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Chemotherapy-induced Ileal Crypt Apoptosis and ileal Microbiome Shape Immunosurveillance and Prognosis of Proximal Colon Cancer

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Abstract

The prognosis of colon cancer (CC) is dictated by tumor infiltrating lymphocytes, including T follicular helper cells (TFH), and the efficacy of chemotherapy-induced immune responses. It remains unclear whether gut microbes contribute to the elicitation of TFH-driven responses. Here, we show that the ileal microbiota dictates tolerogenic versus immunogenic cell death of ileal intestinal epithelial cells (IEC) and the accumulation of TFH cells in CC in patients and mice. Suppression of IEC apoptosis led to compromised chemotherapy-induced immunosurveillance against CC in mice. Protective immune responses against CC were associated with residence of *Bacteroides fragilis* and *Erysipelotrichaceae* in the ileum. In the presence of these commensals, apoptotic ileal IEC elicited $PD1^+$ TFH in an IL-1R1 and IL-12 dependent manner. The ileal microbiome governed the efficacy of chemotherapy and PD1 blockade in CC, independently of microsatellite instability. These findings demonstrate that immunogenic ileal apoptosis contributes to the prognosis of chemotherapy-treated CC.

Introduction

The intestinal mucosa is a dynamic interface between intestinal epithelial cells (IEC), local immune cells and the microbial ecosystem[1]. Mucosal regulatory T cells suppress excessive inflammation but promote the earliest stages of immune prone - carcinogenesis via enhancement of IL-17 production at the expense of IFNγ production[2]. Sustained gut microbial dysbiosis may be a risk factor for the exacerbation of colorectal inflammatory lesions leading to overt carcinogenesis[3,4].Variations in gut microbial communities during colorectal cancer (CRC) progression suggest a role for distinct bacterial species, including *Fusobacterium nucleatum,* enterotoxigenic *Bacteroides fragilis, and Parvimonas micra,* in carcinogenesis[5–8]. The abundance, functional competence and spatial distribution of tumor-infiltrating T lymphocytes (TIL) within the tumor bed (also known as the "immunoscore"[9–11]) dictate the prognosis of CRC[10]. The chemokine CXCL13 and interleukin-21 (IL-21) are pivotal factors in the T follicular helper (TFH)/B cell axis correlating with CRC patient survival[12]. The tumor, immune and/or environmental cues that lead to accumulation of TIL in CRC remain to be elucidated. Previous studies have shown that lymphocyte infiltration is associated with microsatellite instability (MSI), resulting in the generation of truncated peptides produced by frameshift mutations[13–16]. Such neoantigens may predispose patients with MSI^{high} CRC to clinical benefit from immune checkpoint inhibitors (ICI)[17]. Oxaliplatin (OXA) is the backbone of the chemotherapeutic regimen routinely used to treat patients with CC; it can induce immunogenic cell death (ICD) and elicit anti-cancer immune responses[9,18,19]. Surprisingly, combinations of OXA with ICI have not proven superiority over OXA-based chemotherapy alone in advanced CRC so far[20]. Cytotoxicants not only target tumor cells but also affect dividing crypt -derived IEC, thereby perturbing the local microbiota[21]. Hence,

studying the interplay between IEC, commensalism, and immune cells during chemotherapy may reveal mechanistic features governing TIL function against CRC.

Here we show that the immunogenicity of oxaliplatin chemotherapy in proximal CC (pCC) relies on two biological features: (1) antigenicity provided by the caspase 3/7-dependent apoptosis of crypt-derived IEC and (2) the adjuvanticity of selected ileal bacterial families or species (*Erysipelotrichaceae, B. fragilis*). These features cooperate to elicit TFH immune responses protective against tumor progression.

Results

Ileal apoptosis dictates the prognosis of colon cancer in mice and patients

Given the gastrointestinal side effects and mucosal cytotoxicity associated with chemotherapy in patients[22], we monitored ileal cell apoptosis by quantifying levels of cleaved caspase-3 (cCasp3) in the healthy colon and ileal mucosae. These tissues were harvested during right hemicolectomies in patients with proximal colon cancer (pCC) who had or had not received pre-operative OXA-based chemotherapy (Extended data Fig. 1a-c). Surprisingly, apoptosis was induced by chemotherapy only in the crypts of ilea, but not in the villi, lamina propria (LP) or healthy colons adjacent to tumors, in two independent cohorts (Supplementary Table 1, Fig. 1a-b, Extended data Fig. 2a-b). Moreover, higher baseline levels of cCasp3 in ileal crypts (≥ median value) were associated with favorable prognosis in stage IV pCC patients in univariate analyses (Fig. 1c), while immunoscore was not, as previously reported[8]. Chemotherapy did not increase the overall abundance of TILs (Extended data Fig. 2c) but enriched tumor beds with $CD4^+Bcl6^+$ TFH cells in the discovery and validation cohorts (Fig. 1d) while reducing ileal LP TFH cells (Extended data Fig. 2d). The density of tumoral $CD4^+Bcl6^+$ TFH correlated with TILs and with ileal crypt apoptosis, but was negatively

correlated with their residence in the ileal LP (Extended data Fig. 2e-g). The composition of the distal (fecal) microbiota has been associated with CRC gene profiling[23] and distinguishes benign from malignant colon tumors[24]. We next analyzed changes in the ileal microbiota - harvested in the healthy ileal mucosae lining upstream from Bauhin's valve (refer to Extended Figure 1a)- that were associated with ileal IEC apoptosis using 16S rRNA gene sequencing of 70 ileal mucus layers at the family and species taxonomic rank levels (Extended data Fig. 1b). Ileal apoptosis correlated with the relative abundance of *Erysipelotrichaceae*, but negatively correlated with that of *Fusobacteriaceae* families (Fig. 1e). The overrepresentation of *Fusobacteriaceae* family members in the ilea was also negatively correlated with TIL abundance in pCC (Fig. 1f). A higher ileal relative abundance of *Bacteroides fragilis* (considering either tertiles or ventiles) selectively predicted long term survival in these 70 patients, contrasting with other frequent *Bacteroides spp.* (such as *B. uniformis, B. caccae)*, or *Prevotellaceae* family members that were associated with dismal prognosis in univariate analyses (Fig. 1g, Supplementary Tables 2-3). Interestingly, *Bacteroidaceae* and *Erysipelotrichaceae* resided in the same cluster and negatively correlated with *Prevotellaceae* in a non-supervised hierarchical clustering of ileal taxonomic families (Supplementary Fig. 1). Hence, ileal apoptosis in stage IV pCC patients is associated with significant variations of the local microbiome, as well as a redistribution of TFH to the tumor bed that may influence clinical outcome.

To further investigate the mechanistic links between ileal apoptosis, microbiota and tumor immunosurveillance, we utilized the MC38 mouse model of MSI^{high} colon carcinoma (CC)[25]. Pre-treatment with broad spectrum antibiotics (ATB) (Supplementary Fig. 2a) reduced the efficacy of OXA against subcutaneous (s.c.) MC38 (Fig. 2a, Supplementary Fig. 2b), and prevented the release of anti-microbial peptides into feces (Extended data Fig. 3a-b), suggesting a role for the gut microbiota in the local and extra-intestinal effects of OXA. While

OXA triggered apoptosis of both ileal and colonic IEC, there was significantly more apoptosis of the former, as described for CC patients, and only in the presence of an intact microbiome (Fig. 2b,c). Accordingly, baseline proliferation of ileal IEC, which significantly exceeded that of colonic IECs, was markedly affected by OXA therapy (Extended data Fig. 3c,d).

To further study the impact of patient microbiota on the efficacy of OXA, we utilized an avatar model[26], in which the intestines of germ free mice were colonized with human proximal colonic content (Extended data Fig. 1a) from 12 pCC patients (Supplementary Table 4). Two weeks later, these mice were inoculated with s.c. MC38 and another week later treated with OXA (Extended data Fig. 3e). While the majority (8/12) of patient microbiota resulted in antitumor efficacy of OXA comparable or superior to that observed in mice reared in specific pathogen-free (SPF) conditions (henceforth referred to as “avatar responders (aR)”), 4 patients' microbiota induced complete resistance to this immunogenic chemotherapy (“avatar non-responders (aNR)”) (Fig. 2d,e, Supplementary Fig. 2c). We performed 16S rRNA gene sequencing on the avatar feces and compared the mouse metagenomic profiles to that of the original patient sample (Supplementary Fig. 2d, illustrating the 12 clinically relevant families described in Fig. 1). The diversity of bacterial engraftment was driven by the host, with no significant differences among all recipients at family or species taxonomic rank levels (Supplementary Fig. 2e illustrating all the species available in the dataset). However, PLS-DA supervised analysis revealed that some species were differentially associated with aR versus aNR. In particular, *Paraprevotella clara* was strongly related to the aNR status (Extended data Fig. 3f, Supplementary Fig. 2f). Feces from avatar recipients (post-FMT) harvested before starting OXA treatment also revealed increased colonization with *B. fragilis* in aR compared with aNR by quantitative PCR (Fig. 2h). We performed immunological phenotyping of T cell infiltrates in lymphoid organs (spleen, tdLN), with the aim to dissect the interplay between Treg, TH17, Tc1 and TFH as illustrated in our cohorts (Fig. 1) and as

previously described[2]. There was a significant increase of ICOS expressing $CXCR5^{hi}PD1^{hi}CD4^{+}$ TFH in tdLN of OXA-treated aR tumor bearers (Fig. 2f,g; Extended data Fig. 3g) with a concomitant decrease of $CCR6^{+}CXCR3^{-}CD4^{+}$ TH17-like cells (Extended data Fig. 3h).

Consistent with patient data, MC38 bearing mice exhibited OXA-induced ileal apoptosis and their intestinal microbiome composition governed the TFH/TH17 balance in tdLN.

Immunogenicity of caspase 3/7-dependent ileal apoptosis against colon cancer

We next sought to validate the relevance of ileal apoptosis for cancer immunosurveillance in other CC models. To do this, we immunized naïve C57BL/6J or BALB/c mice using a syngeneic vaccine composed of primary (non-malignant) ileal or colonic IEC harvested from OXA- (or PBS-) treated syngeneic littermates, against a minimal tumorigenic dose (MTD) of MC38 or the MSS CC model CT26[27,28], respectively (Fig. 3a). We observed that ileal (and to a lesser extent colonic) IEC conferred partial protection against MC38 but not sarcoma nor cholangiocarcinoma in C57BL/6 mice, while ileal (and not colonic) IEC protected BALB/c mice against CT26 (but not breast cancers) (Fig. 3b-d, Extended data Fig. 4a,b, Supplementary Fig. 3a). This vaccine conferred a long-term protection with immunological memory lasting at least 12 weeks post-vaccination (Fig. 3e, Supplementary Fig. 3b). IEC from mice pretreated *in vivo* with OXA were more efficient in reducing CC growth than untreated IEC (Fig. 3b,c). Only apoptotic IECs (annexin-V^{+}) but not necrotic cells (obtained after freezing-thawing cycles) were immunogenic (Extended data Fig. 4c,d, Supplementary Fig. 3c). Crypt- but not villi-derived dying IEC conveyed protection against MC38 (Extended data Fig. 4e, Supplementary Fig. 3d). Of note, the mitotically active $Lgr5^{+}$ intestinal stem cells were dispensable for the immunogenicity of IEC (Extended data Fig. 4f,g).

