Intestinal inflammation targets cancer-inducing activity of the microbiota

Janelle C. Arthur¹, Ernesto Perez-Chanona¹, Marcus Mühlbauer¹, Sarah Tomkovich¹, Joshua M. Uronis¹, Ting-Jia Fan¹, Barry J. Campbell², Turki Abujamel³,⁴, Belgin Dogan⁵, Arlin B. Rogers⁶, Jonathan M. Rhodes⁶, Alain Stintzi⁵, Kenneth W. Simpson⁵, Jonathan J. Hansen¹, Temitope O. Keku¹, Anthony A. Fodor⁷, and Christian Jobin¹,*

¹Department of Medicine, Pharmacology and Immunology-Microbiology, University of North Carolina at Chapel Hill, NC, USA
²Department of Gastroenterology, University of Liverpool, Liverpool, United Kingdom
³Ottawa Institute of Systems Biology, Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ontario, Canada
⁴Department of Medical Technology, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia
⁵Clinical Sciences, Cornell University, Ithaca, NY, USA
⁶Lineberger Comprehensive Cancer Center, Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, NC, USA
⁷Department of Bioinformatics and Genomics, University of North Carolina at Charlotte, NC, USA

Abstract

Inflammation alters host physiology to promote cancer, as seen in colitis-associated colorectal cancer (CRC). Here we identify the intestinal microbiota as a target of inflammation that impacts the progression of CRC. High-throughput sequencing revealed that inflammation modifies gut microbial composition in colitis-susceptible interleukin-10-deficient (Il10⁻/⁻) mice. Monocolonization with the commensal Escherichia coli NC101 promoted invasive carcinoma in azoxymethane (AOM)-treated Il10⁻/⁻ mice. Deletion of the polyketide synthase (pks) genotoxic island from E. coli NC101 decreased tumor multiplicity and invasion in AOM/Il10⁻/⁻ mice, without altering intestinal inflammation. Mucosa-associated pks+ E. coli were found in a significantly high percentage of inflammatory bowel disease (IBD) and CRC patients. This suggests that in mice, colitis can promote tumorigenesis by altering microbial composition and inducing the expansion of microorganisms with genotoxic capabilities.

Chronic inflammation is a well-established risk factor for several cancers, including colorectal cancer (CRC) (1). Although the mechanism by which chronic intestinal inflammation leads to CRC is still unclear, numerous experimental studies suggest that...
inflammatory cells and their associated mediators such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), IL-23, and reactive oxygen species form a microenvironment favoring the development of CRC, presumably by enhancing DNA damage in epithelial cells (2–4).

In the colon, trillions of commensal bacteria, termed the microbiota, are in close proximity to a single layer of epithelial cells. A critical question is whether these microorganisms actively participate in the process of carcinogenesis. We have previously shown that microbial status modulates development of colitis-associated CRC using the colitis-susceptible II10<sup>−/−</sup> mouse strain (5). To evaluate the effect of inflammation and carcinogenesis on the colonic microbiota, we utilized Illumina HiSeq2000 sequencing targeting the hypervariable V6 region of the 16S rRNA gene in mucosal biopsies and stool samples of II10<sup>−/−</sup> and WT mice, in the presence and absence of the colon-specific carcinogen AOM. Germ-free (GF) II10<sup>−/−</sup> and control WT adult mice were transferred to specific pathogen free (SPF) conditions for 20 weeks. During this timeframe, 100% of II10<sup>−/−</sup> mice develop colitis; with the addition of AOM, 60–80% of mice develop colon tumors (5). WT mice develop neither colitis nor tumors (5). We first compared the luminal microbiota between all II10<sup>−/−</sup> (colitis/cancer) and WT mice (healthy control) and found that the microbiota of II10<sup>−/−</sup> mice clustered apart from those of WT controls (Fig 1A, Fig. S1, ANOSIM (Analysis of Similarity) R=0.925, p=0.002). The altered microbiota of II10<sup>−/−</sup> mice showed reduced richness compared to WT controls (Fig 1B, p<0.0001). Analysis of mucosal biopsies revealed the colonic-adherent microbiota of AOM/II10<sup>−/−</sup> mice with colitis/cancer clustered apart from healthy AOM/WT controls (Fig S2A), with alterations in microbial evenness but not richness (Fig S2B, p=0.023). To determine the impact of AOM on the microbiota in the context of inflammation, we compared the luminal microbiota of II10<sup>−/−</sup> mice with colitis to AOM/II10<sup>−/−</sup> mice with colitis/cancer and found that AOM treatment had no significant effect on luminal microbial composition or richness in II10<sup>−/−</sup> mice (Fig 1C, D). These data suggest that inflammation rather than cancer is associated with the observed microbial shifts.

