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Microbiota manipulation to increase macrophage IL-10 improves colitis and limits colitis-associated colorectal cancer

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ABSTRACT

Inflammatory bowel disease (IBD) is a chronic life-long inflammatory disease affecting almost 2 million Americans. Although new biologic therapies have been developed, the standard medical treatment fails to selectively control the dysregulated immune pathways involved in chronic colonic inflammation. Further, IBD patients with uncontrolled colonic inflammation are at a higher risk for developing colorectal cancer (CRC). Intestinal microbes can impact many immune functions, and here we asked if they could be used to improve intestinal inflammation. By utilizing an intestinal adherent *E. coli* that we find increases IL-10 producing macrophages, we were able to limit intestinal inflammation and restrict tumor formation. Macrophage IL-10 along with IL-10 signaling to the intestinal epithelium were required for protection in both inflammation and tumor development. Our work highlights that administration of immune modulating microbes can improve intestinal outcomes by altering tissue inflammation.

Introduction

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammatory intestinal disease with no cure[1]. It affects almost 2 million people in the United States.[2] IBD is thought to result from unchecked inflammation against the microbiota in a genetically susceptible individual.[3] Although new biologic therapies have been developed, the standard medical treatment fails to selectively control the dysregulated immune pathways involved in chronic colonic inflammation.[4] In IBD patients, changes in the microbiota are found, including expanded pro-inflammatory microbes such as *Ruminococcus gnavus* and proteobacteria, such as *Escherichia coli* and reduced anti-inflammatory microbes such as Bacteroidetes, *Lachnospiraceae*, and *Faecalibacterium prausnitzii*.[5–8] This altered microbiota is thought to support disease progression and inflammatory changes.[9] In parallel, genetic, and

environmental factors can interfere with proper interaction between the host immune system and microbiota leading to enhanced inflammation.[10,11]

IBD patients with poorly controlled intestinal inflammation are at an elevated risk for CRC development.[10–13] Furthermore, chronic intestinal inflammation corresponds with advanced disease stage and decreased survival in CRC.[14,15] Animal models support a causative role for inflammation in CRC with inflammation promoting tumorigenesis.[14]

Numerous groups find the microbiota can both enhance and limit intestinal inflammation. Specifically, we and others find that microbiota-derived signals drive anti-inflammatory processes including macrophage IL-10 production,[16] induction of naive and regulatory T cells,[17,18] and fortification of the epithelial barrier.[19–21] We previously identified that colonization with

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multiple human *E. coli* isolates could replace the microbiota in inducing macrophage IL-10 production, limiting pathology in a model of *Salmonella* infection[16] but it remained unclear if such microbes could further enhance IL-10 and anti-inflammatory effects in the context of the normal microbiota.

Here, we find that colonization with the IL-10-inducing *E. coli*, strain 541–15, increased macrophage IL-10 production but only after intestinal damage. This increased IL-10 production after 541–15 colonization offered protection from intestinal injury as well as limited development of colitis-associated CRC (CAC). These data demonstrate that addition of select microbes is sufficient to shift immune function leading to improved outcomes in models of colitis, and offers insight into the role of gut microbes in intestinal inflammation and cancer.

Results

Intestinal colonization with E. coli *541-15 protects from colitis*

As we previously found that colonization with *E. coli* 541–15 after microbiota depletion with antibiotics was sufficient to induce intestinal CX₃CR1⁺ macrophage production of IL-10 which restricted *Salmonella*-specific Th1 cells,[16] we asked if 541–15 protected from intestinal injury and inflammation in the presence of the steady-state SPF microbiota. We left *E. coli*-free specific-pathogen-free (SPF) C57BL/6 (B6) mice uncolonized or colonized with *E. coli* 541–15 2 days before treatment with dextran sodium sulfate (DSS). In this acute intestinal inflammation model, DSS treatment causes epithelial damage and inflammation in the intestine.[22] *E. coli* colonization was confirmed by qPCR using *E. coli* specific 16s rRNA primers (Figure S1A). *E. coli* 541–15 colonization did not alter the overall composition of the microbiome (Figures S1B-S1D). We found that colonization with *E. coli* 541–15 prevented weight loss and colon shortening, and improved histopathology with less incidence of ulcerations and fewer neutrophil clusters in lamina propria and epithelium (Figures 1A-1d). We also found decreased expression of pro-inflammatory markers lipocalin-2 and myeloperoxidase in fecal samples from *E. coli* 541–15 colonized mice as well as reduced expression of tumor necrosis factor alpha