To analyze the differential immunogenicity of apoptosis versus necroptosis of ileal IEC, we harvested OXA-exposed ileal mucosae from mice in which caspase 3/7 expression was ablated specifically in IECs (*Casp3/7*$^{\Delta IEC}$) or from *Ripk3*-deficient animals to vaccinate naive recipients. The ileal IEC immunizing potential against MC38 cells was lost in the former (Fig. 3f) but not in the latter (Fig. 3g, Supplementary Fig. 3e) mice recipients, underscoring the role of apoptosis but not RIPK3-dependent necroptosis in the immunogenicity of ileal IEC. We next analyzed the impact of ileal IEC apoptosis in the tumor growth-reducing activity of OXA against day 7 established s.c MC38 tumors. The antitumor efficacy and immunostimulatory capacity of OXA in *Casp3/7*$^{\Delta IEC}$ mice was significantly reduced compared with that in *Casp3/7*$^{fl/fl}$ littermate controls (Fig. 3h-i, Supplementary Fig. 3f).

Essential role for TFH in the immunogenicity of ileal apoptosis

Given the accumulation of TFH in tdLN post-OXA in aR tumor bearers (Fig. 2g and Extended data Fig. 3g-h) and in pCC from patients (Fig. 1d), we tested the effects of T cell depletion on the immunizing capacity of apoptotic ileal IEC. The vaccine efficacy relied on $CD4^+$ and $CD8^+$ T cells (Extended data Fig. 5a) and elicited $CXCR5^{hi}PD1^{high}Bcl6^+$ cells expressing high levels of membrane ICOS in the skin dLN (Fig. 4a-c). These TFH were also increased at day 7 post-OXA in mLN of wild-type (WT) mice but not *Casp3/7*$^{\Delta IEC}$ mice, suggesting the crucial role for ileal apoptosis in the accumulation of TFH (Fig. 4d). To evaluate the functional impact of TFH on the immunogenicity of dying ileal IEC, we utilized four approaches. First, we observed that OXA-exposed ileal IEC exhibited a reduced immunizing capacity in a genetically TFH-deficient mouse model, *Cxcr5*$^{-/-}$, compared with WT counterparts (Fig. 4e, Supplementary Fig. 4a). Second, neutralizing the CXCR5 ligand CXCL13 with specific antibodies negatively affected protection by this vaccine (Fig. 4f,

Supplementary Fig. 4b). Finally, in conditional mutant *Bcl6*$^{fl/fl}$*Cre*^{CD4} in which TFH were decreased >90% compared with controls[29], the OXA-exposed IEC vaccine no longer mediated long term protection against a MTD challenge with s.c. MC38 and therapeutic OXA exhibited reduced capacity to induce the accumulation of CXCR3^{+} CD4^{+} or CD8^{+} TIL (Supplementary Fig. 4c-d).

Given that TFH cells are pivotal regulators of the germinal center responses and humoral immunity[30], we explored humoral immune responses and the impact of B cells on the immunizing potential of dying ileal IEC. Elevated serum levels of IgG2b were monitored 3 weeks post-immunization, and this effect was blunted by CXCL13 neutralization and CD4^{+} T cell depletion (Fig. 4g and Extended data Fig. 5a). Systemic B cell depletion, compromised the prophylactic antitumor effects of OXA-exposed ileal IEC (Fig. 4h, Supplementary Fig. 4e).

We conclude that OXA-induced apoptotic cell death of ileal IEC stimulates a TFH response that contributes to the efficacy against CC of the prophylactic vaccine and chemotherapy in avatar mice, and correlates with favorable immunoscores in patients with pCC.

Mechanisms underlying the priming of TFH during oxaliplatin

Intrigued by the impact of the microbiome in avatar mice (Fig. 2e) and the deleterious effect of ATB on the OXA-mediated ileal IEC apoptosis and tumor growth reduction (Fig. 2a-c), we suspected that the ileal microbiome might contribute to the adjuvanticity of the IEC vaccine against CC. In support of this hypothesis, the immunogenicity of OXA-treated ileal IEC harvested from SPF mice was superior to that of IEC from germ-free mice (Fig. 5a, Supplementary Fig. 5a). We next tested the relevance of various damage- or microbe-associated molecular patterns (DAMPs and MAMPs) released from OXA-exposed ileal IEC

for the adjuvanticity of apoptosis. We immunized naïve WT C57BL/6J mice with OXA-treated ileal IEC derived from *Entpd1, Tlr2/Tlr4, Tlr9, Il1αβ* or *Il18* deficient syngeneic mice or OXA-exposed ileal WT IEC in which DAMPs that are usually associated with ICD (ATP, calreticulin and HMGB1) were neutralized by antibodies or pharmacological blockers. The immunogenicity of WT ileal IEC was significantly impaired when TLR2/4 (but not TLR9) or IL-1 (but not IL-18) were removed, or when ATP release was inhibited or purinergic P2 receptors were blocked, yet did not depend on calreticulin nor on HMGB1 (Extended data Fig. 5b-c). Reinforcing the role of specific microbes in the adjuvanticity of the CC vaccine, vaccination with ileal stem cell-derived organoids (which are sterile) treated with OXA accelerated MC38 progression compared with untreated organoids, suggesting that they were tolerogenic (Extended data Fig. 6a, Supplementary data Fig. 5b). Altogether, these findings indicate that ileal IEC exposed to conventional chemotherapy cannot induce a protective anti-cancer immune response in the absence of immunogenic microbiota.

To directly show that the ileal microbiota can restore the immunogenic properties of IEC, we supplemented initially sterile (and hence tolerogenic) OXA-treated ileal organoids with 10 different microbiotas harvested from the ileal mucosae of pCC patients (Extended data Fig. 6b, Supplementary Table 5). Only 6 of the 10 ileal microbiotas were able to restore a relative anticancer protection compared to several negative controls (such as naïve non-immunized mice, mice immunized with sterile OXA-treated organoids or mice immunized with ileal mucosae microbiota without organoids) (Fig. 5b, Extended data Fig. 6c, Supplementary Fig. 5c). To identify the bacterial taxa that contribute to the immunogenicity of ileal IEC apoptosis, we used different and complementary technical approaches, namely, 16S rRNA gene sequencing and culturomics. By 16S rRNA gene sequencing NR showed enrichment in the relative abundance of *Prevotellaceae* at the family taxonomic rank level (Extended data Fig. 6d), and of *Prevotella oralis, Alloprevotella rava, Prevotella clara, Paraprevotella*

xylaniphila at species taxonomic rank level (Extended data Fig. 6e). Moreover, several other species belonging to *Fusobacterium* genus (such as *F. canifelinum, F. periodonticum, F. nucleatum*) were significantly enriched in NR compared with R ileal microflora (Extended data Fig. 6e). In parallel, the prevalence of distinct bacterial species in the ileal microbiota determined by culturomics, pointed to an increase in the abundance of live *Erysipeloclostridium ramosum* (among other species) in the R microbiota (Extended data Fig. 6f). These findings further illustrate the lack of immunogenicity of *Prevotellaceae* and *Fusobacteriaceae* already observed in the larger cohort of 70 antibiotic-naïve pCC patients for whom these taxa were negatively associated with ileal apoptosis, pCC immunoscore and/or prognosis (Fig. 1e-g).

To further characterize the immune response elicited by dying ileal IEC during chemotherapy, we monitored the migration of dendritic cell (DC) subsets 24 hours post-OXA. Migratory DC (defined as $CD11c^{+}$ MHC-I^{hi}) composed mostly of $CD103^{+}CD11b^{-}$ DC (conventional type 1 DC, cDC1) accumulated in mLN along with the upregulation of IL-1β and IL-12 transcripts concomitantly to their depletion from the ileal (but not colonic) lamina propria (Extended data Fig. 6g-i). Importantly, the accumulation of TFH in mLN at 7 days after OXA administration was loss when cDC1 were absent in *Batf3* knockout mice or when the microbiota was perturbed by broad-spectrum ATB treatment (Fig. 5c).

Next, we analyzed the mechanism underlying the adjuvanticity of the ileal bacterial repertoire. Earlier reports demonstrated that recognition of viable bacteria by the innate immune system affected the ensuing helper T cell responses, in particular, the differentiation of TFH. Sensing live bacteria can trigger the release of IL-1β and IL-12p70 by antigen presenting cells and IL-1R1 signaling in TFH[31,32]. Given that IL-1β featured in the cytokine fingerprint of mLN following migration of ileal cDC1 to mLN (Extended data Fig. 6i) and that dying IEC harvested from IL-1-deficient mice were unable to vaccinate against CC

(Extended data Fig. 5b), we performed immunohistochemistry to identify the main source of IL-1β in ileal and colonic mucosae 24 hours after OXA injection. Mononuclear cells residing in the ileal (but not colonic) lamina propria were the unique source of IL-1β (Fig. 5d,e). Of note, IL-1R1 was indispensable for the immunogenicity of ileal apoptotic IEC since systemic administration of an IL-1R1 antagonist abrogated the effects of the vaccine and reduced the IgG2b-based humoral response associated with TFH (Fig. 5f, g). Conversely, supplementation of the ileal stem cell-derived organoids with recombinant IL-1β restored the immunogenicity of OXA-exposed sterile organoids (Fig. 5h) and boosted serum IgG levels (Extended data Fig. 6j). Finally, neutralization of IL-12p40 or IL-12p70 drastically reduced the immunogenicity of dying ileal IEC against MC38 (Fig. 5i, Supplementary Fig. 5d), which is in accordance with the loss of IL-12p40 transcription in mLN from *Batf3*-deficient mice (Extended data Fig. 6k).

Altogether, the priming of TFH is not only dictated by the executioner caspases 3/7 in ileal IEC but also by Batf3^{+} cDC1 that produce IL-1β and IL-12 in mLN, as well as the ileal microbiota that influences both, caspase activation and cytokine production.

