

REPORT

CLINICAL TRIALS

Fecal microbiota transplant promotes response in immunotherapy-refractory melanoma patients

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The gut microbiome has been shown to influence the response of tumors to anti-PD-1 (programmed cell death-1) immunotherapy in preclinical mouse models and observational patient cohorts. However, modulation of gut microbiota in cancer patients has not been investigated in clinical trials. In this study, we performed a phase 1 clinical trial to assess the safety and feasibility of fecal microbiota transplantation (FMT) and reinduction of anti-PD-1 immunotherapy in 10 patients with anti-PD-1-refractory metastatic melanoma. We observed clinical responses in three patients, including two partial responses and one complete response. Notably, treatment with FMT was associated with favorable changes in immune cell infiltrates and gene expression profiles in both the gut lamina propria and the tumor microenvironment. These early findings have implications for modulating the gut microbiota in cancer treatment.

Immunotherapy to inhibit the programmed cell death-1 (PD-1) checkpoint protein in metastatic melanoma patients has demonstrated durable complete response (CR) rates of 10 to 20% (1). However, most of the patients do not respond to PD-1 blockade, and many of the partially responding patients eventually progress (1). Extensive research efforts have been undertaken to overcome the resistance to anti-PD-1 therapy. One of the most promising leads involves modulation of the gut microbiota (2–4), which has been shown to have a profound effect on the development and function of the immune system (5). Although no specific bacterial taxa have been consistently associated with clinical response to immunotherapy (6), fecal microbiota transplantation (FMT)—which transfers the entire gut microbiota from one host to another—has demonstrated promising results in preclinical models (2–4). Compared with mice that received FMT from melanoma patients not responding to anti-PD-1 therapy, mice that received FMT from responders demonstrated increased intratumoral CD8⁺ T cell

infiltration and enhanced overall effectiveness of anti-PD-1 therapy (2, 3). On the basis of these data, we designed a phase 1 clinical trial (NCT03353402) to assess the safety, feasibility, and immune cell impact of FMT and reinduction of anti-PD-1 immunotherapy in patients with refractory metastatic melanoma.

The trial included two FMT donors who had previously been treated with anti-PD-1 monotherapy for metastatic melanoma and had achieved a CR for at least 1 year (table S1 and materials and methods). Patients were considered eligible FMT recipients if they had a diagnosis of metastatic melanoma and had progressed on at least one line of anti-PD-1 therapy. Recipients harboring a BRAF-V600E mutation must have also progressed on BRAF-targeted therapy. As part of the trial's protocol, recipients underwent an initial native microbiota depletion phase in which they were administered with orally ingested antibiotics (vancomycin and neomycin) for 72 hours (Fig. 1A). FMT was then performed by means of both a colonoscopy (protocol day 0) and the administration of oral stool capsules fol-

lowed by reinduction of anti-PD-1 therapy (nivolumab). Six combined treatment cycles composed of anti-PD-1 infusions (nivolumab at standard dose) and additional stool capsules (maintenance FMT) were administered every 14 days until day 90. Each recipient underwent positron emission tomography combined with computed tomography (PET-CT) imaging before the trial and on day 65. Response to treatment was defined as an objective tumor regression per imaging according to iRECIST criteria (response evaluation criteria in solid tumors, modified for immunotherapy) (7). Objective responders and recipients who demonstrated a clinical benefit to the treatment continued anti-PD-1 beyond day 90 as monotherapy and underwent consecutive PET-CTs in intervals of 6 to 8 weeks until disease progression.

Correlative studies included stool, gut, and tumor analyses (see materials and methods). 16S ribosomal RNA (rRNA) gene and metagenomics sequencing were conducted on stool samples that were collected from recipients up to 1 week before the native microbiota depletion phase (defined as pretreatment) and on stool samples collected on days 7, 31, and 65. Donor stool samples were collected during the fecal donation period. Gut and tumor biopsies were collected pretreatment and at days 31 and 70, respectively. Infiltration and activity of immune cells in the tissue samples were assessed using immunohistochemical (IHC) and bulk RNA sequencing (RNA-seq). In cases where no specific gene differed in a statistically significant manner, gene set testing was conducted using the Gene Ontology (GO) dataset. Recipient 2 refused to undergo post-treatment biopsies and withdrew consent immediately after the day-65 imaging assessment, leaving nine available recipients for gut and tumor tissue assessment.

Ten recipients with confirmed progression on anti-PD-1 therapy were enrolled and treated between June 2018 and March 2019 (Table 1 and table S2). Recipients were assigned to receive FMT from one of the two available donors, alternating between donor 1 and donor 2. The median recipient age was 66 years [interquartile range (IQR), 49 to 68 years], the majority were males (70%), and the median elapsed time from the last previous dose of anti-PD-1 to the first dose of the current trial was 113 days

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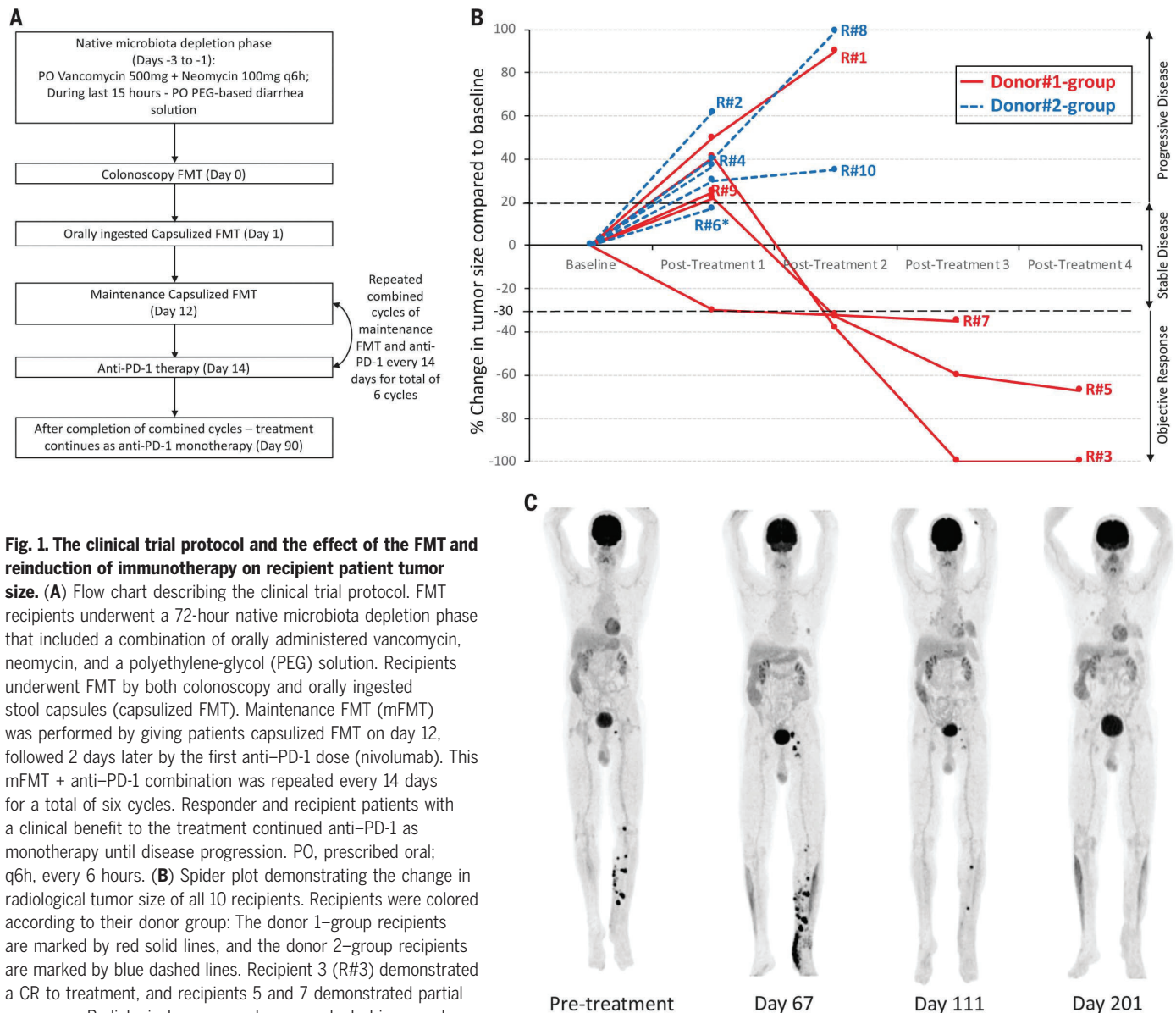


Fig. 1. The clinical trial protocol and the effect of the FMT and reinduction of immunotherapy on recipient patient tumor size. (A) Flow chart describing the clinical trial protocol. FMT recipients underwent a 72-hour native microbiota depletion phase that included a combination of orally administered vancomycin, neomycin, and a polyethylene-glycol (PEG) solution. Recipients underwent FMT by both colonoscopy and orally ingested stool capsules (capsulized FMT). Maintenance FMT (mFMT) was performed by giving patients capsulized FMT on day 12, followed 2 days later by the first anti-PD-1 dose (nivolumab). This mFMT + anti-PD-1 combination was repeated every 14 days for a total of six cycles. Responder and recipient patients with a clinical benefit to the treatment continued anti-PD-1 as monotherapy until disease progression. PO, prescribed oral; q6h, every 6 hours. (B) Spider plot demonstrating the change in radiological tumor size of all 10 recipients. Recipients were colored according to their donor group: The donor 1-group recipients are marked by red solid lines, and the donor 2-group recipients are marked by blue dashed lines. Recipient 3 (R#3) demonstrated a CR to treatment, and recipients 5 and 7 demonstrated partial responses. Radiological assessment was conducted in accordance with the immune response evaluation criteria in solid tumors (iRECIST) (7) and included measurements of target and new target lesions. The asterisk indicates that recipient 6 was excluded from the trial after the first posttreatment imaging study because of unstable metastatic brain disease (hemorrhage into a brain metastasis that was known before inclusion in the trial). (C) Recipient 3 PET-CT imaging. The metastatic lesions, represented as black emission areas, were concentrated in the left leg and groin (inguinal). As a result of the treatment, the metastases had initially increased in size and new lesions appeared (day 67). However, a complete resolution of all lesions was demonstrated in consecutive follow-up imaging studies. The initial tumor size increment was likely caused by the substantial increase in CD8⁺ T cell intratumoral infiltration that was observed in this patient (14 cells/mm² pretreatment versus 736 cells/mm² on day 70; see below, Fig. 3, E to G), a phenomenon known as pseudoprogression (8).

(IQR, 59 to 183 days). The most common PD-L1 expression category in pretreatment tumor biopsies was $\geq 5\%$. This expression category was noted in four recipients, whereas three recipients had no pretreatment PD-L1 expression (table S2). In terms of safety results, the only observed FMT-related adverse event (AE) was mild bloating between days 3 and 15 in one recipient. Several mild (grade 1) immune-related adverse events (irAEs) were observed, mainly arthralgia (table S3). No moderate-to-

severe irAEs (grades 2 to 4) were observed, although five recipients had developed such irAEs during their previous anti-PD-1 treatment lines (table S4).

Objective responses to treatment were demonstrated by three recipients, all of them from the donor 1 group: Recipient 3 achieved a CR, and recipients 5 and 7 achieved partial responses (Fig. 1B, Table 1, and table S5). All responders crossed the progression-free survival milestone of 6 months. Both recipients 3

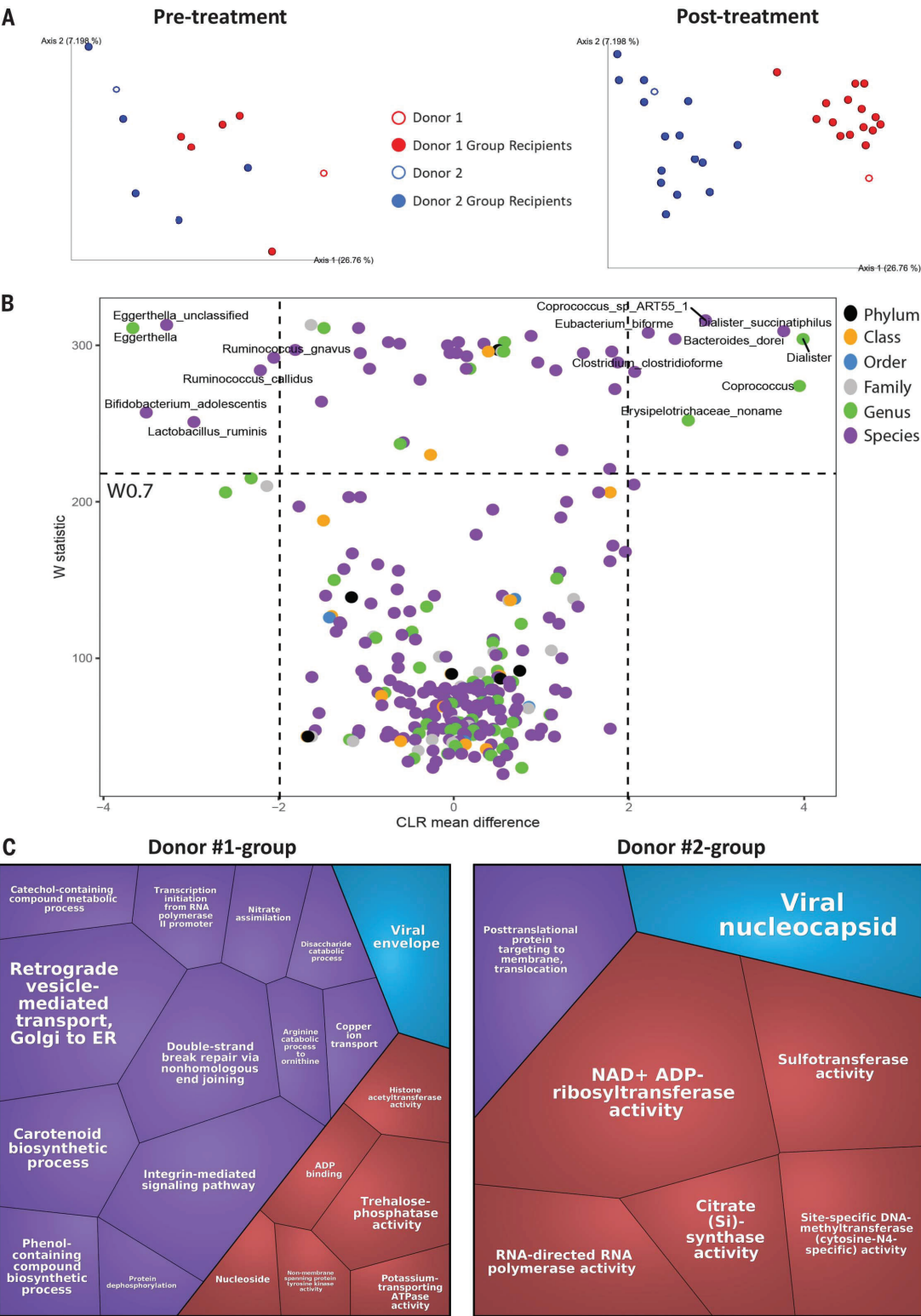
and 5 demonstrated an initial increase in their metastases size followed by regression (Fig. 1, B and C, and fig. S1). This phenomenon is known as pseudoprogression (8) because the increment in metastasis radiological volumes is not caused by tumor proliferation but rather by an influx of antitumoral immune cells into the tumor. Recipient 1 (donor 1 group) demonstrated an initial mixed response with regression of some of lesions, but, overall, the disease had progressed (fig. S2).

Fig. 2. The effect of FMT on gut microbiota composition in metastatic melanoma recipient patients.

(A) Principal components analysis (PCA) plots of patient gut microbiota compositions based on stool 16S rRNA gene sequencing dissimilarity test (β -diversity, unweighted UniFrac). The distance between samples on the plot represents their dissimilarity—the greater the distance between two samples, the higher the dissimilarity of their composition. Recipient patients were grouped according to their donors: Those who received FMT implants from donor 1 are colored in red, and those who received implants from donor 2 are colored in blue. The plots demonstrate no difference between the pretreatment recipient compositions of the two donor groups ($FDR = 0.45$), in contrast to a clear post-treatment donor-based division ($FDR = 0.003$).

