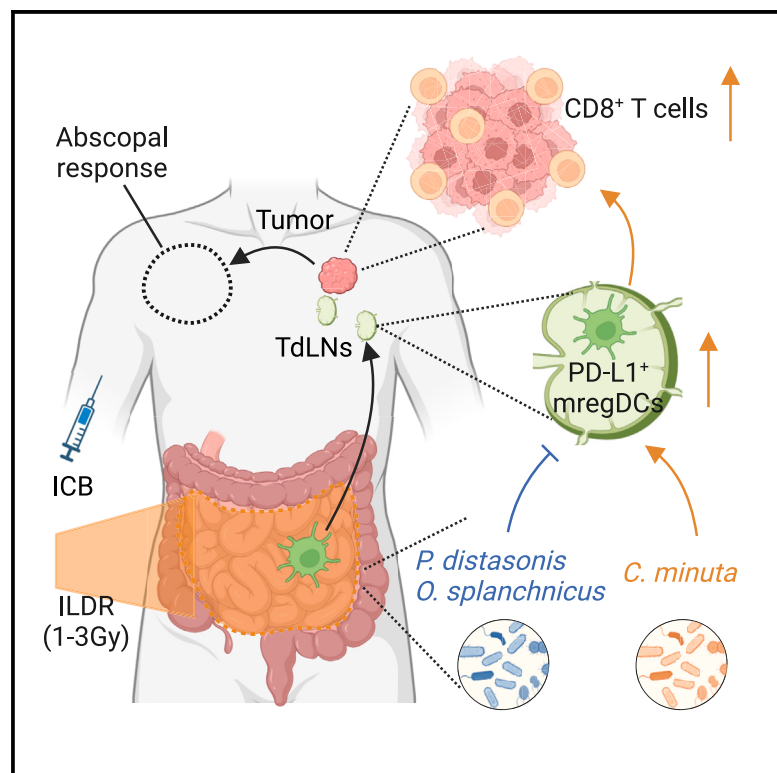


Low-dose irradiation of the gut improves the efficacy of PD-L1 blockade in metastatic cancer patients

Graphical abstract



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In brief

Chen et al. propose a mechanism accounting for the abscopal effects of radiotherapy on distal non-irradiated cancer lesions under immunotherapy. In eight retrospective studies and a prospective trial, the authors show that intestinal low-dose irradiation (ILDOR) synergizes with anti-PD-L1 in a gut microbiota-dependent manner.

Highlights

- Off-target ILDR synergizes with anti-PD-L1 to eradicate non-irradiated tumors
- The abscopal effects of ILDR depend on dosimetry (≥ 1 and ≤ 3 Gy) and volume
- Gut composition governs the abscopal effects of ILDR combined with anti-PD-L1
- ILDR and *Christensenellaceae* influence the emigration of intestinal mregDC to TdLN



Article

Low-dose irradiation of the gut improves the efficacy of PD-L1 blockade in metastatic cancer patients

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SUMMARY

The mechanisms governing the abscopal effects of local radiotherapy in cancer patients remain an open conundrum. Here, we show that off-target intestinal low-dose irradiation (ILDR) increases the clinical benefits of immune checkpoint inhibitors or chemotherapy in eight retrospective cohorts of cancer patients and in tumor-bearing mice. The abscopal effects of ILDR depend on dosimetry (≥ 1 and ≤ 3 Gy) and on the metabolic and immune host-microbiota interaction at baseline allowing CD8⁺ T cell activation without exhaustion. Various strains of *Christensenella minuta* selectively boost the anti-cancer efficacy of ILDR and PD-L1 blockade, allowing emigration of intestinal PD-L1-expressing dendritic cells to tumor-draining lymph nodes. An interventional phase 2 study provides the proof-of-concept that ILDR can circumvent resistance to first- or second-line immunotherapy in cancer patients. Prospective clinical trials are warranted to define optimal dosimetry and indications for ILDR to maximize its therapeutic potential.

INTRODUCTION

Preclinical models suggest that the combination of radiotherapy (RT), including stereotactic ablative radiotherapy (SABR) and im-

mune checkpoint blockade (ICB), mediates synergistic anti-tumor immune responses not only at the local site but also on distal, non-irradiated tumors, known as “abscopal” effects.^{1–3} Hundreds of trials, including ours, have been launched to



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optimize combinations of RT with ICB. While some dedicated clinical studies were positive^{4–11}; other trials were unsuccessful, specifically in patients with head-and-neck, advanced cutaneous tumors or ICB-refractory non-small cell lung cancer (NSCLC).^{12–19}

Irradiation of the gut compromises the epithelial barrier, causing mucosal damage, dysbiosis, and enteritis. Hence, the intestine is subjected to specific dose constraints during abdominopelvic RT.^{20,21} The gut microbiota has emerged as a key modulator of immunotherapy^{22–28} and profoundly influences the clinical outcome of ICB.^{29–32} Intestinal commensals shape systemic immunity and the tumor microenvironment at distal sites through various mechanisms.^{33–41}

Here, we investigated gut-specific off-target effects of irradiation, henceforth called “intestinal low-dose irradiation” (ILDR), on the clinical outcome of metastatic cancer patients treated with anti-programmed death-ligand 1 (PD-L1, also known as CD274) antibodies (Abs). We also explored the mechanisms through which ILDR improves the outcome of PD-L1 blockade in preclinical models.

RESULTS

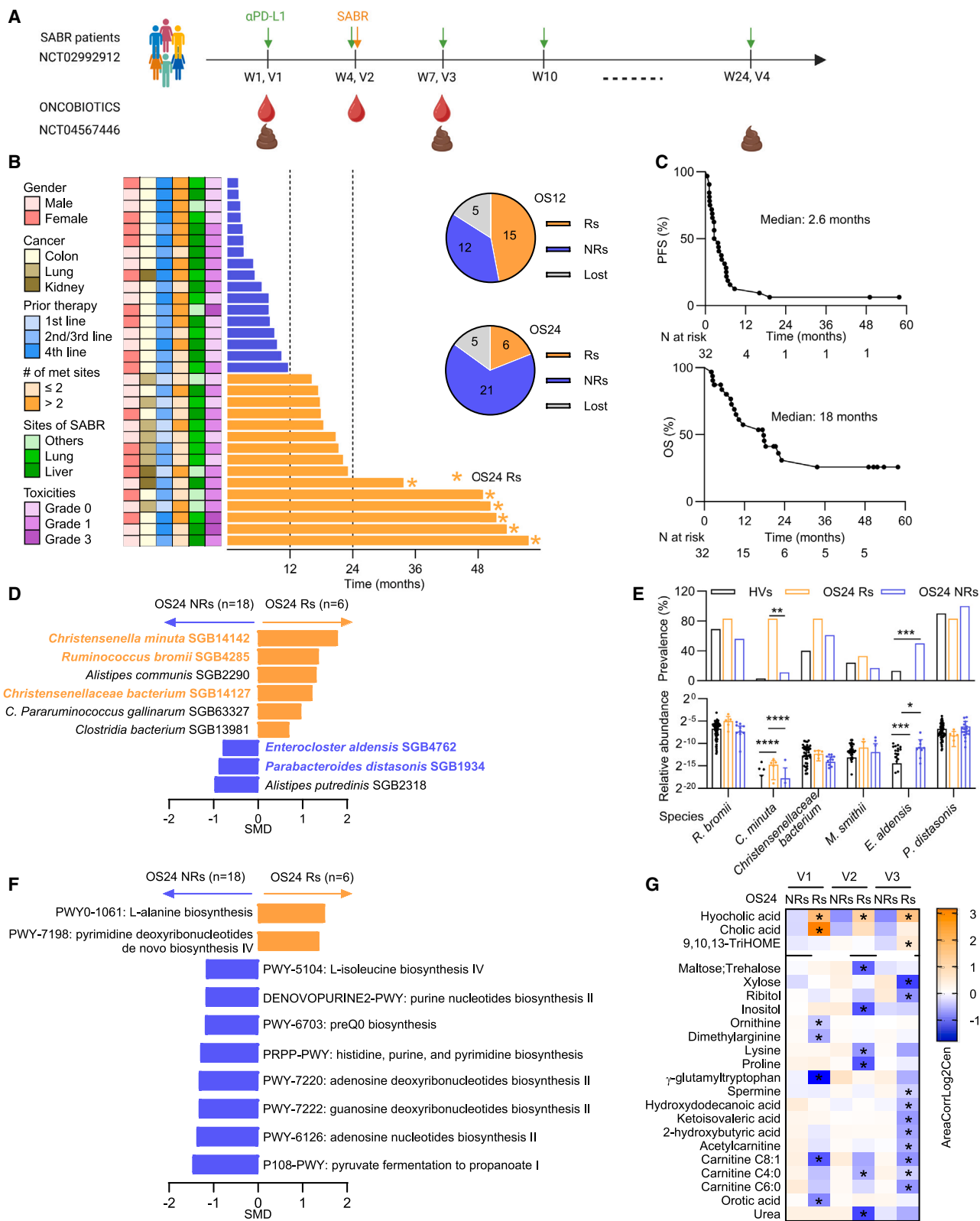
Nineteen percent of advanced cancer patients are long-term survivors after SABR and PD-L1 blockade

Patients with metastatic tumors were enrolled in a multicenter phase 2 “SABR trial” and subjected to SABR in combination

with an anti-PD-L1 antibody (MPDL3280A, α PD-L1). Translational analyses (including serum and stool sample collections) were performed in the context of the ONCOBIOTICS study. SABR (45 Gy in 3 fractions in ≤ 8 days) was delivered to one or more tumor lesions (leaving at least one unirradiated lesion) using 6 MV photons concurrently with the 2nd dose of α PD-L1 (Figure 1A). Between November 2016 and March 2021, 87 patients were recruited. Clinical results of the colorectal cancer subgroup have been published.⁴² Key clinical characteristics and patient survival of the translational cohort ($N = 32$) are illustrated in Figure 1B and listed in Table S1. SABR was mainly delivered to metastases located in the liver (43%), followed by lungs and other organs. The median follow-up was 11 months for the entire group and 49.8 months for the survivors up to May 19, 2023. In total, 47% (15/32) of patients survived the first year (overall survival [OS] ≥ 12 months, OS12 responders [OS12 Rs]) and 19% (6/32) survived beyond 24 months (OS24 Rs) (Figure 1B). The median progression-free survival (PFS) and OS of the entire group was 2.6 and 18 months, respectively (Figure 1C). The tumor objective response rate (ORR) in OS24 Rs was 67% (4/6) as compared to 0% (0/21) in OS24 non-responders (OS24 NRs) ($p = 0.0009$).

Inflammatory, metabolic, and metagenomic profiling of responders to SABR combined with α PD-L1

We performed shotgun metagenomic sequencing (MGS) of 48 fecal samples (29 at first visit [V1], 13 at third [V3], and 6 at fourth



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[V4]). Stools from Rs and NRs (considering both OS24 and OS12) displayed comparable α and β diversities at baseline, and none of the species was differently abundant (all FDR >0.20). Nonetheless, we observed a trend toward a differential enrichment of MGS species in Rs versus NRs at baseline, as described.^{31,36,37,43,44} Members of the *Eubacteriales* order (*Ruminococcus bromii* SGB4285, *Eubacterium rectale* SGB4933, *Ruminococcaceae bacterium* SGB14925) were overrepresented in Rs, while the *Enterocloster* genus (*E. aldensis* SGB4762, *E. bolteae* SGB4758) was dominant in NRs (Figures 1D and S1A). *Parabacteroides distasonis* SGB1934, which is enriched in chemoradiotherapy-resistant rectal cancers,³⁴ stood out in OS24 NRs (Figure 1D). We observed a higher prevalence and/or relative overabundance of *Christensenellaceae* (*Christensenella minuta* and SGB14127) and their associated archaea *Methanobrevibacter smithii*⁴⁵ in OS24 and OS12 Rs, respectively (Figures 1D and S1A). Exploiting the MG database of 124 sex, age and geography-matched healthy volunteers (HVs),⁴⁶ we confirmed that bacterial species prototypically associated with Rs (*C. minuta*, *R. bromii*) were underrepresented in NRs compared to HVs and, vice versa, NR-associated species (*E. aldensis* and *P. distasonis*) were overabundant in NRs (Figure 1E). *C. minuta* was more prevalent in this subset of OS24 Rs than in the 124 HVs (and in publicly available data indicating a mere prevalence of 12.5% of *C. minuta*).

Metabolic pathways were computed from the metagenomes of OS24 (Figure 1F) or OS12 (Figure S1B) Rs versus NRs at V1. Rs harbored fingerprints associated with L-arginine and L-alanine biosynthesis, while NRs displayed enrichment in biosynthesis of guanosine and purine/pyrimidine nucleotide, fatty acids and menaquinol (Figures 1F and S1B). These pathways reportedly impact DNA damage and immune responses.^{34,47,48} At the level of serum metabolomics, OS24 and OS12 Rs displayed higher levels of L-arginine and primary bile acids (BAs, including cholic acid and hyocholic acid) (Figures 1G and S1C), whereas OS12 NRs exhibited a rise in carboxylic acids, mono- or poly-unsaturated fatty acids, shuttle carnitines, menaquinol biosynthesis, and acetylated polyamines (Figures 1G and S1C), as reported for chronic inflammatory disorders including cancers.⁴⁹

A 27-plex enzyme-linked immunosorbent assay was used to measure inflammatory serum factors. Interleukin-7 (IL-7) and interferon- γ (IFN- γ) were the only categorical cytokines (presence/absence) characterizing OS12 Rs and NRs, respectively,

particularly at V1 (Figure 2A). Detection of IL-7 and IFN- γ was close-to-mutually exclusive and associated with favorable and dismal survival, respectively (Figure 2A). No difference of α and β diversity was observed between patients with detectable versus undetectable IL-7 or IFN- γ . Patients lacking IL-7 at V1 displayed increased abundance of OS12 NR oral taxa, *Streptococcus mitis* SGB8163, and species associated with chemoradiotherapy resistance (*B. salyersiae*)³⁴ (Figure 2B). In contrast, *Streptococcus mitis* was overabundant in IFN- γ producers at the expense of health-associated bacteria (*Eubacterium rectale*, *Faecalibacterium prausnitzii*, *G. formicilis*, and *R. faecis*)⁵⁰ (Figure 2B). IL-7 non-producers exhibited an increase in menaquinol biosynthesis (Figure 2B), reported to be linked to disease status.⁵¹ Patients with detectable IFN- γ harbored metabolic pathways associated with sulfur reduction, fatty acid and β -oxidation as well as stearate, palmitoleate and oleate biosynthesis (Figure 2B).