The adjuvanticity of ileal microbiota involved in the efficacy of PD1 blockade

We screened a panel of live immunogenic or tolerogenic commensals isolated from pCC patient ileal microbiotas, identified by MiSeq 16S rRNA gene sequencing and culturomics (as described above in vaccine experiments, Extended data Fig. 6) or by virtue of their correlation with the immunoscore and survival of pCC patients (Fig. 1), for their capacity to trigger the release of IL-1β and IL-12p70 by cDC1-like cells (generated from bone marrow precursors in Flt3L containing media). We found that immunogenic bacteria (i.e. *B. fragilis* –a non-enterotoxigenic spp-, *E. ramosum, A. onderdonkii*) were superior to tolerogenic bacteria (*F.*

nucleatum, P.clara, B. uniformis, S. gallolyticus) in stimulating IL-12p70 production by cDC1 (Extended data Fig. 7a and Supplementary Fig. 6a). To evaluate the immunological and tumor growth control-related relevance of the delicate equilibrium between immunogenic *versus* tolerogenic commensals residing in the ileum at the time of ileal IEC demise, we used three independent strategies. First, tolerogenic OXA-exposed organoids were admixed with different bacteria and evaluated for their capacity to reduce MC38 tumor growth (Fig. 6a). The immunogenic species *B. fragilis* enhanced vaccine efficacy, while the tolerogenic species *F. nucleatum* failed to do so (Fig. 6b), echoing similar results obtained with other immunogenic (*A. onderdonkii, E. ramosum*) or tolerogenic (*B. uniformis, S. gallolyticus*) commensals (Supplementary Fig. 6b). Secondly, we monocolonized germ-free C57BL/6J mice by oral gavage with immunogenic (*E. ramosum or A. onderdonkii*) *versus* tolerogenic (*S. gallolyticus*) bacteria, then harvested OXA-treated ileal IEC to immunize naive SPF counterparts. Only immunogenic bacteria played an adjuvant role in these vaccines protecting mice against MC38 (Supplementary Fig. 6c) and triggering IgG responses (Extended data Fig. 7b) without inducing any ileitis or colitis in vaccinated mice (Supplementary Fig. 6d-e). Thirdly, given that human MSS pCC do not respond to PD-1 blockade in patients[33], and that $PD1^{high}$ expressing TFH could be reinstated in their functional capacity by anti-PD1 mAbs, we subjected mice bearing MSI (MC38) or MSS (CT26) tumors to gavage with immunogenic or tolerogenic commensals at the time of the combined chemoimmunotherapy with OXA and anti-PD1 Ab (Fig. 6c). This treatment was moderately additive against MC38 (MSI) and CT26 (MSS) in SPF mice (Supplementary Fig. 6f). However, *B. fragilis* or *E. ramosum* improved the effects of OXA+anti-PD1 Ab, contrasting with *P. clara* or *F. nucleatum* that blunted the efficacy of the combination therapy (Fig. 6d,f). In parallel, immunogenic bacteria tended to increase serum IgG levels, while tolerogenic bacteria failed to do so (Fig. 6e, Extended data Fig. 7c). Similar results were obtained in a humanized dysbiotic system by

FMT (aNR1, Supplementary Table 4) in which the combination therapy, benefited from immunogenic but not tolerogenic bacteria (Fig. 6g). In the aforementioned settings, we confirmed adequate and comparable colonization of each of these exogenous commensals (Extended data Fig. 7d). Of note, *F. nucleatum* failed to engraft in C57BL6/J mice, but could do so in BALB/c mice (Extended data Fig. 7d, lower panels).

These preclinical data highlight the regulatory role of the ileal microbiota in modulating the anticancer efficacy of immunogenic chemotherapy alone or combined with ICI (Fig. 6h).

Discussion

Altogether, our findings suggest a role for the microbiota in governing the capacity of chemotherapy-induced ileal IEC apoptosis to elicit an anti-tumor immune response in patients with CC (scenario summarized in Figure 6h). In this study, we purposely utilized a subcutaneous CC model to avoid any direct impact of the microbiota on the tumor, which allowed us to dissect the mechanistic interplay between the ileal microbiome and systemic immunomodulation. Ileal apoptosis in GF mice or in TLR2/4 deficient animals failed to elicit protective memory T cell responses against CC. Caspase 3/7-dependent apoptotic cell death of ileal IEC was sensed by $CD103^{+}CD11b^{-}$ Batf3-dependent cDC1 only in the presence of immunogenic commensals, eliciting TFH immune responses in the mLN dependent on IL-1R1 and IL-12. Activation of the executioner caspases in ileal crypts and the presence of immunogenic commensals conferred a favorable prognosis to stage IV pCC patients.

Indeed, ileal IEC apoptosis facilitates the priming and activation of TFH in the mesenteric LN and their accumulation in tdLN. In the absence of $Bcl6^{+}CD4^{+}$ T cells,

CXCR5, CXCL13 or B cells, ileal apoptosis failed to contribute to OXA-induced anticancer immune responses and tumor growth-reduction. Prior reports have supported the role of TFH in human breast and CC immuno-surveillance[12,34] and recent transcriptome analyses confirmed that TFH metagenes are associated with a favorable clinical outcome[35]. The ileal microbiota is essential for the local activation of TFH. Immunogenic commensals are distinguishable from tolerogenic bacteria in that they trigger both IL-1β and IL-12p70 release from migratory DC, thereby increasing bacterial and/or self-antigen-specific TFH or IgG2b responses. Moreover, the density of TFH in patients' pCC correlated with ileal caspase 3 activation and TILs, suggesting that TFH primed during ileal apoptosis may serve helper functions for antitumor effector/memory $CD8^+$ T cell responses recognizing (crypt-derived) self-antigens. Conversely, tolerogenic bacteria elicited the accumulation of $CD4^+$ TH17 cells in tdLN, a parameter that is associated with poor responses to OXA in avatar mice and in pCC patients[36,37].

A variety of microbial structures can elicit TFH responses. An innate signature of microbial viability, more specifically bacterial RNA, could trigger the IL-1β-dependent differentiation of TFH and germinal center B cells[31,38]. Activated $ICOS^+$ TFH cells recirculate in the blood after mucosal vaccination with inactivated enterotoxigenic *Escherichia coli* and represents a biomarker of mucosal memory B cell responses in humans[39]. In line with these reports, our data show that ileal ICD depends on the microbiota and its capacity to trigger the IL-1β-dependent TFH. We and others found that B cells are involved in tumor growth reduction subsequent to vaccination with dying ileal IEC or enforced TMB[40], respectively. Moreover, successful combinatorial regimens and vaccination with apoptotic ileal cells increased IgG2b humoral responses. Nonetheless, the specificity of IgG responses (that may be directed against bacteria or tumor cells)[40–42] and the capacity of immunogenic commensals to invade tumor beds remain unclear and requires further in-depth exploration.

Our findings raise the theoretical possibility of OXA-induced self-reactive $CD8^+$T cell-dependent immunity that spares healthy tissues to specifically combat cancer, presumably due to the tightly controlled tolerance mechanisms in mucosal tissues[43,44]. This concept has been previously described by Ziegler and colleagues in spontaneous CRC, where elevated mitophagy (by genetic loss of *Stat3*) in IEC paved the way to adaptive anti-CC immunity[45].

Our study suggests potential new therapeutic avenues, including ileum IEC-targeted cell death inducers and bacterial adjuvants with the purpose of inducing immune responses against CC (self-) antigens and of reinstating clinical responses to PD1 blockade in MSS tumors. Antibody-drug conjugates, such as those used to target cancer stem cells[46,47], may actually be able to reach these objectives.

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Author Contributions

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Conflict of interest statement: LZ and GK are cofounders of EverImmune, a biotech company devoted to the use of commensal bacteria for the treatment of cancers.

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Figure Legends

Figure 1. Ileal apoptosis and microbiome dictate prognosis in stage IV proximal colon cancer.

a. Representative micrograph pictures of immunohistochemistry for cleaved caspase 3 in ileal and colonic paired specimens from pCC patients without or after preoperative chemotherapy. Scale bars 20 μm. The inset depicts at a higher magnification field at the bottom of an ileal crypt; red arrows indicate cleaved caspase-3 staining. Scale bar: 5 μm. **b**. Statistical analysis of $cCasp3^+$ cells by automated quantification using an algorithm to select crypts. Non-previous chemotherapy ileum n=31, colon n=30; previous chemotherapy ileum n=14, colon n=14. (Validation cohort, patients described in Supplementary Table 1). Each dot represents one pCC patient, box plot center lines correspond to the median value; lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles); and lower and upper whiskers extend from the box to 5 and 95 percentiles, respectively. Two-tailed Mann Whitney U test *p*-value is shown. **c.** Kaplan Meier survival curve of PFS and univariate two-sided Log rank analysis of non-treated stage IV pCC patients from the validation cohort (ileum n=18, colon n=13) segregated in two subgroups according to the median value of $cCasp3^+$ cell density. **d.** Number of $CD4^+Bcl6^+$ double positive cells (TFH)/mm^2 assessed by immunohistochemical stainings in tumors from pCC patients without or after preoperative chemotherapy. Non-previous chemotherapy discovery n=33, validation n=37; previous chemotherapy discovery n=11, validation n=7 (Patients described in Supplementary Table 1). Box plot center lines correspond to the median value; lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles); and lower and upper whiskers extend from the box to 5 and 95 percentiles, respectively. Two-tailed Mann Whitney U test *p*-value is shown. **e.** Correlations between $cCasp3^+$ density in ileal crypts and bacteria families

in the autologous ilea. Each dot represents one pCC patient, n=30. Spearman's rank coefficients of correlation (r_s) and two-tailed *p*-values are shown. The continuous and dotted lines show the regression line and 95% of confidence intervals, respectively. **f.** Heatmap showing correlation coefficients between ileal bacteria families identified by 16S rRNA gene sequencing of ileal mucus and TIL density of $CD3^+$ and $CD8^+$ T cells in the invasive margin (IM) or the core of the tumor (CT) in n=59 paired samples of the discovery cohort. (Supplementary Tables 1 and Extended data Fig. 1b). Spearman's rank coefficients of correlation (r_s) and two-tailed *p*-values<0.05 (*) are depicted. **g.** Kaplan Meier survival curves and univariate two-tailed Log rank analyses of n=70 pCC patients (Supplementary Table 1), segregating the cohort according to tertiles of relative abundance of the corresponding bacteria taxa (Supplementary Table 2).