(B) A volcano plot based on the ANCOM test. The plot compares the relative abundance of specific taxa between the donor 1 group (negative x axis) and the donor 2 group (positive x axis). Each donor group was composed of posttreatment samples of the relevant recipients and the donor sample. Taxa that differed between the groups with $FDR q \leq 0.05$ are presented above the horizontal dashed line. The center log transformation (CLR) mean difference on the x axis is an ANCOM calculation that is used to determine compositional differences in microbial communities. For convenience, only taxa with a mean difference above an absolute

value of 2 are labeled with text. The full list of taxa that significantly differed between the two donor groups is detailed in table S7. (C) Voronoi treemap plots of microbiota GO gene sets that were enriched among the different donor groups' microbiotas. The abundance of gene sets was compared across donors and posttreatment recipient samples. Gene sets that showed statistically significant differences between the donor 1 group and the donor 2 group and had a $\log_2$ differential abundance >1 (table S8) are plotted. The polygon size represents the scale of the $\log_2$ abundance difference—a bigger polygon represents a more abundant pathway. The GO gene sets are also colored according to their GO category: purple for biological processes, light blue for cellular components, and red for molecular functions. ER, endoplasmic reticulum; ADP, adenosine 5'-diphosphate; ATPase, adenosine triphosphatase; NAD⁺, oxidized nicotinamide adenine dinucleotide.



Stool 16S rRNA gene sequencing analysis demonstrated that posttreatment gut microbiota composition of all recipients significantly differed from their baseline [β -diversity, unweighted UniFrac, $P = 0.02$; false discovery rate (FDR) = 0.05; Fig. 2A and figs. S3 and S4]. There was no statistically significant difference between the pretreatment microbiota composition of recipients from the donor 1 group and those of the donor 2 group ($P = 0.36$; FDR = 0.45). However, posttreatment microbiota compositions of the donor 1-group recipients differed from those of the donor 2 group ($P = 0.001$; FDR = 0.003; Fig. 2A). Donor 2 had a higher microbiota richness (α -diversity, Faith's phylogenetic diversity) compared with donor 1. Accordingly, despite similar richness in the pre-

treatment compositions ($P = 0.60$; FDR = 0.77), posttreatment compositions of the donor 2 group demonstrated higher richness than those of the donor 1 group ($P < 0.001$; FDR = 0.001; fig. S5). Metagenomic sequencing was used to identify specific taxa and functional pathways that differed between the trial's groups. Overall comparison between recipient pre- and posttreatment microbiota compositions [analysis of composition of microbiomes (ANCOM) test] showed that posttreatment compositions had a higher relative abundance of the immunotherapy-favorable *Veillonellaceae* family (3) and a lower relative abundance of *Bifidobacterium bifidum*, which was reported to promote immune tolerance via regulatory T cells (9) (figs. S6 and S7). Both donors had

previously reported immunotherapy-favorable features (fig. S8 and table S6) such as high relative abundances of *Lachnospiraceae* (both donors), *Veillonellaceae* (donor 1), and *Ruminococcaceae* (donor 2) (fig. S8) (2–4). Comparison of posttreatment recipient microbiota compositions by their assigned donors demonstrated that the donor 1 group was characterized by a higher relative abundance of taxa like *Bifidobacterium adolescentis* (Fig. 2B), whereas the donor 2 group had a high relative abundance of taxa like *Ruminococcus bromii* (table S7)—both of which have been previously described as immunotherapy favorable (2, 3). The pretreatment microbiota compositions of the three responding patients (recipients 3, 5, and 7) did not differ from the pretreatment

Table 1. Clinical characteristics of patients receiving FMT and reinduction of anti-PD-1 treatment. The time from previous anti-PD-1 treatment dose to the first trial dose was calculated from the most recent anti-PD-1 treatment dose to the day of the first anti-PD-1 treatment in the clinical trial. The percentage of viable tumor was calculated as the percentage of viable tumor out of the entire tumor tissue which was examined in a hematoxylin and eosin (H&E) slide of the tumor biopsy (see materials and methods). Clinical responses were based on the iRECIST (7). Response category “None” represents iRECIST-confirmed progressive disease. Recipient 2 did not consent to undergo repeated tumor and gut biopsies, and hence, the percentage of viable tumor is presented as not available (N/A). Additional clinical data per donor and recipient can be found in the supplementary materials (tables S1 and S2, respectively). D, dabrafenib; T, trametinib; Nivo, nivolumab; Pembro, pembrolizumab; Ipi, ipilimumab; T-VEC, talimogene laherparepvec; TIL, adoptive cell therapy composed of tumor-infiltrating lymphocytes.

FMT donor group	Recipient	Previous treatment lines (in chronological order)	Best response during all previous anti-PD-1 lines	Time from previous anti-PD-1 dose to first trial dose (days)	Percentage of viable tumor during the current trial		Clinical response in the current trial
					Pretreatment	Posttreatment	
Donor 1	1	D+T; Nivo; D+T reinduction; Ipi+Nivo	None	57	100	95	None
Donor 1	3	Pembro	None	66	100	30	Complete
Donor 1	5	Ipi+Nivo	Partial	119	100	<1	Partial
Donor 1	7	Pembro; D+T Nivo (adjuvant); Ipi; Carboplatin + Paclitaxel	Complete	204	80	30	Partial
Donor 1	9	Pembro; Ipi; Pembro reinduction	None	209	80	90	None
Donor 2	2	Nivo (adjuvant)	Stable disease	114	N/A	N/A	None
Donor 2	4	Ipi; Pembro; D+T; Nivo; T-VEC + Nivo; TIL; D+T reinduction; Palbociclib; Carboplatin + Paclitaxel	None	112	85	90	None
Donor 2	6	Ipi+Nivo	Partial	322	100	85	None
Donor 2	8	Ipi+Nivo	Mixed	42	90	100	None
Donor 2	10	Ipi+Nivo	Stable disease	57	100	90	None

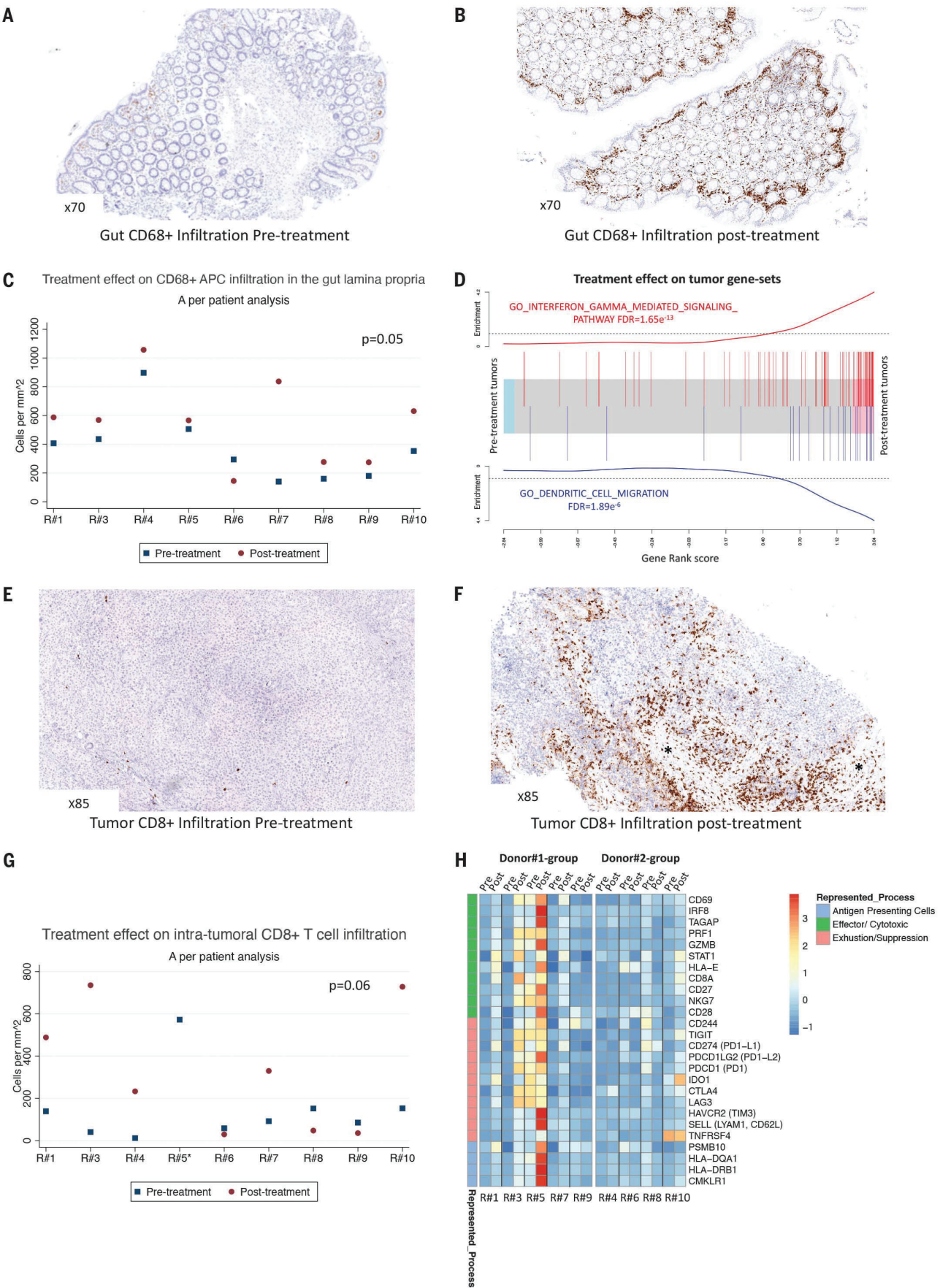


Fig. 3. The effect of FMT and reinduction of immunotherapy on immune activity in the gut and in the tumor microenvironment of metastatic melanoma recipient patients. (A) IHC staining of CD68, representing APCs, conducted on pretreatment sigmoid colon biopsies of recipient 7. (B) IHC staining of CD68⁺ cells conducted on the posttreatment (day 31) sigmoid colon biopsy of recipient 7, which demonstrates a clear increase in CD68⁺ cell infiltration in the gut lamina propria. This infiltration was especially prominent in the subepithelial area, which is physically closer to the gut. (C) An image analysis algorithm was used to quantify the number of CD68⁺ APCs within the gut lamina propria of each recipient patient. A posttreatment increment in CD68⁺ cell infiltration was demonstrated in most recipients ($P = 0.05$). (D) A barcode plot of gene set enrichment among tumor samples. Each bar represents a single gene out of the entire gene set. The plot demonstrates the up-regulation of APC- and T cell–related gene sets among posttreatment tumor samples. The full list of enriched gene sets is detailed in table S12. (E) IHC staining of CD8, representing cytotoxic T cells, conducted on pretreatment tumor metastasis (subcutaneous, left leg) of recipient 3. (F) IHC staining of CD8⁺ T cells conducted on posttreatment biopsy from another subcutaneous

metastasis in the left leg of recipient 3, demonstrating a clear increase in intratumoral CD8⁺ T cell infiltration and immune-induced tumor necrosis (marked by asterisks). (G) An image analysis algorithm was used to quantify the number of CD8⁺-stained T cells within viable tumor tissue for each remote tumor metastasis biopsy. Posttreatment tumor biopsies were preferably taken from the same metastasis used for the pretreatment biopsy or from another metastasis at the same organ. Five recipient patients had increased their intratumoral CD8⁺ T cell infiltration in posttreatment biopsies ($P = 0.06$). The asterisk indicates that recipient 5 achieved a near-pathological CR (<1% viable tumor), and hence their posttreatment CD8⁺ infiltration could not be accurately assessed. (H) Heatmap of tumor immune gene expression. The heatmap illustrates expression dynamics before and after treatment across three representative immune processes—antitumoral effector activity, suppression-exhaustion activity, and APCs activity-abundance. Only members of the donor 1 group demonstrated a posttreatment up-regulation of effector T cell response. Recipient 10 demonstrated a posttreatment up-regulation of the immune checkpoints IDO-1 and TIGIT without an effector response. Scale represents the z-score of gene counts.

microbiota compositions of the rest of the cohort. When the responders' posttreatment compositions were compared with the posttreatment compositions of the other two nonresponders from the donor 1 group (recipients 1 and 9), four taxa differed in a statistically significant manner (fig. S9A). The responders had a higher relative abundance of *Enterococcaceae*, *Enterococcus*, and *Streptococcus australis* and a lower relative abundance of *Veillonella atypica*. However, when the abundance of these specific taxa was assessed in the entire patient cohort (fig. S9B), there were some nonresponders and even pretreatment samples with similar dynamics. Hence, no clear association between those taxa and clinical response to therapy was established. Functional metabolic data were based on annotation of genes to the MetaCyc database (direct measurements of metabolite levels were not conducted). The functional metabolic data demonstrated that the donor 1 group up-regulated the lactose and galactose degradation I pathway [log-fold change (logFC) = 1; FDR = 0.015], whereas the donor 2 group up-regulated the formaldehyde assimilation II (logFC = 2.2; FDR = 3.93×10^{-6}), the formaldehyde oxidation I (logFC = 2.4; FDR = 0.001), and the creatinine degradation I (logFC = 1.4; FDR = 0.014) pathways. Metagenomics GO gene sets that significantly differed between the microbiota of the two donor groups are illustrated in Fig. 2C (table S8). Comparison between the posttreatment microbiota composition of the responding patients (recipients 3, 5, and 7) and the other two nonresponding patients (recipients 1 and 9) among the donor 1 group showed no significant functional or metabolic differences.

Gut sample analysis of all available FMT-recipient patients demonstrated a posttreatment up-regulation of gene sets that were related to the presentation of peptides by antigen-presenting cells (APCs) via major

histocompatibility complex (MHC) class I and interleukin-1–mediated signaling (FDR = 0.014 and 0.038, respectively; table S9). Analysis per donor group demonstrated that the donor 1–group recipients up-regulated additional gene sets related to APC activity, innate immunity, and interleukin-12 (table S10). By contrast, the donor 2–group recipients did not up-regulate any immune-related gene sets (table S11). Per-patient analysis demonstrated an increased lamina propria infiltration of CD68⁺ cells, representing APCs, from an overall pretreatment median of 353 to 569 cells/mm² posttreatment ($P = 0.05$; Fig. 3, A to C, and fig. S10). The CD68⁺ infiltration was concentrated in the subepithelial area, where the proximity to the gut lumen is the highest. All available recipients increased the posttreatment CD68⁺ infiltration except for recipient 6 (donor 2 group, nonresponder patient). Notably, gut sample analysis did not demonstrate a statistically significant increase in T cell infiltration in the gut lamina propria (fig. S11) or T cell–related gene set enrichment.

Tumor sample analysis of all available recipients demonstrated posttreatment up-regulation of multiple immune-related gene sets (Fig. 3D and table S12), such as interferon- γ –mediated signaling pathway (FDR = 1.65×10^{-13}), T cell activation (FDR = 3.27×10^{-12}), MHC class II protein complex (FDR = 9.31×10^{-13}), dendritic cell differentiation (FDR = 5.15×10^{-9}), and T helper 1-type immune response (FDR = 1.06×10^{-6}). Although these immune-related gene sets remained enriched in the donor 1 group–only analysis (table S13), no immune-related gene sets were statistically significantly enriched among tumor samples of the donor 2 group (table S14). Per-patient analysis demonstrated increased posttreatment intratumoral CD8⁺ T cell infiltration among five patients (recipients 1, 3, 4, 7, and 10) with an overall pretreatment median of 89 cells/mm²

versus 282 cells/mm² posttreatment ($P = 0.06$; Fig. 3, E to G, and fig. S12). Recipient 5 achieved a near-pathological CR, as posttreatment viable tumor tissue composed <1% of the entire biopsy, and recipients 3 and 7 achieved partial pathological response (Table 1). Assessment of commonly investigated genes related to intratumoral immune activity demonstrated that the posttreatment tumors of recipients 1, 3, 5, and 7 up-regulated effector-related genes with some reciprocal exhaustion responses (Fig. 3H). Recipient 10, however, up-regulated exhaustion-related genes without an effector response.