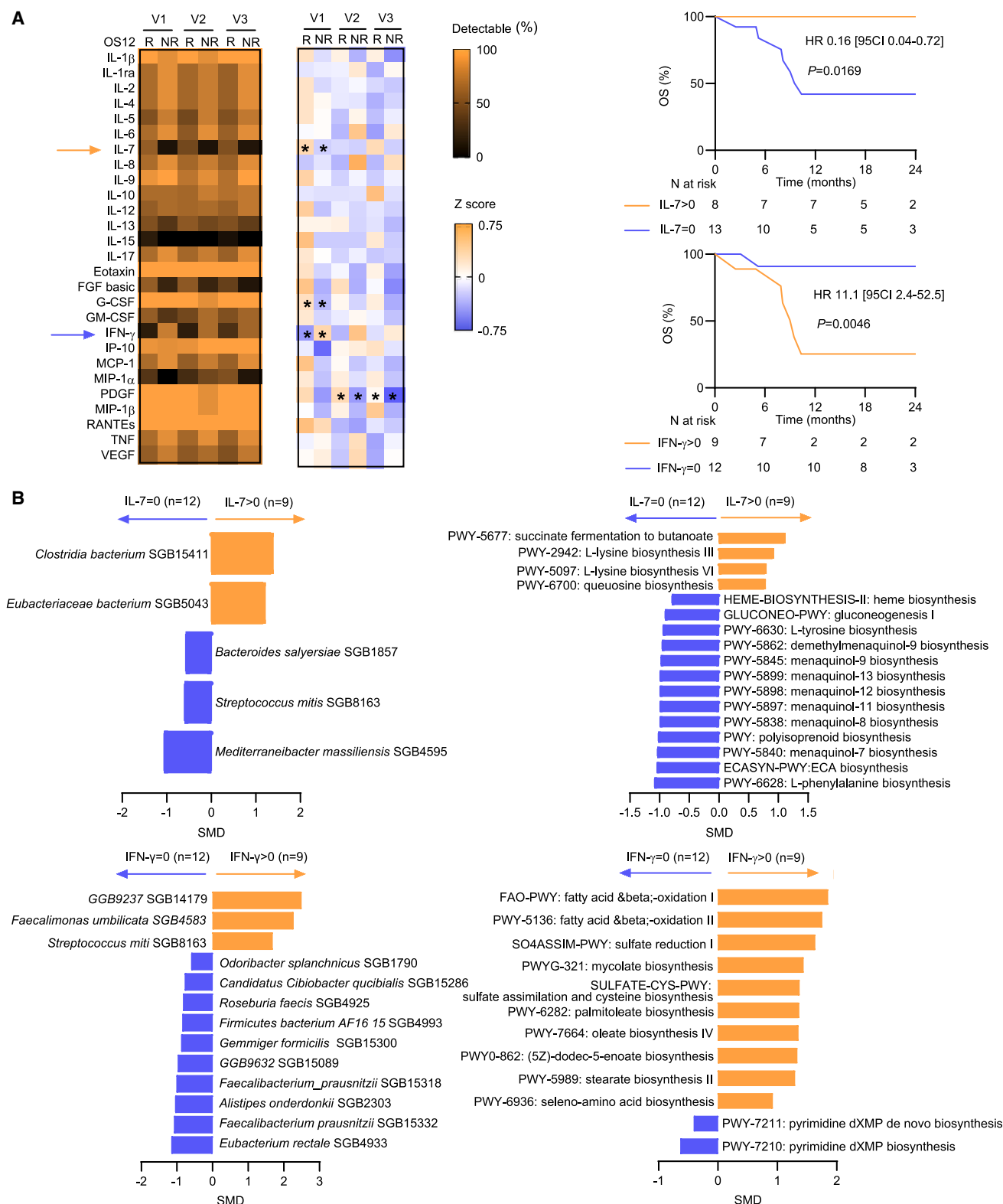
ILDR is associated with clinical responses to PD-L1 blockade

Thirteen patients (41%) enrolled in the SABR trial were exposed to incidental ILDR (representative dosimetry in Figure 3A) with a median RT dose of 3.3, 1.0, and 1.3 Gy to duodenum, jejunum/ileum, and colon, respectively, as calculated from the dose-volume histograms (DVHs) in the SABR treatment plans (Table S1). Patients with or without ILDR presented comparable grade 1 toxicities (62% vs. 58% respectively, Table S1). Patients with ILDR more frequently attained OS24 R status than patients without ILDR (38% [5/13] vs. 5% [1/19], $p = 0.0289$). Multivariate Cox regression analysis revealed that ILDR was an independent prognostic factor of survival (Figure 3B, HR = 0.22, $p = 0.028$), outcompeting serum IL-7 (Figure 3B, HR = 0.14, $p = 0.061$) and MSI-H (Figure 3B, HR = 1.13, $p = 0.9$).

Driven by this unanticipated result, we extended our analysis to all patients ($N = 87$) enrolled in the SABR- α PD-L1 trial with a ≥ 2 -year follow-up (up to May 19, 2023), as well as to 7 independent validation cohorts of cancer patients subjected to SABR or fractionated RT in combination with ICB or chemotherapy worldwide. A total of 388 patients were included in these trials (Tables S1, S2–S4, S5, and S6). Most patients with incidental ILDR received abdominopelvic RT, while patients without ILDR received thoracic or head-and-neck RT (Figure 3C). Hence, we focused on the 38 patients with abdominopelvic metastases (Table S2), 26 of whom (69%) bore metastases in liver or soft

Figure 1. Long-term survivors during SABR and anti-PD-L1 combinational regimen harbor a healthy microbiome profile

(A) Scheme of the SABR trial (NCT02992912) and sample collection (in the context of ONCOBIOTICS study [NCT04567446]). W, week; V, visit.
(B) Clinical characteristics of the 32 patients enrolled in the translational part of SABR trial. #, number; OS12, overall survival at 12 months; OS24, overall survival at 24 months; Rs, responders; NRs, non-responders.
(C) Kaplan-Meier curves of progression-free survival (PFS) and OS.
(D) Differential abundance of the fecal microbiota of OS24 Rs ($n = 6$) versus NRs ($n = 18$) at V1. Bars show the standardized mean difference (SMD).
(E) Comparisons of prevalence and relative abundances of key metagenomic species (MGSs) in OS24 Rs ($n = 6$) or NRs ($n = 18$) compared with gender, sex and geography matched healthy volunteers (HVs, $n = 124$). Data on the lower panel are shown as mean $\pm$ SD.
(F) Differential abundance analysis of gene-encoded metabolic pathways computed from the metagenome of OS24 Rs versus NRs at V1.
(G) Differential metabolite enrichment in the targeted metabolomics analysis of serum samples collected at 3 time points (For Rs, $n = 5$ at V1, $n = 6$ at V2, $n = 6$ at V3; For NRs, $n = 15$ at V1, $n = 19$ at V2, $n = 15$ at V3). Data in the heatmap represent the relative abundance of metabolites (calculated as AreaCorrLog2Cen). Metabolites with statistical significance between OS24 Rs and NRs are indicated with an asterisk.
Statistical analysis: Mann-Whitney U test without p value correction (D and F) and with (G) and Kruskal-Wallis H test (E). p values < 0.05 were considered significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.
See also Figure S1 and Table S1.



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tissues, which are associated with dismal prognosis.^{52,53} Strikingly, patients experiencing ILDR displayed improved OS compared with patients without (Figure 3D, HR = 0.27, $p = 0.0006$, Table S2) and ILDR independently predicted favorable OS (Table S2). Patients receiving ILDR at 1 Gy on 92–417 cc of the entire intestine (EI) tended to display a better survival than those receiving less (<92 cc) or more (>417 cc) irradiation (Figure 3D, $p = 0.0572$).

Consistently, in the ARTIC trial evaluating the combination of RT and ICB in metastatic cancers (Table S5), patients exposed to <3 Gy ILDR (mean dose of EI) tended to display superior survival (median OS undefined) than patients without ILDR (13.6 months, $p = 0.0764$) or with ≥ 3 Gy ILDR (18.93 months, $p = 0.0643$) (Figure S2A).

The National Cancer Institute (NCI) trial enrolled 59 patients with advanced pancreatic ductal adenocarcinoma (aPDA), most of whom (90%) received incidental ILDR (Table S3). Patients in whom the small intestine (SI) was exposed to 1 Gy within 7–588 cc exhibited superior survival than patients receiving 1 Gy on a smaller or larger volume (Figure 3E, $p = 0.0061$). A similar clinical benefit was observed for a volume within the window of 11–670 cc when 1 Gy ILDR of the large intestine (LI) was considered (Figure 3E, $p = 0.0034$). Notably, the optimal volume appeared inversely proportional to the optimal dose (Figures S2B–S2E). EI V1Gy together with RT dose was independent predictors of patient survival (Table S3).

The ABIMMUNE trial recruited 44 patients with metastatic squamous cell carcinoma (SCC) from esophagus, cervix, and anal canal to receive SABR combined with cytotoxic T lymphocyte associated protein 4 (CTLA-4) and PD-L1 co-blockade (Table S5). Here, the optimal and protective ILDR dose was set at 0.25 Gy at the intermediate volume of 42–311 cc for SI, that was more beneficial for OS than >311 cc ($p = 0.07$, Figure 3F). LI irradiation at <197 cc was associated with longer survival compared with LI irradiation of ≥ 197 cc ($p = 0.0203$, Figure 3F). When ILDR threshold was set at 4 Gy, LI irradiation (>0 cc) became deleterious compared with no ILDR (Figure S2F).

In the Verona cohort of advanced NSCLC patients (Table S4), ILDR set up at 0.25 Gy with an intermediate volume (9–88 cc for SI, $p < 0.05$, and 39–702 cc for LI, $p = 0.06$ respectively, Figure 3G) correlated with favorable outcomes. EI V0.25 Gy at 39–206 cc proved to be an independent predictor of survival (Table S4). In the Firenze lung cancer cohort, the protective ILDR window was found at a higher volume (>603 cc at 1 Gy for SI) (Figure 3H, $p = 0.0174$, Table S5).

In addition to ICB, we investigated the role of ILDR in patients receiving chemoradiotherapy in the IMMUNE002 trial enrolling 65 primary cervical SCC patients (Table S6) that were subjected to external beam radiotherapy (EBRT, 46 Gy/23F, 2 Gy/F/day, 5 days/week) in combination with concurrent chemotherapy followed by brachytherapy. Patients exhibiting complete regression (CR) received higher ILDR doses on the colon than patients

experiencing partial regression (PR) (1.19 ± 0.18 Gy vs. 1.00 ± 0.17 Gy respectively, $p < 0.0001$). Cervical cancer patients receiving <1 Gy ($N = 16$) had an increased risk of not experiencing CR after EBRT compared with those receiving >1 Gy ($N = 49$) (RR = 2.667, $p = 0.0371$) (Figure 3I).

A correlation between colonic ILDR dose and anal cancer response to concomitant chemotherapy was also observed in the BISQUIT trial (Table S6). Incomplete tumor regressions (non-CR) were only found in patients receiving <1 Gy colonic daily dose compared with patients receiving a colonic ILDR >1 Gy (15% vs. 0%) (Figure S2G).

In conclusion, in 8 small cohorts of patients receiving RT combined with ICB or immunogenic chemotherapy, incidental ILDR administered at 0.25–3 Gy conferred survival benefits to advanced cancer patients.

ILDR administered at 1 Gy but not 4 Gy is associated with clinical benefit to PD-L1 blockade in mice

We treated C57BL/6J mice bearing subcutaneous MC38 colon cancers with tumor sparing ILDR+ α PD-L1 (Figure 4A). Animals subjected to 1 Gy ILDR alone displayed no survival benefit, and α PD-L1 alone resulted in transient effects, meaning that all tumors relapsed. However, 1 Gy ILDR+ α PD-L1 cured 30% of the animals (Figure 4B), contrasting with, lower (0.25 Gy) or higher (4 Gy) ILDR doses, which failed to do so (0% tumor-free mice) (Figure 4C). SABR delivered onto the liver was not effective (Figure 4D). The beneficial role of incidental ILDR was also observed in a breast cancer model induced by administration of carcinogens to C57BL/6J mice (Figure S3).⁵⁴ A total of 37 mice with primary breast tumor developing either in the cervical ($N = 22$) or abdominal ($N = 15$) mammary fat pad were subjected to mock ($N = 18$) or SABR (20 Gy $\times$ 2F or 20 Gy $\times$ 3 F, $N = 19$) delivered to the first primary tumor (Figure S3A). In response to mock SABR, mice with cervical or abdominal breast cancers displayed comparable OS and time to secondary (TTS) tumor, a surrogate marker of abscopal effects (Figure S3B). However, delivery of SABR onto abdominal lesions (but not cervical ones) significantly improved OS (median 60 days vs. 41 days, $p = 0.0285$) as well as TTS (median 48 days vs. 5 days, $p = 0.0116$) (Figure S3C). In conclusion, incidental ILDR boosts the efficacy of PD-L1 blockade in mice.

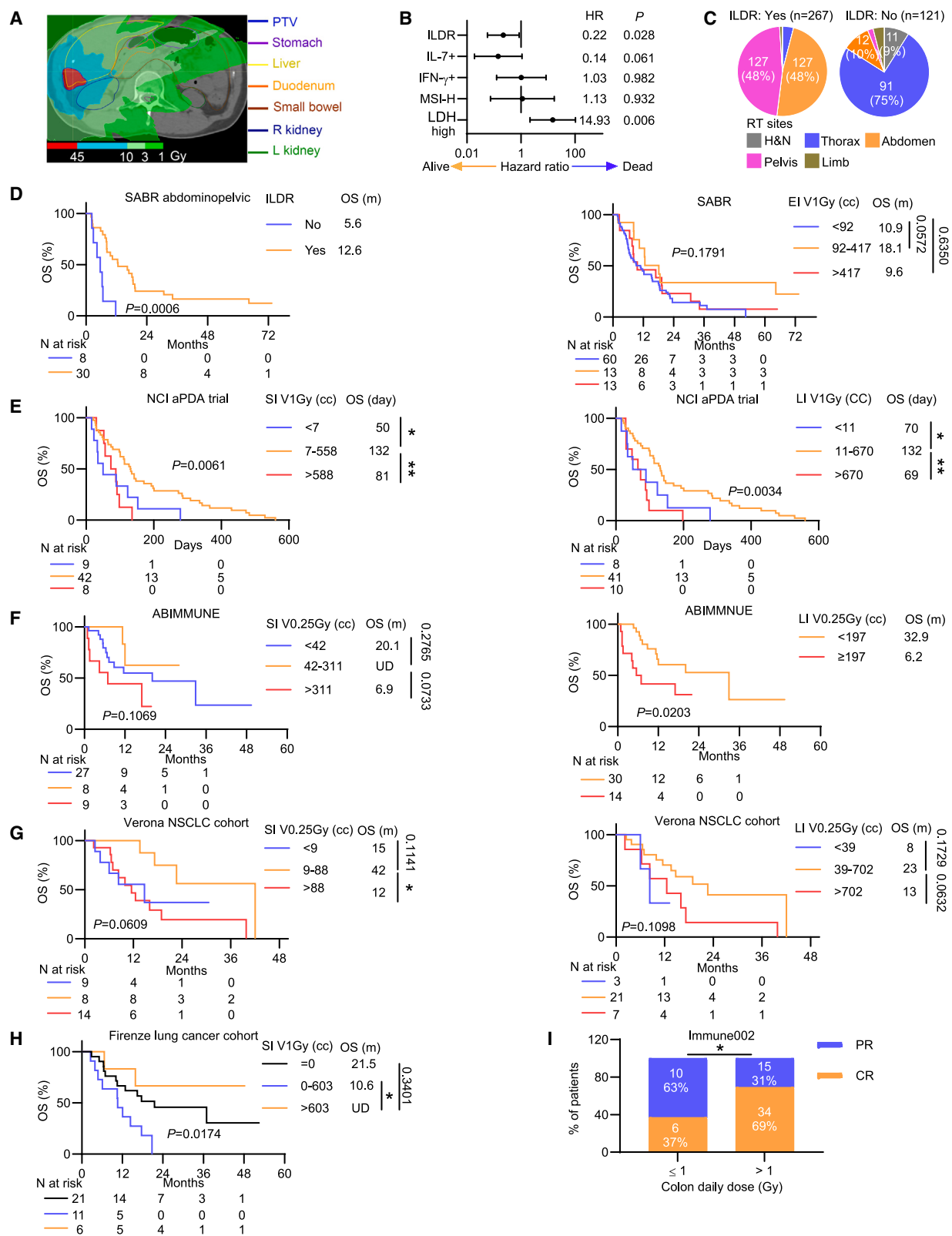
Immunostimulation by Christensenellaceae and immunosuppression by Bacteroidales in ILDR

We selected 8 patient stool samples to perform fecal microbial transplantation (FMT) by gavage into mice that were then subcutaneously inoculated with MC38 and treated by ILDR+ α PD-L1 (Figure 4A). This combination treatment was efficient in mice colonized with fecal samples from 4 out of 8 donors (Figure 4E, $p = 0.0007$ in pooled favorable FMT; Figure 4F, $p = 0.9736$ in pooled deleterious FMT). The immunogenic and tolerogenic donor groups tended to display distinct taxonomic compositions

OS12 Rs and OS12 NRs. Cytokines with statistical significance in concentration are indicated with an asterisk. Kaplan-Meier survival plots in patients with or without detectable serum IL-7 or IFN- γ at first visit (V1) (right). The hazard ratio (HR) of death is shown.