Figure 2. Impact of the microbiome in the efficacy of OXA in MC38 tumor bearers.

a. Tumor size for OXA and PBS -treated mice in water controls or under continuous ATB treatment. Each dot represents one mouse, the mean ± SEM tumor size of 5 independent experiments. Water PBS, n=30; water OXA, n=30; ATB PBS, n=33; ATB OXA, n=30. **b-c.** Representative micrographs (b) and statistical analysis (c) of the automated quantification of positive staining of cleaved caspase 3, in paired healthy ilea and colons in MC38 s.c. tumor bearers untreated or treated with OXA (with or without concomitant ATB treatment) at 24h. Means ± SEM depicted. Water groups n=20 and ATB groups n=5. Scale bar: 50 μm. **d.** Avatar MC38 model system (experimental setting as in extended data figure 2e) using pCC patients colon content to colonize GF C57BL/6J recipients. Representative tumor growth curves for aR and aNR. Tumor size over time is represented as mean ± SEM for natural tumor growth (PBS, n=5) or after OXA treatment (n=4) for one FMT **e.** Box-plots showing the % of decrease in tumor size per day of OXA treatment relative to the tumor size in corresponding

PBS group for all aR and aNR tested (red and blue dots, respectively). Dotted line shows the SPF average response. Box plot center lines correspond to the median value; lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles); and lower and upper whiskers extend from the box to the smallest or largest value, correspondingly. aR1 PBS n=5, OXA n=4; aR2 PBS n=4, OXA n=3; aR3 PBS n=5, OXA n=5 ; aR4 PBS n=5, OXA n=4; aR5 PBS n=6, OXA n=6; aR6 PBS n=7, OXA n=6; aR7 PBS n=5, OXA n=4; aR8 PBS n=5, OXA n=6; aNR1 PBS n=5, OXA n=4; aNR2 PBS n=7, OXA n=6; aNR3 PBS n=5, OXA n=4; aNR4 PBS n=6, OXA n=6. Details of each FMT donor patient in Supplementary Table 4. **f**. Heatmap representation of flow cytometric analyses of T cell immunophenotyping in spleens and tdLN as a means fold ratio between OXA and PBS treated groups at sacrifice. Pooled data from experiments shown in (e). Spleen samples: aNR PBS n=23, OXA=19; aR PBS n=35, OXA=35. tdLN samples: aNR PBS n=19, OXA n=20; aR PBS n=22, OXA n=30. Only for $CXCR5^{hi}PD1^{hi}/CD4^{+}$ and $CD19/CD45^{+}$ markers: aNR PBS n=19, OXA=22; aR PBS n=19, OXA=20. **g.** TFH cells in treated versus untreated aR versus aNR. Each dot represents one mouse aNR PBS n=19, OXA=20; aR PBS n=19, OXA=22. Means ± SEM depicted. **h.** *B. fragilis*-specific qPCR determination of the relative abundance of the bacterium in fecal material collected at baseline (before treatment). Graph depicting pooled data from 4 aNR and 8 aR in which *B fragilis* was present (aNR n=25; aR n=37). Each dot represents one mouse. Means ± SEM depicted. Statistics: mixed-effect modeling with a specific software (Methods) for longitudinal tumor growth analysis (e,d) and Mann-Whitney U test used to compare two independent groups (after Kruskal-Wallis was implemented for multiple groups) (a,c,f,g,h). Two-tailed *p*-values are shown in the figures, for (f): **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

Figure 3. Protective role of intestinal caspases -3 and -7 in the cell death of ileal IEC against colon cancer.

a. Experimental setting of vaccination with dying intestinal cells against CC. **b**. Vaccination of naïve C57BL/6J mice using ileal or colonic IEC which were or were not exposed to OXA to protect against syngeneic transplantable colon cancer, MC38. Tumor growth curves (left panels) showing one representative experiment (each group n=5, means ± SEM depicted) and tumor size at day 21 of 2-3 pooled experiments (right panels, 6-10 mice/group, ileum PBS n=27, ileum OXA n=25, colon PBS n=15, colon OXA n=13). Each data point represents one mouse, means ± SEM depicted. **c**. Vaccination of naïve BALB/c mice using ileal or colonic IEC which were or were not exposed to OXA to protect against syngeneic transplantable colon cancer, CT26. Tumor growth curves (left panels, naïve n=6, ileum OXA n=8, colon OXA n=8, means ± SEM depicted) showing one representative experiment and tumor size at day 21 of 2-3 pooled experiments (right panels, 6-10 mice/group ileum PBS n=15, ileum OXA n=14, colon PBS n=16, colon OXA n=14). Each data point represents one mouse, means ± SEM depicted. **d-e**. Long term protective antitumor immunity using ileal IEC-based immunization. Tumor rechallenge at day 14 (d) after complete tumor rejection in (a-c) settings using 10 times the minimal tumorigenic dose (MTD) of autologous MC38 versus the MTD of irrelevant syngeneic cancer cells (MCA205 and CCA) or at week 12 post-immunization (e). Percentages of tumor-bearing mice post-implantation of various malignancies (d). n=6 mice/group in 3 independent experiments for each cell line. For tumor growth kinetics (e), means ± SEM are depicted. n= 6 and 10 mice/group for naïve and vaccinated groups, respectively. **f-g**. Vaccination experiment using WT littermates or genetic variants, *Casp3/7*$^{\Delta IEC}$ (f) or *Ripk3*$^{\Delta IEC}$ (g), as ileal IEC donors to immunize WT hosts. Means ± SEM are depicted. n=6 mice/group showing 1 representative experiment out of 2 independent experiments yielding similar results. **h.** Tumor growth kinetics of MC38 treated

or not treated with OXA at 7 days post-implantation in *Casp3/7*$^{\Delta IEC}$ mice compared with *Casp3/7*$^{fl/fl}$. One representative experiment out of 2 independent experiments yielding similar results is shown. *Casp3/7*$^{fl/fl}$ PBS n=5, *Casp3/7*$^{fl/fl}$ OXA n=5, *Casp3/7*$^{\Delta IEC}$ PBS n=6, *Casp3/7*$^{\Delta IEC}$ n=7. Means ± SEM are depicted. **i.** Flow cytometric analyses of various T cell subsets $CD3^+$, $CD8^+$, and $CD8/CD4^+Foxp3^+$ ratios at 10 days post-OXA. One representative experiment out of two yielding similar results is shown. *Casp3/7*$^{fl/fl}$ PBS n=7, *Casp3/7*$^{fl/fl}$ OXA n=7, *Casp3/7*$^{\Delta IEC}$ PBS n=7, *Casp3/7*$^{\Delta IEC}$ n=8. Each data point represents one mouse, means ± SEM depicted. Statistics: mixed-effect modeling with a specific software (Methods) for longitudinal tumor growth analysis (b left panel, c left panel, e, f, g, h) and Mann-Whitney U test used to compare two independent groups (after Kruskal-Wallis was implemented for multiple groups) (b right panel, c right panel, i). Two-tailed *p*-values are shown in the figures.

Figure 4. Mandatory role of TFH in the immunogenicity of dying ileal IEC.

a-b. Representative flow cytometry dot plot depicting percentages of TFH (defined as $CXCR5^{hi}PD1^{hi}CD4^+$ cells in the viable $CD3^+CD45^+$ population) from dLN (a) and overlay of mean fluorescence intensities of Bcl6 expression in the $CXCR5^{hi}PD1^{hi}CD4^+$ population (b). Black line histogram corresponds to staining with the isotype control Ab. One experiment. **c.** Flow cytometry analyses of percentages of TFH in vaccinated draining LN defined as defined in (a-b), 7 days after the 2nd immunization. Each data point represents one mouse, means ± SEM are depicted. n=6 mice/group. **d.** Flow cytometry analysis of percentages of TFH as defined in (a-b) in mLN of tumor bearers from the indicated genetic background treated or not with OXA and sacrificed at day 21. Pooled data from two independent experiments. *Casp3/7*$^{fl/fl}$ PBS n=11, *Casp3/7*$^{fl/fl}$ OXA n=11, *Casp3/7*$^{\Delta IEC}$ PBS n=18, *Casp3/7*$^{\Delta IEC}$ n=13. Each dot represents one mouse mLN. Means ± SEM are depicted. **e-f.** Dying (OXA-exposed) ileal IEC vaccination as in Fig. 3a. in WT C57BL/6 mice or littermates harboring *Cxcr5* genetic

defects (e) or co-treated with neutralizing anti-CXCL13 (f) or isotype control Ab. After vaccination, mice were challenged with the MTD of MC38 2 to 3 weeks later. Tumor growth kinetics are depicted as means ± SEM. n=6 mice/group. **g**. ELISA monitoring of serum levels of IgG2b at 4 weeks at mice sacrifice, each dot representing one mouse in the experiment of sc vaccination using OXA-exposed ileal IEC in the presence of depleting or neutralizing antibodies (anti-CD4, anti-CXCL13 or isotype control Abs). A representative experiment out of two is depicted, comprising n=6 mice/group. Two-sided Wilcoxon signed-rank test. **h.** Vaccination experiment as in (f) but using B cell depleting Abs. Tumor growth kinetics are depicted in a representative experiment. Statistics: unless otherwise specified, mixed-effect modeling with a specific software (Methods) for longitudinal tumor growth analysis (e, f, h) and Mann-Whitney U test used to compare two independent groups (after Kruskal-Wallis was implemented for multiple groups) (c, d). Two-tailed *p*-values are shown in the figures.

Figure 5. Role of Batf3 DC, IL-1R and IL-12 in the elicitation of TFH during ileal cell death.

a. Tumor growth kinetics (means ± SEM) of MC38 after vaccination with OXA-exposed ileal IEC from SPF versus GF mice. One representative experiment (with n=6 mice/group) is depicted out of 3 yielding similar results. **b.** Refer to experimental setting in extended data Figure 5b. Representative tumor growth curve in mice immunized with ileal mucosae-derived microbiota alone (left) and OXA-IEC plus ileal mucosae-derived microbiota (right) from the same patient conveying immunogenicity (5 mice/group). Means ± SEM are depicted. **c.** FACS determination of $CXCR5^{hi}PD1^{hi}CD4^{+}$ T-lymphocytes from mLN of MC38 tumor bearing WT versus *Batf3* KO mice or WT animals treated with ATB (as in Fig. 2a), at day 7 post-OXA ip. Two experiments were performed and each dot represents one mLN. n=5 mice/group. Each data point represents one mouse, means ± SEM depicted. **d.** Representative micrographs of

immunohistochemical analyses of IL-1β of the lamina propria in paired ilea and colons from WT mice treated or not with OXA at 24h. Scale bar: 50µm. **e.** Statistical analysis of IL-1β$^+$ cells by automatic quantification. Samples size in Ileum and Colon: water PBS n=5, water OXA n=5, ATB PBS n=4, ATB OXA n=6. Each data point represents one sample, means ± SEM depicted. **f-g.** Tumor sizes at sacrifice (means ± SEM) of MC38 after vaccination with OXA-exposed ileal IEC from SPF mice treated or not with IL-1R1 antagonist (f) and concomitant monitoring of serum IgG2b levels by ELISA (g, Two-sided Wilcoxon signed-rank test). Pooled data from two independent experiments are shown. Naïve n=11, IEC-OXA Iso n=12, IEC-OXA αIL1R1 n=12. Each data point represents one mouse. **h.** Tumor size at day 21 of MC38 after vaccination with OXA-exposed ileum crypt-stem cell derived organoids as donors to immunize WT hosts, with or without supplementation of rIL-1β. Pooled data from two independent experiments. Naïve n=11, ctrl n=11, rIL-1β n=10. Each data point represents one mouse, means ± SEM depicted. **i.** Tumor growth kinetics of MC38 after vaccination with OXA-exposed ileal IEC from SPF mice with isotype control or neutralizing anti-IL-12p40 or anti-IL-12p70 Abs. Pooled data from two independent experiments are shown. Naïve n=12, Isotype n=12, anti-IL-12p40 n=12, anti-IL-12p70 n=6. Each data point represents one mouse, means ± SEM depicted. Statistics: unless otherwise specified, mixed-effect modeling with a specific software (Methods) for longitudinal tumor growth analysis (a, b, i) and Mann-Whitney U test used to compare two independent groups (after Kruskal-Wallis was implemented for multiple groups) (c, e, f, h). Significant two-tailed *p*-values are shown in the figures.