This study demonstrates that the combination of FMT from a CR donor and reinduction of anti-PD-1 therapy in refractory metastatic melanoma patients is safe, feasible, and potentially effective. FMT is considered a common treatment for recurrent *Clostridioides difficile* colitis, with a well-established safety profile (10), and its safety has been demonstrated even in immunocompromised patients (11). Nevertheless, the lack of FMT-related complications in this study among immunocompetent metastatic patients treated with repeated FMTs was reassuring. Notably, the combination of FMT and reinduction of anti-PD-1 therapy appeared safe and resulted in some objective clinical responses. Out of 10 anti-PD-1 refractory recipients, three demonstrated clinical responses, including one CR. A similar trial of FMT and anti-PD-1 reinduction in refractory melanoma patients has reported preliminary results of one objective partial response and one stable disease among the first three patients (12). Because the FMT-recipient patients were not treatment naïve, there is a possibility that these clinical outcomes are caused by delayed responses to previous anti-PD-1 treatments. However, this possibility is unlikely, as Ribas *et al.* have reported that delayed response rates in metastatic melanoma patients who continued

anti-PD-1 therapy beyond RECIST-confirmed disease progression were <8% (13). Similarly, Betof Warner *et al.* have reported that response rates of metastatic melanoma patients who were reinduced with anti-PD-1 monotherapy were 5 out of 34 (<15%) (14). These results were possibly an overestimation because three of five responders in that study had an elapsed previous-to-reinduction dose time of at least 12 months (14). Such a prolonged time period might enable the reemergence of immunotherapy-susceptible tumor clones. In our study, the median previous-to-reinduction dose time was only 113 days among the entire cohort and 119 days among the three responders. Moreover, the inclusion criterion of our trial was disease progression on previous anti-PD-1 lines based on iRECIST. According to RECIST 1.1, partial responses or CRs may be deemed unconfirmed pending follow-up, but the classification of progressive disease is always considered final (15). However, immunotherapies might sometimes lead to pseudo-progression (8), as seen in recipients 3 and 5. iRECIST was designed to distinguish between unconfirmed and confirmed disease progression (7) (table S5). Hence, it is possible that the use of iRECIST in those previous publications would have resulted in even lower postfailure response rates.

This higher-than-expected clinical response rate can be explained by the correlative immunological data. Tumor-infiltrating dendritic cells (DCs) have a crucial role in trafficking T cells into tumors (16, 17). Multiple papers from mouse-model studies have demonstrated that microbiota modulation promoted the infiltration of DCs into remote tumors, which resulted in the activation of both T helper 1 cells via interleukin-12 (4, 18) and cytotoxic CD8⁺ T cells (19–21). The same findings were demonstrated in our human FMT trial. Because the donors' microbiotas were transplanted into the recipients' guts, it is plausible to assume that the immune activation cascade started in the gut. The donor 1-group recipients demonstrated increased posttreatment gut infiltration and activity of APCs. Geva-Zatorsky *et al.* have assessed the immune response to colonization of different commensal gut microbes and demonstrated that the local effect of microbes in the gut was mostly on the innate immunity cells (22), which could later migrate into the lymphatic system (23). Notably, some of the donor 2-group recipients also increased their posttreatment gut APC infiltration, although as a group, their RNA-seq findings were not statistically significant. Overall, the recipients who increased their posttreatment intratumoral CD8⁺ T cell infiltration also increased their APC gut infiltration. It is unlikely that the increment in CD8⁺ T cell infiltration was caused by the mere anti-PD-1 administration, because Chen *et al.* have used pre- and

on-treatment tumor biopsies to demonstrate that nonresponding patients undergoing anti-PD-1 therapy did not increase their intratumoral CD8⁺ infiltration (24). However, microbiota-driven gut APC activation would not necessarily yield enhanced intratumoral CD8⁺ activity. Impaired antigen presentation machinery within the tumor cells themselves is a well-known anti-PD-1 resistance mechanism that usually results in lack of intratumoral CD8⁺ T cell infiltration (25). The tumor from recipient 9 demonstrated such an antigen presentation impairment (fig. S13). Moreover, even the presence of high intratumoral infiltration of CD8⁺ T cells sometimes fails to translate into a clinical response. Tumors with high CD8⁺ T cell infiltration can be refractory if the T cell infiltration is ineffective, for example as a result of CD8⁺ T cell exhaustion after exposure to additional immune checkpoints (26). Recipient 10 had overexpression of these molecules, such as IDO-1 (Fig. 3H). Recipient 1, who demonstrated increased intratumoral CD8⁺ T cell activity, had an initial regression in some metastases but eventually progressed as a result of an unknown cause. These tumor characteristics of different patients emphasize the wide context of clinical responses to immunotherapy and that beneficial microbiota composition is not the only factor in treatment response.

The microbiota composition of the two donors and the posttreatment recipients from both donor groups were characterized by high relative abundances of taxa that were previously associated with response to immunotherapy. Yet, the three responding recipients were solely part of the donor 1 group. The reason for this dissonance is unclear. However, this study was statistically powered to assess safety and was not designed to compare efficiency between donors. Lack of clinical responders among the donor 2 group does not necessarily implicate that clinical responses could not be observed in a larger cohort. Moreover, our inability to pinpoint specific response-inducer microbiota characteristics echoes the inconsistency among previous observational studies (6). As the characteristics of optimal microbiota compositions of donors and recipients remain elusive, the design and implementation of future microbiome modulation clinical trials must be carefully considered. Numerous considerations must be taken into account when contemplating strategies to modulate gut microbes, including diet (27). Studies in preclinical models incorporating microbiota into germ-free mice avatars may yield insight into both microbe and host factors. Nonetheless, in light of the decades-based safety profile of FMTs (10), promising results in preclinical models (2–4, 18, 19, 21), and findings that suggest treatment effectiveness in our current clinical trial, clinical institutions should not be deterred by the lack of sufficient mechanistic

knowledge to examine the clinical potential of FMTs in the setting of well-designed and supervised human trials. This is especially true for refractory patients, in whom the risk-to-benefit ratio of FMTs appears favorable.

One limitation of this clinical trial arises from the use of antibiotics as part of the pre-FMT preparation. Antibiotic preparation was adopted because it has enhanced the FMT ability to modulate microbiota composition in reported murine models (28). The vancomycin-neomycin protocol has been reported as an effective pre-FMT protocol in humans (29). Because all of our recipients underwent the exact same pre-FMT protocol, we believe that the use of antibiotics did not affect the observed immune and clinical outcomes. However, this possibility cannot be ruled out in the current study design.

FMT from CR donors and reinduction of anti-PD-1 therapy in refractory metastatic melanoma patients was demonstrated to be safe and feasible. In some patients, this treatment increased the intratumoral immune activity, which was translated into objective clinical responses. These findings support the concept of overcoming resistance to immunotherapy by modulating the gut microbiota.

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J.A.W. **Competing interests:** I.Y. is a medical adviser for Mybiotix Ltd. G.B.-B. received honoraria and travel support from MSD, Roche, BMS, Novartis, and Medison. Y.S.-S. received honoraria from MSD, Roche, BMS, and Novartis. N.A. received honoraria from MSD, Roche, BMS, Novartis, and Medison. R.S.-F. received honoraria from MSD and BMS. J.S. received honoraria from MSD, Roche, BMS, and Novartis; serves on the advisory boards of MSD, BMS, and Novartis; owns shares of 4c BioMed; and is partially employed by 4cBiomed. J.A.W. is an inventor on a U.S. patent application (PCT/US17/53.717) submitted by the University of Texas MD Anderson Cancer Center that covers methods to enhance immune checkpoint blockade responses by modulating the microbiome; reports compensation for speaker's bureau and honoraria from Imedex, Dava Oncology, Omniprex, Illumina, Gilead, PeerView, Physician Education Resource, MedImmune, Exelixis, and Bristol-Myers Squibb; serves as a consultant and/or advisory board member for Roche/Genentech, Novartis, AstraZeneca, GlaxoSmithKline, Bristol-Myers Squibb, Merck, Biothera Pharmaceuticals, and Microbiome DX; and receives research support from GlaxoSmithKline, Roche/Genentech, Bristol-Myers Squibb, and Novartis. G.M. received honoraria from MSD, Roche, BMS, and Novartis; received research grants from Novartis and BMS (not related to this study); serves on the advisory board of MSD, NucleAI, and Biond

Biologics; holds IP and shares of Kitov and 4cBiomed; and is partially employed by 4cBiomed. The rest of the authors declare no competing interests. **Data and materials availability:** Microbiome 16S rRNA and metagenomics sequencing data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under BioProject ID PRJNA678737. The human gut and tumor RNA-seq data have been deposited at the NCBI Gene Expression Omnibus (GEO) under BioProject ID GSE162436.

SUPPLEMENTARY MATERIALS

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Supplementary Materials for

Fecal microbiota transplant promotes response in immunotherapy-refractory melanoma patients

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This PDF file includes:

Materials and Methods
Supplementary Text
Figs. S1 to S13
Tables S1 to S5 and S15
Captions for Tables S6 to S14
References

Other Supplementary Material for this manuscript includes the following:
(available at science.sciencemag.org/cgi/content/full/science.abb5920/DC1)

Tables S6 to S14 (.csv)
MDAR Reproducibility Checklist (.pdf)

Materials and Methods

Patients:

Fecal implant donors were two metastatic melanoma patients who underwent anti-PD-1 monotherapy due to metastatic melanoma and achieved complete response (CR) for over a year (Table S1). Since the optimal microbiota composition is currently unknown, microbiota composition characteristics were not part of the donor screening and donors were selected based only on their clinical response. Donors' exclusion criteria were based on the Israeli Ministry of Health guidelines for fecal donor safety screening (Memo number 19/2017), which incorporated the American(30) and the European(31) guidelines.

Fecal implant recipients were metastatic melanoma patients who failed at least one line of anti-PD-1 treatment, either as monotherapy or in combination with other drugs. Recipients with a BRAFV600E mutation also had to fail targeted anti-BRAF treatments. Recipient inclusion and exclusion criteria were detailed in Table S15. Major inclusion criteria were:(A) an immune Response Evaluation Criteria in Solid Tumors (iRECIST)(7) -confirmed progressive disease on previous anti-PD-1 treatment line; (B) metastatic melanoma with viable tumor tissue comprising at least 75% of the examined area in the pre-treatment tumor biopsy(32);and (C) a washout period of at least four weeks from the previous anti-PD-1 treatment line to the first day of the treatment protocol. Ten out of the fifteen patients who entered the trial's screening phase were qualified as eligible recipients. Five patients failed the screening process due to: rapid deterioration in performance status (n=2); new unstable metastatic brain disease (n=1); patient preference to participate in another clinical trial (n=1); and new diagnosis of another malignancy during the screening process (Transitional Cell Carcinoma of the bladder, n=1).

Assignment of donors to recipients was conducted on the basis of "first in first out" – the first donor who complete the donation process (Donor#1) was assigned to the first enrolled recipient. Donor#2 was assigned to the next consecutive recipient, Donor#1 assigned again to the third recipient and so on.

FMT implant preparation

Donor feces were collected after the completion of the safety screening phase. By that time, both donors had demonstrated CR for at least one year. Donor's feces were processed under Good Manufacturing Processing conditions at the Center for Microbiome Research at Shamir Medical Center (Assaf Harofeh), Israel. The manufacturing process was carried out in ambient air as previously described(33). Briefly, a fecal suspension was generated using normal saline without preservatives. Materials were sequentially sieved to remove particulate material. Colonoscopy-based implants were derived from approximately 50g of fecal matter. The final slurry was concentrated 3-fold by centrifugation and re-suspended in sterile saline with 10% glycerol added as a bacterial cryoprotectant.

For capsule-based implants, commercially available acid-resistant hypromellose capsules (DRCaps, Capsugel, USA) were used. The capsulized FMT dose was composed of 12 capsules, containing concentrated material derived from a mean of 15g of fecal matter. The slurry was concentrated by centrifugation and re-suspended in saline at 1/10 volume of the initial sample with 10% glycerol added as a bacterial cryoprotectant. Fecal matter solution was then pipetted in size 0 capsules (650µL) which were closed and then secondarily sealed in size 00 capsules.

All implants were stored frozen at temperature monitored -80°C until use.

Study design and FMT procedure:

This trial was a single-center phase I study. The study was approved by the Sheba Medical Center Institutional Review Board (approval reference number SMC17-3956) and conducted in accordance with Good Clinical Practice. The first primary end points was the incidence of FMT-related and immunotherapy-related adverse events (irAEs, according to the Common Terminology Criteria for Adverse Events, version 5.0)(34). The second primary end point was microbiota modulation - ideal engraftment from a single donor would result in identical microbiota composition between that donor and recipients; In the case of two donors, ideal engraftment would result in two distinctive recipient-donor compositions. Hence, successful microbiota modulation was defined as grouping of the recipient post-treatment microbiota compositions into two distinct clusters based on their donor's composition. The secondary end point was the change in intra-tumoral CD8⁺ T cell infiltration in remote, non-GI metastases. An exploratory end point was incidence of objective responses according to iRECIST.

After providing written informed consent, recipients underwent pre-trial whole body 18F-fluorodeoxyglucose positron emission tomography combined with computed tomography (PET-CT) imaging, a biopsy from the sigmoid colon and from a non-GI metastasis. After these pre-trial tests, recipients entered a "native microbiota depletion phase" (see Figure 1A in the manuscript's main text) consisting of oral antibiotic treatment with vancomycin 500mg and neomycin 1000mg q6h for 72 hours(29). During the last 24 hours of the depletion phase, recipients underwent bowel preparation with an exclusively clear liquid diet and three liters of oral polyethylene-glycol (PEG) solution.

Up to four hours from the last dose of the PEG solution, recipients underwent a full

colonoscopy with deep intubation of the terminal ileum. During bowel intubation with the colonoscope (Olympus 190-series, Olympus, Japan) residual stool was suctioned to achieve maximal mucosal cleansing. After reaching 20cm into the terminal ileum, a 100mL fecal implant suspension was administered via a catheter (MAG-15 snare tube, Olympus, Japan) in the following sequence: 20mL in the terminal ileum, 40mL in the cecum and right colon, 20mL in the transverse colon and 20mL in the descending colon. After procedure completion, the recipients remained lying on prone Trendelenburg position for at least 4 hours to maintain the FMT suspension in the bowel. The day of the colonoscopy-based FMT was considered as day 0 of the trial protocol.

The following day, recipients underwent an additional FMT, via orally ingested stool capsules (capsulized FMT), to ensure exposure of the entire digestive tract to the donor's microbiota, as the large and small bowel have different metabolic and immune environments(35, 36). After the capsulized FMT, recipients were suggested to follow a diverse Mediterranean diet (see Supplementary Text below). On day 12, recipients ingested an additional dose of capsulized FMT (maintenance FMT, mFMT) which was followed two days later by the first anti-PD-1 drug (Nivolumab, BMS, 3mg/kg, q2w). This combined cycle of mFMT and anti-PD-1 was repeated every 14 days (mFMT on day 26 and drug on day 28 etc.) for a total of six cycles. Each recipient received FMT implants that originated from the same single donor. After completion of six combined cycles, in case the recipient demonstrated an objective response or a clinical benefit, treatment was continued with anti-PD-1 monotherapy.

Post-treatment assessments included repeated: (A) stool sampling at days 7, 31 and 65;(B) sigmoid colon biopsy at day 31;(C) PET-CT on day 65 and (D) tumor biopsy on day 70 – preferably from the same metastasis used for the baseline biopsy, or from another metastasis in

the same organ. iRECIST measurements were performed by an expert radiologist (S.R.) using the MintLesion™ software (Mint Medical, Germany).

Stool sample collection and DNA extraction:

Stool samples were collected using a kit composed of sterile 30mL stool container with a built-in spoon (De-Groot Medical, Israel), a sampling diaper and a cooler bag. Stools were collected once from donors during their donation period, and repeatedly from recipients up to one week prior to the native microbiota depletion phase and on days 7, 31 and 65. The time points of days 31 and 65 were selected since FMT clinical trials usually sample patients one and two months post FMT(31, 37, 38). Day 7 was select as a time point to assess early changes in post-FMT composition(39). Patients were requested to sample their own stool at home, up to 24 hours before their arrival to the clinic. The containers were frozen in the patients' home freezer (-20°C) and transported to the clinic in the cooler bag. The containers were frozen upon receipt in a -80°C temperature monitored freezer.