We then hypothesized that inflammation-induced changes in microbial composition includes the expansion of bacteria within the Proteobacteria phylum, as several members have been associated with colitis and CRC (6–9). Analysis of phylum-level distribution revealed that inflammation in II10<sup>−/−</sup> mice was associated with significantly increased levels of luminal Verrucomicrobia, Bacteroidetes, and Proteobacteria compared to WT controls (Fig S3). Although Verrucomicrobia significantly differed between groups, this phylum is not well characterized, restricting detailed molecular analysis. Within Proteobacteria, however, the Gammaproteobacteria class, Enterobacteriales order, and Enterobacteriaceae family were all significantly more abundant in II10<sup>−/−</sup> mice (Fig 1E–H). Because *E. coli* are members of the family Enterobacteriaceae and adherent-invasive *E. coli* have been associated with human IBD and CRC (8, 10–13), we determined by quantitative PCR if *E. coli* was more abundant in the context of inflammation in II10<sup>−/−</sup> mice. We found that relative to WT mice, the luminal microbiota of II10<sup>−/−</sup> mice exhibited a ~100-fold increase in *E. coli* (Fig 1I). AOM treatment did not affect *E. coli* abundance (Fig S4A). Total bacterial loads between WT and II10<sup>−/−</sup> mice did not differ (Fig S4B), nor did levels of the common commensal *Lactobacillus* (Fig S4C).

To determine the causative effect of commensal *E. coli* on CRC, we administered AOM to GF II10<sup>−/−</sup> mice mono-associated with either the commensal murine adherent-invasive *E. coli* NC101 or the human commensal *Enterococcus faecalis* OG1RF, both of which cause aggressive colitis in II10<sup>−/−</sup> mice (14). As expected, both *E. coli* NC101 and *E. faecalis* mono-associated, AOM-treated II10<sup>−/−</sup> mice developed severe colitis (Fig 2A). Despite similar levels of colitis, 80% of *E. coli* mono-associated mice developed invasive mucinous...
adenocarcinoma, whereas *E. faecalis* mono-associated mice rarely developed tumors (Fig 2B–D). Colonic cytokines involved in inflammation and carcinogenesis including *Il6, Tnfα, Il1b, Il18, Il17* and *Il23* were not significantly different between AOM-treated *E. coli* and *E. faecalis* mono-associated mice (Fig 2E, S5, Table S1–2). In addition, infiltrating CD3+ T cells, F4/80+ macrophages, and Ly6B.2+ monocytes and neutrophils were similar between AOM-treated *E. coli* and *E. faecalis* mono-associated mice (Fig. S6). These observations demonstrate that in addition to inflammation, bacteria-specific factors may be required for the development of colitis-associated CRC.

We hypothesized that *E. coli* NC101 has carcinogenic capabilities not shared by *E. faecalis*. Several members of the family *Enterobacteriaceae*, including select *E. coli* strains of B2 phyotype, harbor a ~54kb polyketide synthases (*pks*) pathogenicity island that encodes multi-enzymatic machinery for synthesizing a peptide-polyketide hybrid genotoxin named Colibactin (15–18). A bioinformatics BLAST search of the *E. coli* NC101 genome (accession NZ_AEFA00000000) revealed the presence of *pks* and the absence of other known *E. coli* genotoxins Cif, CNF and CDT. Using PCR and sequencing (15), we detected the *pks* island in *E. coli* NC101, but not *E. faecalis* or non-colitogenic *E. coli* K12 (Fig 3A). To determine if *E. coli* *pks* is associated with human CRC or IBD, we screened mucosa-associated *E. coli* strains isolated from colorectal tissue specimens of 35 patients with IBD, 21 with CRC, and 24 non-IBD/non-CRC controls (11). CRC specimens could not be obtained from IBD-associated CRC patients as these patients typically undergo colectomy upon diagnosis of dysplasia. Although 5 of the 24 (20.8%) non-IBD/non-CRC controls harbored *pks*+ *E. coli*, the genotoxic island was detected in 14 of 35 (40%, p<0.05) IBD patients and in 14 of 21 (66.7%, p<0.001) CRC patients (Figure 3B, Table S3). This suggests *pks*+ bacteria are associated with chronic intestinal inflammation and CRC and may impact carcinogenesis.