(B) Study of the functional pathways encoded by the gut microbiota in SABR according to serum cytokine profiling. Differential abundance analysis performed for the fecal microbiome of patients with IL-7 > 0 pg/mL versus IL-7 = 0 pg/mL, or IFN- γ > 0 pg/mL versus IFN- γ = 0 pg/mL at V1. Idem for the differential abundance analysis of gene-encoded metabolic pathways computed from the metagenome. Bars show standardized mean differences (SMD).

Statistical analysis: Mann-Whitney U test without p value correction (A, left and B) and log rank test (A, right). p values < 0.05 were considered significant.



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(ANOSIM $p = 0.053$, PERMANOVA $p = 0.08$, Figure S3D) with species clustering according to donor-specific traits (Figure S3D). Notably, a few species of the SABR response-associated *Christensenellaceae* (*Christensenella hongkongensis* SGB14143, *Luoshenia tenuis* SGB7265, and *Gehongia tenuis* SGB82545) were only found in the immunogenic group (Figure S3E). The prevalence of these species appeared higher in patients compared with HVs (Figure S3F).⁴⁶ Thus, stool composition at baseline impacts therapeutic success.

We next assessed fluctuations in indigenous bacterial species in murine feces during the combinatorial regimen. 16S rRNA amplicon sequencing, followed by principal-component analysis (PCA) of longitudinal beta-diversities comparing V2 (post- α PD-L1 initiation) and V4 (post-ILDR+ICB) (Figure S4A), as well as between V2 and V5 (post-ILDR+4 injections of α PD-L1) (Figure S4B), revealed significant differences (ANOSIM $p = 0.32$, PERMANOVA $p = 0.02$ for V2 vs. V4; ANOSIM $p = 0.11$, PERMANOVA $p = 0.0008$ for V2 vs. V5). Moreover, the PCoA was different between the two groups ([1 Gy ILDR+ α PD-L1] versus [4 Gy ILDR+ α PD-L1]) at V4 (ANOSIM $p = 0.006$, PERMANOVA $p = 0.003$) but not at V5. At V5, the PCA was significantly different between 1 Gy ILDR+ α PD-L1 versus 1 Gy ILDR+isotype control Abs (ANOSIM $p = 0.005$). The addition of 1 Gy, but not 4 Gy, ILDR to α PD-L1 favored the growth of immunogenic species such as the xylanolytic *Lactrimispora aerotolerans*^{55,56} and *Odoribacter splanchnicus*, which protects mice against colitis and CRC⁵⁷ and extends longevity⁵⁸ (Figure S4C), and *Ruminococcus flavefaciens*⁵⁹ (Figure S4D). Conversely, 4 Gy ILDR expanded tolerogenic taxa, particularly the BA deconjugating species *Muribaculum intestinale*⁶⁰ and *Culicoidibacter larvae* (Figure S4E). The relative abundance of *Christensenellaceae* exhibited see-sawing fluctuations in mice treated with α PD-L1 (not with the isotype control Ab) alone or together with ILDR (Figure S4F), in line with the capacity of ICB to induce commensal translocation.⁶¹ qPCRs confirmed that 1 Gy ILDR increased the relative abundance of *E. rectale* and *R. bromii*, but not that of the NR-related immunosuppressive *Enterocloster clostridioformis* (Figures S4G and S4H). Of note, 1 Gy ILDR resulted in the upregulation of antimicrobial peptides such as *Reg3g* and *Defa5* mRNAs in ileal tissues *in vivo* (Figure S4I), and mouse crypt stem cell-derived 3D organoids (Figure S4J).

Out of seven *Christensenellaceae* strains, only *C. minuta* 601 significantly boosted the anti-cancer effects of ILDR+ α PD-L1

(Figures 4G–4I). Oral gavage of *C. minuta* 601 did not alter tumor growth kinetics in response to 1 Gy ILDR alone (Figure 4G), but significantly ($p = 0.0473$) prolonged survival after treatment with 1 Gy ILDR+ α PD-L1 (Figure 4H). Another strain of *C. minuta* (spp-2) mediated non-significant beneficial effects. Instead, other species, such as *O. splanchnicus*, *B. fragilis*, or *B. thetaio-taomicron* decreased the ILDR effect (Figure 4I). Microbes from the *Bacteroidales* order, reportedly attenuate radiotherapy-induced DNA damage by supplying nucleotides for DNA repair.³⁴ Similarly, patients with higher abundance of *Parabacteroides distasonis* tended to exhibit shorter OS in the SABR trial (Figure S5A, $p = 0.06$), correlating with metabolites of the guanosine biosynthesis pathway but anticorrelating with purine-pyrimidine degradation, as reported (Figure S5B).³⁴ *P. distasonis* was undetectable by qPCR in the stools of C57BL/6J mice (Figure S5C). Gavage with *P. distasonis* resulted in qPCR-validated colonization (Figure S5C) and failed to impact tumor growth in untreated mice (Figure S5C) but reduced the effect of 1 Gy ILDR+ α PD-L1 (Figures 4I and S5C, $p = 0.03$), while increasing plasma deoxyguanosine concentrations (Figure S5D). These results indicate that the presence of NR-related commensals compromises the therapeutic effect of ILDR+ α PD-L1, contrasting with the positive impact of *C. minuta*.

1 Gy ILDR enhanced α PD-L1 effects by eliciting gut emigration of PD-L1 expressing intestinal dendritic cells and effector CD8⁺ T cells

Depletion of CD8⁺, but not CD4⁺ T cells abrogated the synergistic anti-tumor effects of 1 Gy ILDR+ α PD-L1 (Figure 5A). IL-7, the OS12 R-related cytokine known to maintain lymphocyte survival and to block TGF- β immunosuppressive effects,^{62,63} was undetectable in mouse plasma samples after ILDR+ α PD-L1. *In vitro* irradiation of murine ileal organoids stimulated IL-7 secretion by 48 h in a dose-dependent manner (Figure S5E). In contrast, in the context of PD-L1 blockade, ileal tissues from mice subjected to 4 Gy ILDR+ α PD-L1 tended to display lower *Il7* transcripts compared to those from mice treated with α PD-L1 alone (Figure S5F). Blockade of IL-7R with neutralizing Abs failed to interfere with the curative effects of ILDR+ α PD-L1 (Figure S5G). We immunophenotyped tumor-infiltrating lymphocytes (TILs) from mice treated with isotype or α PD-L1 Ab with or without 1 or 4 Gy ILDR at day 11 (Figures S6A and S6B). Both 1 and

Figure 3. Intestinal low-dose irradiation (ILDR) is associated with clinical benefit and long-term survival in eight independent cohorts

- (A) Representative dosimetric image of ILDR in a liver metastasis (planning treatment volume [PTV] in blue) bearing patient undergoing SABR.
(B) Multivariate analysis of prognosis factors that are independently associated with the death hazard ratio (HR) in the translational part of SABR trial ($N = 32$). Data represent the mean $\pm 95\%$ confidence intervals.
(C) Enumeration and proportions of different radiotherapy (RT) sites with or without off-target ILDR among patients enrolled in 8 independent clinical trials, including trials abbreviated as SABR, ABIMMUNE (NCT03212469), NCI aPDA (NCT02311361), Verona NSCLC cohort, Firenze lung cancer cohort, ARTIC (DRKS00032390), Immune002 (NCT05188716), and BISQUIT (NCT03870607).
(D) Kaplan-Meier survival curves of SABR patients with RT delivered to abdominopelvic metastases with or without ILDR and effects of ILDR volumes when evaluated as the entire intestine (EI) receiving 1 Gy (V1Gy) in the whole cohort of SABR patients. m, months; OS, overall survival.
(E) Kaplan-Meier survival curves of patients with different volumes of V1Gy delivered to the small intestine (SI) or large intestine (LI) in the NCI aPDA trial.
(F) Kaplan-Meier survival curves of patients with different V0.25Gy onto the SI or LI in the ABIMMUNE trial. UD, undefined.
(G) Kaplan-Meier survival curves of patients with different V0.25Gy onto SI or LI in the Verona cohort.
(H) Kaplan-Meier survival curves of patients with different V1Gy onto the SI in the Firenze lung cancer cohort.
(I) The percentages of partial (PR) and complete (CR) responses among cervical cancer patients in the Immune002 trial receiving daily ≤ 1 or >1 Gy off-target irradiation on the colon are annotated.

Statistical analysis: Cox proportional hazard regression analysis (B), log rank test (D–H), and Fisher's exact test (I). p values < 0.05 were considered significant.

* $p < 0.05$; ** $p < 0.01$.

See also Figure S2 and Tables S1, S2–S4, S5, and, S6.

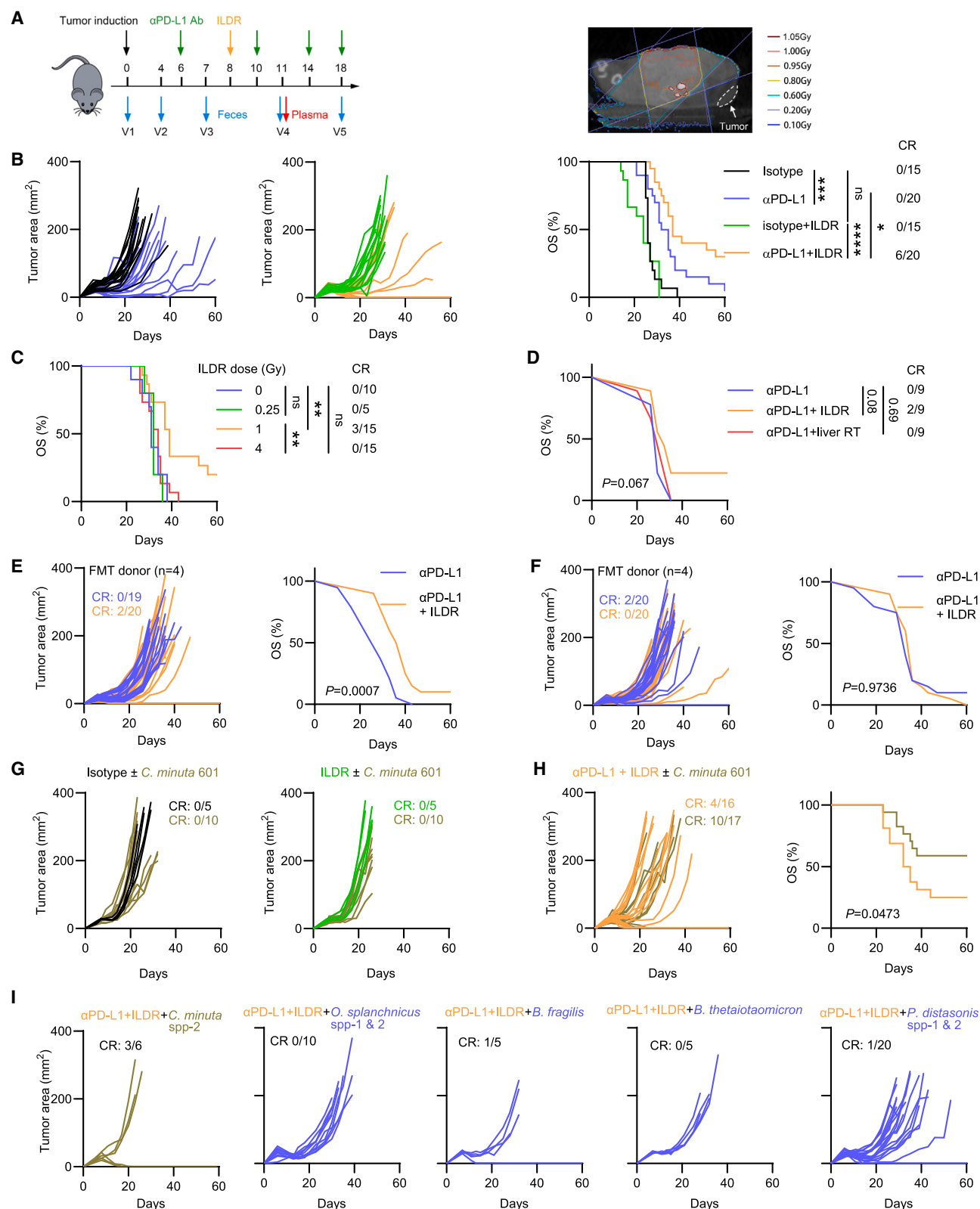


Figure 4. The synergistic anti-cancer effects of ILDR and PD-L1 blockade involve distinct gut commensals in tumor-bearing mice

(A) Experimental setting for the combination of ILDR and anti-PD-L1 Abs in MC38 colon cancer-bearing mice and timelines of sample harvesting. MC38 bearing C57BL/6J mice were subjected to intraperitoneal (i.p.) injections of isotype control Ab versus α PD-L1 Ab and abdominal ILDR (with increasing dosing of 0.25, 1, or

(legend continued on next page)

4 Gy ILDR increased infiltration of MC38 tumors by CD8⁺ T cells (Figure 5B), regulatory T cells (Tregs, Figure S6C) and CD8⁺CD44⁺PD-1⁺CXCR6⁺ memory T cells (Figures S6D and S6E). In contrast, 4 Gy, but not 1 Gy, increased the frequency of CD8⁺ T cells expressing the exhaustion marker TOX (Figures 5C and S6F). Moreover, the systemic recirculation of enterotrophic Tregs and RORγt⁺ Tregs (Tr17) cells was reduced after 1 Gy (but not as much after 4 Gy) ILDR, causing an increased CD8/Treg ratio of intestinal T cell emigrants in TILs (Figures 5D, 5E, and S6G).

Optimal ICB outcome requires intestinal dendritic cells (DCs) emigration to mesenteric lymph nodes (mLNs).^{36,37,61} RT may enhance DC antigen-presentation, but direct high-dose (10–30 Gy) irradiation of DCs impairs their function.^{64,65} We observed that the percentage of mature dendritic cells enriched in immunoregulatory molecules (mregDCs, defined as CD45⁺lineage[−]CD11c⁺MHC-II⁺PD-L1⁺CD40⁺)⁶⁶ among total DCs or CD45⁺ cells increased in mLNs and tumor-draining lymph nodes (TdLNs) after ILDR (Figures 5F and S7). We also injected the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) into mLNs to track the translocation of activated DCs from mLNs to TdLNs, which was stimulated by ILDR (Figures 5G and 5H). Gavage with *C. minuta* further increased the accumulation of DC expressing CCR7 into TdLNs (Figure 5I).