Purelink Microbiome DNA purification kit (Invitrogen, USA) was used for extracting DNA from stool samples. The kit was used according to the manufacturer's instructions, including a two-minutes use of a bead-beater (BioSpec, USA).

16S rRNA gene sequencing analysis:

The extracted DNA underwent a Polymerase Chain Reaction (PCR) using 515-Forward and 806-Reverse primers for the V4 variable region of the bacterial 16S rRNA gene. PCR reactions were performed using the Primestar taq polymerase (Takara, Japan). The PCR protocol included 30 cycles of denaturation (95°C), annealing (55°C) and extension (72°C), with a final

elongation at 72°C. PCR products were purified by AMPure magnetic beads (Beckman Coulter, USA) and quantified using Pico-green dsDNA quantitation kit (Invitrogen, USA). DNA was pooled at equal concentrations (50ng/μl) from each sample, loaded on 2% E-Gel (Thermo Fisher, USA) and purified again by the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany). Purified DNA was sequenced using the Illumina MiSeq platform at the Genomic Center of the Azrieli Faculty of Medicine, Bar Ilan University, Israel.

Sequenced DNA was analyzed using Qiime2(40) (version 2018.11). Sequences were demultiplexed and features with read number below 0.001% of the total read frequency were removed from the analysis. A sampling depth of 22,445 sequences per sample was used and optimal alpha-rarefaction curves were achieved. Taxonomy was determined using Greengenes classifier(41). A phylogenetic tree was generated and Beta diversity was calculated using Unweighted UniFrac distance matrices(42). Alpha diversity was calculated using Faith's Phylogenetic Diversity test(43). Unweighted UniFrac distances dot-plot was created using Prism (GraphPad, USA), version 8.2.1.

Metagenomic and functional microbiota analysis:

Since the Genomic Center of the Azrieli Faculty of Medicine, do not perform metagenomic shotgun sequencing, this sequencing was carried out at CosmosID Inc. (Rockville, MD, USA, <https://www.cosmosid.com/>), using the same DNA employed for the 16S rRNA gene profiling (see above). AT CosmosID, extracted DNA samples were quantified using Qubit 4 fluorometer and Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, USA). DNA libraries were prepared using the Nextera XT DNA Library Preparation Kit and Nextera Index Kit (Illumina, USA) following the manufacturer's protocol with slight modifications. The

standard protocol was used for total DNA input of 1ng. Genomic DNA was fragmented using a proportional amount of Illumina Nextera XT fragmentation enzyme. Combinatory dual indexes were added to each sample followed by 12 cycles of PCR to construct libraries. DNA libraries were purified using AMPure magnetic Beads (Beckman Coulter, USA) and eluted in QIAGEN EB buffer (Qiagen, The Netherlands). DNA libraries were quantified using Qubit 4 fluorometer and Qubit™ dsDNA HS Assay Kit and were pooled together for sequencing on the Illumina HiSeqX. Raw reads from metagenomics samples were analyzed by CosmosID metagenomic software (CosmosID Inc., USA) to reveal strain level identification of microbes in the specimen as described elsewhere(44-46). Briefly, the system utilizes a high-performance data-mining k-mer algorithm and highly curated dynamic comparator databases that rapidly disambiguate millions of short reads into the discrete genomes or genes engendering the particular sequences. This query of reads enables the sensitive yet highly precise detection and taxonomic classification of microbial reads. The resulting statistics are analyzed to return the fine-grain taxonomic and relative abundance estimates for the microbial datasets. MetaPhlAn2 was used to classify taxonomic reads as described previously(47). ANalysis of Composition of Microbiomes (ANCOM) test(48) was used for the differential taxonomical analysis with abundance datasets of all taxonomic levels between groups using the FDR cutoff of 0.05(49). The ANCOM test provides W statistic score for each taxon and logical indicators demonstrating whether a taxon is differentially abundant under 0.9, 0.8, 0.7 and 0.6 cutoffs. We followed default cutoff setting (0.7) to detect the taxa that satisfy $FDR \leq 0.05$. The ANCOM test also provides CLR (the conditional likelihood ratio test) F statistic score that essentially accounts the effect size difference of each taxon between two groups. Higher F and W scores indicate higher chances for the taxa to be truly different across groups. Volcano plots were generated with W statistic on the

y-axis, and the F score (CLR mean difference) on the x-axis using an R package ggplot2(50). Heatmaps were created using "pheatmap" for R (RStudio version 1.1.463, based on R version 3.5.1).

For functional analysis, Initial QC, adapter trimming and preprocessing of metagenomic sequencing reads were performed using BBduk. The quality-controlled reads were then subjected to a translated search using Diamond against a comprehensive and non-redundant protein sequence database, UniRef 90. The UniRef90 database, provided by UniProt, represents a clustering of all non-redundant protein sequences in UniProt, such that each sequence in a cluster aligns with 90% identity and 80% coverage of the longest sequence in the cluster. The mapping of metagenomic reads to gene sequences are weighted by mapping quality, coverage and gene sequence length to estimate community wide weighted gene family abundances(51). Gene families were then annotated to MetaCyc reactions (Metabolic Enzymes) to reconstruct and quantify MetaCyc metabolic pathways in the community(51). Furthermore, the UniRef_90 gene families are also regrouped to GO terms in order to get an overview of GO functions in the community. To facilitate comparisons across multiple samples with different sequencing depths, the abundance values are normalized using Total-sum scaling (TSS) normalization to produce "Copies per million" (CPM, analogous to TPMs in RNA-Seq) units. The CPM normalized values were used as an input table for ALDEx2(52) package (version 3.10, using RStudio as described above). Random instances of centered log-ratio transformed values were generated (using 1,000 Monte-Carlo instances drawn from a Dirichlet distribution). The instances were assessed using the Wilcoxon. Statistically significant differed pathways were defined as those with $FDR \leq 0.05$. Abundance differences were calculated as $\log_2(\text{average}$

abundance of group1) - $\log_2$ (average abundance of group2). Pathways with $\log_2$ abundance difference <1 were filtered out.

Voronoi treemaps were created using www.proteomaps.net (53). Custom treemap file (.tmp) was created based on the statistically significant GO pathways. Data files were generated for each donor-group. The $\log_2$ abundance difference was set as the polygon size parameter.

Historical observational pre-anti-PD-1 cohort:

The Supplementary Figures included 16S rRNA gene sequencing data of an historical cohort of metastatic melanoma patients who provided stool samples prior to initiating anti-PD-1 therapy at the Sheba Medical Center. Patients in this cohort did not undergo FMT, as this cohort was purely observational and was not related to a clinical trial.

The historical observational cohort was composed of 49 immunotherapy-naïve patients: 29 patients underwent anti-PD-1 monotherapy while 20 underwent a combination of anti-PD-1 and anti-CTLA-4 therapy; and of three patients who had failed previous anti-PD-1 mono- or combinational therapy and were designated for anti-PD-1 re-induction. Stool samples were collected up to one week prior to initiation of the anti-PD-1 therapy, or the re-induction of therapy in case of the refractory patients. Clinical response to treatment was determined after six months from treatment initiation, based on patient medical records. In the anti-PD-1-naïve group: among anti-PD-1 monotherapy patients, 16 patients responded, and 13 patients did not respond; among combined anti-PD-1 + anti-CTLA-4, 12 patients responded and 8 did not. All three patients who failed previous anti-PD-1 therapy did not respond to re-induction; two of them failed monotherapy and one failed combinational therapy.

Sample collection and analysis methods of the historical observational cohort was not identical to those of the FMT clinical trial methods. These are the key differences: (1) the stool home collection kit was composed of sterile nylon swabs in a dry tube (FLOQSwabs, Copan, Italy), in contrast to 30mL stool container tube used in the FMT study, a sampling diaper and a cooler bag. The kit could be frozen in the patients' home freezer (-20°C) for 48 hours until it was delivered by the patients to the clinic. (2) DNA was extracted from the swabs using DNeasy PowerSoil HTP 96 Kit (Qiagen, The Netherlands), in contrast to the PureLink kit (Invitrogen, USA) used for the FMT trial. In addition, DNA extraction of the historical-observational cohort was conducted via the Tecan's Freedom EVOware handler robot (Tecan, Switzerland). (3) Sequences were demultiplexed and features with read number below 0.001% of the total read frequency were removed from the analysis, similarly to the 16S rRNA gene sequencing analysis of the FMT trial's samples. However, in the historical-observational cohort, this filtration lead to a sampling depth of 4,835 reads only, in contrast to 22,445 in the FMT trial's cohort. The rest of the historical observational cohort 16S rRNA gene sequencing analysis methodology was similar to that of the FMT cohort.

Immunohistochemistry analysis of gut and tumor biopsies

Tissues that were acquired by gut and tumor biopsies were fixed in formalin for up to 24 hours and embedded into paraffin blocks using the Tissue-Tek VIP A61 (Sakura, Japan). Blocks were sectioned at 4µm and a positive control was added on the right edge of the slides. The slides were warmed up to 60°C for 1hour and were processed by a fully automated protocol using a Benchmark XT module (Ventana Medical Systems Inc., USA). After sections were dewaxed and rehydrated, a CC1 Standard Benchmark XT pretreatment for antigen retrieval (Ventana Medical Systems Inc., USA) was selected for stains against CD4 (1:50, Spring Bioscience, E1294, USA)

and CD8 (1:50, Biocare medical, CRM311C, USA). CD68 (1:50, DAKO, M0876, Denmark) underwent a Protease 2 (Ventana Medical Systems Inc., USA) pretreatment for 8 minutes. Detection was performed with iView DAB Detection Kit (Ventana Medical Systems Inc., USA) and counterstaining with Hematoxylin (Ventana Medical Systems Inc., USA).

Benchmark Ultra staining module was used for IHC stains against PD-L1(54). Briefly, after sections were dewaxed and rehydrated, a CC1 Standard Benchmark Ultra pretreatment for antigen retrieval (Ventana Medical Systems Inc., USA) was selected for PD-L1 IHC 22C3 pharmDX (DAKO, SK006). 130 µl of prediluted antibody were titrated on sections and incubated for 2 hours at 37°C. PD-L1 was detected with UltraView DAB Detection Kit (Ventana Medical Systems Inc., USA) and the signal was amplified with Amplification Kit (Ventana Medical Systems Inc., USA). Sections were counterstained with Hematoxylin (Ventana Medical Systems Inc., USA).

After the action of the automated stainer was completed, all slides were dehydrated in graded ethanols (70%, 96%, and 100%). Before cover-slipping, sections were cleared in Xylene and mount with Entellan.

Stained slides were scanned using Aperio VERSA Digital Pathology Scanner (Leica Biosystems, Germany). For each CD4, CD8, and CD68 slides, five fixed size (500X500µm) regions of interests (ROI) were marked by expert pathologists (D.D.N and D.R., supervised by I.B.) who were blinded to both clinical (responder/non-responder) and chronological (pre/post-treatment) annotations. For gut biopsies, only areas of the lamina propria and epithelium were marked. For tumor biopsies, only viable tumor areas in the tumor center were marked. Peri-lymphatic or peri-vascular areas, as well as tumor margin were not marked as ROIs. For CD8

and CD68 slides, stained cells within the ROIs were automatically counted using adjusted versions of the default algorithm ImageScope "Nuclear V9" (Leica Biosystems, Germany). The algorithm was adjusted, and its accuracy tested by expert pathologists (D.R. and D.D.N.) (55). The algorithms produced the number of stained cells per area (1mm^2). For CD4 slides, due to a background stain which impaired the algorithm ability to correctly count cells, cells were counted manually within the ROI by D.D.N, who were blinded to the slides clinical and chronological annotations. Pre and post-treatment counts were compared using two-tailed Paired T-Test (STATA version 15.0, $p \leq 0.05$).

PD-L1 stains were also assessed in blinded fashion. Stain results were not provided in the form of stained cells per mm^2 . Instead, the entire slide was assessed by D.D.N. together with a PD-L1 specialist (Dr. Efrat Ofek, Pathological Institute, Sheba Medical Center) according to the acceptable expression categories of $<1\%$, $\geq 1\%$, $\geq 5\%$, $\geq 10\%$, $\geq 25\%$, $\geq 50\%$ (56).

For calculation of viable tumor ratio, H&E slides of each tumor biopsy were examined by D.D.N. in the same blinded fashion. D.D.N. assessed the percentage of viable tumor tissue out of the entire tissue in the examined H&E slide.

RNA sequencing analysis

RNA sequencing analysis was based on FFPE material from the recipient biopsies. Prior to each biopsy block sectioning, the microtome was meticulously cleaned using a 70% ethanol solution followed by RNAase AWAY™ spray (Thermo Fischer, USA), and the water in the water plate were replaced. All gut and tumor biopsy blocks were sectioned 10 times, each section was $6\mu\text{m}$ thick. An expert pathologist (I.B.) reviewed the H&E slides of each of the tumor

biopsies to assess for the presence of healthy tissue. Healthy tissues were present in part of the pre-treatment sample of Recipient#3 and the post-treatment sample of Recipient#1. For those two patients, the tumor area was marked on their respective H&E slides. Following the section process, the marked tumor area was grossly dissected using a scalpel (blade No. 15) into sterile 1.5mL vials. For other tumor and gut blocks, the entire FFPE materials were sectioned directly into sterile 1.5mL vials. A separate vial was used for each of the samples. The vials were sealed with parafilm and were kept in -80⁰C until RNA extraction and sequencing. Sectioned FFPE samples could spend up to 6 months in deep freeze.

For RNA extraction and sequencing, FFPE samples were shipped in -80⁰C packages to Otogenetics Corporation (Georgia, USA). Extraction of RNA from FFPE was conducted using RNeasy FFPE kit (Qiagen, The Netherlands). The integrity and purity of total RNA were assessed using Agilent Bioanalyzer. cDNA library was generated using Illumina truseq stranded cDNA library preparation kit (Illumina, USA, Cat# 20020594) using 500ng of total RNA. The quality, quantity, and size distribution of the Illumina libraries were determined using an Agilent Tapestation 2200. The libraries were then sequenced using an Illumina NovaSeq 6000 sequencer according to the standard protocols. Paired-end 150 nucleotide reads were generated and checked for data quality using FastQC version 0.11.2 (Babraham Institute, UK). The reads were trimmed using TrimGalore version 0.5.0 (Babraham Institute, UK) to remove the adapters and low-quality reads, as well as poly A tail and low-quality base pairs at the 3' ends of the reads. Reads of length shorter than 20 bp were removed from further analysis. Alignment of the trimmed fastq files to the human genome Hg38 (version 38.98) was performed via STAR version 2.6.0a (Cold Spring Harbor Laboratory, USA) using the following parameters: STAR --outFilterType BySJout --outFilterMultimapNmax 20 --alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --

outFilterMismatchNmax 999 --outFilterMismatchNoverLmax 0.6 --alignIntronMin 20 --alignIntronMax 1000000 --alignMatesGapMax 1000000 --outSAMattributes NH HI NM MD --outSAMtype BAM SortedByCoordinate. Quantification of the gene expression was performed against Ensembl GTF (version 38.98) using STAR gene quantification mode.

After read count tables were obtained for each sample, analysis was conducted using R studio version 1.1.463 (R version 3.5.1) according to standard Limma and edgeR packages workflow(57). Briefly, Ensembl IDs were matched with ENTREZ IDs, gene symbol and gene names using the Homo.sapiens package. Reads with Counts Per Million (CPM) values of 2 or less and reads that appeared in only one sample were filtered out. Normalization was performed by the Trimmed Mean of M-values (TMM) method. Gene set testing was conducted using the Camera method. Gene sets database was the MSigDB C5 Gene Ontology (GO), which was downloaded from the Broad Institute website (<http://software.broadinstitute.org/gsea/index.jsp>) during November 2019. Threshold for statistically significant differed genes and gene sets was $FDR \leq 0.05$.

Heatmaps were produced using the pheatmap package. Representative genes for the tumor inflammatory heatmap were selected based on previous publications which assessed tumor inflammation, T cell effector and T cell exhaustion signatures(58-60). Representative genes for the immune exclusion heatmap were selected based on a landmark article reviewing anti-PD-1 resistance mechanisms(61). The used values for both Heatmap were reads after filtering and TMM normalization. Colors and scales were according to a calculated Z-score.

Supplementary Text

Recommended post-FMT diet

Recommended foods for daily ingestion (fresh, cooked or baked):

- Jerusalem artichokes, onion, garlic, banana, asparagus, leeks.

