. coli* were isolated in our laboratory from the feces of patients with melanoma. A colony of each bacterium was inoculated into fresh ABB media and grown for 48 hours prior to the start of growth experiments.

Fluo-Castalagin Labeling Experiments on Bacterial Isolates

Stationary-phase cultures were diluted into fresh ABB media (4 mL/condition) and grown to early–mid exponential phase ($OD_{600} \geq 0.2$). Bacteria were washed in a defined minimum medium (MM; see recipe in Supplementary Materials and Methods) and then resuspended in 176 µL of MM.

For labeling experiments at 0°C, bacteria were incubated for one hour in an ice bath prior to the addition of fluo-castalagin. For competition experiments, castalagin was added to a final concentration of 200 µmol/L prior to the addition of fluo-castalagin. The fluo-castalagin probe was added to a final concentration of 2 µmol/L and incubated with bacteria for one hour at 37°C (or 0°C for the ice condition). After incubation, bacteria were washed twice in PBS and then analyzed by flow cytometry and epifluorescence microscopy (detailed below).

Flow Cytometry Acquisition for Fluo-Castalagin Labeling Experiments

Flow cytometry analysis of fluo-castalagin–labeled bacteria was performed on an LSRFortessa cytometer (BD Biosciences) equipped with a blue (488 nm) laser. The blue 695/40-area channel was used as a background channel. A gate was established above the unstained population in the blue 530/30-area channel, and the signal for 100,000 events was measured. Bacteria within the gate were considered to be labeled by fluo-castalagin.

Epifluorescence Microscopy Acquisition for Fluo-Castalagin Labeling Experiments

Fluo-castalagin–labeled bacteria were loaded onto a slide with 0.2% Gelzan (Sigma) pads. Epifluorescence microscopy imaging of fluo-castalagin–labeled bacteria was performed using a Nikon Ti2 microscope equipped with a 60× CFI Plan Apochromat phase contrast objective with a numerical aperture of 1.40. Fluorescein imaging used a SpectraX illuminator (Lumencor) with an FITC filter set (470/24 nm excitation, 525/50 nm emission). Images were captured with a Hamamatsu Orca Fusion CMOS camera. Images were processed in FIJI as follows: images were cropped, the background from the fluorescence channel was subtracted, and an identical lookup table was applied to the fluorescence channels.

16S rRNA Analysis

Isolated DNA was analyzed using 16S rRNA gene sequences to investigate the microbial composition in fecal samples. The detailed procedure is described in Supplementary Materials and Methods.

Shotgun Sequencing Analysis

Isolated DNA was analyzed using shotgun sequencing to investigate the microbial composition in fecal samples. The detailed procedure is described in Supplementary Materials and Methods.

CC Trial in Non–Cancer Patients

As part of a CC clinical trial study (NCT04058392), two participants living with HIV with undetectable viremia were invited to take CC capsules (total 1 g, Natural Traditions) per day for four weeks in addition to their antiretroviral therapy. Feces were collected by each participant before and after four weeks of CC intake, and were kept at 4°C for a maximum of 16 hours before freezing at –80°C.

The CC study was approved by Health Canada, the Natural and Non-prescription Health Products Directorate, and the research ethics board of the McGill University Health Centre (study number 2020-5903). Each participant provided written informed consent before entering the study. The study is conducted in accordance with the Declaration of Helsinki.

Statistical Analysis

The Mann–Whitney *U* test was used to determine significant differences among the different groups using alpha diversity, which shows the diversity in each individual sample. DESeq2 (35) was used to perform differential abundance analysis at the genus level. All *P* values of differential abundance analyses have been adjusted for multiple comparisons with the Benjamini–Hochberg method. The Spearman rank correlation test was obtained using GraphPad Prism 8 and was used to compare continuous variables between the flow cytometry analysis parameters and the significant bacteria identified with differential abundance analysis in the water versus CC, water/αPD-1 versus CC/αPD-1, water/αPD-1 versus castalagin/αPD-1, and water/IsoPD-1 versus castalagin/IsoPD-1 groups for the MCA-205 and E0771 tumor models. *P* values were two-sided with 95% confidence intervals: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The castalagin antitumor dose–response curve was constructed by fitting data to a baseline-adjusted variable slope dose–response equation in GraphPad Prism 8. Fisher exact test was used for the analysis of the frequency of metabolizers and nonmetabolizers of castalagin in the NR and R cohort patients in GraphPad Prism 8.