We performed targeted metabolomics of plasma collected at day 11 (3 days after ILDR) from mice treated with ILDR+αPD-L1 (Figure 5J). As compared to αPD-L1 alone, 1 Gy ILDR+αPD-L1 enhanced alpha-tocopherol and cholesterol, but reduced 2-(OH)-3-methylbutyric and isobutyric acids (Figure 5J). These data are in line with the reported inhibitory effects of butyrate on RT-mediated anti-tumor immune responses,^{67,68} as well as the stimulatory effect of cholesterol and alpha-tocopherol on the DC-T cell crosstalk.^{40,69} Next, using a DC-T cell co-culture system,⁷⁰ we examined the dose (0–6 Gy) effects of ionizing radiation (IR) on the capacity of DCs pulsed with ovalbumin (OVA) protein to stimulate IL-2 secretion by the OVA_{257–264} peptide-specific, H-2K^b-restricted T cell hybridoma (B3Z) (Figure 5K). Of note, IR doses that appeared optimal in ILDR (1–3 Gy) significantly augmented antigen presentation by DCs, but doses outside of this range (0.25, 4, or 6 Gy) failed to do so (Figure 5L). Next, we used the same experimental system to screen 96 metabolites including bile acids, sterols, and carnitines. Strikingly,

cholesterol and several bile acids (tested at 5 μg/mL) augmented antigen presentation by DCs (Figure 5M). Altogether, these *in vitro* findings suggest that ILDR mediates its CD8⁺ T cell effector-stimulatory effects via metabolic effects on DCs.

ILDR reduced lactate accumulation while increasing immunogenic indoles and secondary bile acids in plasma

Plasma metabolomics of tumor bearing mice treated by ILDR+αPD-L1 revealed a drop in immunosuppressive lactic acid-related metabolites⁴¹ (Figures 6A and 6B). In our mouse colony, the relative abundance and prevalence of *Christensenella* species were variable (Figure 6C), and *C. massiliensis* correlated with the secondary bile acids deoxycholic acid (DCA) and tauro-lithocholic acid (Figure 6D), as reported.⁷¹ Using our DC-T cell co-culture system,⁷⁰ we observed that two secondary BAs, DCA and ursodeoxycholic acid (UDCA) (tested at 10 μg/mL), significantly increased DC antigen presentation (Figure 6E). Importantly, in patients, we observed a 30% fecal prevalence of *C. minuta*, that was stable over the course of the SABR protocol (Figure 6F), marginally correlating with a secondary BA taurodeoxycholic acid (Figure 6G), as well as with several known immunostimulatory metabolites,⁷² namely, arginine, indole-3 propionate and methyl indole-3 acetate.

Taken together, these findings suggest that 1 Gy ILDR boosts the immunostimulatory effects of PD-L1 blockade by favoring the exodus of CCR7⁺PD-L1⁺ mregDC from mLNs to TdLNs in the context of gut residence of *Christensenella* species and metabolic rewiring, limiting immunosuppressive anaerobic glycolysis in favor of immunostimulatory secondary BAs and indoles.

Phase 2 ILDR-01 study in cancer patients who are refractory to PD-1 blockade

We initiated a phase 2 trial enrolling patients with advanced metastatic solid tumors who had progressed after first- or second-line immunotherapy (Figure 7A). Here, we report on the clinical results of the first 10 patients receiving 1 Gy ILDR (Figure 7B), but no cancer-targeted RT, followed by at least 1 cycle of ICB (Table S7). Most of them displayed grade 1–2 toxicities during treatment (Table S7). One patient among the 10 experienced grade 3 liver toxicity and recovered after ceasing ICB. The

4 Gy). The image on the right illustrates a representative abdominal tomography showing 1 Gy ILDR by three 220 kV X-ray beams with isodose curves on the transverse plane. The tumor (indicated in dashed white circle) was spared from all beams.

(B) Tumor growth kinetics for each individual mouse in the control and αPD-L1 Ab groups, individual growth curves for ILDR and ILDR+αPD-L1 groups (left), Kaplan-Meier survival curves (right) and numbers of complete tumor eradications are shown. Each experiment comprised 5–6 mice/group and was repeated at least 3 times, the graphs gathering all experiments.

(C) Kaplan-Meier survival curves and log rank test of mice subjected to αPD-L1 Ab in combination with a range dose of ILDR (0–4 Gy). *N* = 5–15 mice/group from at least two independent experiments.

(D) Kaplan-Meier survival curves and log rank test of mice subjected to αPD-L1 Ab in combination with either 1 Gy ILDR or liver RT. *N* = 9 mice/group from two independent experiments.

(E and F) Mice were subjected to FMT derived from 8 donors, prior to αPD-L1 with or without ILDR. Mice were grouped according to their benefit from the additional ILDR; with benefit (*N* = 4 donors, E) vs. without (*N* = 4 donors, F). Tumor growth kinetics from two independent experiments is shown on the left with the Kaplan-Meier survival of curves of all mice on the right.

(G and H) Mice subjected to oral gavage of NaCl or *C. minuta* 601 (on day-4, 7, 9, and 11) were treated with isotype Ab, isotype Ab+1 Gy ILDR (G) or αPD-L1 Ab+ILDR (H). Tumor growth kinetics and survival curves are shown. Several independent experiments containing 5–6 mice/group were concatenated.

(I) Mice were colonized with a variety of bacteria species prior to αPD-L1+ILDR treatment, including *C. minuta* (spp-2), *O. spianchnicus* (two different strains, spp-1 and -2), *B. fragilis*, *B. thetaiotaomicron*, and *P. distasonis* (spp-1 and -2). Several independent experiments with 5–6 mice/group were concatenated. Statistical analysis: log rank test (B–F and H); *p* values < 0.05 were considered significant. ns: *p* > 0.05, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. See also Figures S3–S5.

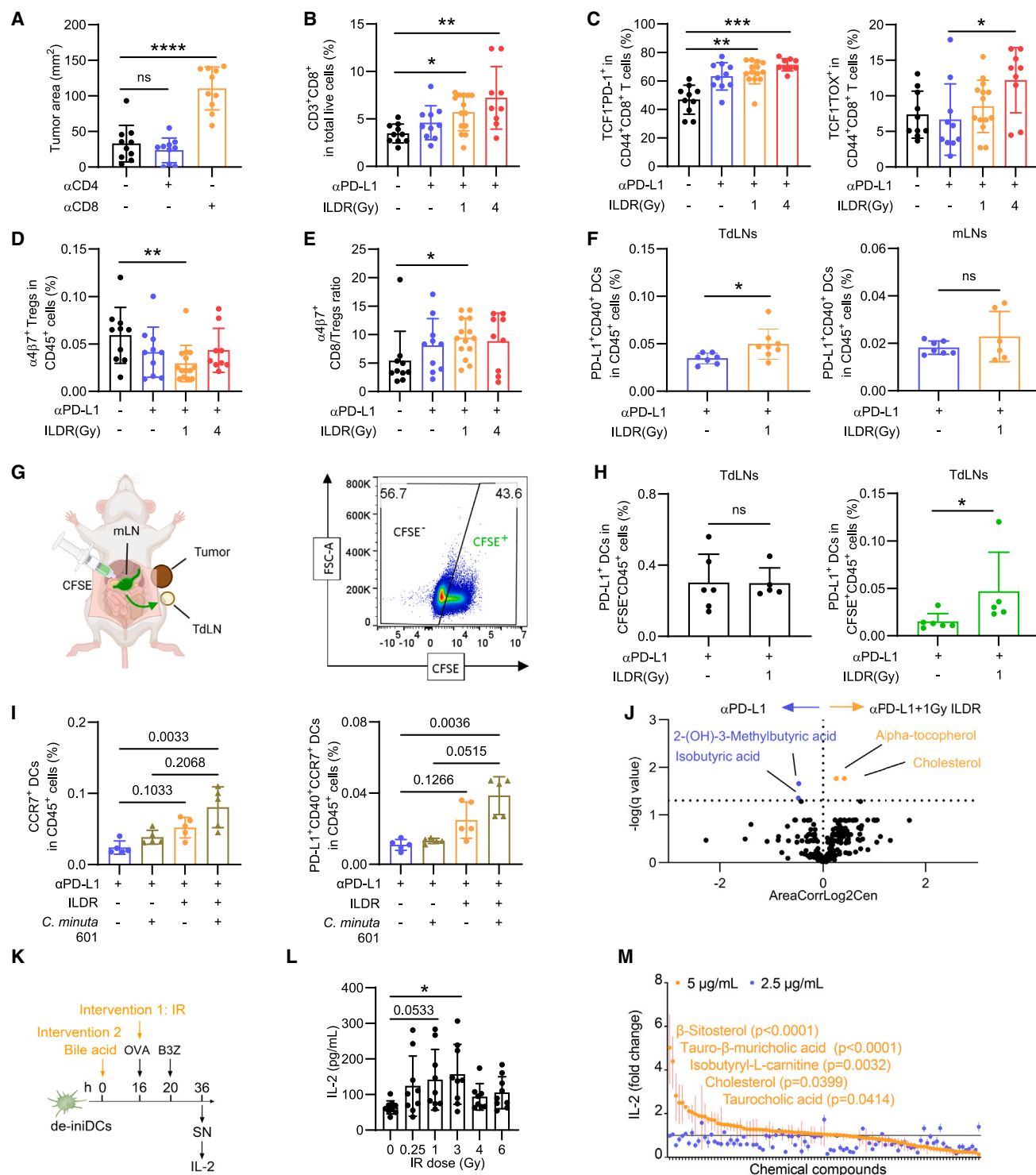


Figure 5. mregDC-CD8⁺ T cell-dependent effects of the combinatorial regimen of ILDR with αPD-L1

(A) Isotype control, anti-CD4 or anti-CD8 antibodies (Abs) were injected i.p. into MC38-bearing mice undergoing 1 Gy ILDR+αPD-L1 Ab following the same experimental setting as in Figure 4A. Tumor sizes at day 21 are depicted. *N* = 10 mice/group from two independent experiments. (B–E) MC38-bearing mice were subjected to isotype Ab or αPD-L1 with 0–4 Gy ILDR. Tumors were harvested at day 11 and analyzed by flow cytometry for immune cell profiling. The percentages of CD3⁺CD8⁺ T cells within total live cells are shown (B). (C) The percentages of TCF1⁺PD-1⁺ and TCF1⁺TOX⁺ cells (see Figure S6F for representative dot plots) among CD44⁺CD8⁺ T cells. (D) The percentages of α4β7⁺ Tregs (CD3⁺CD4⁺CD25⁺Foxp3⁺) within CD45⁺ cells and their ratio over enterotrophic α4β7⁺CD8⁺ T cells are depicted (E). *N* = 9–14 mice/group from two independent experiments.

(legend continued on next page)

waterfall plot of the best tumor response (Figure 7C), spider plot depicting the evolution of the target lesion (Figure 7D) and swimmer plot of each patient outcome (Figure 7E) revealed 2 cases of PR, 5 cases of stable disease (SD) and 3 cases of progressive disease (PD). Of note, the first PR case started after 2 cycles (1.5 months) of ICB and has been lasting over 8 months. The second PR case was initially classified as SD at 1.5 months and gradually transformed to PR at 6 months. A representative magnetic resonance imaging of a scalp lesion before and after 3 months of combinatorial therapy is shown in Figure 7F. The thoracic CT scan of a second patient presenting regression of thoracic lymph node metastasis is depicted in Figure 7G. We conclude that ILDR may circumvent ICB resistance in patients with advanced cancer.

DISCUSSION

Our findings indicate that incidental or deliberate ILDR has a beneficial role in tumor control by ICB. It is crucial to note that the survival-extending effect of ILDR occurs exclusively at a low-dose range (≥ 1 and ≤ 3 Gy) with a bell shape curve for the appropriate irradiation volume. This dose effect of RT on normal tissues is reminiscent of a study reporting that only an intermediate RT dose induced a secretory pathway in lung resident *Scgb1a1*⁺ club cells enhancing the therapeutic efficacy of ICB against NSCLC.⁷³

Patients who have simultaneous abdominal and extra-abdominal lesions might gain more pronounced survival benefits from SABR when IR is delivered to abdominal tumor deposits and hence causes ILDR as an “accidental” byproduct. When treating abdominal metastases, one might consider deliberately delivering ILDR within the (likely non-toxic) ≥ 1 and ≤ 3 Gy range. ILDR could also be useful for patients with exclusively extra-abdominal lesions. Apparently, microbial factors affect the efficacy of ILDR. This is suggested by the predominant gut dysbiosis in SABR OS24 NRs, as well as the detrimental effect of *P. distasonis* and bacteria from the *Bacteroidales* order on the efficacy of ILDR+ α PD-L1 in mice. In contrast, *Christensenellaceae* (such as *C. minuta*, specifically strain 601, and potentially *C. massiliensis*) were associated with OR24 Rs, boosted the trafficking of mregDCs to TdLNs, and improved the anti-cancer efficacy of

ILDR+ α PD-L1. Hence, analyses of the gut microbiota prior to treatment initiation might help to select patients that are likely to benefit from this combination treatment. Patients suffering from dysbiosis might require additional interventions such as FMT.^{22–24}

PD-1/PD-L1 blockade alone can drive the proliferation and differentiation of stem cell-like tissue resident CD8⁺ T lymphocytes, resulting in the expansion of exhausted progenitor cells in mice.^{74,75} Addition of ILDR to α PD-L1 resulted in a substantial increase in CD8⁺ T cell infiltration of tumors. ILDR increased CXCR6 expression in TCF1⁺CD44⁺PD-1⁺ exhausted effector T cells, which is critical for their survival in the tumor microenvironment.⁷⁶ One Gy (but not 4 Gy) ILDR also reduced the unwanted homing of enterotropic Tregs to tumor lesions. In contrast, 4 Gy (but not 1 Gy) ILDR increased T cell exhaustion defined by the upregulation of TOX in TCF1⁺CD44⁺PD-1⁺CD8 TILs. Consistently, high-dose (16.0–18.4 Gy) IR of gut and kidney has been reported to augment TOX expression in TILs, accompanied by rerouting of proinflammatory monocytes from the tumor to colon.⁷⁷ Lastly, 1 Gy (but not 4 Gy) ILDR activated the DC-T cell cross-talk *in vitro*. ILDR induced the trafficking of CCR7⁺PD-L1⁺ DC from mLNs to TdLNs, which was exacerbated by *C. minuta*, and unleashed by PD-L1 blockade. The immunostimulatory effects of such a migration have been demonstrated in the context of CTLA-4+PD-1 co-blockade.⁶¹

The data presented here suggest that ILDR mediates distal immunostimulation, most probably through microbiota-triggered shifts in systemic metabolism. ILDR affects cholesterol (and consequently BA) metabolism, that in turn increases DC antigen presenting function. Cholesterol accumulation in DCs may enhance antigen presentation and T priming.^{78,79} In contrast, NRs patients harboring increased abundance of lipids, fatty acids and their oxidized products may be resistant to the immunostimulatory effects of ILDR on DC, as previously shown.^{67,80–82} Similarly butyrate produced by the intestinal ecosystem reportedly inhibits the stimulator of IFN gene pathway in DC, dampening RT-elicited CTL-mediated immunity.^{67,68} Similarly, ILDR+PD-L1 blockade reduces lactic acid derivatives that mediate resistance to chemoradiotherapy.⁴¹ *C. minuta* expresses high levels of bile salt hydrolases, resulting in the stimulation of fatty acid oxidation while inhibiting triglyceride and hepatic fatty acid production, in accordance with the

(F) The percentage of mature dendritic cells enriched in immunoregulatory molecules (mregDCs) in CD45⁺ cells in tumor draining lymph nodes (TdLNs, left) or mesenteric LNs (mLNs, right) collected from mice 3 days after α PD-L1 with or without 1 Gy ILDR are represented in bar graphs, each dot representing one mouse. The results of a typical experiment containing 7–8 mice/group are depicted.