Recommended foods for general ingestion:

- *Vegetables of all colors, preferably fresh but can be cooked or baked:*
 - Cucumber, tomato, red pepper, pepper orange, yellow pepper, green pepper, carrots, lettuce, beet, radish, herbs, squash, pumpkin, spinach, broccoli, cabbage, cauliflower, asparagus and kohlrabi.
- *Beans:*
 - Green and yellow beans, peas, lentils, fava bean, chickpeas, mung bean
- *Essential fatty acids:*
 - Olive oil, Tahini (sesame paste), avocado, fish, walnuts, flax, Chia seeds
- *Dairy products:*
 - Hard cheese, cottage cheese, Labneh (strained yogurt), natural yogurt
- *Fruit:*
 - Banana, seasonal fruits
- *Proteins:*
 - Hard boiled eggs, omelet, fish 2-3 times a week, unprocessed chicken and beef.
- *Bread:*

- Whole-wheat bread

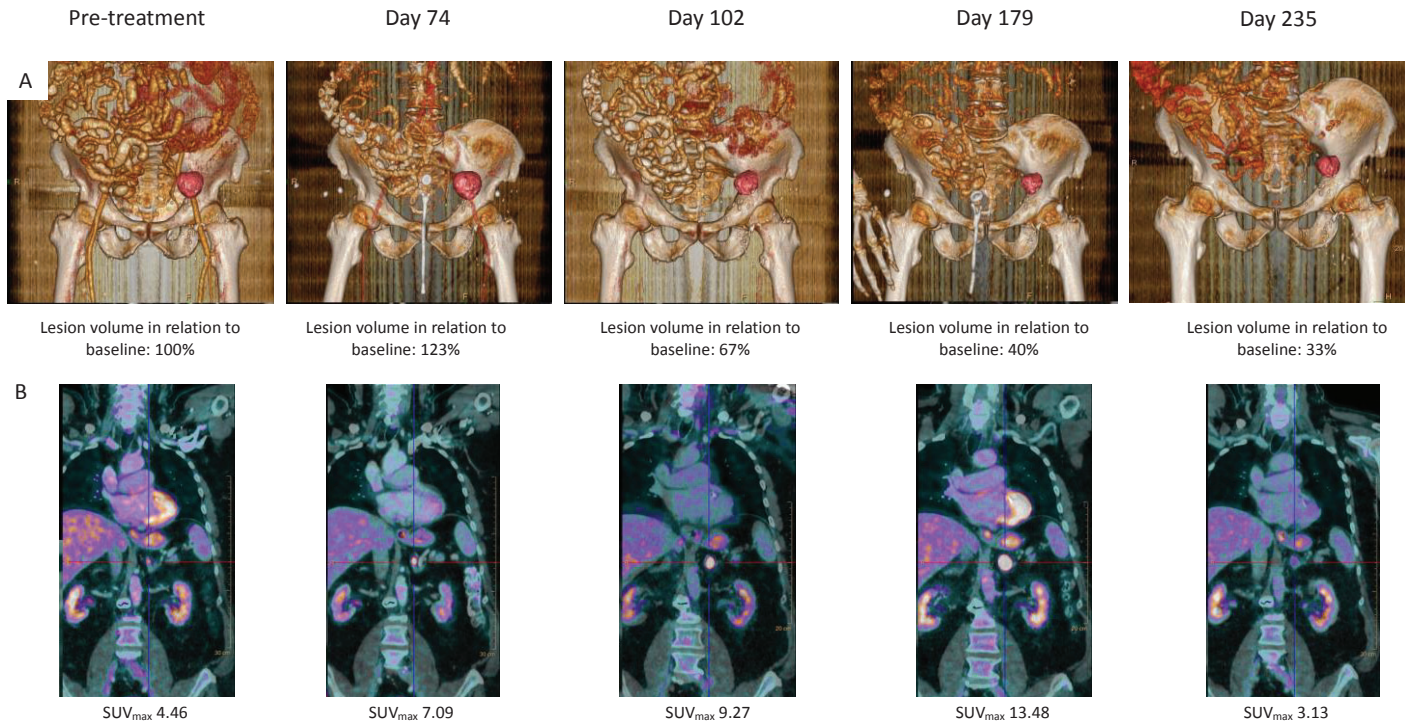


Fig. S1. Evidence for pseudo-progression in Recipient#5's imaging studies. (A) Three-dimensional reconstruction of Recipient#5 metastatic lesion in a left iliac lymph node. This reconstruction demonstrated an initial increase in the node size, from a baseline volume of 35cm³ to 43cm³ on day 74. Subsequent imaging studies demonstrated a total decrease of 67% in the volume of this lymph node in comparison to baseline at day 235. The same lymph node was sampled as part of the trial's protocol, and the immune analyses demonstrated a near pathological complete response with increased T cell activity in the post-treatment biopsy (Fig. 3H, main text) – hence confirming the diagnosis of pseudo-progression. Reconstructions and volume calculation were conducted using the Philips Multi-modality Tumor Tracking® application (Philips Healthcare, The Netherlands). (B) Coronal sections of a combined Positron Emission Tomography and Computed Tomography (PET-CT) image of Recipient#5's adrenal lesion. The

combined image demonstrated an increase in maximal Standardized Uptake Volume (SUV_{max}) from baseline until day 179, with a significant decrease on day 235. The change in SUV_{max} was accompanied by a change in volumes, as baseline volume was 0.6cm^3 , day 179 volume was 7cm^3 while day 234 volume was only 2.2cm^3 . The radiological dynamics of these two lesions were compatible with pseudo-progression(8). All radiological calculations and measurements were performed by an expert radiologist (S.R.).

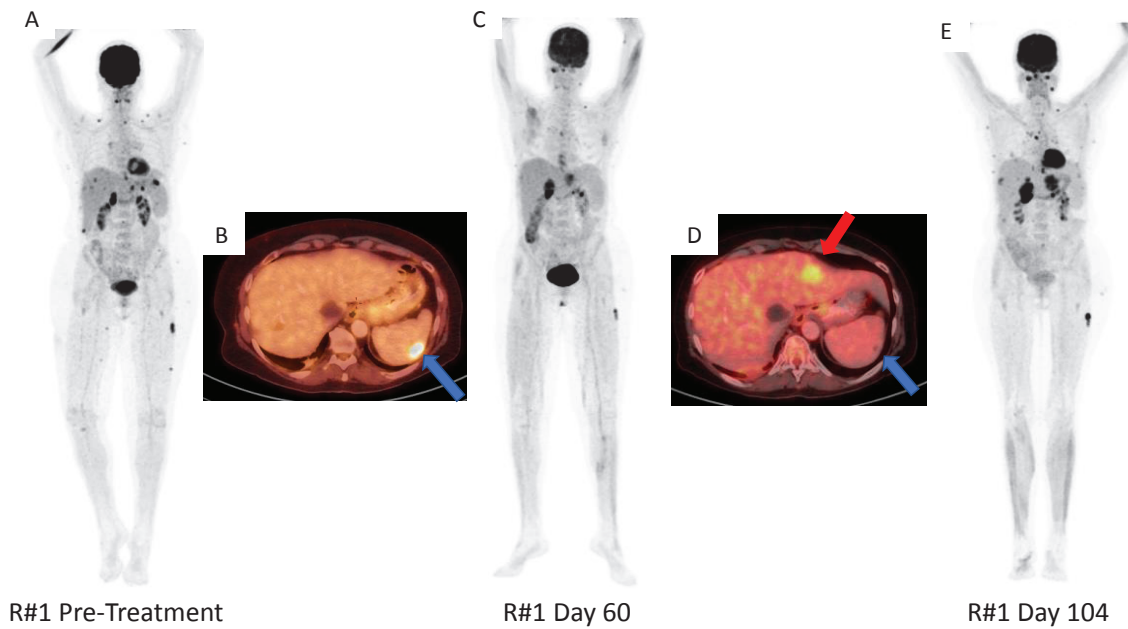


Fig. S2. Evidence for Recipient#1 mix radiological response. (A) Pre-treatment positron emission tomography combined with computed tomography (PET-CT) of Recipient#1 demonstrated multiple metabolically active metastatic lesions (represented as black emission areas). (B) Pre-treatment PET-CT slide demonstrating metabolically active spleen metastasis (blue arrow). (C-D) Repeated PET-CT at day 60 demonstrating a regression of multiple metastases, including the spleen metastasis, with progression of other lesions and appearance of new liver metastasis (red arrow). (E) Repeated PET-CT at day 104 demonstrated a confirmed overall disease progression.

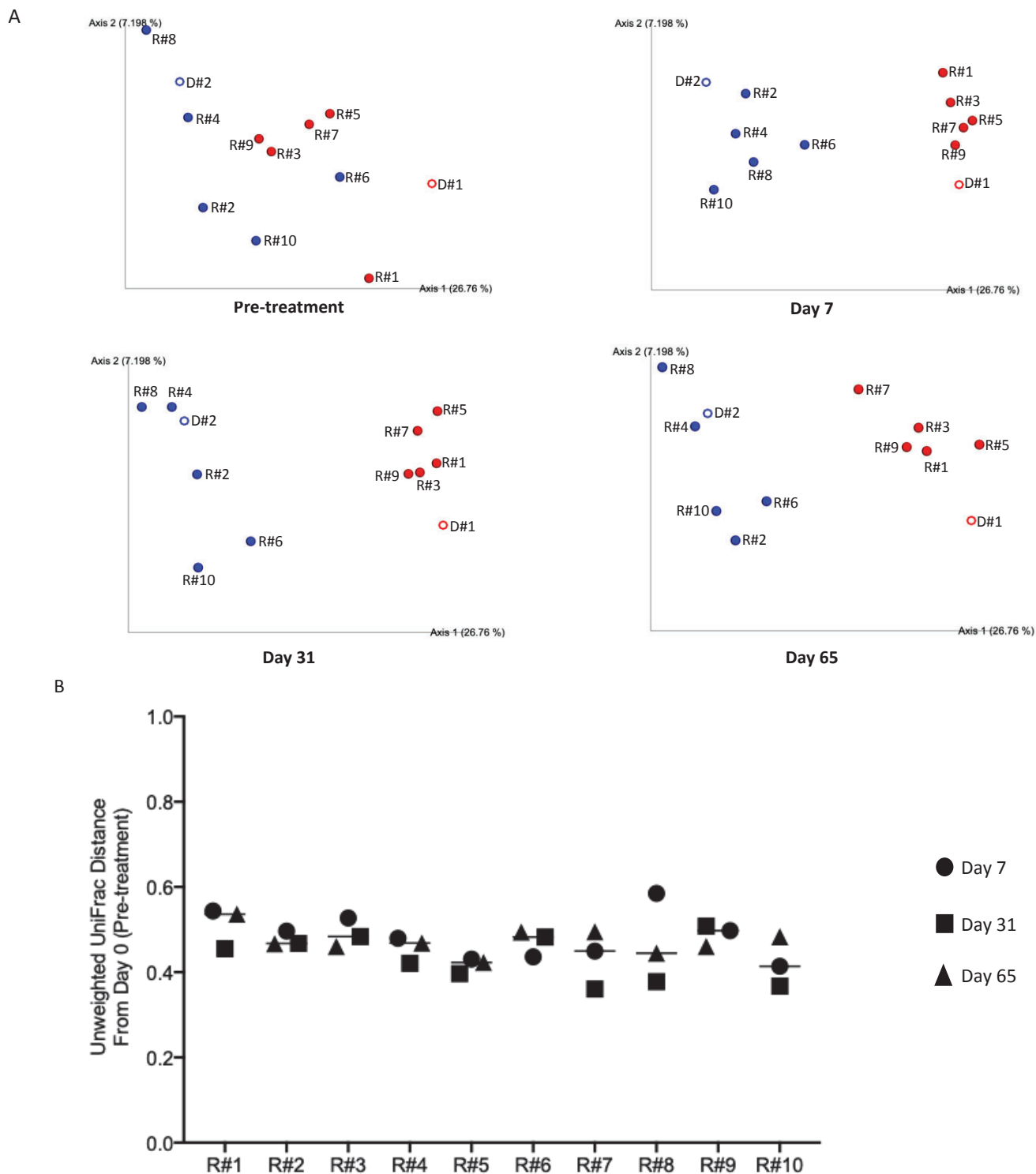


Fig. S3. Dynamics of recipient gut microbiota compositions over time. (A) These Principal Component Analysis (PCA) plots of gut microbiota compositions were based on stool 16S rRNA gene sequencing dissimilarity analysis (beta-diversity, Unweighted UniFrac distances). The

distance between samples on the plot represents their dissimilarity – the greater the distance between two samples, the higher the dissimilarity of their composition. Donors (D#) were represented in the plot as rings, while Recipients (R#) were represented in plots as spheres. Recipient were grouped according to their donors – those who received fecal implants from Donor#1 were colored in red while those who received fecal implants from Donor#2 were colored in blue. The dynamics in microbiota composition was demonstrated by the different distances from the donors in pre-treatment and each post-treatment days: 7, 31, and 65. (B) A dot-plot presenting the dissimilarity of post-treatment samples per recipient, based on 16S rRNA gene sequencing Unweighted UniFrac distances. The distance from the pre-treatment sample was calculated for all post-treatment samples (days 7, 31, and 65) per recipient. The Unweighted UniFrac distance scale (Y-axis) ranges from 0 to 1, as 0 represents identical microbiota compositions and 1 represents completely dissimilar compositions. The horizontal line represents the average post-treatment distance. This plot demonstrated that the dissimilarity from pre-treatment remained similar for most recipients during the post-treatment period, demonstrating the stability of microbiota modulation by the treatment. Specific taxa which differed between pre- and post-treatment groups were presented in Fig. S6 (below).

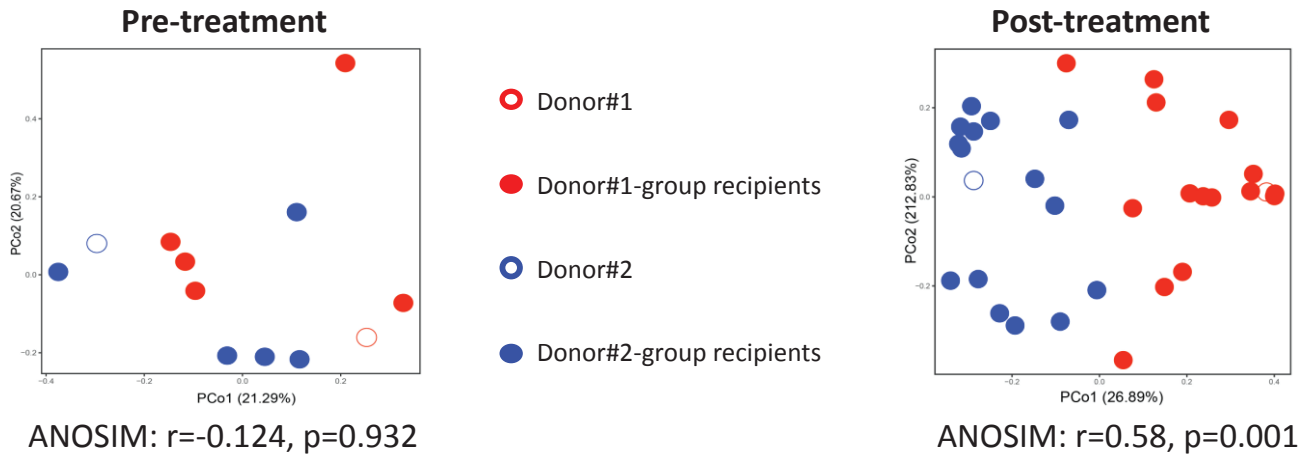


Figure S4. Microbiota dissimilarity assessment based on meta-genomic sequencing. These Principal Component Analysis (PCA) plots of gut microbiota compositions were based on stool metagenomic sequencing. Using Bray-Curtis matrixes, the dissimilarity of microbiota compositions between the two donor groups were assessed in the pre- and post-treatment phases. In accordance with the 16S rRNA gene sequencing results (Fig. 2A, main text), there was no statistically significant difference between the microbiotas of the donor-groups in the pre-treatment phase. ($p=0.932$). However, post-treatment, microbiota compositions of Donor#1-group differed from those of Donor#2-group ($p=0.001$). The statistical analysis was based on the ANOSIM test, and the p value was calculate using 999 permutations.