Figure 6. Immunogenic ileal commensals boost the anticancer effects of immunotherapies against colon cancer.

a. Experimental setting of vaccination with organoids. Ileal stem cell derived- organoids were exposed to distinct commensals and OXA *ex vivo* prior to s.c. injection to immunize naive mice against MC38. **b.** MC38 tumor sizes at sacrifice from two independent experiments performed as indicated in the experimental setting (a.). Naïve n=10, *B. fragilis* n=16, *F. nucleatum* n=15. Each data point represents one mouse, means ± SEM depicted. **c-f.** Experimental setting (c) of combinatorial therapeutic regimen against day 7 established MC38 (d) and CT26 (f) tumor bearing mice reared in SPF conditions and supplemented with oral gavage (with various commensals) prior to i.p. administration of OXA. Tumor growth kinetics for each mouse in a representative experiment out of two yielding similar results. Each group comprised n=6 mice followed until sacrifice of the control group. Detailed tumor growth kinetics in groups treated with OXA or anti-PD1 alone or combination of both are presented in Supplementary Figure 6f. **e**. ELISA monitoring of IgG responses in serum of mice treated as in (c.) for MC38/C57BL6J and in Extended data Fig. 6c for CT26 in BALB/c mice. Two-sided Wilcoxon signed-rank test. **g**. Id. as in (f) but combinatorial regimen performed after FMT of one aNR patient (aNR1, Supplementary Table 4). Isotype n=5, OXA+αPD1 n=6, OXA+αPD1+*B. fragilis* n=6, OXA+αPD1+*F. nucleatum* n=6. Each data point represents one mouse. **h.** *Proposed model on the role of the ileal microbiome in regulating the balance between tolerance and immunity of dying intestinal epithelial cells.* Chemotherapeutic agents (such as oxaliplatin) act not only on the tumor cells but also on cycling ileal IEC of the crypts. Ileal crypt IEC undergo Casp3/7-dependent cell death which is tolerogenic, i.e does not induce TFH nor B cell maturation, but instead promotes TH17 accumulation in tdLN, when the ileal microbiome is dominated by *Fusobacteriaceae* (such as *F. nucleatum*) or *Prevotellaceae* (such as *P. clara*) family members. Conversely, ileal crypt IEC undergo Casp3/7-dependent cell death which becomes immunogenic, i.e inducing TFH, B cell maturation with class-switched antibody IgG(2b) production and activation of

(crypt/bacterium antigen-specific) $CD8^+$Tc1 cell subsets, when the ileal microbiome is dominated by *Erysipelotrichaceae family members* (such as *E. ramosum*) or *B. fragilis*. Local immunity is triggered by the migration of lamina propria $CD103^+CD11b^-(Batf3^+)$ DCs to the mLN where they elicit an IL-1β and IL-12-dependent TFH immune response. $PD1^{high}$ TFH may respond to anti-PD1 Ab in the setting of OXA-induced crypt cell death, paving the way to the additive/synergistic effects of the combinatorial regimen when immunogenic bacteria dominate the ileal microbiome. We surmise that TFH play an helper role for the effector functions of self-antigen reactive $CD8^+$ T cells or facilitate IgG-mediated ADCC responses against cancer cells.

Statistics: unless otherwise specified, mixed-effect modeling with a specific software (Methods) for longitudinal tumor growth analysis (d, f, g) and Mann-Whitney U test used to compare two independent groups (after Kruskal-Wallis was implemented for multiple groups) (b). Significant two-tailed *p*-values are shown in the figures.

Methods

Patients' characteristics and clinical study details for discovery cohort. The inclusion criteria for the study were patients with colon adenocarcinoma who underwent right hemi-colectomy plus ileal resection (last 10 centimeters). Patients were not on antibiotics at the moment of surgery. Three different health cancer centers participated in the study: Gustave Roussy Cancer Campus (Villejuif, France), Centre G-F Leclerc (Dijon, France) and Hôpital Bicêtre (Le Kremlin-Bicetre, France). Clinical studies were conducted according to the ethical guidelines and approval of the local CCPPRB. The study received the legal ethic authorization by the Ministère de l'Enseignement Supérieur et de la Recherche (AC-2013-1884). For feces collection, the study name was “Oncobiotics”, B2M ethics protocol number PP: 15-013. Written informed consent in accordance with the Declaration of Helsinki was obtained from all patients. Patients' clinical characteristics are detailed in Table S1.

Patients' characteristics and clinical study details for validation cohort. The inclusion criteria for the study were patients with colon adenocarcinoma who underwent right hemi-colectomy plus ileal resection (last 10 centimeters). Samples were retrieved from formalin-fixed paraffin embedded (FFPE) tissue from the pathology archives. Two different health centers participated in the study: Centre G-F Leclerc (Dijon, France) and Istituto Nazionale dei Tumori, Italy. Patients' clinical characteristics are detailed in Table S1.

Patients’ clinical categorization and follow up. The information pertaining to tumor staging (TNM staging system of the American Joint Committee on Cancer –AJCC) and MSI status was obtained in pathology reports. Information pertaining to cancer recurrence, death and causes of death was obtained from hospital records. All observations were censored at loss to

follow-up and at the end of the study period. OS was measured from the date of surgery to the date of death from any cause. Progression free survival (PFS) was measured from the date of surgery to the date of local or regional recurrence, distant metastases or death.

Preparation of patients' samples for the study. Samples were collected at the time of surgery (Extended data Fig. 1a-b). Luminal content from proximal colon: approximately 5 g of luminal content from the right colon were collected. 1g of material was transferred into a sterile cryotube and immediately frozen at -80^{o}C for 16S rRNA gene sequencing. The remainder was suspended at 0.1g/ml in Brain-Heart infusion broth (BHI) with 15% glycerol, homogenized and divided in 10 ml aliquots for FMT experiments. Mucus adherent to the mucosae: After washing the surgical specimen, a non-involved surface of the mucosae from the ileum and colon (distant from the tumor) was scraped using glass slides. 1g of mucosal material was transferred into a sterile cryotube and immediately frozen at -80^{o}C for 16S rRNA gene sequencing. When possible, the remainder of the specimen was suspended at 0.1g/ml in BHI with 15% glycerol. Mucosal biopsies: approximately 4 cm^2 of ileal and non-tumoral colonic tissue (distant from the tumor) that included at least the submucosa were removed, cut into small pieces (approximately 1 cm^2). All samples were FFPE after collection. Samples where technical analysis was performed are detailed in Extended data Fig. 1b.

Tumor immune profiling by Immunoscore. Immune histological analysis of lymphocyte infiltration was performed on paraffin-embedded tumor samples. Two adjacent 4 µm sections were deposited on positively charged slides (SuperFrost +, Menzel Gläser, Germany). Immunoblots were performed using protocols optimized for the XT Benchmark (Ventana, USA). Dewaxing and rehydration were carried out on the automaton as well as unmasking

with Cell Conditioning 1 (Ventana, USA), EDTA buffer, pH8. CD3 monoclonal antibody, clone 2GV6 (ventana, USA) was incubated 20min at 37°C and the CD8 monoclonal antibody, clone C8 / 144B (Dako) was incubated for 30 min at 37 ° C. The ultraview DAB development system (Ventana, USA) was used to visualize Diaminobenzidine; a counter-staining of the tissue is performed by incubating 4min, Hematoxilin II (ventana, USA). Slides were scanned using the high-resolution slide scanner, NanoZoomer HT2.0 (Hamamatsu photonics, Japan) at a 20x magnification with a resolution of 0.45 μm / pxl. Images of the scanned slides were imported into the Developer XD image analysis software (Definiens, Germany). Immune quantification of $CD3^+$ and $CD8^+$ was analyzed separately in the core of the tumor (CT) and the invasive margin (IM).

Cell culture, reagents and tumor cell lines. MC38, MCA-205 (syngeneic from C57BL/6J mice), and CT26 or 4T1 cell lines (syngeneic from BALB/c mice) were purchased from ATCC. CCA cell line was a kind gift from Professor Gregory J. Gores[48]. Cells were cultured in RPMI 1640 containing 10% FBS, 2mM L-glutamine, 100 IU/ml penicillin/streptomycin, 1mM sodium pyruvate and MEM non-essential amino acids. All reagents were purchased from Gibco-Invitrogen (Carlsbad, CA, USA). All cell lines were cultured at 37°C with 5% CO_2 and regularly tested to be free of mycoplasma contamination.