(G and H) At 2 days after α PD-L1 $\pm$ ILDR, mice were subjected to intra-mLN injection of carboxyfluorescein succinimidyl ester (CFSE) (G). Twenty-four hours later mice were sacrificed and the percentages of PD-L1⁺ DCs in CFSE⁺ or CFSE⁺CD45⁺ cells were assessed. A representative dot plot showing CFSE⁺ cells gated on CD45⁺ cells is shown on the right. (H) The percentage of PD-L1⁺ DC in CFSE⁺ or CFSE⁺CD45⁺ cells is depicted. $N = 5$ –6 from a typical experiment.

(I) Mice were treated with α PD-L1 $\pm$ ILDR $\pm$ oral gavage of *C. minuta* 601. The infiltration of CCR7⁺ or PD-L1⁺CD40⁺CCR7⁺ DC (gated on CD45⁺ cells) in TdLNs was evaluated and compared. Results of a typical experiment containing 5–6 mice/group are depicted.

(J) Volcano plot comparing α PD-L1 vs. 1 Gy ILDR+ α PD-L1 subgroups of mice with respect to the abundance of plasma metabolites at day 11. $N = 10$ mice/group in 2 independent experiments pooled together.

(K–M) *In vitro* screening of metabolites on a DC-T cell assay. Differentiated de-iniDCs were treated by IR (0–6 Gy) (intervention 1) or a metabolite library (at 2.5 μ g or 5 μ g/mL) for 16 h (intervention 2), then pulsed with OVA protein (1 mg/mL) for 4 h and incubated with B3Z hybridoma cells for 16 h (K), when IL-2 in the supernatant was measured by ELISA (L and M). The results of two independent experiments containing 9 biological replicates/group are depicted in (L). IL-2 concentrations in intervention 2 were normalized to the mean values of dimethyl sulfoxide-treated controls. Data represent means $\pm$ SD in one representative experiment out of three.

Data are shown as mean $\pm$ SD in (A–F, H, I, L, and M). Statistical analysis: one-way ANOVA (B), Kruskal-Wallis H test (A, C–E, I, and L), Mann-Whitney U test (F, H, and M), and unpaired multiple t test with the two-stage Benjamini, Krieger and Yekutieli procedure to control FDR ($q < 0.05$) (J). p values < 0.05 were considered significant. ns: $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

See also Figures S5–S7.

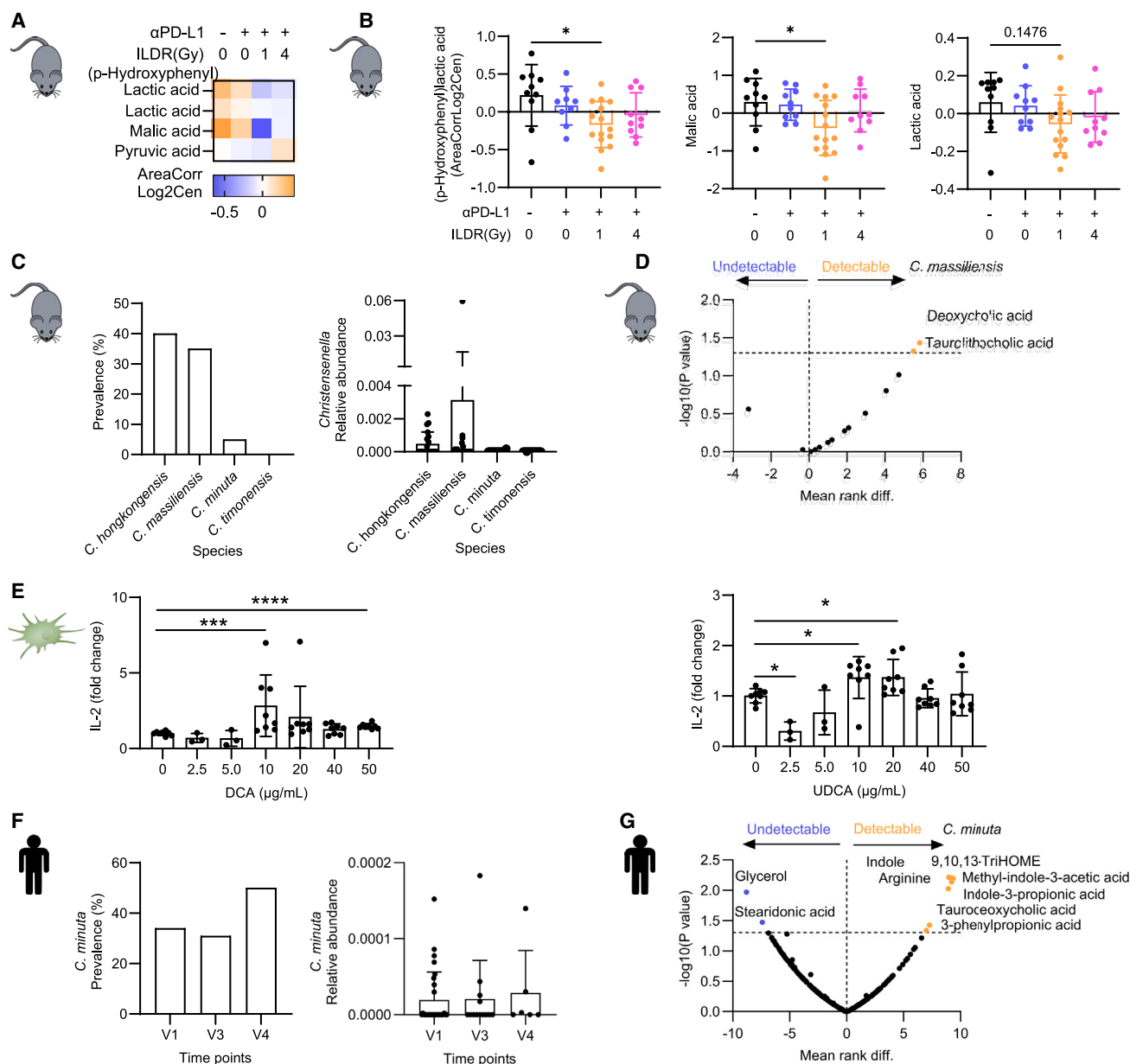


Figure 6. *C. minuta*-associated metabolites increase DC cross-presentation

(A and B) Plasma metabolomics focusing on anaerobic glycolysis metabolites. Lactic acid and afferent metabolites in the plasma of mice subjected to isotype control Ab or 1 or 4 Gy ILDR+αPD-L1 (from the experiment in Figure 4B). Heatmap (A) and dot plots with comparisons (B). $N = 9-14$ mice/group from two independent experiments.

(C and D) Prevalence (C, left) and relative abundance (C, right) of *Christensenella* species in fecal samples from mice in Figure 4B ($n = 20$ from at least 3 independent experiments). (D) Volcano plot aligning the significant FC differences and p values between subgroups of mice with ($n = 7$) or without ($n = 13$) detectable *C. massiliensis* for the relative abundances of biliary salts at day 11.

(E) Impact of deoxycholic acid (DCA) or ursodeoxycholic acid (UDCA) on OVA presentation by DCs. IL-2 concentrations in experimental groups were normalized to the mean values of the control group (0 μg/mL) for fold changes. $n = 3-8$ from two independent experiments.

(F and G) The prevalence (F, left) and relative abundance (F, right) of *C. minuta* in human fecal samples collected from SABR patients at multiple time points (see Figure 1, $n = 29$ at first visit [V1], $n = 13$ at V2, $n = 6$ at V3). (G) Volcano plot of serum metabolites in SABR patients with ($n = 11$) or without ($n = 21$) detectable fecal *C. minuta*.

Metabolites with statistical significance are depicted in orange/blue in (D) and (G).

Data are shown as mean $\pm$ SD in (B, C [right], E, and F [right]). Statistical analysis: one-way ANOVA (B, left and middle), Kruskal-Wallis H test (B, right), multiple Mann-Whitney U tests without p value correction (D and G), and Mann-Whitney U test (E). p values < 0.05 were considered significant. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

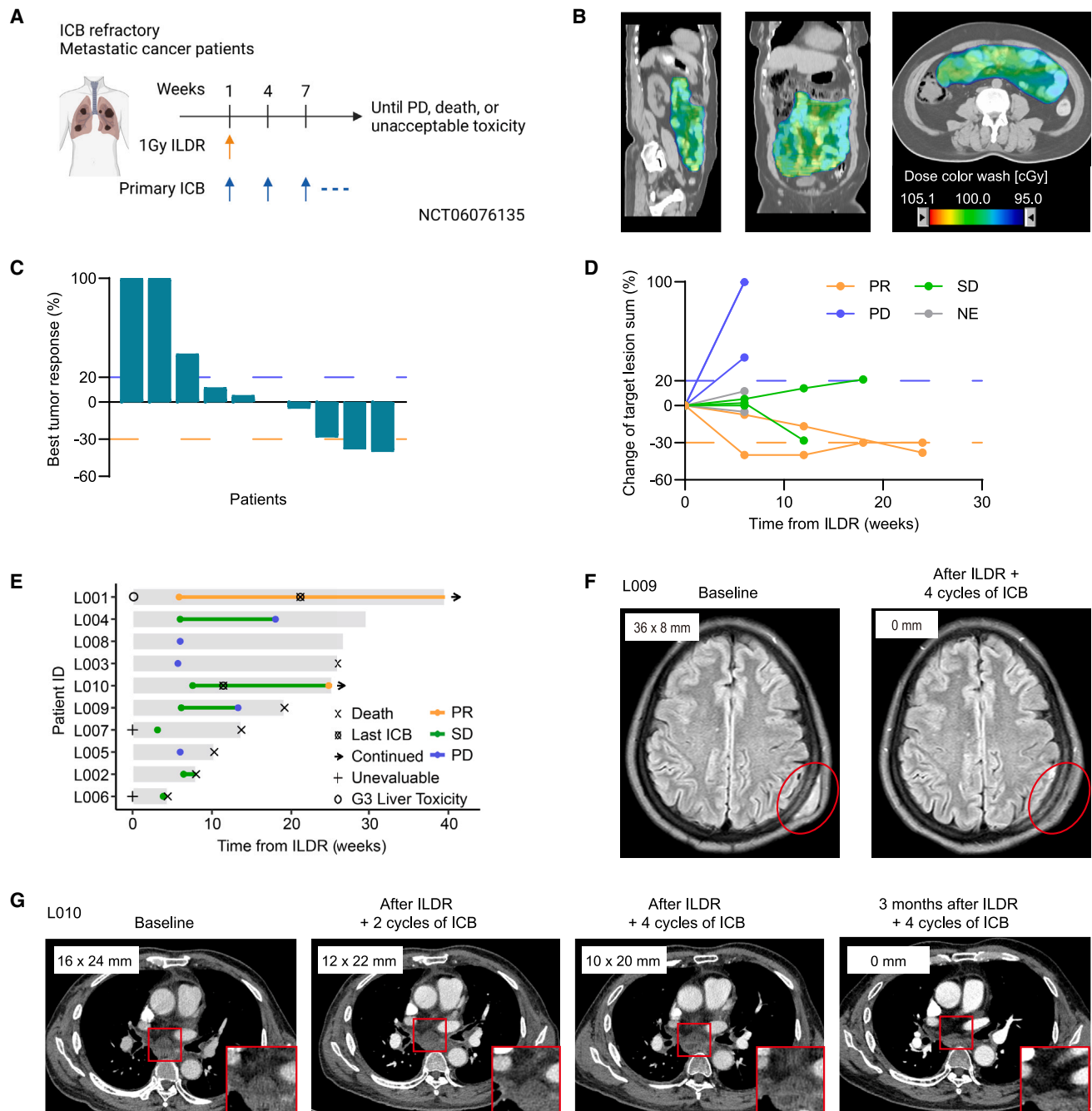


Figure 7. 1 Gy ILDR circumvented resistance to ICB in a phase 2 prospective trial

(A) Design of the phase 2 prospective trial combining ILDR and ICB.

(B) Representative dosimetry of 1 Gy ILDR delivered onto the jejunum and ileum but sparing the liver.

(C–E) Evaluation of the clinical efficacy on the first 10 enrolled patients. Waterfall plot (C), spider plot (D) of the best tumor response (by RECIST 1.0) and swimmer plot (E) of patient responses over time after ILDR+ICB. PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable.

(F) Representative MRI images (T_2 weighted spectral pre-saturation inversion recovery combined with fluid-attenuated inversion recovery) of a scalp metastasis (red circle) in patient L009, taken before and after ILDR+4 cycles of PD-1 blockade.

(G) Representative CT images of a mediastinal lymph node metastasis (red square) in patient L010, taken before and after ILDR+ICB.

See also Table S7.

metabolic profile of Rs in SABR.⁷¹ Altogether, ILDR might enhance the anti-tumor CD8⁺ T cell response by improving the metabolic fitness of intestinal DCs.

Aside from the taxonomic composition, other surrogate markers of gut dysbiosis were found associated with resistance to ILDR+ α PD-L1. Hence, a high ratio IFN- γ over IL-7 in serum was correlated with unfavorable outcome in patients. However, neutralization of IL-7R failed to compromise the outcome of ILDR+ α PD-L1 in mice, perhaps reflecting interspecies differences in this pathway.⁸³ The association between high serum levels of IFN- γ and dismal prognosis appears counterintuitive and may reflect the context-dependent role of IFN- γ in anti-tumor immunosurveillance.^{62,74,84–87}

Limitations of the study

There are several limitations to this work. The analysis of the impact of ILDR relied on 8 retrospective cohorts affected by heterogeneity (cancer type, tumor burden, age/sex, tumor mutational profile, prior lines of therapies, and PD-L1 expression). The optimal oncological indication and dosimetry of ILDR could not be determined due to the small sample size in these eight independent retrospective studies, calling for larger prospective validation trials. The precise mechanisms underlying the abscopal effects of IR-associated ILDR in conjunction with chemo- or immunotherapy require further exploration and it remains to be determined which segment of the small or large intestine should be targeted for optimizing ILDR. All in all, this study suggests that ILDR+ α PD-L1 warrants further investigations on both the translational and clinical levels.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Dr Eric Deutsch (eric.deutsch@gustaveroussy.fr).

Materials availability

We will share all unique/stable reagents generated in this study upon request from the [lead contact](#) with a completed materials transfer agreement (MTA).