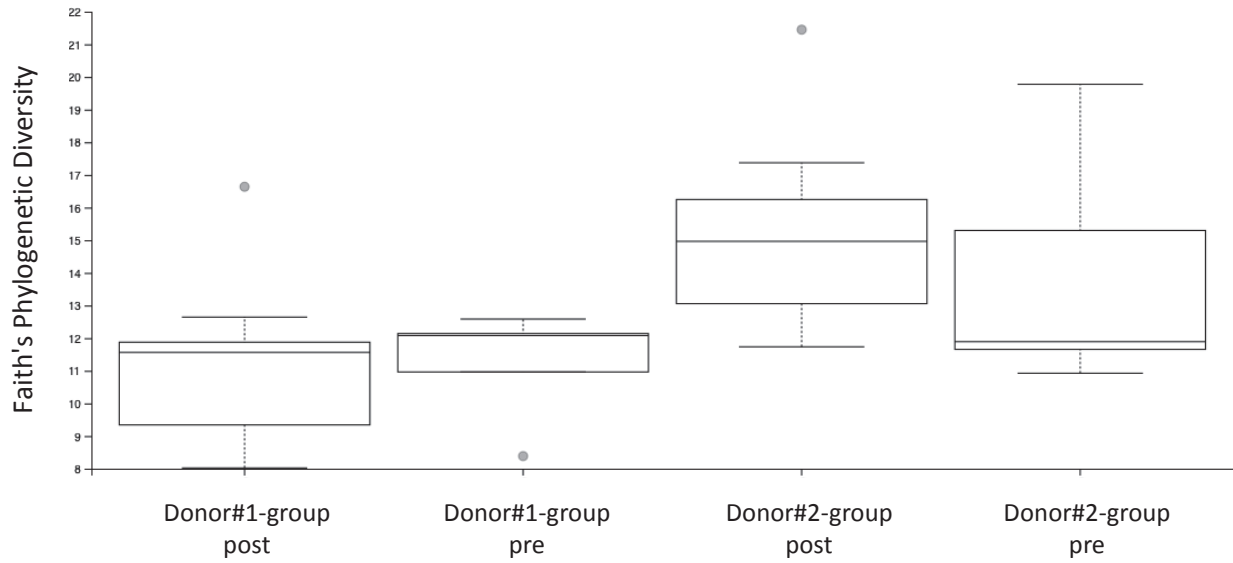


Figure S5. Comparison of microbiota richness (alpha diversity) per donor and donor-group. Microbiota richness (an alpha diversity test) measures the number of different taxa per microbiota composition; A higher number of different taxa yields higher richness. Microbiota richness assessment was conducted via the Faith's Phylogenetic Diversity (PD) test. Comparison of Faith's PD score between pre-treatment samples of Donor#1-group and Donor#2-group demonstrated no statistically significant difference ($p=0.60$, $FDR=0.60$). However, a comparison of the post-treatment richness demonstrated a superior richness of Donor#2-group ($p<0.001$, $FDR<0.001$).

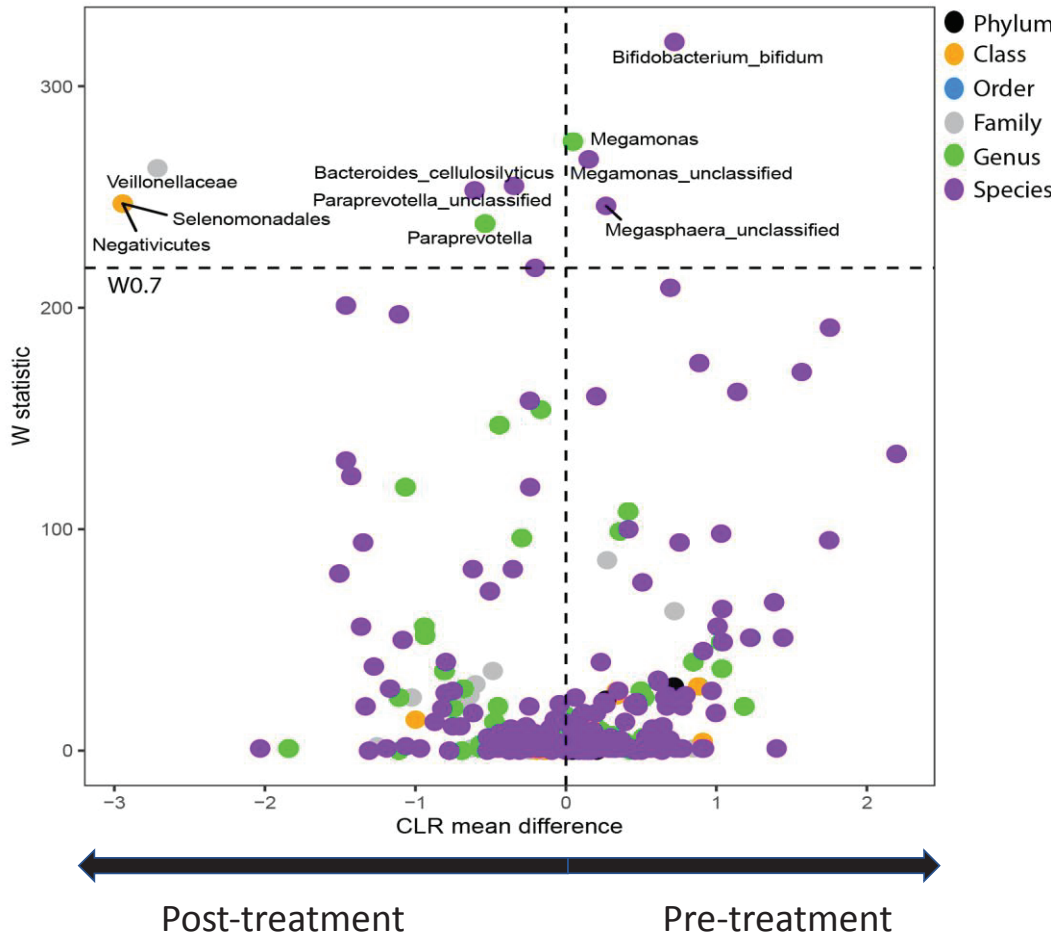


Figure S6. Taxonomical differences between pre- and post-treatment gut microbiota compositions. This volcano plot was based on metagenomic sequencing, using the ANalysis of Composition of Microbiomes (ANCOM) test. The plot compared between pre-treatment recipient stool samples (pre-treatment group) to post-treatment recipient and the donor stool samples (post-treatment group). Taxa which differed between the groups with FDR $q \leq 0.05$ were presented above the horizontal dash line (corresponding with the W statistic Y axis). The center log transformation (CLR) mean difference on the X-axis is an ANCOM calculation which is used to determine compositional differences in microbial communities.

Historical observational cohort – Genus level taxa bar-plot (16S)



B

FMT interventional cohort – Genus level taxa bar-plot (16S)



C

Historical observational cohort – Dissimilarity assessment (Beta-diversity, Unweighted UniFrac)

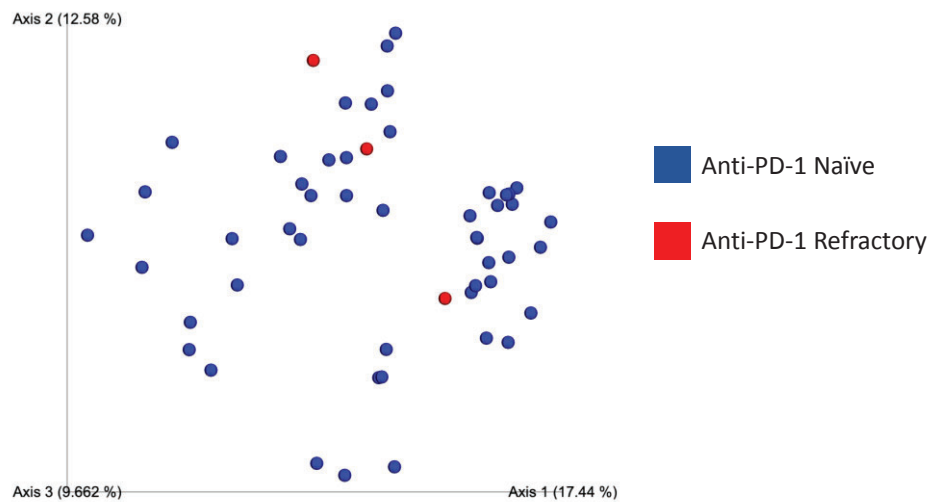
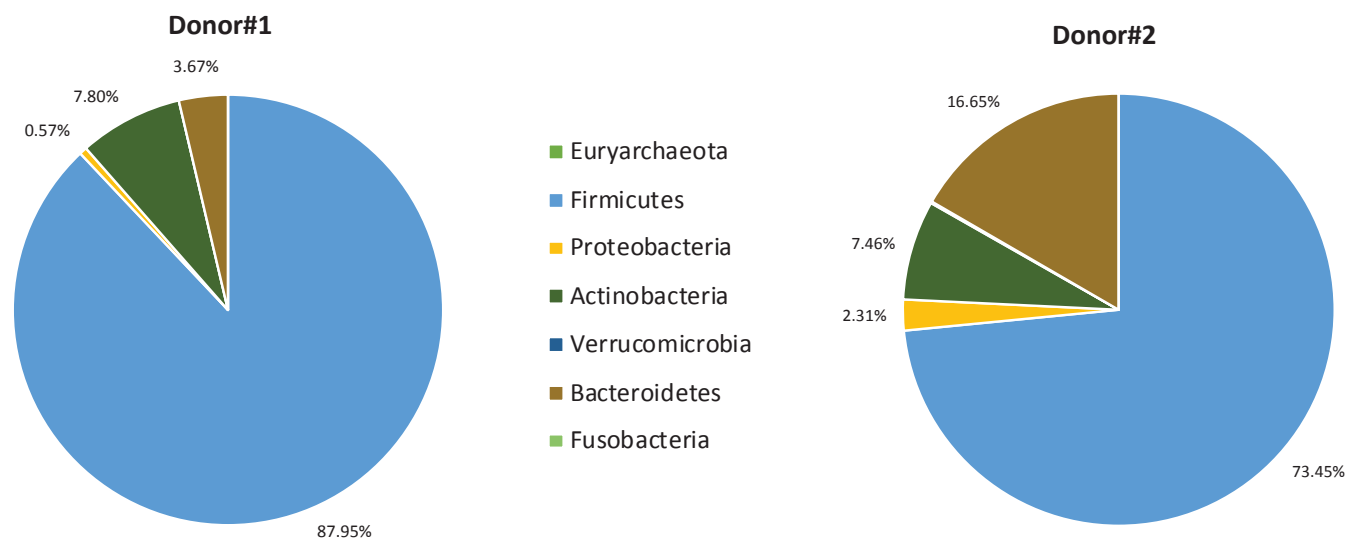


Fig. S7. Comparison of pre-treatment gut microbiota compositions between FMT recipients and an historical cohort of metastatic melanoma patient who underwent anti-PD-1 therapy without FMT. FMT recipients in the current clinical trial were anti-PD-1 refractory metastatic melanoma patients. As part of the clinical trial, they underwent a combination of Fecal Microbiota Transplantation (FMT) and re-induction of anti-PD-1 therapy. As a comparative resource, Figure S7 included data on pre-treatment gut microbiota compositions (based on stool samples) of an historical cohort of metastatic melanoma patients who underwent anti-PD-1 therapy without FMT. (A) 16S rRNA gene sequencing analysis taxa bar-plot of 52 metastatic melanoma non-FMT (historical observational) patients: 49 were anti-PD-1 naïve patients and three were anti-PD-1 refractory. The taxa bar-plot demonstrate the gut microbiota composition at the genus taxonomy level. Sample annotation was based on response to anti-PD-1 therapy - In the Anti-PD-1-naïve group 28 patients responded and 21 patients did not. All three Anti-PD-1-refractory patients did not respond to the anti-PD-1 re-induction. Since

this was an historical cohort, the method of stool collection and DNA extraction were different from that of the FMT interventional cohort (see Supplementary Methods above). Hence, the microbiota compositions of the two cohort could not be directly statistically compared. (B) To enable a non-direct comparison between the cohorts, a taxa bar-plot of the Genus taxonomical level was created for the pre-treatment samples FMT-interventional cohort, based on 16S rRNA gene sequencing analysis. For ease of use, both of the Genus levels taxa bar-plots (panels A and B) contain legend for only the 32 most common taxa on that level. (C) To assess for a potential difference in pre-treatment microbiota compositions between anti-PD-1 naïve versus refractory patients, a Principal Component Analysis (PCA) plot was created based on a dissimilarity test (beta-diversity, Unweighted UniFrac distances) of the historical cohort data. With the caveat of a small sample size, the Unweighted UniFrac test demonstrated no difference between the pre-anti-PD-1 microbiota compositions of Anti-PD-1-naïve and Anti-PD-1-refractory patients (FDR=0.337).

A

Donor gut microbiota composition - Phylum level



B

Donor gut microbiota composition – Family level

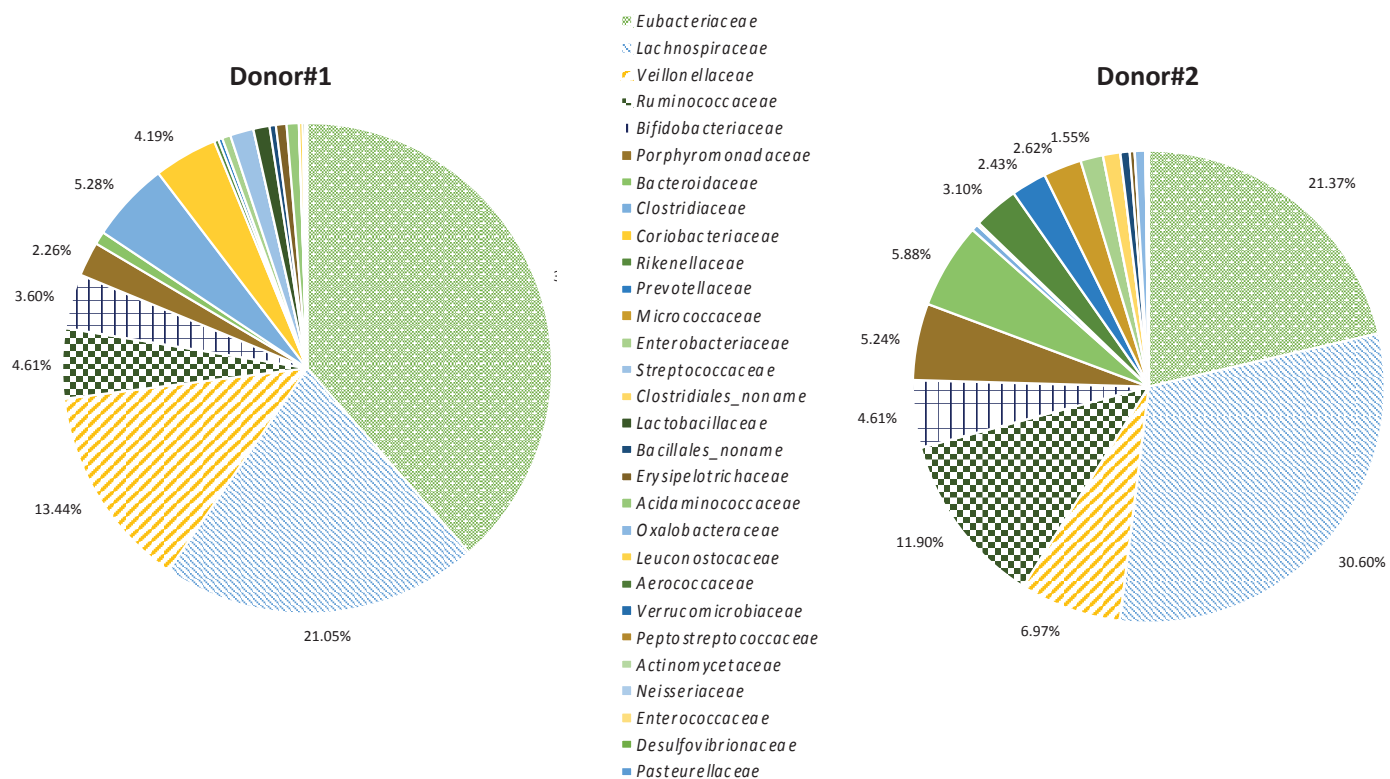
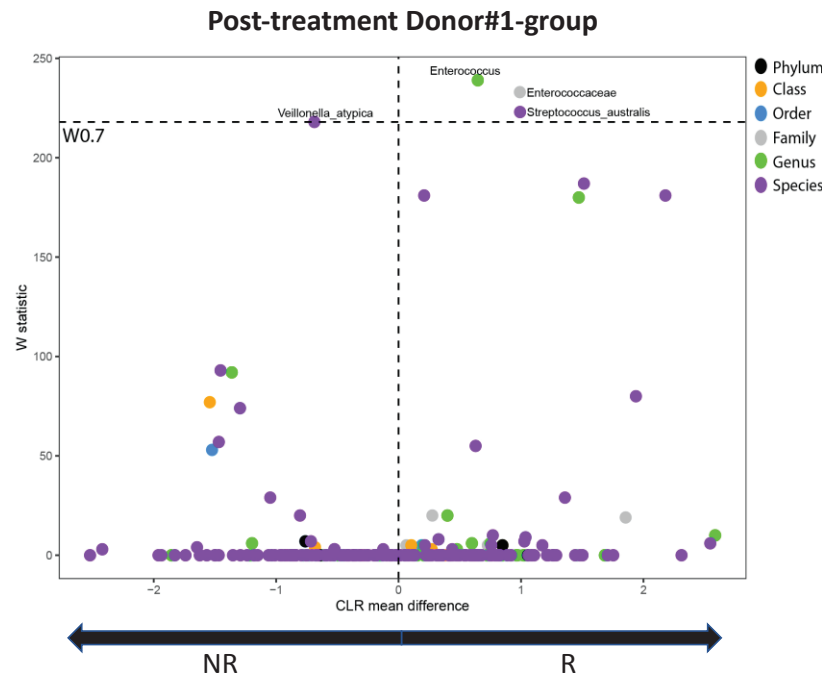