Mice. All animal experiments were carried out in compliance with French and European laws and regulations. The local institutional board (Ministère de la Recherche, de l'Ensiegnement Supérieur et de l'innovation, MESRI) approved all mouse experiments (permission numbers: 2014-071-1124 and 2017-020-8964). Female C57BL/6J and BALB/c mice were purchased from Harlan (France) and Janvier (France), respectively. Mice were used between 7 and 14 weeks of age. Germ-free C57BL/6J mice and *Il1ab*$^{-/-}$, *Il18*$^{-/-}$*Cd39/Entpd1*$^{-/-}$, *Tlr2/4*$^{-/-}$ and *Tlr9*$^{-/-}$

(all C57BL/6J genetic background) and WT littermates from the same breeding zones were obtained from the facility located at CDTA (Cryopreservation, Distribution, Typage et Archivage, Orléans, France). Lgr5-EGFP-IRES-creERT2 mice were used for lineage-tracing of Lgr5-expressing stem cells of the small intestine and were purchased from The Jackson Laboratory (JAX stock #008875)[49]. Homozygous *Cxcr5*-deficient mice, B6.129S2(Cg)-Cxcr5^{tm1Lipp}/J, were a kind gift from Dr. Reinhold Förster (Hannover, Germany)[50]. *Bcl6$^{fl/fl}$Cre^{CD4}* mice[29] were a kind gift from Dr. James A. Harker (Imperial College London, UK). *Batf3*-deficient mice, B6.129S(C)-Batf3^{tm1Kmm}/J, were obtained from the animal facility of University of Erlangen (Germany). In these mice the first 2 exons of the transcription factor BatF3 have been removed inactivating this important transcription factor for cDC1 DCs[51]. Casp3$^{fl/fl}$; Casp7$^{fl/fl}$; Villin-Cre Tg (*Caspase3/Caspase7* IEC double KO), RIPK3$^{-/-}$; Nnt$^{Mut/Mut}$ (*Rip3k* KO) and respective WT littermate controls were obtained by Dr. Peter Vandenabeele and some experiments were conducted in the animal facility of VIB-UGent Center for Inflammation Research, Ghent, Belgium. Caspase-3$^{fl/fl}$ and Caspase-7$^{fl/fl}$ mice were generated using respectively ES clone HEPDO716_4_G05 and EPD0398_5_E02 (C57BL/6N) from the International Mouse Phenotyping Consortium (IMPC). The neomycin selection cassette was removed using FLPe deleter mice[52]. Intestinal specific targeting was achieved by crossing to Villin Cre mice[53]. All other mouse experiments were performed at the animal facility in Gustave Roussy Cancer Campus where animals were housed in specific pathogen-free conditions or were maintained in isolators for germ-free and FMT experiments.

Antibiotic treatments. Mice were treated with an antibiotic solution (ATB) containing ampicillin (1 mg/ml), streptomycin (5 mg/ml), and colistin (1 mg/ml) (Sigma-Aldrich), with or without the addition of vancomycin (0.25 mg/ml), which were added in the sterile drinking water of mice. Solutions and bottles were changed 3 times and once weekly, respectively.

Antibiotic activity was confirmed by analyzing feces for the presence of bacterial DNA by qPCR (see below) and by cultivating fecal pellets resuspended in BHI+15% glycerol at 0.1 g/ml on COS (*Columbia Agar* with 5% *Sheep Blood*) plates for 48h at 37°C in aerobic and anaerobic conditions, weekly. Duration of ATB treatments were slightly different based on the experimental settings. In brief, to compromise the efficacy of Oxaliplatin (OXA) with ATB, mice were treated for 2 weeks prior to tumor implantation and continuously throughout the experiment.

Prophylactic and therapy setting in tumor models. *Subcutaneous model of MC38*. Syngeneic C57BL/6J mice were implanted with 1×10^6 MC38 WT cells subcutaneously and treated intraperitoneally (*i.p.*) when tumors reached 20 to 30 mm^2 in size with 10mg/kg OXA or vehicle (PBS). The composition of the commensal gut microbiota in the treated and non-treated groups was maintained synchronized by cohousing. Tumor size was routinely monitored every 3 days by means of a caliper. *Immunizations with IEC isolated from the gut.* Donor mice were treated with OXA (10mg/kg) i.p. for 6h to induce intestinal epithelial cell (IEC) death. Control animals were treated with vehicle (PBS) alone. The composition of the commensal gut microbiota in the treated and non-treated groups was maintained synchronized by cohousing. At 6h post-OXA (or PBS), mice were euthanized and the ileum and/or colon were collected to isolate IEC. One million IECs from ilea or colons were then injected s.c. into the left flank of the recipient naive mice. The procedure was repeated once, 7 days later. Tumor challenge was performed on the right flank 7 days after the last immunization, with minimal tumorigenic doses (MTD, doses which obtain 100% of tumor incidence in naïve mice, i.e. MC38 (1×10^6 cells), MCA205 (0.8×10^6 cells), CT26 (1×10^6 cells) and 4T1 (0.3×10^6 cells)). In indicated experiments, IEC were pretreated with 100mM pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS, Sigma), 200

µM 2,4-Dinitrophenol (DNP, Sigma), or vehicle for 20 min at 4°C. Treated cells were washed 3 times with cold PBS before injection, or IEC were co-injected with neutralizing anti-HMGB1 Ab (ab18256,Abcam), anti-Calreticulin Ab (NB600-101, Novus Biologicals) or Rabbit IgG Isotype Control (NBP2-24891, Novus Biologicals) at 10µg per injection.
In indicated experiments, T cell depletion was performed by i.p. injections of anti-CD4 (clone GK1.5, 200µg/mouse), anti-CD8 mAbs (clone 53–6.72; 200µg/mouse), anti-CXCL13 (clone 143614R, 500µg/mouse, from R&D) anti-IL12p40 (clone C17.8; 500µg/mouse), anti-IL12p70 (clone R2-9A5; 500µg/mouse), or respective isotype controls (LTF-2, 2A3, rat IgG2a, rat IgG2b). Depletion treatment started 1 day before 1st vaccination and was repeated at the same dose for the second vaccination. B cells depletion was performed as previously described[54]. Briefly, mice were injected i.p. with the following mixture of monoclonal antibodies at 150µg/mouse each: rat anti–mouse CD19 (clone 1D3), rat anti–mouse B220 (clone RA36B2, from Biolegend), and mouse anti–mouse CD22 (clone CY34). After 48h, the mice were injected with a secondary antibody mouse anti–rat kappa immunoglobulin light chain (clone MAR 18.5) at 150 g/mouse. (all antibodies from BioXCell, unless otherwise indicated). *Immunizations with IEC from ileum-derived organoids***.** Organoids were treated with 10 µg/ml OXA for 3h. In indicated experiments, IECs were admixed with pasteurized ileal mucus harvested from patient samples as adjuvants, were then injected s.c. (10^6 IEC cells and 50µl of mucosal material at 500mg/ml) into the left flank of the recipient mice. The procedure was repeated once, 7 days later. Tumor challenge was performed on the right flank 7 days after the last immunization. *Alistipes onderdonkii, Erysipelatoclostridium ramosum, Bacteroides uniformis* and *Streptococcus gallolyticus* isolates were isolated in our laboratory from ileal mucosal material from patients used in vaccination experiments. In the indicated experiments, organoids were treated with 10 µg/ml OXA for 3h in the presence of 10^6 pasteurized bacteria/ml. *Immunotherapy.* Mice were treated intraperitoneally (i.p.) with anti-

PD-1 (250µg/mouse; clone RMP1-14, BioXcell), or the appropriate isotype control (clone 2A3) at 4 days after OXA (or PBS) treatment. 4 total injections every 3 days were performed.

Gut dissociation to harvest IEC. Ilea and/or colons were collected and fat tissue, Peyer's patches and feces were removed. Intestines were cut longitudinally and then cut transversally into small pieces into a tube. Pieces were transferred into a new 50ml tube with 20ml of IECs medium (PBS, 5% FCS, 5mM EDTA and 1mM DTT), vortexed and shaken at 37°C for 15 min. Cell suspensions were collected in a new tube, filtered with a cell strainer (100µm), centrifuged, resuspended in PBS, and stored on ice until use. In indicated experiments villi and crypt cells were isolated as previously described[55]. Briefly, pieces of ileum were washed in cold PBS and villi cells were recovered by mechanical isolation, by means of repetitive pipetting. This procedure was repeated until limpid supernatant was obtained. The remaining ileal tissue was incubated in crypt chelating buffer (IEC medium) to recovered crypt cells as described above.

Organoid cultures. Crypts were isolated and enriched from the ileum of 8-12 week old C57BL/6J mice as previously described[55] with the following modifications. Briefly, washed pieces of ileum were incubated in crypt chelating buffer (2mM EDTA in PBS) for 30 minutes on ice. Following the removal of crypt chelating buffer, fragments were vigorously rinsed 3 times with PBS containing 10% FCS and filtered through a 70-µm cell strainer (BD Bioscience). Crypts were pelleted, washed with Advanced DMEM/F12 (ADF) (Invitrogen), resuspended in 1mL of Matrigel growth factor reduced basement membrane matrix (Corning) and 50uL drops were pipetted into a 24 well plate. Crypts were overlayed with ADF containing the following: 100 U/mL penicillin G sodium, 100µg/mL streptomycin sulphate, 2mM L-glutamine, 10mM HEPES, 1x N2 supplement, 1x B27 supplement, 50ng/mL mEGF,

100 ng/mL mNoggin (Peprotech, Hamburg, Germany), N-acetylcysteine (Sigma) (reagents from Invitrogen unless otherwise indicated) and 10% conditioned medium of R-Spondin-1 transfected HEK 293T cells.

FMT experiments. Frozen fecal samples collected from proximal colon were thawed and thoroughly vortexed. Large particulate material was allowed to settle by gravity. 200µl of supernatant was administered in a single dose by oral gavage into germ-free mice. Additionally, an extra 100µl was topically applied onto the fur of each animal. The resulting gnotobiotic mice were maintained in positive pressurized isolators with irradiated food and autoclaved water. Two weeks after FMT, tumor cells were injected subcutaneously and mice were treated with OXA or vehicle (PBS) as described above. Baseline colonization was assessed by 16S rRNA sequencing of fecal gene amplicons within each group of mice before starting any treatment (refer to Supplementary Fig. 2). *Immunotherapy*. SPF mice were treated with ATB cocktail for 3 days (to sterilize the gut) that was discontinued 24h before performing the oral gavage with the patient sample. After two weeks, tumor was inoculated and bacterial gavage, OXA and anti-PD1 injections performed as described for the SPF model.

Gut colonization with dedicated commensal species.

We used several converging biological and/or clinical readouts to classify bacteria that fall into each of these two "immunogenic" versus "tolerogenic" categories: (i) in vitro assays using Flt3-derived DC: no/low IL-12 associated with "tolerogenic"bacteria; (ii) in vivo experiments using ip OXA+oral gavages of bacteria: no raise in serum IgG/IgG2b responses, no increase of TFH in tdLN but instead, accumulation of TH17 cells in tdLN associated with "tolerogenic"bacteria; (iii) in vivo experiments using FMT following by therapeutic

OXA+oral gavages or vaccination with organoids ± bacteria: no control of MC38 tumor growth associated with "tolerogenic"bacteria; (iv) correlative studies between 16S rRNA sequencing of ileal gene amplicons and progression free survival (PFS) in the whole cohort of pCC patients: no significant impact on PFS associated with "tolerogenic" bacteria.
Isolated species tested were isolated from samples for this study by culturomics. Species were grown on COS plates in aerobic or an anaerobic atmosphere created using anaerobic generators (Biomerieux) at 37°C for 24-72h. Colonization of SPF, GF, or avatar (FMT) mice was performed by oral gavage with 100 µl of suspension containing 10^9 bacteria in PBS. For bacterial gavage, suspensions of 10^9CFU/ml were obtained using a fluorescence spectrophotometer (Eppendorf) at an optical density of 1 measured at a wavelength of 600 nm. Two bacterial gavages were performed for each mouse, the first, 24h before the treatment with OXA and then 24h after the treatment. The efficacy of colonization was confirmed by qPCR performed on fecal DNA with specific primers for bacterial spp (see below) or culturing the feces 48h after the first gavage (Supplementary Fig. 6).