Data and code availability

Human metagenomic sequencing data are available in the European Nucleotide Archive (ENA): [PRJEB85676](https://www.ebi.ac.uk/ena/record/PRJEB85676). 16S rRNA sequencing data of murine fecal samples are available in GenBank: [PRJNA1058463](https://www.ncbi.nlm.nih.gov/genbank/PRJNA1058463). Human and mouse metabolomic data are available in Mendeley Data: <https://doi.org/10.17632/3rmd9h4hgx.1>. All code used for the data analysis are listed in the [key resources table](#). Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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DECLARATION OF INTERESTS

L. Zitvogel founded EverImmune and is the SAB president of EverImmune. L. Zitvogel had grant support from Daichi Sankyo, Kaleido, 9 m, and Pileje. G.K. has been holding research contracts with Daiichi Sankyo, Eleor, Kaleido, Lytx Pharma, PharmaMar, Osasuna Therapeutics, Samsara Therapeutics, Sanofi, Sutro, Tollys, and Vascage. G.K. is on the Board of Directors of the Bristol

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STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat IgG2b isotype control Ab (clone LTF-2)	Bio X Cell	RRID:AB_1107780
Rat IgG2a isotype control Ab (clone 2A3)	Bio X Cell	RRID:AB_1107769
Anti-mouse PD-L1 Ab (clone 10F.9G2)	Bio X Cell	RRID:AB_10949073
Anti-mouse CD4 Ab (clone GK1.5)	Bio X Cell	RRID:AB_1107636
Anti-mouse CD8 Ab (clone 53-6.7)	Bio X Cell	RRID:AB_1107673
Anti-mouse IL7R (clone A7R34)	Bio X Cell	RRID:AB_1107590
anti-mouse CD16/32 Ab (clone 93)	Thermo Fisher Scientific	RRID:AB_467134
BV421 anti-mouse CXCR6 (SA051D1)	BioLegend	RRID:AB_2616760
BV421 anti-mouse CD274 (MIH5)	BD Bioscience	RRID:AB_2738911
Vioblu anti-mouse CD4 (REA604)	Miltenyi Biotec	RRID:AB_2733943
BV605 anti-mouse CCR7 (4B12)	BioLegend	RRID:AB_2715777
BV610 anti-mouse CD44 (IM7)	BioLegend	RRID:AB_2562451
BV650 anti-mouse CD25 (PC61)	BioLegend	RRID:AB_11125760
FITC anti-mouse CD8 α (53-6.7)	BioLegend	RRID:AB_312744
FITC anti-mouse CD11c (N418)	BioLegend	RRID:AB_313775
PerCP anti-mouse CD19 (6D5)	BioLegend	RRID:AB_2072926
PerCP anti-mouse NK-1.1 (PK136)	BioLegend	RRID:AB_2132707
PerCP anti-mouse CD3 ϵ (145-2C11)	BioLegend	RRID:AB_893317
PE anti-mouse TCF-1 (C63D9)	Cell Signaling Technology	RRID:AB_2798483
PE anti-mouse α 4 β 7 (REA457)	Miltenyi Biotec	RRID:AB_2811331
PE anti-mouse I-A/I-E (M5/114.15.2)	BioLegend	RRID:AB_313323
PE-Cy7 anti-mouse PD-1 (RMP1-30)	BioLegend	RRID:AB_572016
PE-Cy7 anti-mouse ROR γ t (B2D)	Thermo Fisher Scientific	RRID:AB_2784671
APC anti-mouse TOX (REA473)	Miltenyi Biotec	RRID:AB_2751485
APC anti-mouse Foxp3 (FJK-16s)	Thermo Fisher Scientific	RRID:AB_469457
APC anti-mouse CD40 (3/23)	BioLegend	RRID:AB_1134072
APC anti-mouse CD11c (N418)	BioLegend	RRID:AB_313779
Alexa Fluor 700 anti-mouse CD45 (30-F11)	BioLegend	RRID:AB_493715
APC Vio770 anti-mouse CD3 ϵ (REA606)	Miltenyi Biotec	RRID:AB_2733115
APC-Cy7 anti-mouse Ly-6G (1A8)	BioLegend	RRID:AB_10640819
APC-Cy7 anti-mouse Siglec-F (S17007L)	BioLegend	RRID:AB_2904295
Purified anti-mouse IL-2	BioLegend	RRID:AB_315292
Biotin anti-mouse IL-2	BioLegend	RRID:AB_315298
Bacterial and virus strains		
<i>P. distasonis</i> spp1	This paper	NA
<i>P. distasonis</i> spp2	This paper	NA
<i>C. minuta</i> 601	This paper	NA
<i>C. minuta</i> spp2	This paper	NA
<i>O. splanchnicus</i> spp1	This paper	NA
<i>O. splanchnicus</i> spp2	This paper	NA
<i>B. fragilis</i>	This paper	NA
<i>B. thetaiotaomicron</i>	This paper	NA

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Fecal samples from patients enrolled in the SABR trial	This paper	NA
Serum samples from patients enrolled in the SABR trial	This paper	NA
Fecal samples from the ONCOBIOTICS study	This paper	NA
Chemicals, peptides, and recombinant proteins		
Zombie Aqua Fixable Viability dye	BioLegend	423102
RPMI 1640 Medium	Life Technologies SAS	11875093
FBS	Dutscher	S1900-500
Penicillin/streptomycin	Life Technologies SAS	15140122
Sodium pyruvate	Life Technologies SAS	11360039
Nonessential amino acids	Life Technologies SAS	11140-035
TrypLE	Life Technologies SAS	12604013
Matrigel	Corning	734-1101
Advanced DMEM/F12	Life Technologies SAS	12634028
L-glutamine	Life Technologies SAS	25030081
HEPES	Life Technologies SAS	15630056
N2 supplement	Life Technologies SAS	17502048
B27 supplement	Life Technologies SAS	17504044
mEGF	Invitrogen	PGM8043
mNoggin	PeproTech	250-38
N-acetylcysteine	Sigma-Aldrich	A9165-5G
Primocin	InvivoGen	ant-pm-1
Y27362	Tocris	129830-38-2
Zeocin	InvivoGen	ant-zn-1
RBC lysis buffer	BioLegend	420301
Collagenase IV	Thermo Fisher Scientific	17104019
Hyaluronidase	Sigma-Aldrich	H3506
DNase I	Millipore	260913
EDTA (0.5 M), pH 8.0, RNase-free	Thermo Fisher Scientific	10135423
Sodium Azide	Sigma-Aldrich	S2002
albumin from chicken egg white (OVA)	Sigma-Aldrich	A5503
Recombinant murine GM-CSF	PeproTech	315-03
β -mercaptoethanol	Sigma-Aldrich	M3148
Dexamethasone	Sigma-Aldrich	D0700000
Doxycycline Hyclate	Sigma-Aldrich	D3000000
Recombinant murine IL-2_ELISA standard	BioLegend	575409
ELISA Coating Buffer (5x)	BioLegend	421701
HRP-Avidin	BioLegend	405103
1-Step Ultra TMB-ELISA Substrate Solution	Life Technologies	34028
Bile Acid/Carnitine/Sterol Metabolite Library of Standards	Sigma-Aldrich	BACSMLS-1EA
Medroxyprogesterone acetate (MPA, M) pellets	Innovative Research of America	NP-161
7,12-dimethylbenz[a]anthracene (DMBA)	Sigma-Aldrich	D3254
Corn oil	MilliporeSigma	C8267
Noradrenaline	Kind gift of the pharmacy of Gustave Roussy Institute	
Ursodeoxycholic acid	Thermo Fisher Scientific	208590250
Deoxycholic acid	Sigma-Aldrich	30960-25G

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Bio-Plex Pro Human Cytokine 27-plex Assay	Bio-Rad	M500KCAF0Y
QIAamp Fast DNA Stool Mini Kit	QIAGEN	51604
RNeasy Mini Kit	QIAGEN	74104
RNase-Free DNase Set	QIAGEN	79254
Transcriptase inverse SuperScript™ III	Thermo Fisher Scientific	18080093
TaqMan Gene Expression MasterMix	Thermo Fisher Scientific	4370074
SYBR PowerUp MasterMix	Thermo Fisher Scientific	A25741
eBioscience™ Foxp3/Transcription Factor Staining Buffer Set	Thermo Fisher Scientific	00-5523-00
CellTrace™ CFSE Cell Proliferation Kit	Thermo Fisher Scientific	C34554
Deposited data		
Metagenomics data from the SABR trial	This study	European Nucleotide Archive: PRJEB85676
Metabolomic data from the SABR trial	This study	Mendeley Data: https://doi.org/10.17632/3rmd9h4hgx.1
Metabolomic data from the murine experiment	This study	Mendeley Data: https://doi.org/10.17632/3rmd9h4hgx.1
16S rRNA-Seq of murine fecal samples	This study	GenBank: PRJNA1058463
Experimental models: Cell lines		
MC38	Sigma-Aldrich	SCC172
Inducible immortalized dendritic cells	Richter, C.	NA
B3Z hybridoma T cells	Sebastian Amigorena	NA
Murine ileal organoid from C57BL/6J	This paper	NA
Experimental models: Organisms/strains		
C57BL/6J	Envigo	C57BL/6J0laHsd
C57BL/6J	The Jackson Laboratory	RRID: IMSR_JAX:000664
Oligonucleotides		
TaqMan probes for <i>Ii7</i> (Mm01295803_m1)	Thermo Fisher Scientific	4453320
TaqMan probes for <i>Reg3g</i> (Mm00441127_m1)	Thermo Fisher Scientific	4453320
TaqMan probes for <i>Defa5</i> (Mm00651548_g1)	Thermo Fisher Scientific	4453320
TaqMan probes for <i>Ppia</i> (Mm02342430_g1)	Thermo Fisher Scientific	4453320
qPCR primer forward for <i>R. bromii</i> : GGCGGCYTRCTGGGCTTT	Thermo Fisher Scientific	10629186
qPCR primer reverse for <i>R. bromii</i> : CCAGGTGGATWACTTATTGTGTAA	Thermo Fisher Scientific	10629186
qPCR primer forward for <i>E. rectale</i> : GCGGTRCGGCAAGTCTGA	Thermo Fisher Scientific	10629186
qPCR primer reverse for <i>E. rectale</i> : CCTCCGACACTCTAGTMCGAC	Thermo Fisher Scientific	10629186
qPCR primer forward for <i>P. distasonis</i> : GGACACGTCCCGCACTTAT	Thermo Fisher Scientific	10629186
qPCR primer reverse for <i>P. distasonis</i> : TTCTGAGAGGAAGGTCCCCC	Thermo Fisher Scientific	10629186
qPCR primer forward for <i>E. clostridioforme</i> : CCGCATGGCAGTGTGTGAAA	Thermo Fisher Scientific	10629186
qPCR primer reverse for <i>E. clostridioforme</i> : CTGCTGATAGAGCTTTACATA	Thermo Fisher Scientific	10629186
qPCR primer forward for <i>C. minuta</i> : GTAATACGTAGGGAGCAAGC	Thermo Fisher Scientific	10629186

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
qPCR primer reverse for <i>C. minuta</i> : CCCTCTCCTGTACTCAAGTC	Thermo Fisher Scientific	10629186
qPCR primer forward for universal bacteria: ATGGCTGTCGTCAGCT	Thermo Fisher Scientific	10629186
qPCR primer reverse for universal bacteria: ACGGGCGGTGTGTAC	Thermo Fisher Scientific	10629186
Software and algorithms		
R studio	https://posit.co/download/rstudio-desktop/	NA
Prism	GraphPad	RRID:SCR_002798
IBM SPSS Statistics	IBM	RRID:SCR_016479
Flowjo	BD Bioscience	RRID:SCR_008520
Python v.3.8.2	https://www.python.org/	NA
MetaPhlAn	https://github.com/biobakery/MetaPhlAn	NA
HUMANn 3	https://hutterhower.sph.harvard.edu/humann/	NA
ape_5.7-1	https://github.com/emmanuelparadis/ape.git	NA
DiscrMiner_0.1-29	https://cran.r-project.org/src/contrib/Archive/DiscrMiner/	NA
vegan_2.6-4	https://github.com/vegandevs/vegan.git	NA
Sci-Kit learn package v1.0.1	https://scikit-learn.org/stable/	NA
MMUPHin	https://www.bioconductor.org/packages/release/bioc/html/MMUPHin.html	NA
Seaborn v0.11.2	https://seaborn.pydata.org/whatsnew/v0.11.2.html	NA
The GRMeta R package for metabolomics analysis	@Github/Kroemerlab/GRMeta	NA
MuriPlan	XStrahl	NA
Other		
SARRP	XStrahl	NA
XRad 320	X-ray Precision	NA

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Clinical studies and participants

The SABR trial (NCT02992912)

Patients with advanced colorectal cancer (CRC), renal cell carcinoma, non-small cell lung carcinoma (NSCLC) and sarcomas were enrolled in an international, multi-center single-arm phase 2 trial (sponsor protocol N: CSET 2335/2015, EudraCT N: 2015-005464-42). Atezolizumab (anti-PD-L1 Ab, 1200 mg every 3 weeks until progression or unmanageable toxicity) was delivered in combination with concurrent SABR (recommended dose of 45Gy in 3 fractions, adapted upon normal tissue tolerance constraint) at the 2nd cycle of atezolizumab. The primary endpoint of this trial was to determine the 1-year progression-free survival (PFS) rate under combined SABR and anti-PDL1 atezolizumab therapy using RECIST v1.1. The secondary and exploratory endpoints include PFS rate, objective response rate, toxicity, the functional imaging changes and systemic immunologic anti-tumor response. The first patient was enrolled on 15 November 2016, while the last patient was enrolled on 11 March 2021. In total, 138 patients were recruited into this study. The clinical data of the colorectal cancer cohort have been published.⁴² Thirty-two participants were enrolled in the translational part of this study. Participant details are summarized in Table S1. A total of 48 fecal samples were collected from these patients at baseline V1 (n = 29), after SABR V3 (n = 13) or at 6 months since the 1st cycle of α PD-L1 V4 (n = 6). Serum samples were collected at V1 (n = 25), before the 2nd cycle of α PD-L1 V2 (n = 30), V3 (n = 25) and V4 (n = 13). Translational analyses were performed in the context of ONCOBIOTICS study (see below for details) in Gustave Roussy. The dose of intestine (duodenum, small intestine, and colon) was extracted from the Dose-volume histogram (DVH) of SABR treatment plan of these patients.

The only notable (albeit not significant) difference of clinical characteristic between patients who survived >24 months (OS24) and then were considered as responders (Rs) and non-responders (NRs) resided in tumor microsatellite instability (MSI), MSI-High tumors being detected in 3/4 evaluated OS24 Rs versus 1/10 evaluated NRs (5 patients being lost during follow-up) (Table S1). OS24 Rs presented more toxicities (Table S1). Patients who did not survive at 12 months (OS12 NRs) differed from OS12 Rs by the more frequent colorectal origin of their primary cancer (92% vs 40%), and high (>224.5 U/L) lactate dehydrogenase (LDH) serum levels (83% vs 20%) (Table S1). No significant difference was found in other routine clinical and biological features (e.g., circulating

lymphocytes) between Rs and NRs (Table S1). Patients receiving incidental ILDR did not differ in their clinical characteristics from patients not receiving ILDR, except that the ILDR group was more frequently subjected to liver SABR (62% vs 32%, $P=0.0276$, Table S1), and had a higher tumor burden ($9.0\pm3.2\text{cc}$ vs $6.4\pm3.3\text{cc}$, $P=0.0398$) and larger planning treatment volume (PTV) ($155\pm145\text{cc}$ vs $42\pm43\text{cc}$, $P=0.0004$). Of note, most MSI-H patients (4/5, 80%) had ILDR (Table S1).

The ONCOBIOTICS study

Translational analyses (and stool sample collections) were performed (and preplanned) under the study ONCOBIOTICS (sponsor protocol N: CSET 2017/2619, ID800 RCB N: 2017-A02010-53) according to the ethical guidelines and approval of the local ethical committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (CCPPRB) of the Kremlin Bicêtre Hospital). As specified in page 2 of the protocol (attached PDF), ONCOBIOTICS started in 2015 but has been registered on [ClinicalTrials.gov](https://clinicaltrials.gov) with the NCT number: NCT04567446 only in 2018.