Fig. S8. Donors' gut microbiota composition. Gut microbiota compositions at the (A) Phylum and (B) Family taxonomy levels of the two donors, based on metagenomic sequencing. The pie charts demonstrate the difference between the two donors. For example, Donor#1 had higher relative abundance of *Veillonellaceae* while Donor#2 had higher relative abundance of *Ruminococcaceae*. The colors were ranked by abundance – the most abundant taxa appear first; The abundance ranking is the same for both donors. In panel (B), for reader convenience, the five most abundant taxa were represented by both different colors and pattern. For examples, *Eubacteriaceae* (dotted light green) was the most abundant family, follow by *Lachnospiraceae* (light blue thin lines) and *Veillonellaceae* (orange thick lines). Microbiota compositions at the Genus and Species taxonomy levels were detailed in Table S6.

A



B

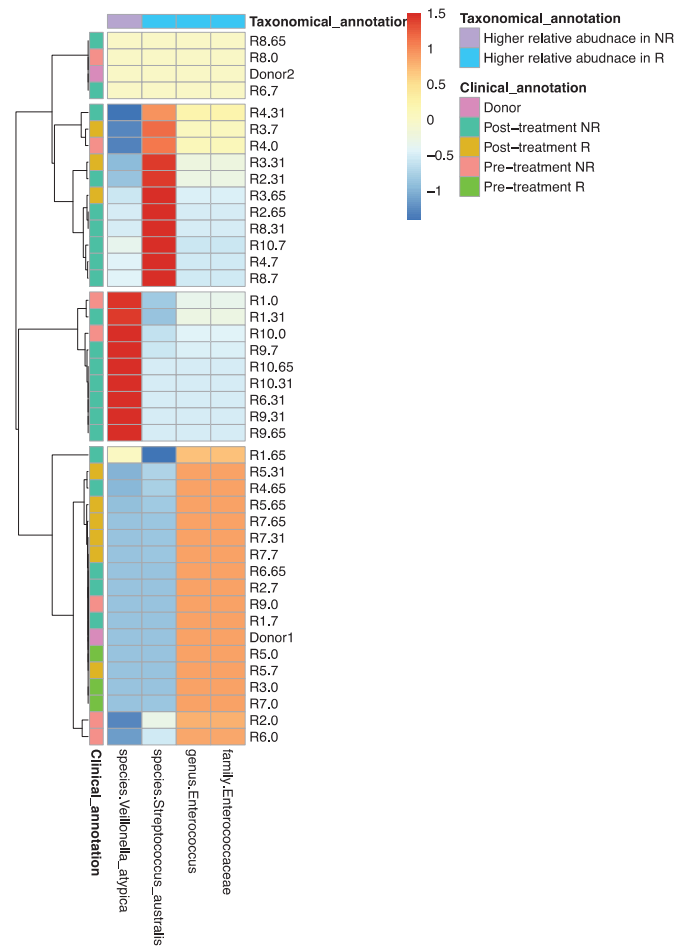
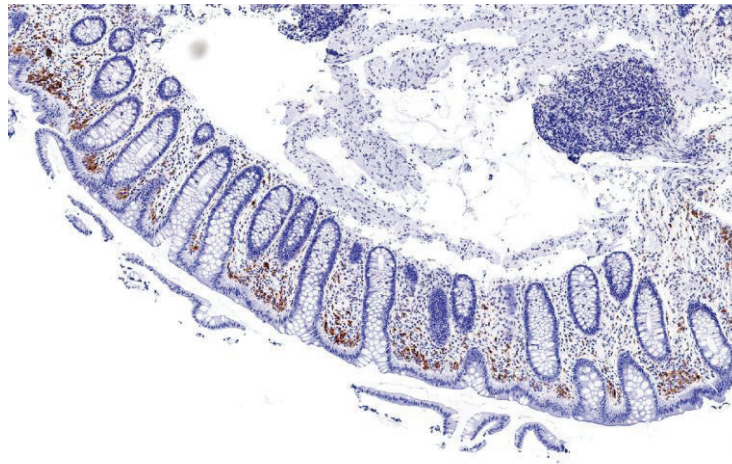


Fig. S9. Taxonomical characterization of the trial's responders. Recipients #3,5,7 achieved objective responses to treatment. All of them were part of Donor#1-group. There was no statistically significant difference between the pre-treatment gut microbiota compositions of those three responders and the pre-treatment compositions of the rest of the cohort. (A) A volcano plot based on metagenomic sequencing, comparing the post-treatment gut microbiota compositions of the three responders to the post-treatment compositions of the other two recipients from that donor group who failed to respond (Recipients #1,9). This comparison was based on the the ANalysis of Composition of Microbiomes (ANCOM) test. Four taxa significantly differed (FDR $q \leq 0.05$) between the responders (R) to the non-responders (NR). R had higher relative abundance of *Enterococcaceae*, *Enterococcus*, and *Streptococcus australis*. NR had high relative abundance of *Veillonella atypica*. (B) Heatmap comparing the relative abundance of those four taxa across all of the trial's microbiota samples; Scale represents the Z-score of post-MetaPhlan2 abundance table. As there were some non-responders and even pre-treatment samples with similar dynamics to those described in panel A, a clear association with clinical response to treatment could not be established to any of the taxa.

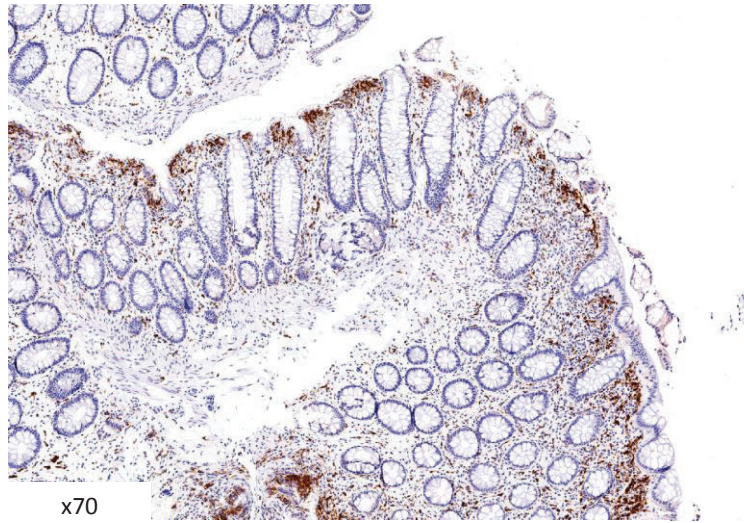
A



x70

R#10 Gut CD68+ Infiltration Pre-treatment

B



x70

R#10 Gut CD68+ Infiltration post-treatment

Fig. S10. Treatment effect on antigen presenting cells (APC, CD68⁺ cells) infiltration in the gut lamina propria of Recipient#10 (Donor#2-group). (A) Immunohistochemical (IHC) stain of CD68, representing antigen presenting cells (APC), conducted on pre-treatment sigmoid colon biopsy of Recipient#10. (B) IHC stain of CD68⁺ cells conducted on post-treatment (day 31) sigmoid colon biopsy of Recipient#10, demonstrating an increase in CD68⁺ cells in the gut

lamina propria, especially in the sub-epithelial area, which is physically closer to the presence of the new microbiota. A similar increment in the lamina propria infiltration of CD68⁺ cells was demonstrated in Recipient#7 and was described in Fig. 3A-B (main text). A per recipient analysis of CD68⁺ cells infiltration in the gut lamina propria was described in Fig. 3C.

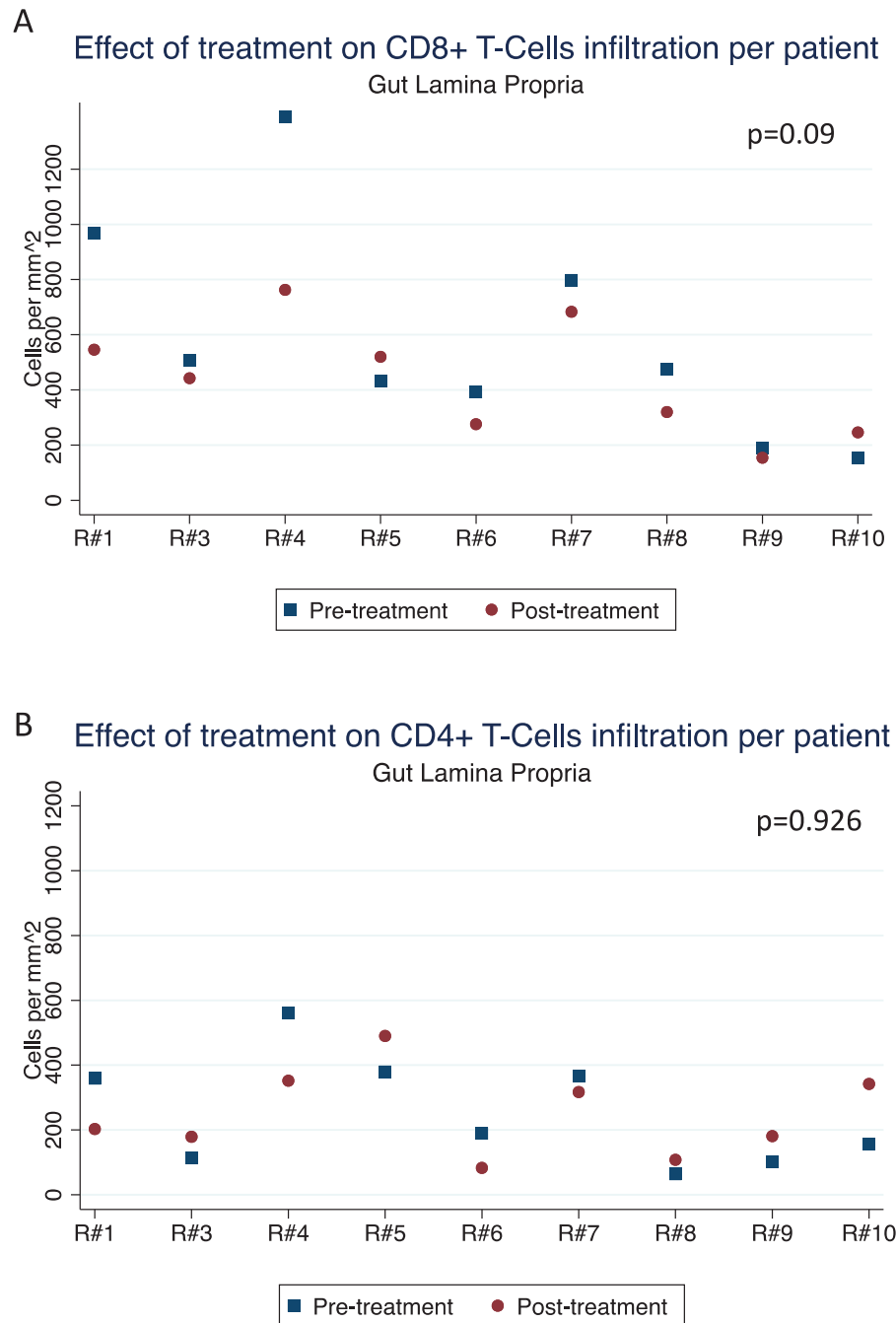


Fig. S11. Treatment effect on CD8⁺ and CD4⁺ T cells infiltration in the gut lamina propria.

Image analysis algorithms were used to count the number of Immunohistochemically (IHC) stained cells within the lamina propria of each sigmoid colon biopsy. (A) Counts of IHC stains

against CD8⁺ cells, representing cytotoxic T cell infiltration (B) Counts of IHC stains against CD4⁺ cells, representing T-helper-cell infiltration. Both T cell stains failed to demonstrate a consistent post-treatment change (p=0.09 and p=0.926, respectively, paired t-test). R – Recipient.

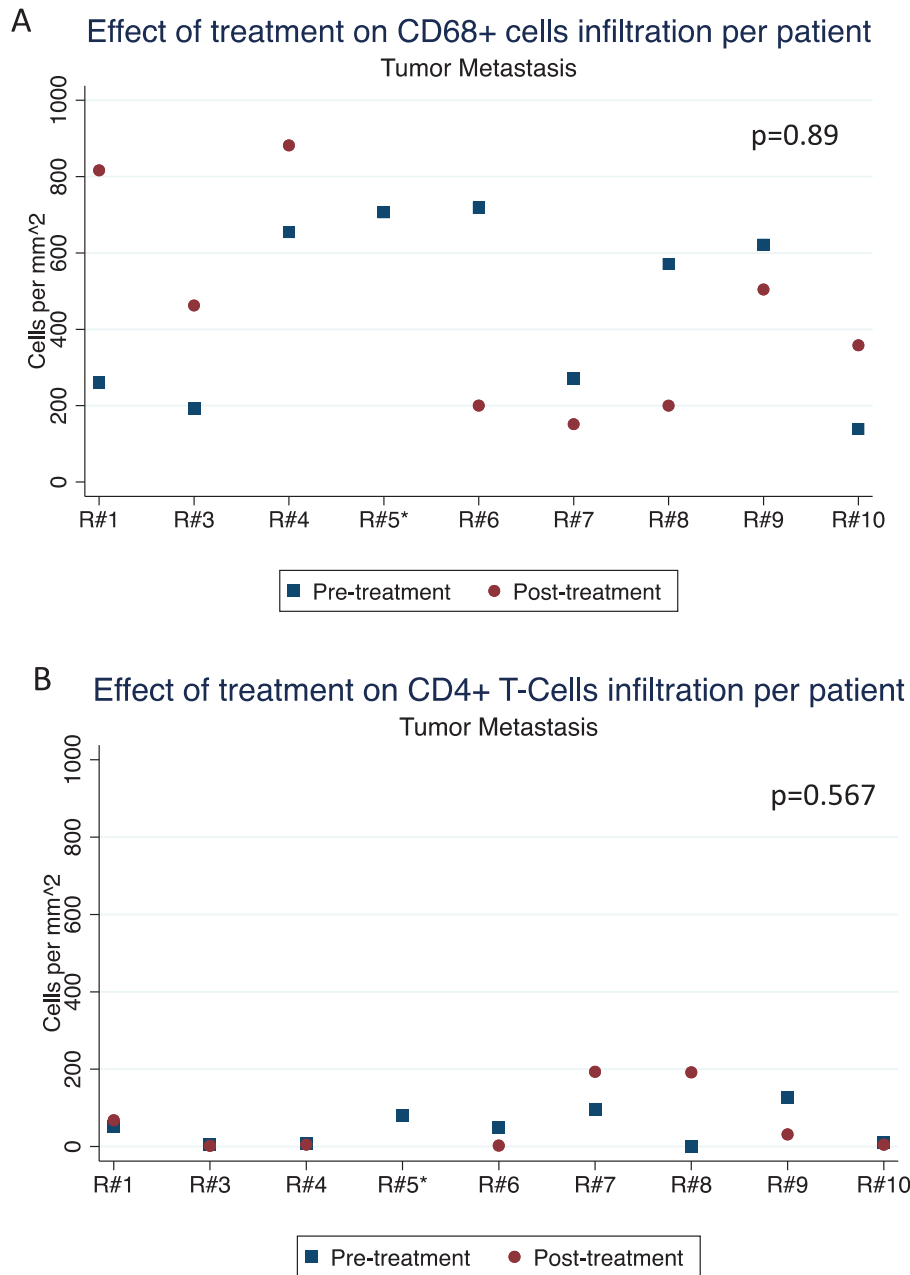


Fig. S12. Treatment effect on CD68⁺ and CD4⁺ cells infiltration in the tumor. Image analysis algorithms were used to count the number of Immunohistochemically (IHC) stained cells within viable tumor tissue for each remote tumor metastasis biopsy. Post-treatment tumor biopsies were preferably taken from the same metastasis used for the pre-treatment biopsy, or from another metastasis at the same organ. (A) Counts of IHC stain against CD68⁺ cells, representing antigen

presenting cells (APC), demonstrating no consistent change intra-tumoral APC infiltration (p=0.89, paired t-test); (B) Counts of IHC stain against CD4⁺ cells, representing T-helper-cell infiltration, demonstrating no consistent change intra-tumoral CD4⁺ cells infiltration (p=0.567, paired t-test). R – Recipient. * - Recipient#5 developed a near complete pathological response (<1% viable tumor tissue); Since the infiltration analysis was conducted only on immune cells which infiltrated viable tumor tissue, it could not be conducted on Recipient#5's post-treatment tumor sample.