Bacteria identification by qPCR.

Total gDNA from fecal pellets was extracted with QIAamp Fast DNA Stool Mini Kit (Qiagen) following manufacturer's protocol. gDNA was analyzed by real-time quantitative PCR (RT-qPCR) using the Universal Master Mix II (Invitrogen) or PowerUp SYBR Green Master Mix (Invitrogen) according to the manufacturer's instructions using the 7500 Fast Real Time PCR system or Quant Studio 3 (Applied Biosystems). Expression was normalized to the expression of the total bacterial load determined by Universal 16S RNA gene by means of the $2^{-\Delta Ct}$ method. All primers were from Thermo Fisher.

Target	PCR system	Primers and probes	Oligo sequence	Ref
Universal *16S rRNA*	TaqMan	Forward	CGGTGAATACGTTCCCGG	56
		Reverse	TACGGCTACCTTGTTACGACTT	

		Probe	FAM-CTTGTACACACCGCCCGTC-MGB	
Bacteroides fragilis	SYBR green	Forward	TGATTCCGCATGGTTTCATT	57
		Reverse	CGACCCATAGAGCCTTCATC	
Erysipelatoclostridium ramosum	SYBR green	Forward	GTGACCGTATTAAAAGTGCCT	58
		Reverse	TACCGTCACTCGGCTAC	
Fusobacterium nucleatum	TaqMan	Forward	CAACCATTACTTTAACTCTACCATGTTCA	59
		Reverse	GTTGACTTTACAGAAGGAGATTATGTAAAAATC	
		Probe	FAM-TCAGCAACTTGTCCTTCTTGATCTTTAAATGAACC -MGB	
Paraprevotella clara	SYBR green	Forward	GGATGTGTTTGTCTTTCCGC	60
		Reverse	CTACCCATCGTYGCCTTGG	

Characterization of mLN immune gene expression profile by real-time quantitative PCR Analysis. Total RNA from mLN was extracted with RNeasy Mini Kit (Qiagen) and then reverse transcribed into cDNA with the SuperScript III Reverse Transcriptase and the RNaseOUT™ Recombinant Ribonuclease Inhibitor (Life Technologies, Saint Aubin, France), in the presence of random primers (Promega, Charbonnieres, France) and the Deoxynucleoside Triphosphate Set, PCR grade (Roche Diagnostics, Meylan, France). cDNA was analyzed by real-time quantitative PCR (RT-qPCR) with the TaqMan method with TaqMan® Gene Expression Assays using the Universal Master Mix II (Invitrogen) according to the manufacturer's instructions using the 7500 Fast Real Time PCR system (Applied Biosystems). Expression was normalized to the expression of the housekeeping gene *Ppia* by means of the $2^{-\Delta Ct}$ method. All primers were from TaqMan® Gene Expression Assay (Thermo Fisher). Mouse primers: *Ppia* (Mm02342430_g1); *Il12a* (Mm00434165_m1); *Il12b* (Mm00434174_m1); *Il2* (Mm00434256_m1); *Il4* (Mm00445259_m1); *Il6* (Mm00446190_m1); *Il21* (Mm00517640_m1); *Tgfb1* (Mm01178820_m1); *Il1b* (Mm00434228_m1); *Il1a* (Mm00439620_m1); *Tnf* (Mm00443258_m1); *Il10* (Mm01288386_m1); *Il23a* (Mm00518984_m1); *Ifng* (Mm01168134_m1).

ELISA. For quantification of lipocalin-2 from feces, samples were resuspended (at 100 mg/ml) in PBS containing 0.5% Tween 20 and incubated with shaking for 20 min at 37°C. Samples were then centrifuged for 10 min at 12,000 rpm. Supernatants were collected and stored at -20°C until analysis. Lipocalin-2 levels were measured using the Duoset murine Lcn-2 ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. For quantification of serum immunoglobulins, sera were collected and stored at -20°C until analysis. Ig levels were measured using the IgG (Total) and IgG2b Mouse Uncoated ELISA Kits (Thermofisher) following the manufacturer's instructions.

Flow cytometry analyses. Tumor draining lymph nodes (tdLN) were harvested at the end of the experiment for the FMT model. Mesenteric lymph nodes (mLN) were harvested 7 days post-OXA treatment for gut immunology analysis. Lymph nodes were crushed in RPMI medium or, for DC subset analysis, digested with Collagenase D (Worthington) and DNAse I (Roche) for 30 min at 37°C, as previously described[61]. Subsequently, the cells were meshed through a 100 μm cell strainer to retrieve single cell suspensions. In the next step, erythrocytes were lysed by incubation in ACK lysis buffer and filtered through a 40 μm cell strainer. IEC suspensions were obtained from ileum-derived organoids by means of 3 wash dissociation method, as described above. In all cases, 2-10 million cells were pre-incubated with purified anti-mouse CD16/CD32 (clone 93; eBioscience or clone 2.4G2) for 20 minutes at 4°C, before membrane staining. For intracellular staining, the Foxp3 staining kit (eBioscience) was used and dead cells were excluded using the Live/Dead Fixable Yellow dead cell stain kit (Life Technologies). For extracellular stainings, DAPI was used to exclude dead cells. Anti-mouse antibodies (and clones) used for phenotyping were purchased from eBiosciences, BD and Biolegend: CD3e (145-2C11 and 17A2), CD4 (GK1.5 or RM4-5), CD8a (53-6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD45 (30-F11), CD45R/B220

(RA3-6B2), CD103 (2E7), CD161b/c (PK136), CD172a (P84), CD317 (927), FOXP3 (FJK-16s), CXCR3 (FAB1685P), CX3CR1 (SA011F11), CCR6 (140706), CCR9 (W-1.2), Ly-6C (HK1.4), PD-1 (29F.1A12), ICOS (11-9942-82), CXCR5 (2G8), MHC-II/I-A/I-E (M5/114.15.2), Siglec-H (551), TNFα (MP6-XT22), XCR1 (ZET), CALR (Polyclonal). Streptavidin PE, Annexin V-APC, 7AAD, 4',6-Diamidino-2-Phenylindole (DAPI), and Propidium Iodide (PI) were from BD Pharmingen. Samples were acquired on Cyan ADP 9 colors cytometer (Beckman Coulter), 13 color Cytoflex (Beckman Coulter), or 18 color LSR Fortessa SORP (BD) and analyses were performed with FlowJo software (Tree Star, Ashland, OR, USA).

Intestinal Ki67 staining and evaluation. OXA was injected i.p. at a dose of 10 mg/kg. Mice were euthanized 72h later and small and large intestines were collected and formalin fixed for subsequent immunohistochemistry. FFPE sections from small and large intestine were deparaffinized and rehydrated through a series of graded alcohols and distilled water. Antigen retrieval was performed by pre-treating sections with citrate buffer (pH 6.0, Dako REAL) for 20 min in a microwave (700W) and then for 30 min at RT. Endogenous peroxidase activity was inhibited by treating sections with 3% hydrogen peroxidase for 30 min. Sections were blocked with 1X Teng-T for 30min at RT. The primary polyclonal Rabbit antibody (Ab), Ki67 (NCL ki67p, Novocastra, 1:1000) was incubated overnight, followed by the secondary Ab, biotinylated anti-rabbit (BA-1000, Vector) for 1h. Peroxidases were detected with di-amino-benzidine-peroxidase substrate kit (Vectastain Elite, PK6100, Vector), and counterstained with Mayer's haematoxylin and alcian blue.

Immunohistochemistry staining and analysis of cleaved caspase-3 expression. Composition and cellularity were assessed using a hematoxylin, eosin & safran stain (H&E).

3µm-thick sections of formalin-fixed, paraffin-embedded ileum and colon from treated and untreated mice were mounted on poly-L-lysine-coated slides, deparaffinized and rehydrated through a series of graded alcohols and distilled water. Antigen retrieval was performed by pre-treating sections with 0.01 M sodium citrate buffer (pH 6.0, Diapath) for 30 min in a 98°C water bath. Endogenous peroxidase activity was inhibited by treating sections with 3% hydrogen peroxidase (#S202386, DAKO) for 10 min. Sections were blocked with IHC/ISH Super Blocking Solution (#PV6122, LeicaBiosystem) for 10 min. The primary polyclonal rabbit antibody (Ab), Cleaved Caspase-3 (Asp175) (#9661, Cell Signalling, dilution 1:100) was incubated for 1h, followed by the secondary Ab, PowerVision Poly-HRP anti-Rabbit IHC Detection Systems (#PV6114, LeicaBiosystem) for 20 minutes. Peroxidases were detected with Di Amino Benzidine-peroxidase substrate kit (DAKO), and counterstained with Mayer's haematoxylin. Images displayed in the figures were acquired as whole slide images (WSIs) with a slide scanner Olympus VS120 at 20x objectives. For detecting murine crypts, a deep-learning based method was used. Briefly, pathologists trained neural network in order to perform the semantic segmentation of villi, crypts, lamina propria and muscle in murine ileum and colon. WSIs were cropped into 256 x 256 pixels tiles. Blank tiles and tiles with only debris or uninterpretable pieces of tissue were excluded by pathologists. Cropped tiles were processed by the neural network-based semantic segmentation model and crypts images were obtained. QuPath software was used to detect DAB positive cells[62] and the cell density of positive cleaved caspase-3 cells in crypts was calculated.