Data Availability Statement

The raw read data generated from the RNA-seq, metagenomic sequencing, and 16S rRNA sequencing performed in this study are deposited under BioProject ID's PRJNA783624 (RNA-seq and metagenomic sequencing) and PRJNA706824 (16S rRNA sequencing). The newly constructed 5.0M nonredundant mouse gut microbial gene catalog and associated Metagenomic Species Pan-genomes reconstructed thereof are available through dataverse INRAE-MGP (https://doi.org/10.15454/L11MXM).

Authors' Disclosures

M. Messaoudene reports a patent for G17004-00006-AD (use of castalagin or analogs thereof for anticancer efficacy and to increase the response to immune-checkpoint inhibitors) pending. R. Pidgeon reports grants and personal fees from Canadian Institutes of Health Research, grants from Weston Family Foundation and Canadian Glycomics Network (GlycoNet), and personal fees from Fonds de recherche du Quebec (Santé; FRQS) during the conduct of the study; other support from Microprediction (cofounder from 2018–2019) outside the submitted work; and a patent for G17004-00006-AD (use of castalagin or analogs thereof for anticancer efficacy and to increase the response to immune-checkpoint inhibitors) pending. S. Isnard reports grants from Canadian HIV Trials Network during the conduct of the study. D. Raoult reports other support from Eurofins and Culturetop outside the submitted work. G. Kroemer reports grants from Agence Nationale de Recherche, Institut Nationale du Cancer, Ligue National contre le Cancer, and Cancéropôle Ile de France during the conduct of the study; grants from Daiichi Sankyo, Eleor, Kaleido, Lytix Pharma, PharmaMar, Samsara, Sanofi, Sotio, Vascage, and Vasculox/Tioma outside the submitted work; and is on the Board of Directors for the Bristol Myers Squibb Foundation France and is a scientific cofounder of Everimmune, Samsara Therapeutics, and Therafast Bio. A. Elkrief reports other support from Canadian Institutes of Health Research, Royal College of Physicians and Surgeons of Canada, and the Henry R. Shibata Award outside the submitted work. A. Marette reports grants from Weston Foundation during the conduct of the study. B. Castagner reports a patent for G17004-00006-AD (use of castalagin or analogs thereof for anticancer efficacy and to

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increase the response to immune-checkpoint inhibitors) pending. B. Routy reports grants from Davoltera, Kaleido, and Vedanta outside the submitted work, as well as a patent for G17004-00006-AD (use of castalagin or analogs thereof for anticancer efficacy and to increase the response to immune-checkpoint inhibitors) pending. No disclosures were reported by the other authors.

Authors' Contributions

M. Messaoudene: Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing. **R. Pidgeon:** Resources, formal analysis, and methodology. **C. Richard:** Formal analysis, validation, and methodology. **M. Ponce:** Investigation. **K. Diop:** Investigation. **M. Benlaifaoui:** Investigation. **A. Nolin-Lapalme:** Formal analysis. **F. Cauchois:** Investigation. **J. Malo:** Investigation. **W. Belkaid:** Investigation. **S. Isnard:** Resources. **Y. Fradet:** Writing–review and editing. **L. Dridi:** Investigation. **D. Velin:** Formal analysis, investigation, writing–review and editing. **P. Oster:** Formal analysis and investigation. **D. Raoult:** Resources. **F. Ghiringhelli:** Resources, writing–review and editing. **R. Boidot:** Investigation and methodology. **S. Chevrier:** Investigation. **D.T. Kysela:** Investigation. **Y.V. Brun:** Investigation, writing–review and editing. **E. Falcone:** Resources. **G. Pilon:** Investigation. **F. Plaza Onate:** Software, investigation, methodology. **O. Gitton-Quent:** Software, investigation, methodology. **E. Le Chatelier:** Software, investigation, and methodology. **S. Durand:** Resources, formal analysis, investigation, and methodology. **G. Kroemer:** Resources, methodology, writing–review and editing. **A. Elkrief:** Writing–review and editing. **A. Marette:** Resources, investigation, methodology, writing–review and editing. **B. Castagner:** Resources, formal analysis, investigation, methodology, writing–review and editing. **B. Routy:** Conceptualization, resources, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing.

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