supporting reduced colonic inflammation (Figures 1E, 1f and S1E). As intestinal inflammation is linked with increased gut permeability, we assayed for intestinal permeability after DSS treatment by gavaging mice with fluorescein isothiocyanate (FITC) conjugated dextran (FITC-dextran) and measuring serum FITC-dextran levels.[23] DSS-treated mice had increased serum FITC-dextran levels that were reduced in 541–15 colonized mice demonstrating decreased intestinal permeability (Figure 1g). Decreased permeability reflects improved intestinal barrier integrity, likely the consequence of lower intestinal inflammation and improved repair.[24–26] We also characterized immune infiltration in DSS-treated *E. coli* 541–15 colonized mice. In the colon lamina propria, *E. coli* 541–15-colonized mice had decreased immune infiltration (Figure 1h) with decreased Th1 cells, CTLs, and neutrophils as well as increased Tregs and no change in macrophages or monocytes (Figures 1I-1m). Supporting these findings, in the mesenteric lymph node (MLN), *E. coli* 541–15-colonized mice had decreased IFNγ-producing CD4⁺ T cells and ILCs (Figure S1F). Our mice are segmented filamentous bacteria (SFB) free and so we do not see T helper 17 (Th17) cell responses, and these are not altered by 541–15 colonization (Figure S1F). To test if 541–15 enhanced IL-10 production in the presence of the SPF microbiota, we colonized *E. coli*-free SPF IL-10-GFP reporter mice (Vert-X) with 541–15 and left untreated or treated with 2% DSS for 7 days and used flow cytometry to detect IL-10⁺ cells.[27,28] As previously described[27,28] we find CX₃CR1⁺ macrophages to be the main source of IL-10 (Figure S2A). However, 541–15 colonization did not enhance IL-10 production in the presence of the SPF microbiota (Figure S2B). In contrast, after DSS treatment, we find increased IL-10⁺ cells in the colon of 541–15 colonized mice with increased IL-10 expression by CX₃CR1⁺ macrophages (Figures 2A and 2b).

Intestinal colonization with E. coli *541–15 prevents colitis by inducing IL-10 production by CX₃CR1⁺ macrophages which targets the intestinal epithelium*

IL-10 deficient mice develop microbiota-driven spontaneous colitis and also show increased sensitivity to DSS.[29] To assess the requirement for IL-10 in colitis
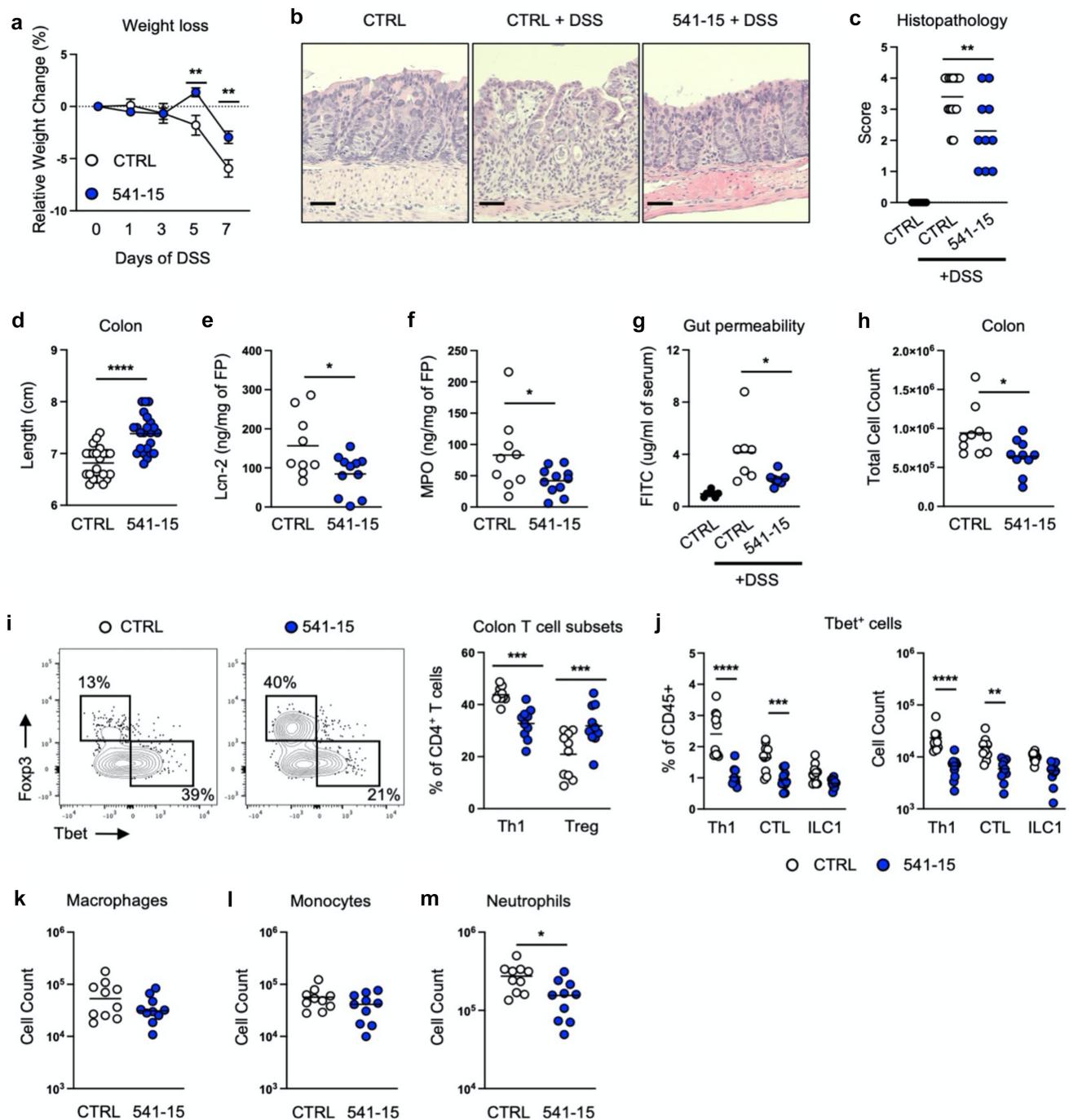
Figure 1. Intestinal colonization with *E. coli* 541–15 protects from colitis. Wildtype mice were colonized with *E. coli* 541–15 or gavaged with LB broth (CTRL) and 3 days later 2% DSS was provided in drinking water. At day 7 of DSS treatment, samples were collected. (a) Relative weight change. (b) Representative H&E staining of distal colons. Scale bars represent 50 µm. (c) Histopathology scores. (d) Colon lengths. (e) Lipocalin-2 (Lcn-2) and (f) Myeloperoxidase (MPO) concentration in fecal pellets. At day 7 mice were gavaged with FITC-dextran and 3 hours later blood was collected. (g) FITC concentration in serum. Colon lamina propria cells were isolated. (h) Number of immune cells. (i) Flow cytometry and frequencies of Th1 and Tregs. (j) Frequencies and counts of total Th1 cells, CTLs, and ILC1s. Counts of (k) Macrophages, (l) Monocytes, and (m) Neutrophils. Each replicate is a biologically independent sample. Individual dots represent samples from individual mice. Data are shown as individual values and mean, compared by two-tailed unpaired t-test or two-way ANOVA with Fisher's LSD post hoc test. The results are representative of at least two independent experiments. *P < .05 was considered statistically significant; **P < .01; ***P < .001; ****P < .0001. See also Figure S1.