The ABIMMUNE trial (NCT03212469)

This phase I/II single arm multicenter study (sponsor protocol N: CSET 2016/2454, EudraCT N: 2016-003293-40) included a two-step safety run (dual combination of durvalumab [D]+SABR then triple combination of D+ tremelimumab [T]+SABR). D (1500 mg IV every 4 weeks (Q4W), for up to 13 dose) +- T (75mg/Q4W for up to 4 doses). To evaluate the safety of D +/- T in combination with SABR, then modified following manufacturer instruction to 300 mg IV single dose at C1D1) and SABR (9 Gy x 3 fractions from C1D15) were administered in patients with advanced squamous cell carcinomas (head and neck, lung, esophagus, cervix, vagina, vulva or anus). Each study step was, started after dose-limiting toxicity (DLT, based on CTCAE-V4, within 8 weeks from C1D1) assessment of combination-related toxicities. The phase I part aimed evaluated the safety of D +/- T in combination with SABR. The phase II part assessed the abscopal response rate following treatment with D+T in combination with SABR. The first patient was enrolled on June 20, 2017, while the last patients were enrolled on November 3, 2022. The data from 44 patients enrolled at our center were included in this analysis. Participant details are summarized in Table S5.

The ARTIC trial (ID: DRKS00032390)

In this multi-center study, patients with solid tumors (mostly lung or kidney) who received combined RT-Immune checkpoint inhibitor (ICI) treatment were included for analysis. All patients had advanced diseases and were subjected to ICI and RT to progressive or symptomatic sites, leaving at least one non-irradiated lesion. Of the 98 eligible patients, 16 receiving fractionated RT had incidental intestinal irradiation. These patients together with the additional 16 patients without intestinal irradiation (matched for gender, age, primary disease, and RT dose) were included in this report (N = 32). Intestinal doses were collected from the DVHs of these patients. Data for intestinal segments were not available. Participant details are summarized in Table S5.

The Immune002 trial (NCT05188716)

A total of 64 patients with locoregionally advanced cervical cancer were recruited to a prospective observation trial approved by the Ethics Committee of the Cancer Hospital of Shantou University Medical College (sponsor protocol N: 2021016). Written informed consent was obtained from all enrolled patients. Participant details are summarized in Table S6. Patients were subjected to external beam radiotherapy (EBRT) in combination with concurrent platinum-based chemotherapy, followed by brachytherapy. Tumor response was evaluated immediately after EBRT according to the RECIST 1.0 criteria as previously described.⁸⁸ Local tumor response was assessed immediately after EBRT through the internal speculum and bimanual examinations. All patients had objective response and were segregated into two groups: patients with complete regression (CR) versus patients with partial response (PR). The dosimetric data of small intestine, colon and rectum were collected from these patients. Of note, patients with or without CR had similar tumor coverage in RT dosimetry (D95, 46Gy vs 46Gy, $P=0.9608$).

The BISQUIT trial (NCT03870607)

The Brazilian cohort was composed of a subset of 33 patients who were enrolled to an open label randomized phase II trial that evaluates the efficacy of pre and probiotics administration during chemoradiation for localized anal carcinoma ($\geq$ T2N0M0). Participant details are summarized in Table S6. Consenting patients were randomized 1:1 to receive pre and probiotics (Simbioflora) starting one week prior to and throughout chemoradiation until response evaluation at 8 weeks post treatment or conventional chemoradiation. Patients with active infection requiring antibiotics or those who received antibiotics within 30 days of enrollment were excluded. Tumor response in these patients at 6 months was assessed according to the RECIST criteria. The dosimetry of the colon was collected. Of note, patients with or without CR displayed comparable tumor coverage in RT dosimetry (D95, 53Gy vs 55Gy, $P=0.3657$).

The National Cancer Institute (NCI) advanced pancreatic ductal adenocarcinoma (aPDA) trial (NCT02311361)

Results from this trial have been published.⁸⁹ This phase I, two-cohort, four-arm, open-label clinical trial with a two-arm extension cohort was conducted at the NCI between March 2015 and August 2019. The trial consisted of two cohorts. One cohort received durvalumab 10 mg/kg every 2 weeks, which was continued for a total of 12 months or until progressive disease (PD) was confirmed by immune-related response criteria. Patients in the other cohort received durvalumab 1,500 mg and tremelimumab 75 mg every 4 weeks for 4 doses, followed by 1,500 mg durvalumab monthly for an additional 8 months or until PD was confirmed by immune-related response criteria. Each cohort included two separate radiation dose levels (DLs), which differed by the quantity of radiation (1 x 8Gy or 5 x 5 Gy). Imaging studies were performed by contrast-enhanced CT scans every 8 weeks. Participant details are summarized in Table S3.

The Verona lung cancer study

This study, promoted by the University of Verona and approved by the Ethical Committee (sponsor protocol N: 1839CESC), investigates the immune microenvironment in patients with advanced non-small cell lung cancer (NSCLC) treated with immunotherapy, either alone or with chemotherapy/radiotherapy. Data from 31 patients (participant details in Table S4) were reviewed from December

2019 to December 2023, including their simulation CTs, radiotherapy (RT) plans, and Dose Volume Histograms (DVHs). In cases where bowel regions weren't separately contoured, new Regions of Interest (ROIs) were contoured by a Radiation Oncologist (V.D.) and a Radiation Therapist (A.G.). Post-RT, DVHs were retrospectively analyzed, focusing on parameters like mean doses for small and large bowel (Dmean) and volumes (V0.25Gy to V4Gy). No alterations in treatment quality were made, and all information was saved in the institutional database. Pembrolizumab was the most common immunotherapy used, and 61% of patients showed no molecular alterations.

The Firenze lung cancer study

Data from 38 patients treated with immunotherapy at Radiotherapy Unit of University of Florence were reviewed. Selection criteria were: diagnosis of lung cancer (SCLC or NSCLC), immunotherapy and sequential or concomitant radiotherapy. 18 of them underwent abdominal radiotherapy combined with immunotherapy; 20 underwent extra-abdominal radiotherapy combined with immunotherapy. Primary disease, age, gender, ECOG, prior lines of therapy, number of metastases at the time of immune-radiotherapy were collected. Radiotherapy sites, radiotherapy schemes as well as immunotherapy scheme and timing of combination (concomitant/sequential) were registered, tumor objective response, progression free survival and overall survival were collected. For patient treated with abdominal radiotherapy dosimetric data were collected: bowel, small intestine and large intestine Dmean, V0.25Gy, V1Gy, V4Gy (cc) were registered. Participant details are summarized in [Table S5](#).

The ILDR-01 prospective study (NCT06076135)

This is a researcher-initiated, two-stage, prospective phase II trial entitled "Intestinal low-dose radiotherapy combined with immunotherapy in immune-resistant metastatic solid tumors (ILDR-01)". The trial was approved by the Ethics Committee of the Cancer Hospital of Shantou University Medical College, China (sponsor protocol N: 2023109), and initiated in December 2023. Patients with metastatic cancer that have progressed in response to anti-PD-1 therapy are enrolled in this study and subjected to ILDR targeting the jejunum and ileum (1Gy/1F in stage I for 16 patients and 2-3Gy/2-3F in stage II for 44 additional patients) followed by the primary ICB reintroduction one day later. The primary immunotherapy regimen that each patient has developed resistance to is re-administered every 3 weeks until disease progression, patient death, or unacceptable toxicity. Primary endpoints include disease control rate, objective response rate by RECIST/iRECIST criteria, time to disease progression after ILDR (PFS2) and abscopal response. The first patient was enrolled in December 2023. Clinical results of the first 10 patients consecutively recruited with follow-up until September 30, 2024 are reported in the current study. Written informed consent was obtained from all enrolled patients. Participant details are summarized in [Table S7](#).

Animals

Female, 6-7 weeks old, wild type (WT) C57BL/6J mice were purchased from Envigo (France) and housed in specific pathogen-free conditions at the animal facility of Gustave Roussy in France. In indicated experiment, WT C57BL/6J female mice of 6-7 weeks of age were obtained from The Jackson Laboratory and housed under standard conditions at Weill Cornell Medical College (New York, NY) in line with institutional requirements, on a 12 light/12 dark light cycle, 20-25°C, and 30% humidity. All animal experiments were performed in compliance with French and European laws and regulations and approved by the local institutional animal ethic board and French Ministère de la Recherche (permission number: 2023_047_43699).

Cell lines

MC38 cell line (syngeneic from C57BL/6J mice) were purchased from Sigma and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 IU/mL penicillin/streptomycin, 1mM sodium pyruvate and nonessential amino acids. Mycoplasma test in cell culture medium was routinely performed and all tests were negative. Cells were used within passage number 8.

The inducible immortalized dendritic cells (iniDCs) were a gift from Cornelia Richter and colleagues and cultured in basic DC medium: RPMI-1640 medium supplemented with 10% decompartmented FBS, 1 mM sodium pyruvate, 10 mM HEPES, 1X penicillin/streptomycin, 50 μ M β -mercaptoethanol and 10 ng/mL recombinant GM-CSF. IniDCs cells were immortalized under the induction of Dex/Dox (dexamethasone at 100 nM + doxycycline hyclate at 2 μ M) and differentiated into immature DCs ("de-iniDCs") upon removal of Dex/Dox.

The B3Z hybridoma T cells were kindly provided by Sebastian Amigorena and maintained with DC medium supplemented with β -mercaptoethanol (50 μ M).

Murine intestinal organoids

Crypt-derived organoids were generated from ileal tissues of 8-10 weeks old C57BL/6J female mice as described previously.³⁶ They were cultured in advanced DMEM/F12 medium supplemented with 100 U/mL penicillin/streptomycin, 2mM L-glutamine, 10 mM HEPES, 1X N2 supplement, 1X B27 supplement, 50 ng/mL mEGF, 100 mg/mL mNoggin (PetroTech), N-acetylcysteine (Sigma-Aldrich) (reagents from Invitrogen unless otherwise indicated) and 10% conditional medium of HA-R-Spondin1-Fc 293T Cells.

METHOD DETAILS

Metagenomic sequencing of fecal samples

Fecal samples were prospectively collected from patients enrolled in the ONCOBIOTICS ancillary protocol (Sponsor Protocol N: CSET 2017/2619, ID800 RCB N: 2017-A02010-53) at multiple time points (V1, before the 1st dose of α PD-L1 Ab; V3, after

SABR and before the 3rd dose of α PD-L1; V4, at 6 months post the 1st dose of α PD-L1) following the International Human Microbiome Standards (IHMS) guidelines. The stools were subjected to total DNA extraction and sequencing with Ion Torrent technology following MetaGenoPolis (INRAE), France as previously reported.^{29,37,43} Microbiome taxonomic and functional composition of each sample were estimated via MetaPhlAn 4 (run with Jan21 database) and HUMAnN 3 profiling tools, respectively.

16S rRNA-Seq and analysis of murine fecal samples

Fecal samples were collected from individual mice enrolled in indicated experiments and subjected to DNA extraction using the QIAamp Fast DNA Stool Mini Kit (QIAGEN) according to the manufacture's protocol. Fecal DNA samples were sequenced at IHU Méditerranée Infection, targeting the V3–V4 regions of the 16S rRNA gene with Illumina MiSeq technology.

Raw FASTQ files were analyzed with DADA2 pipeline v.1.14 for quality check and filtering on a Workstation Fujitsu Celsius R940 (Fujitsu, Tokyo, Japan). Raw reads (12460891 in total, on average 78866 per sample) were filtered (2377392 in total, on average 15047 per sample) and 2849 Amplicon Sequence Variants (ASV) were found with a 99% coverage for all samples. Bioinformatic and statistical analyses on recognized ASV were performed with Python v.3.8.2 (<https://www.python.org/>). Each ASV sequence underwent a nucleotide Blast using the National Center for Biotechnology Information (NCBI) Blast software (ncbi-blast-2.3.0) and the latest NCBI 16 S Microbial Database (<ftp://ftp.ncbi.nlm.nih.gov/blast/db/>). After blasting, the 2849 ASVs were merged into 392 species. Of them, 142 species with $\geq 20\%$ prevalence were considered for subsequent statistical analyses.

Metabolomic analyses of serum/plasma samples

Samples were treated following the protocol described previously (Claudia Grajeda-Iglesias, et al. Aging (Albany NY) 2021 Mar 2;13(5):6375-6405) to extract metabolites. Briefly, 50 μ L of serum were vortexed with 500 μ L of ice-cold extraction mixture (MeOH/water, 9/1, -20°C , with a cocktail of internal standards) during 5 minutes, and then centrifuged (10 min at 15000 g, 4°C). Several fractions were split to be analyzed by different liquid and gas chromatographies coupled with mass spectrometers (LC/MS and GC/MS) as previously described.⁹⁰ Widely targeted analysis by GC-MS/MS was performed on a 7890A gas chromatography (Agilent Technologies) coupled to a QQQ (triple quadrupole) 7000C (Agilent Technologies). Polyamines, short chain fatty acids and bile acids analyses were performed by LC-MS/MS with a 1290 UHPLC (Ultra-High Performance Liquid Chromatography) (Agilent Technologies) coupled to a QQQ 6470 (Agilent Technologies). Widely pseudo-targeted analysis by UHPLC-HRMS (Ultra-High Performance Liquid Chromatography – High Resolution Mass Spectrometer) was performed on a Dionex U3000 / Orbitrap q-Exactive (Thermo Fisher Scientific) coupling. All targeted treated data were merged and cleaned with a dedicated R (version 4.0) package (@Github/Kroemerlab/GRMeta).

Multiplex enzyme-linked immunosorbent assay (ELISA)

Serum samples were prospectively collected from patients enrolled in the SABR trial at multiple time points (V1, before the 1st dose of α PD-L1 Ab; V2, before the 2nd dose of α PD-L1 and SABR; V3, after SABR and before the 3rd dose of α PD-L1; V4, at 6 months post the 1st dose of α PD-L1). The concentration of 27 cytokines/growth factors (IL-1 β , IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, FGF basic, G-CSF, GM-CSF, IFN- γ , IP-10, MCP-1, MIP-1 α , PDGF, MIP-1 β , RANTES, TNF α , VEGF) in these samples was assessed using a Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad) according to the manufacturer's protocol. Briefly, 50 μ L of serum samples were incubated with magnetic capture beads for 30 minutes, washed then incubated with detection antibody for 30 minutes, wash again, incubated with streptavidin-PE prior to assessment on the Bio-Plex 200 reader (Bio-Rad).

Combination of intestinal low -dose irradiation (ILDR) and PD-L1 blockade in tumor models

A total of 1×10^6 MC38 cells in 100 μ L PBS was inoculated subcutaneously into the right flank of 8–10 weeks old C57BL/6J mice on day 0. Mice were subjected to intraperitoneal (i.p.) injection of isotype control (clone LTF-2, 10 μ g/g of mouse body weight) or α PD-L1 Ab (clone 10F.9G2, 10 μ g/g of mouse body weight) on day 6, 10, 14 and 18. Mice were also treated with mock or ILDR with the indicated dose on day 8. Mice were monitored for a minimum of 2 months since tumor inoculation for tumor growth kinetics, body weight and other adverse signs (e.g. bloody feces). In indicated experiment, fecal samples were collected from individual mice at day-1 before tumor inoculation (day0), day4, 7, 11 and 18, whereas plasma was obtained on day 11 via submandibular sampling.

ILDR in the mouse model

ILDR in the murine tumor model was performed using the Small Animal Radiation Research Platform (SARRP, XStrahl). Mice were anesthetized with 2.5% of isoflurane on supine position. They were first subjected to a whole-body CT scan using cone beam computed tomography following by three-dimensional reconstruction of these CT images on the Treatment planning system (MuriPlan). An ILDR treatment plan was designed to deliver the prescribed RT dose (0.25, 1 or 4Gy) to the intestinal zone while sparing the subcutaneous tumor on the flank. This was achieved by a 3-beam treatment plan at gantry angles of -135° , 70° and -10° , respectively with the isocenter placed on the center of the bowel area. The size of beam field was set at 14mm x 12mm with the moving variable collimator (MVC) encompassing the intestinal zone between liver and the bladder on coronal/sagittal plane. Tumors were spared from all beams with an approximate 5mm margin on the transverse plane. The out of field dose on the edge of these beams is $\leq 10\%$ as indicated by dosimetry calculation. ILDR was delivered using 220 kV X-ray at a dose rate of 3.021 Gy/min. Mice with mock irradiation were subjected to anesthesia for a similar period with the beam off.