Immunohistochemistry staining, scanning, and analysis of double staining BCL6-CD4. Mouse anti-human BCL6 monoclonal Ab (DAKO, M7211, clone PG-B6p, 20 µg/ml) and rabbit anti-human CD4 monoclonal Ab (Spring, clone SP35) were performed on 3µm-thick sections of formalin-fixed, paraffin-embedded ileum, colon and adenocarcinoma of colon

from the same patients with a Benchmarck Ultra automated immunostainer (Ventana, Gustave Roussy Cancer Campus). Antigen retrieval was performed by incubating slides in CC1 buffer (EDTA pH 8.0) for 92 min at 95°C. Then the BCL6 antibody was incubated for 1h20 at room temperature and the CD4 antibody for 1h at 37°C. Antibodies were detected by respectively Amplification OptiView kit (Roche, #760-099) and OptiView DAB (Roche, #760-700) and Amplification kit (Roche, #760-080) and UltraView RED (Roche, #760-501). Finally, the sections were counterstained by Hematoxylin I (Roche, #760-2021) and bluing reagent (Roche #760-2037). Images for analysis were acquired as whole slide images (WSI) with a slide scanner Zeiss Axio Scan.Z1 and Olympus VS120. To classify and quantify BCL6 and/or CD4 positive cells, QuPath software was used[62]. ROIs were defined firstly by 'Simple Tissue Detection' function and modified by hand in each WSI. After detecting all cells in ROIs, we interactively trained QuPath to classify $CD4^{-}BCL6^{+}$ cells using morphology and intensity measurements of all detected cells. $CD4^{+}BCL6^{+}$ cells were quantified manually. Cell density was calculated by dividing the total number of cells in each population by area of ROIs.

Immunohistochemistry staining and analysis of IL-1β in murine ileum and colon.

Composition and cellularity were assessed using a hematoxylin, eosin & safran stain (H&E). 3µm-thick sections of formalin-fixed, paraffin-embedded ileum and colon from mice were mounted on poly-L-lysine-coated slides, deparaffinized and rehydrated through a series of graded alcohols and distilled water. Antigen retrieval was performed by pre-treating sections with 0.01 M sodium citrate buffer (pH 6.0, Diapath) for 30 min in a 98°C water bath. Endogenous peroxidase activity was inhibited by treating sections with 3% hydrogen peroxidase (#S202386, DAKO) for 10 min. Sections were blocked with IHC/ISH Super Blocking Solution (#PV6122, LeicaBiosystem) for 10 min. The primary monoclonal mouse antibody (Ab), IL-1b (3A6) (#12242S, Cell Signalling, dilution 1:50) was incubated for 1h,

followed by the mouse antibody enhancer and secondary Ab, Klear Mouse HRP-Polymer DAB Detection System (#D52-18, Golden Bridge International, Mukilteo, WA) for 15 min, respectively. Peroxidases were detected with DAB Plus Substrate System (#TA060HDX, ThermoFisher), and counterstained with Mayer's haematoxylin. Images displayed in the figures were acquired as whole slide images (WSIs) with a slide scanner Olympus VS120 at 20x objectives. For mouse tissue analysis, QuPath software was used[62]. ROIs were defined firstly by 'Simple Tissue Detection' function and modified by hand in each WSI. DAB positive cells within the ROIs were quantified and the cell density of positive IL-1b cells was calculated.

Intestinal toxicity analysis. For histological analysis, longitudinal sections were counterstained with hematoxylin, eosin & safran stain (H&E). For histological quantitative analysis, inflammatory foci, appearance of the submucosa, length of villi, and the thickness of lamina propria were scored for each section by a pathologist (P.O.). The score was defined as: 0 = normal, 0 + = focal and minor lesions; 1 = diffuse and minor lesions; 2 = diffuse, minor and major lesions; 3 = major lesions with areas containing only connective tissue.

Culturomics analyses. The bacterial diversity of the ileal mucus samples used for the vaccination experiments was explored using a culturomics approach[63,64]. Each sample was inoculated in aerobic and anaerobic blood culture bottles. Ten-fold serial dilutions of the liquid cultures were subsequently plated on 5% sheep blood enriched Columbia agar (BioMerieux, Marcy l'Etoile, France) and incubated respectively in aerobic conditions for 48h and in anaerobic conditions for one week. Obtained colonies were subcultured and routinely identified using a Matrix Assisted Laster Desorption Ionization Time-of-Flight Mass Spectrometer (MALDI-TOF MS, Microflex, Bruker Daltonics, Bremen, Germany)[65]. In case

of a failed routine identification, the colony was identified by sequencing the 16S rRNA gene. For each bacterial taxon, a mean frequency was calculated in two groups defined according to the response to the vaccination. A relative frequency difference was calculated for each species in order to determine which species were enriched or depleted. Statistical significance of the relative frequency difference was determined using uncorrected chi-square test, comparing the proportion of each taxon in the two groups.

16S rRNA gene sequencing. Characterization of metagenomic communities was performed through amplification and sequencing of hyper-variable regions.

Human samples: gDNA extraction, library preparation and sequencing were conducted at GATC Biotech AG (Konstanz, Germany). Amplification was performed using region-specific primers that target conserved regions flanking the variable regions, V3-V5. Sequencing was performed with Illumina MiSeq technology.

Mouse samples: Total gDNA from fecal pellets was extracted with QIAamp Fast DNA Stool Mini Kit (Qiagen) following the manufacturer's protocol. Sequencing of obtained DNA was performed at IHU Méditerranée Infection (Marseille, France) targeting the V3–V4 regions of the 16S rRNA gene with Illumina MiSeq technology.

Microbiota characterization. Raw FASTQ files were analyzed with Mothur pipeline v.1.39.5 for quality check and filtering (sequencing errors, chimerae) on a Workstation DELL T7910 (Round Rock, Texas, United States). Raw reads (213457810 in total, on average 652776 per sample) were filtered and clustered into Operational Taxonomical Units (OTUs), followed by elimination of low-populated OTUs (till 5 reads), downsampling (the real rarefaction step) of each sample to the number of reads present in the less-populated sample, and by de novo OTU picking at 97% pair-wise identity using standardized parameters and

SILVA rDNA Database v.1.19 for alignment. In all, 1001 OTUs were identified. Sample coverage was computed with Mothur and resulted to be on average equal to 99% for all samples (mean±SDM, 99.4%±0.5%), thus meaning a suitable normalization procedure for subsequent analyses. ANalysis Of SIMilarity (ANOSIM, which represents the difference of datasets' centroids) with 999 permutations or, when requested, Spearman correlation coefficient, were computed with SciKit-Bio package v.0.4.1. Linear discriminant analysis Effect Size (LEfSe) analysis was employed on bacterial species relative abundances which were statistically different after Mann-Whitney U test (for pairwise comparison) or Kruskal-Wallis test (for multiple comparison). Benjamini-Hochberg two-stage false detection rate (FDR) at 10% was then applied, with the additional constraint in leaving only the species which were represented by at least 5 datapoints.

OTUs species assignment and multivariate statistical analyses. Bioinformatic and statistical analyses on recognized OTUs were performed with Python v.2.7.11. The most representative and abundant read within each OTU (as evidenced in the previous step with Mothur v.1.39.5) underwent a nucleotide Blast using the National Center for Biotechnology Information (NCBI) Blast software (ncbi-blast-2.3.0) and the latest NCBI 16S Microbial Database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/). We used an optimized workflow encompassing both SILVA database alignment/classification and NCBI BLAST with the highest Evalue and percentage identity. This approach allowed us to resolve difficult genera such as *Bacteroides* at species-level. A matrix of bacterial relative abundances was built at each taxon level (phylum, class, order, family, genus, species) for subsequent multivariate statistical analyses. Raw matrix (tabular) data were first normalized then standardized using QuantileTransformer and StandardScaler methods from Sci-Kit learn package v0.20.3. Normalization using the output_distribution='normal' option transforms each variable to a

strictly Gaussian-shaped distribution, whilst the standardization results in each normalized variable having a mean of zero and variance of one. These two steps of normalization followed by standardization ensure the proper comparison of variables with different dynamic ranges, such as bacterial relative abundances. Measurements of alpha diversity (within sample diversity) such as observed_otus and Simpson index, were calculated at OTUs level using the SciKit-learn package v.0.4.1. Exploratory analysis of beta diversity (between sample diversity) was calculated using the Bray-Curtis measure of dissimilarity calculated with Mothur and represented in Principal Coordinate Analyses (PCoA), while for Hierarchical Clusterization Analysis (HCA) 'Bray-Curtis' metrics and 'complete linkage' method were used implementing in-house scripts (Python v.2.7.11). In order to compare the microbiota taxa with gene expression datasets, a multivariate statistical Spearman correlation analysis (and related *p*-values) was performed with in-house Python scripts. Mann-Whitney U and Kruskall-Wallis tests were employed to assess significance for pair-wise or multiple comparisons, respectively, taking into account a *p*-value less than or equal to 0.05 as significant after Benjamini-Hochberg two-stages false detection rate (FDR) at 10%.

Statistical analysis. Data analyses and representations were performed either with the statistical environment R (http://www.R-project.org/), Microsoft Excel (Microsoft Co., Redmont, WA, USA) or Prism 6 (GraphPad, San Diego, CA, USA). Tumor growth in mouse models was analyzed with dedicated software (https://kroemerlab.shinyapps.io/TumGrowth)[66]. Briefly, data was subjected to a linear mixed effect modeling applied to log pre-processed tumor surfaces. *p*-values were calculated by jointly testing whether both tumor growth slopes and intercepts (on a log scale) were different between treatment groups of interests. In FMT experiments, comparisons between the efficacy of OXA for each FMT are derived from the estimated slope between treatment

contrasting OXA-PBS for each FMT-treated mice. Contrasts were transformed to be interpreted as % improvement of the tumor size per day of treatment. The Mann-Whitney U test was used to compare two independent groups, while non-parametric Kruskal-Wallis was implemented for multiple groups. For comparisons between patient groups, the Mann Whitney U test *p*-value was used. Chi-square test was used in cases of categorical variables to test differences in proportions between groups. Survival curves were estimated using the Kaplan-Meier product limit method. Cox models were estimated with PFS and OS as outcome based on each gene or cell measurement values. All tests were two-tailed, and *p*-values <0.05 were considered statistically significant.

Data Availability

All raw sequencing data can be found at the NCBI Sequence Read Archive (accession number: PRJNA478491; "Ileal Apoptosis and Microbiome Shape Immunosurveillance and Prognosis of Proximal Colon Cancer", https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA478491, or https://www.ncbi.nlm.nih.gov/sra/PRJNA478491).

For both patients (submission ID SUB4218459) and mice (submission ID SUB6655852), raw fastq.gz files and de-aggregated and de-identified metadata are provided at the following SRA Run Selector link: https://www.ncbi.nlm.nih.gov/Traces/study/?query_key=4&WebEnv=NCID_1_50416433_130.14.22.76_5555_1583484624_2234475021_0MetA0_S_HStore&o=acc_s%3Aa.

All BioSample (PATIENTS_Metadata.csv, MICE_Metadata.csv) and Run Metadata files (PATIENTS_Run.csv, MICE_Run.csv) are also provided as supplementary information to ease metadata availability.

Reporting Summary

Further information on research design is available in the Nature Research Reporting

Summary linked to this Article.

Online Methods References

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