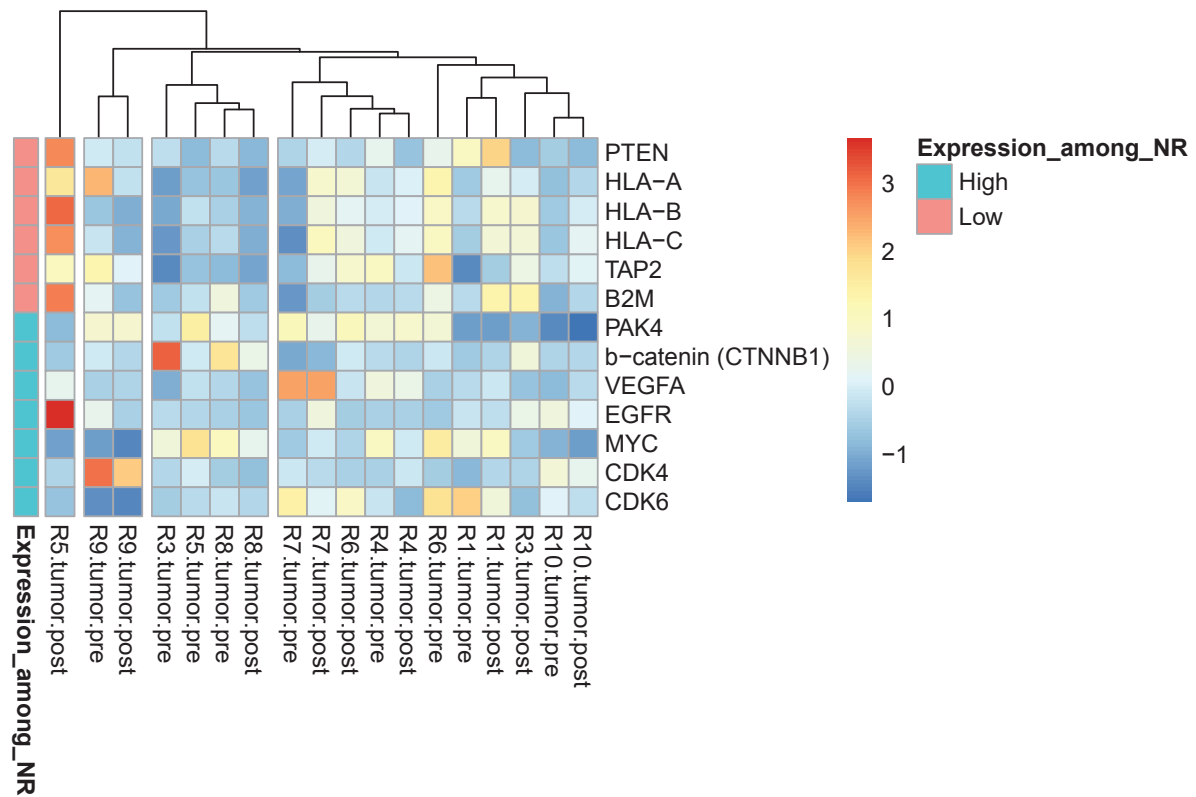


Fig. S13. Immune-exclusion gene expression in tumor samples. The heatmap represented the expression of genes that were associated with intrinsic resistance to anti-PD-1 therapy(61).

Resistance mechanisms for anti-PD-1 therapy may originate in mutated, deleted, or over-express genes that impair either antigen presentation machinery of tumor cells or T cells infiltration. This heatmap demonstrated that Recipient#9 expressed high levels of CDK4, which were previously associated with the lack of intra-tumoral T cell infiltration and decreased antigen presentation(62). Indeed, Recipient#9 antigen presentation related genes were lowly expressed. PAK4, which was also associated with decreased intra-tumoral infiltration of T cells and dendritic cells(63), was highly expressed among most of the pre-treatment and of non-responder post-treatment samples. Recipient#5, who was a responder with a near-pathological complete response in his post-treatment biopsy, demonstrated high expression of antigen presenting

machinery – corresponding to the high immune activity demonstrated by Figure 3H. Scale represents the Z-score of TMM normalized gene counts. NR – Non-responders; The "Expression among NR" categories were determined according to published literature.

Age	Gender	BRAF V600E mutation	Previous treatment lines	Anti-PD-1 therapy	Stage	# of Disease Sites	Time in complete response (months)
59	M	Mutated	Vemurafenib	Nivolumab	M1d	3	12
41	F	Mutated	Vemurafenib + Cobimetinib	Nivolumab	M1c	2	14

Table S1. Clinical characteristics of FMT donors. FMT – Fecal Microbiota Transplantation;

M – Male, F- Female. Stage was determined according to the American Joint Cancer Committee 8th Edition(64).

Recipient #	Age	Gender	BRAF V600E	Stage	Baseline LDH	Sum of target lesion diameter (mm)*	% of Pre-treatment PD-L1 stained cells†	Number of cycles during previous anti-PD-1 line#
1	66	F	Mutated	M1d	High	136	≥5%	9
2	70	M	WT	M1b	High	145	N/A	5
3	78	M	WT	M1a	Normal	44	0	10
4	69	F	WT	M1a	Normal	120	0	6
5	66	M	WT	M1a	Normal	38	≥10%	30
6	33	M	Mutated	M1d	Normal	220	≥5%	7
7	66	M	Mutated	M1c	High	132	≥5%	35
8	65	M	WT	M1c	Normal	33	≥5%	10
9	35	F	WT	M1c	High	125	0	8
10	44	M	WT	M1c	Normal	85	≥1%	11

Table S2. Extended clinical characterization of FMT recipients. M – Male, F- Female, LDH

– Lactate dehydrogenase, WT – Wild Type. Stage was determined according to the American Joint Cancer Committee 8th Edition(64). * Sum of target lesion diameters was calculated according to the iRECIST (immune Response Evaluation Criteria in Solid Tumors(7)). † - The percentage of positively stained Programmed cell Death Ligand 1 (PD-L1) cells was assessed and reported according to the acceptable expression categories of <1%, ≥1%, ≥5%, ≥10%, ≥25%, ≥50%(56). # - Number of anti-PD-1 cycles during the previous and most recent anti-PD-1 treatment line. Recipient#2 refused to undergo repeated tumor and gut biopsies and was thus not included in the correlative tissue analysis.

irAE	Incidence during mFMT period (Day ≤ 90)	Incidence after mFMT period (Day > 90)
Rash – G1	10%	0%
Myalgia – G1	10%	10%
Arthralgia – G1	30%	40%
Fatigue – G1	10%	10%
Chills – G1	10%	10%
Hepatobiliary disorder, other – G1	10%	0%
Vitiligo – G1	10%	10%

Table S3. Incidence of immune related adverse events (irAEs) during the current trial.

mFMT – maintenance Fecal Microbiota Transplantation; G - Grade. irAEs and their grades were determined according to the Common Terminology Criteria for Adverse Events, CTCAE, version 5.0(34).

Recipient #	irAEs during previous treatment line	irAEs in the current trial
1	None	None
2	None	None
3	Arthralgia – G2	None
4	Diarrhea – G3	None
5	Hyperthyroidism – G2; Hepatobiliary disorder, other – G3	None
6	None	None
7	None	None
8	Hemolysis – G3; Myalgia – G3	None
9	None	None
10	Arthralgia – G3; Hepatobiliary disorder, other – G2	None

Table S4. Incidence of moderate to severe (grade 2-4) immune related adverse events (irAEs) during pre-trial anti-PD-1 treatment lines in comparison to the current trial.

Data on pre-trial irAEs were extracted from the recipient medical records. G - Grade. irAEs were determined according to the Common Terminology Criteria for Adverse Events, CTCAE, version 5.0(34).

Recipient #	Response by RECIST 1.1(15) at the first post-treatment imaging study	Response by iRECIST(7) at the first post-treatment imaging study	Best response by iRECIST(7)
1	PD	iuPD	icPD
2	PD	iuPD	icPD
3	PD	iuPD	iCR
4	PD	iuPD	icPD
5	PD	iuPD	iPR
6	PD	iuPD	icPD
7	Unconfirmed PR	iPR	iPR
8	PD	iuPD	icPD
9	PD	iuPD	icPD
10	PD	iuPD	icPD

Table S5. Radiological response to treatment according to both RECIST 1.1 and iRECIST.

Best response was determined as the best immune Response Evaluation Criteria in Solid Tumors (iRECIST) response a recipient had achieved during the study. i – iRECIST based; iu – iRECIST unconfirmed; ic – iRECIST confirmed.



Table S6. Genus and Species levels profiling of FMT donors. Relative abundance of Genus and Species taxa were presented per donor, based on metagenomic sequencing. Data are presented in percentage, and each donor column for Genus/Species sums up to 100%. FMT – Fecal Microbiota Transplantation.



Table S7. Specific taxa which significantly differed in comparison of post-treatment microbiota compositions of the two donor-groups. Profiling differing taxa was based on metagenomic sequencing. The abundance of these taxa was compared between the two donor-groups using the ANalysis of Composition of Microbiomes (ANCOM) test. Negative mean abundance difference – taxa which were relatively more abundant in post-treatment stool samples of Donor#1-group recipients and Donor#1 himself; Positive mean abundance difference – taxa which were relatively more abundant in post-treatment stool samples of Donor#2-group recipients and Donor#2 herself. Statistical significance was defined as FDR $q \leq 0.05$.



Table S8. GO gene sets which were significantly enriched among the post-treatment microbiota compositions of the two donor-groups. Functional microbiota analysis was based on metagenomic sequencing. Microbiota genes were re-grouped to GO gene sets. The abundance of these gene sets was compared between the two donor-groups. Positive abundance difference – gene sets which were enriched in post-treatment stool samples of Donor#1-group recipients and Donor#1 himself; Negative abundance difference – pathways which were enriched in post-treatment stool samples of Donor#2-group recipients and Donor#2 herself. Statistical significance was defined as FDR $q \leq 0.05$. Pathways with $\log_2$ abundance difference < 1 were filtered out of the analysis.



Table S9. Gene sets which were significantly enriched among all available post-treatment gut samples in comparison to their baseline. Gene sets were based on the Gene Ontology (GO) dataset. Statistical significance was defined as FDR $q \leq 0.05$. Up – gene sets which were enriched among all post-treatment gut samples; Down - gene sets which were enriched among all pre-treatment gut samples. Recipient#2 did not consent to undergo repeated tumor and gut biopsies and was thus not included in the tissue analysis.



Table S10. Gene sets which were significantly enriched among Donor#1-group post-treatment gut samples in comparison to their baseline. Gene sets were based on the Gene Ontology (GO) dataset. Statistical significance was defined as FDR $q \leq 0.05$. Up – gene sets which were enriched among Donor#1-group post-treatment gut samples; Down - gene sets which were enriched among Donor#1-group pre-treatment gut samples.



Table S11. Gene sets which were significantly enriched among available Donor#2-group post-treatment gut samples in comparison to their baseline. Gene sets were based on the Gene Ontology (GO) dataset. Statistical significance was defined as FDR $q \leq 0.05$. Up – gene sets which were enriched among Donor#2-group post-treatment gut samples; Down - gene sets which were enriched among Donor#2-group pre-treatment gut samples. Recipient#2 did not consent to undergo repeated tumor and gut biopsies and was thus not included in the tissue analysis.



Table S12. Gene sets which were significantly enriched among all available post-treatment tumor samples in comparison to their baseline. Gene sets were based on the Gene Ontology (GO) dataset. Statistical significance was defined as FDR $q \leq 0.05$. Up – gene sets which were enriched among all post-treatment tumor samples; Down - gene sets which were enriched among all pre-treatment tumor samples. Recipient#2 did not consent to undergo repeated tumor and gut biopsies and was thus not included in the tissue analysis.



Table S13. Gene sets which were significantly enriched among Donor#1-group post-treatment tumor samples in comparison to their baseline. Gene sets were based on the Gene Ontology (GO) dataset. Statistical significance was defined as FDR $q \leq 0.05$. Up – gene sets which were enriched among Donor#1-group post-treatment tumor samples; Down - gene sets which were enriched among Donor#1-group pre-treatment tumor samples.



Table S14. Gene sets which were significantly enriched among available Donor#2-group post-treatment tumor samples in comparison to their baseline. Gene sets were based on the Gene Ontology (GO) dataset. Statistical significance was defined as FDR $q \leq 0.05$. Up – gene sets which were enriched among Donor#2-group post-treatment tumor samples; Down - gene sets which were enriched among Donor#2-group pre-treatment tumor samples. Recipient#2 did not consent to undergo repeated tumor and gut biopsies and was thus not included in the tissue analysis.

Inclusion Criteria	Exclusion Criteria
Able to provide written informed consent	Presence of contra-indications to colonoscopy.
A histologically confirmed diagnosis of metastatic melanoma	Allergies to food or antibiotics
A viable tumor tissue comprising at least 75% of the pre-treatment tumor biopsy examined area	Metastasis in the gastro-intestinal tract
Failed at least one line of anti-PD-1 therapy and targeted therapy for BRAF-V600E patients.	Inability to swallow capsules
An iRECIST confirmed disease progression on last previous anti-PD-1 treatment line	Current participation in a study of another investigational agent
ECOG Performance Status ≤ 2	Has a diagnosis of immunodeficiency or is receiving systemic immunosuppression in a dose higher than the equivalent to > 10 mg Prednisolone daily
Age ≥ 18 years	Chronic kidney disease or serum creatinine level ≥ 1.5 upper normal limits
ASA score ≤ 2	History of bleeding disorder, current platelet count $< 50,000$ or INR > 1.5
A washout period of at least four weeks from the previous treatment line to the first day of the treatment protocol	History of inflammatory bowel disease
	History of major abdominal surgery
	Pregnant or breastfeeding, or expecting to conceive or father children within the projected duration of the trial, starting with the pre-screening or screening visit through 120 days after the last dose of trial treatment
	Active metastatic brain disease

Table S15. Inclusion and exclusion criteria for potential recipients in the current clinical

trial (NCT03353402). iRECIST – immune Response Evaluation Criteria in Solid Tumors;

ECOG - Eastern Cooperative Oncology Group; ASA - American Society of Anesthesiologists;

INR - International Normalized Ratio.

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