Fecal microbiota transplant (FMT) in C57BL/6J mice

Mice were subjected to antibiotics (ATBs) treatment prior to FMT between Day-18 and Day-15, which consisted of ampicillin (final 1mg/mL), streptomycin (5mg/mL) and colistin (1mg/mL) in drinking water. Fecal samples collected from patients in the ONCOBIOTICS study were used for FMT. FMT was performed twice for each mouse on Day-14 and Day-10. As described before, frozen fecal samples were brought back to room temperature and resuspended in NaCl at a density of 0.1g/mL. Mice were placed in a new cage. A total of 200 μ L feces suspension were transferred by oral gavage into each ATB pre-treated recipient using animal feeding needles. In addition, another 100 μ L were applied on the fur.

Gut colonization of specific bacterial species in C57BL/6J mice

Bacteria strains used in this experiment were isolated from intestinal mucosa tissue or fecal samples obtained from patients via culturomics and stored in the bacterial biobank as previously described.³⁶ In total 8 strains were used, including *C. minuta* 601, *C. minuta* spp2, *O. splanchnicus* spp1 & spp2, *B. fragilis*, *B. thetaiotaomicron*, *P. distasonis* spp1 & spp2. They were grown on Columbia agar with 5% sheep blood plates in an anaerobic atmosphere created using anaerobic generators (Biomérieux) and harvested at exponential growth for their gut colonization in C57BL/6J mice. In indicated experiments, mice were oral gavage with 100 μ L of NaCl or suspension containing 10^8 bacteria (obtained using a fluorescence spectrophotometer at an optical density of 1 measured at a wavelength of 600 nm) on day 4, 7, 9 and 11 for *C. minuta* (both 601 and spp2), and on day 5 and 7 for other species. The efficacy of colonization was confirmed by qPCR performed on fecal DNA with specific primers 48 h after the first gavage.

Depletion of CD4⁺ or CD8⁺ T cells in mice

In indicated experiments, mice were subjected to i.p. injection of isotype control Ab (Rat IgG2b, clone LTF-2 50 μ g + Rat IgG2a, clone 2A3 50 μ g), α CD4 Ab (clone GK1.5, 100 μ g/mouse) or α CD8 Ab (clone 53-6.7, 100 μ g/mouse) on day 5, 7, 9 and 13. The efficacy of T cell depletion was validated by flow cytometry assessment on blood samples obtained from these mice on day 11 via submandibular sampling.

IL-7R neutralization in mice

In indicated experiments, mice were subjected to i.p. injection of isotype control Ab (Rat IgG2a, clone 2A3 200 μ g/mouse) or α IL7R Ab (clone A7R34, 200 μ g/mouse) on day 5, 7, 9 and 13.

Tracing migration of dendritic cells (DCs) from the gut to tumor-draining lymph nodes (TdLNs)

Evaluation of emigration of DC from mesenteric LNs (mLNs) to TdLNs has been previously described.³⁷ Briefly, at 48 hours after ILDR $\pm$ α PD-L1, MC38-bearing C57BL/6J mice were subjected to analgesics preparation by subcutaneous injection of Meloxicam (2.5mg/Kg of mouse body weight), and anesthesia by inhalation of 2.5% isoflurane. The abdominal area was disinfected with Iodine and sprayed with lidocaine. The abdominal wall and peritoneum were cut at the midline to access the mLNs. mLNs were exposed and gently mobilized on a sterile surgical gauze soaked in sterile saline. The two most prominent mLNs were injected with 5 μ L carboxy-fluorescein succinimidyl ester (CFSE) solution (CFSE stock diluted in PBS to a final concentration of 100 μ M) in a 30G insulin syringe. The intestine was then repositioned, and the abdominal wall was closed by a continuous stitch with 5-0 coated Vicryl plus suture on peritoneum and the muscle layer, and individual metal clips on the skin.

qPCR assessment of abundance of bacterial species in murine fecal samples

Fecal samples were obtained from mice in indicated experiments and subjected to DNA extraction using the QIAamp Fast DNA Stool Mini Kit (QIAGEN). The quality and quantity of DNA were measured by Nanodrop (Thermo Fisher Scientific). The abundance of indicated bacteria species in these samples was analyzed by qPCR using SYBR green and specific primer pairs (see [key resources table](#)). Reactions were run on ABI QuantStudio 5 Real-time PCR System using the standard condition (Thermo Fisher Scientific). Following the amplification, the threshold cycle values of targeted bacterial species and universal bacteria (reference for normalization) were recorded. The relative abundance of bacteria species was calculated using the Δ Ct method.

RT-qPCR assessment of gene expression in mouse ileal tissues

Ileal tissues were harvested from mice in indicated experiments and subjected to RNA extraction using the RNease mini kit with DNase treatment (both from QIAGEN) to remove potential contamination of genomic DNA. The quality and quantity of mRNA were measured by Nanodrop. RNA samples were then reverse transcribed and analyzed by RT-qPCR using TaqMan probes specific for targeted genes (Thermo Fisher Scientific). The following genes were analyzed in this study: *Ilf7* (Mm01295803_m1), *Reg3g* (Mm00441127_m1), *Defa5* (Mm00651548_g1) and *Ppia* (Mm02342430_g1).

Flow cytometry assessment

Tumor and blood samples were harvested from mice. Tumor were minced on the petri dish by scalpels and incubated in dissociation buffer (RPMI medium containing 2mg/mL collagenase IV, 0.2mg/mL Hyaluronidase and 150U/mL DNase I) at 37°C for 45 minutes, then filtered through 100 μ M cell strainer, incubated with 1X RBC lysis buffer (BioLegend) on ice for 5 minutes, then run through the 40 μ M cell strainer. Following washing, cells were resuspended in FACS buffer (1X PBS containing 2% FBS, 1mM EDTA and 0.1% sodium azide) at a density of 2×10^6 cells/100 μ L. These cells were first incubated with anti-mouse CD16/32 Ab (clone 93, Thermo

Fisher Scientific) and Zombie Aqua Fixable Viability dye (BioLegend) dye on ice for 10 minutes, then a mixture of antibodies targeting surface markers. Following washing, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to the manufacture's protocol. They were then stained with antibodies recognizing intracellular markers. Anti-mouse fluorescence-conjugated Abs (clone) used for phenotyping were purchased from BioLegend, Thermo Fisher Scientific, Miltenyi Biotec, Cell Signaling Technology and BD Bioscience as follows: BV421 CD274 (MIH5), BV421 CXCR6 (SA051D1), Vioblu CD4 (REA604), BV605 CCR7 (4B12), BV610 CD44 (IM7), BV650 CD25 (PC61), FITC CD8 α (53-6.7), FITC CD11c (N418), PE TCF-1 (C63D9), PE α 4 β 7 (REA457), PE I-A/I-E (M5/114.15.2), PerCP CD19 (6D5), PerCP NK1.1 (PK136), PerCP CD3 ϵ (145-2C11), PE-Cy7 PD-1 (RMP1-30), PE-Cy7 ROR γ t (B2D), APC TOX (REA473), APC Foxp3 (FJK-16s), APC CD40 (3/23), APC CD11c (N418), Alexa Fluor 700 CD45 (30-F11) and APC Vio770 CD3 ϵ (REA606), APC-Cy7 Ly6G (1A8), APC-Cy7 Siglec-F (S17007L). Upon washing, cells were resuspended in FACS buffer and analyzed by Cytotflex (Beckman). Flow cytometry data were analyzed by Flowjo (BD Bioscience) and the graphs were generated using Prism (GraphPad).

SABR in the spontaneous breast cancer model

Endogenous mammary tumors were established per conventional procedures.⁵⁴ In brief, 6–7 weeks old female C57BL/6J mice were implanted subcutaneously with 50 mg slow-release (90 days) medroxyprogesterone acetate (MPA, M) pellets (Innovative Research of America). Mice were then administered with 1 mg 7,12-dimethylbenz[a]anthracene (DMBA, D; Sigma-Aldrich) in 200 μ L corn oil (Millipore Sigma) once a week on weeks 1, 2, 3, 5, 6 and 7 after implantation of the MPA pellet.⁵⁴ Mice were then routinely monitored for the emergence of neoplastic lesions along the mammary lines. Tumor surface was calculated as the area of an ellipse ($A = \text{longest diameter} \times \text{shortest diameter} \times \pi/4$), as per common procedures.⁵⁴ Mice with primary tumor area reaching 15–45 mm² (d0) were randomized into subgroups to receive mock or SABR (20Gyx2F or 20Gyx3F). Following treatment, mice were monitored for tumor growth and the presence of secondary tumor. Mice were euthanatized when reached ethical endpoints (e.g. the cumulative tumor surface $\geq 180\text{--}200\text{mm}^2$, or in the presence of evident signs of toxicity or stress).

Murine ileal organoid ionizing radiation experiments

For IL-7 induction, murine organoids were seeded in 6-well plates with Matrigel (Corning) on day 0. On day 5, these organoids were subjected to mock or a range dose (0.25–6Gy) of 320 kV X-ray at a dose rate of 0.87Gy/minute using the XRad320 (X-ray Precision) irradiator. Culture medium was collected from the wells at 48 hours after IR. The concentration of murine IL-7 in these media was assessed by a commercial ELISA kit (Thermo Fisher Scientific) according to the manufacture's guideline.

For induction of *Reg3g* and *Defa5*, as previously described,⁴⁴ ileal organoids were seeded in 6-well plates on day 0. On day 5, Noradrenaline (kind gift of the pharmacy of Gustave Roussy) was added to the medium to a final concentration of 100 μ M. These organoids were then subjected to 0–4Gy IR. Twenty-four hours following IR, organoids were harvested and assessed by RT-qPCR using the following TaqMan probes: *Reg3g*, *Defa5* and *Ppia*.

The DC-B3Z co-culture system

de-iniDCs were collected by trypsinization and diluted with DC medium containing β -mercaptoethanol and GM-CSF. Cells were seeded in 12-well plates (5×10^5 cells/well) for a dose range (0–6Gy) of IR (intervention 1), or in 96-well μ -bottom tissue-culture plates for the treatment with a metabolite library of 96 compounds including bile acids/carnitines/sterols (at two concentrations i.e 2.5 μ g and 5 μ g/mL) for 16 hours (intervention 2), or in range concentration (0–50 μ g/mL) of deoxycholic acid (DCA) or ursodeoxycholic acid (UDCA) (Intervention 3) then pulsed with OVA protein (1mg/mL) for 4 hours and incubated with CTL (B3Z hybridoma cells) for 16 hours. The supernatant of cell culture was collected and the concentration of IL-2 in these supernatants was assessed by customized ELISA.

QUANTIFICATION AND STATISTICAL ANALYSIS

The following software were used for statistical analysis: R studio and Python v.3.8.2 for bioinformatics analyses of metagenomic sequencing, 16S rRNA-Seq and metabolomic data; IBM SPSS Statistics for multivariate Cox proportional hazard regression analysis; Flowjo (BD Bioscience) for analyses of flow cytometry data; Prism (GraphPad) for the remaining analyses and graph plotting.

MGS analysis: Shannon index was calculated by applying the diversity function available in the Vegan R package. PERMANOVA analysis was performed applying the *adonis2* function (Vegan R package) with default parameters and principal coordinate decomposition was computed via the *pcoa* function available in the *ape* R package. We used the Mann–Whitney U test to estimate differential abundant microbiome features for each of the considered conditions. Only microbiome features that were present in at least 5 samples in one of the two tested sets were included in the analysis. Adjusted P-values (Q) are computed via the Benjamini-Hochberg procedure to control for false discovery rate (FDR). All the reported associations for differential abundance analysis presented $P < 0.05$ and $Q \geq 0.2$. Standardized mean difference (SMD, Cohen's d) was computed after arcsine square root transformation. Spearman correlation analysis was performed to detect microbial pathways involved in nucleotide metabolism associated with *Parabacteroides distasonis* SGB1934.

16S rRNA-Seq analysis: Data matrices were first transformed with pseudocount and centered-log-ratio (CLR), then normalized and standardized using QuantileTransformer and StandardScaler methods from Sci-Kit learn package v1.0.1 (<https://scikit-learn.org/stable/>). Measurements of α diversity was calculated at species level using the SciKit-learn package v1.0.1. Exploratory analysis

of β -diversity was calculated using the Bray-Curtis measure of dissimilarity and represented in Principal Coordinate Analyses (PCoA), along with methods to compare groups of multivariate sample units (analysis of similarities - ANOSIM, permutational multivariate analysis of variance - PERMANOVA) to assess significance in data points clustering. PCoA and the other analysis was performed on batch-effect corrected data with MMUPHin. Batch-corrected data were assessed by two-way interaction terms in the Mixed Model with Principal Variance Component Analysis (PVCA) and visualized with Uniform Manifold Approximation and Projection (UMAP), both implemented within BatchServer (<https://lifeinfor.shinyapps.io/batchserver/>). In order to visualize a non-supervised clusterization as PCoA, we implemented with custom scripts (Python v3.8.2, Seaborn v0.11.2) a Hierarchical Clustering Analysis (HCA) with 'Bray-Curtis' metrics and 'complete linkage' method. We implemented Partial Least Square Discriminant Analysis (PLS-DA) and the subsequent Variable Importance Plot (VIP) as a supervised analysis wherein the VIP values (order of magnitude) are used to identify the most discriminant bacterial species among the cohorts. Mann-Whitney U test and P values, without FDR, was used for a fixed sample size as previously described and Kruskal-Wallis tests were employed to assess significance for pairwise or multiple comparisons, respectively, considering a P value < 0.05 as significant. All P values were corrected for multiple hypothesis testing using a two-stage Benjamini-Hochberg FDR at 10%. Lineplot temporal kinetics of bacterial species relative abundances were computed for each timepoint and depicted with Seaborn v0.11.2, representing data variance with 95% confidence intervals.

Other analyses: Kaplan-Meier survival curves were generated using Prism and the statistical difference between groups was compared using the Log-rank test. Multivariate analysis and hazard ratio evaluation were performed using the Cox proportional hazard regression model. Comparisons of proportions between groups and evaluation of relative risk were performed using the Fisher's exact test. Each dot in graphs represent individual patient, mouse or biological replicate. Bars represent mean $\pm$ standard deviation (SD) unless otherwise indicated. Data were subjected to normal distribution test (D'Agostino & Pearson test) prior to mean comparisons. Mean comparisons in two independent groups were performed using the unpaired student's t test when data followed a normal distribution and the Mann-Whitney U test when they did not or their normality cannot be evaluated. Mean comparisons in ≥ 3 groups were performed using One-way ANOVA in normally distributed data or Kruskal-Wallis H test in data without normal distribution. Multiple comparisons of means were performed using the Mann-Whitney U test with or without P value correction as indicated, or unpaired multiple t-test with the two-stage Benjamini, Krieger & Yekutieli procedure to control FDR ($q < 0.05$). Mean comparisons of paired data without normal distribution was performed using the Wilcoxon matched-pairs signed rank test. Correlation analysis in data without normality was performed using the Spearman's analysis. Statistical details (numbers, experimental replications, data representations) of experiments and P value details are provided in the figure legends. P values < 0.05 were considered to be significant.