To functionally link *pks* with the development of CRC, we created an isogenic *pks*-deficient *E. coli* NC101 strain (NC101Δ*pks*). Absence of *pks* did not affect bacterial growth in vitro (Fig. S7) nor did it impair colonization capacity in vivo (10⁹–10¹⁰ per 200g stool pellet, 4–6 mice/group). As *pks* from strains of extraintestinal pathogenic *E. coli* can elicit mammalian DNA damage (15, 16), we tested the ability of *E. coli* NC101 *pks* to induce a DNA damage response. We infected the non-transformed rat intestinal epithelial cell line IEC-6 with NC101 or NC101Δ*pks* and assessed levels of phosphorylated histone H2AX (*γH2AX*), a surrogate marker of DNA damage (19–21). WT NC101 induced *γH2AX* in ~30% of cells, whereas NC101Δ*pks* induced *γH2AX* in <5%, a level equivalent to that induced by non-colitogenic *E. coli* K12 (Fig 3C). Consistent with these results, we observed that WT NC101 induced a 3-fold increase in the percent of cells arrested in G2/M phase relative to untreated and NC101Δ*pks* infected cells (Fig 3D). These experiments demonstrate that *pks* alone has the capacity to induce DNA damage and indicate that the genotoxic island does not block the initiation of DNA damage response. This led us to hypothesize that *pks* would also promote tumorigenesis in vivo.

To test this hypothesis, we mono-associated GF *Il10−/−* mice with *E. coli* NC101 or NC101Δ*pks*, with or without AOM treatment, and assessed inflammation and tumorigenesis. The absence of *pks* did not affect the severity of colonic inflammation in *Il10−/−* mice with colitis (12wk no AOM), or colitis/cancer (14wk+AOM and 18wk+AOM) (Fig 4A). Similarly, colon tissue pro-inflammatory cytokine transcripts and immune cell infiltration were not significantly different between mice mono-associated with NC101 vs. NC101Δ*pks* (Fig S8, S9; Table S4). Importantly though, at both 14wk+AOM and 18wk +AOM, the absence of *pks* was associated with significantly reduced neoplastic lesions (Fig 4B). At 14wk+AOM, high grade dysplasia (HGD) or invasive carcinomas were present in 5/8 mice mono-associated with NC101, whereas only 1/8 NC101Δ*pks* mono-associated...
mice developed HGD. At 18wk+AOM, the absence of *pks* did not affect mouse survival or tumor size, however, macroscopic tumor burden and carcinoma invasion were significantly decreased (Fig 4C–F, S10). In addition, all 9 NC101 mono-associated mice developed invasive carcinoma, with 4/9 fully invading the muscularis propria and serosa. In contrast, 0/9 NC101Δ*pks* mono-associated mice exhibited full invasion. This likely suggests that the presence of *E. coli* *pks* accelerates progression from dysplasia to invasive carcinoma. In the absence of AOM, GF *Il10*−/− mice colonized with NC101 for 21 weeks developed only mild dysplasia (Fig. S11A). GF WT mice mono-associated with *E. coli* NC101 and treated with AOM developed neither inflammation nor dysplasia/tumors (Fig. S11B), suggesting that this bacterium is not carcinogenic in the absence of inflammation. Together these data indicate that the absence of *pks* reduces the tumorigenic potential of *E. coli* NC101 without altering colonic inflammation.

To evaluate the impact of *pks* on host DNA damage *in vivo*, we measured colocyte γH2AX+ nuclear foci (*γ*-foci) in AOM/*Il10*−/− mice mono-associated with NC101 vs. NC101Δ*pks* for 14 weeks (19–21). The abundance of *γ*-foci+ colocytes/crypt was significantly reduced in AOM/*Il10*−/− mice mono-associated with *E. coli* NC101Δ*pks* vs. *E. coli* NC101 (Fig 4G). We detected a 5-fold reduction in *γ*-foci+ colocytes/crypt in *E. coli* NC101 mono-associated AOM/WT mice relative to *E. coli* NC101-associated AOM/*Il10*−/− mice (Fig 4G). This suggests that both host inflammation and *E. coli*-derived *pks* act in concert to create a host microenvironment that promotes DNA damage and tumorigenesis in AOM/*Il10*−/− mice.

Although the etiology of colitis associated CRC is multifactorial, this work indicates that chronic inflammation targets the intestinal microbiota and can induce the expansion of microbes, including *E. coli*, that influence CRC in mice. The carcinogenic effect of *E. coli* NC101 *pks* clearly demonstrates that genotoxic microorganisms promote CRC in the presence of the carcinogen AOM in *Il10*−/− mice. It remains to be seen if NC101 and other *pks*-harboring bacteria have similar effects in other models of colitis-associated CRC. An increased prevalence of *pks*+ *E. coli* in IBD and CRC patients may suggest a cancer-promoting role in human CRC. We propose a model in which inflammation creates an environment that supports carcinogenesis through its effects on both the host and the microbiota. In this two-hit model, inflammation targets the microbiota to foster the expansion of bacteria with genotoxic potential, such as *pks*+ bacteria. In parallel, inflammation creates an opportunity for *pks*+ bacteria to adhere to the colonic mucosa by decreasing protective mucins and antimicrobial peptide production (22, 23), a process prevented by natural barrier function present in non-inflamed WT mice. The genotoxic effect of *pks* requires bacteria-host cell contact (15, 16), thus an environment in which bacteria can more readily access the epithelium could result in increased delivery of the *pks* product Colibactin to epithelial host cells. This would explain the lack of cancer in *pks*+ *E. coli*-associated WT mice. Although other microbes likely participate in the progression of CRC, our findings highlight the complex effects of inflammation on both microbial composition/activity and the host’s ability to protect itself from a dysbiotic microbiota.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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References and Notes


Fig 1.
Inflammation alters fecal microbial community structure. (A–B) Luminal microbiota of Il10−/− vs. WT mice. (A) Operational taxonomic unit (OTU) abundances were standardized by total, square root transformed, and assembled into a Bray Curtis similarity matrix to generate a multidimensional scaling (MDS) plot, where in these plots each symbol represents the microbiota of an individual mouse analyzed by Illumina sequencing of 16S V6 region. Il10−/− vs. WT comparison by ANOSIM (Analysis of Similarity), R=1 is maximum dissimilarity. (B) Richness, mean + SEM of cage means, 5–6 cages/group, 2–4 mice per cage, t test. (C–D) Luminal microbiota of AOM/Il10−/− vs. Il10−/− (C) MDS plot, AOM/Il10−/− vs. Il10−/− comparison by ANOSIM. (D) Richness, mean + SEM of cage...
means, 2–3 cages/group, 2–4 mice per cage, t test. (E–G) Standardized transformed abundance, median + SEM of cage means, 5–6 cages/group, 2–4 mice per cage, Mann Whitney U. (H) MDS plot depicting luminal microbiota of \( \text{Il}10^{−/−} \) vs. WT, overlaid with \( \text{Enterobacteriaceae} \) abundance depicted by circle size. (I) \( E. \text{coli} \) \( \Delta C_t \) relative to total bacteria (16S). Each symbol depicts one mouse, line at median, Mann Whitney U.
Fig 2.
Colonization of germ-free mice II10−/− mice with E. coli (E.c.) or E. faecalis (E.f.) differentially impacts tumorigenesis without affecting inflammation. (A) Histologic inflammation scores, t test. (B) Macroscopic tumor counts; two tailed Mann Whitney. (C) Percent of mice with invasive adenocarcinoma; Fisher’s exact test. (D) Representative H&E histology. (A–D): Mean + SEM, 7–12/group, single experiment. (E) Colonic cytokine mRNA expression relative to GF II10−/−; Mean + SEM, 4/group, t test.
Fig 3.
Pks+ *E. coli* strains are associated with CRC and DNA damage. (A–B) PCR screen for pks (A) using primers targeting the left (1824bp) and right (1413bp) ends of the pks island in *E. coli* strains NC101 and K12, and *E. faecalis* (E.f.), and (B) using primers targeting the right end and ClbB gene of the pks island in mucosa-associated *E. coli* isolated from human colorectal tissue specimens. Binomial test *p<0.05, ***p<0.001. (C–D) NC101 pks induces (C) γH2AX (MOI 20, 4hr) in IEC-6 cells; Mean + SEM, ANOVA + Tukey, and (D) G2 cell cycle arrest (MOI 100, 24hr). A, C–D are representative of 3 experiments.

<table>
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<th>Disease</th>
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<td>5</td>
<td>5</td>
<td>20.8</td>
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</table>

1 Mucosa-associated *E. coli* was isolated from colon tissue specimens  
2 Differences in # ClbB+Pks+ patients vs controls were assessed using the binomial test  
3 Control = non-CRC, non-IBD patients (see Table S3)
Fig 4. Deletion of pks reduces the tumorigenicity, but not inflammatory potential, of E. coli NC101 in AOM/Il10−/− mice. (A) Histologic inflammation and (B) neoplasia scores in 3 cohorts of mice at weeks 12, 14+AOM, 18+AOM, (C–F) 18wks+AOM: (C) Histologic invasion score, (D) Macroscopic tumor number, (E) Mean macroscopic tumor diameter in each mouse (F) Representative H&E histology. (A–F) Each symbol represents data from one mouse, line at mean, pairwise comparisons by t test. (G) Cells/crypt with ≥4 γH2AX foci, and γH2AX IHC (400x). Each symbol represents data from mouse, ANOVA + Tukey, line at mean, arrowheads indicate γfoci+ cells.