Figure 2. Intestinal colonization with *E. coli* 541–15 prevents colitis through IL-10 induction. Vert-X mice were colonized with *E. coli* 541–15 or gavaged with LB broth (CTRL) and 3 days later 2% DSS was provided. At day 7 of DSS treatment, samples were collected. Flow cytometry and frequencies of (a) total IL-10+ immune cells from the colonic lamina propria and (b) identification of IL-10 + immune cells. IL-10 deficient (IL-10-/-) and wildtype (WT) littermate mice were colonized with *E. coli* 541–15 or gavaged with LB

protection downstream of 541–15 colonization, we left IL-10 wildtype and knockout (IL-10$^{-/-}$) littermate mice uncolonized or colonized with *E. coli* 541–15 before DSS treatment. As above, 541–15 colonization improved colitis in wildtype mice (Figures 2C-2g and S2C-S2H). However, in IL-10 knockout mice 541–15 colonization did not improve colitis outcomes including no effect on colon length, histopathology scores, or immune infiltration (Figures 2C-2g and S2C-S2H), supporting the requirement of IL-10 in protection after 541–15 colonization.

To assess the role of CX$_3$CR1$^+$ macrophages in 541–15 protection from colitis, we used mice where the diphtheria toxin receptor (DTR) is expressed from the *Cx3cr1* locus (CX3-DTR mice).[30] In these mice, all CX$_3$CR1$^+$ cells are depleted after diphtheria toxin (DT) injection. Colonization of these mice with 541–15 before DSS treatment did not induce changes in colon length or infiltrating immune cells (Figures S3A-S3D). This is likely because DT treatment depleted both CX$_3$CR1$^+$ monocytes that drive pathology in colitis and CX$_3$CR1$^+$ macrophages that are important for barrier repair.[31] Therefore, we used mice where a floxed-stop cassette upstream of the DTR was inserted into the *Cx3cr1* locus (CX3-STOP-DTR). Crossing these mice to CD11c-Cre allows for depletion of CX$_3$CR1$^+$ macrophages and dendritic cells (DCs) but not monocytes or other CX3CR1-expressing cells after DT injection.[32] After 541–15 colonization and DSS treatment, mice lacking CX$_3$CR1$^+$ macrophages and DCs had shorter colons with increased immune infiltration including more Th1 cells, CTLs, ILC1s, and fewer Tregs as compared to littermate controls (Figures S3E-S3H).

We next used mice lacking IL-10 production by CX$_3$CR1$^+$ cells to test if CX$_3$CR1$^+$ cell IL-10 was required for colitis protection after *E. coli* 541–15 colonization. For these experiments, mice with a conditional allele for IL-10 were crossed to the tamoxifen-inducible CX$_3$CR1-CreERT2.[33] Tamoxifen treatment results in loss of IL-10 production by CX$_3$CR1$^+$ cells in IL-10$^{flox/-}$

compared to IL-10$^{flox/+}$ littermate control CX$_3$CR1-CreERT2 mice. As compared to controls, in DSS treated 541–15 colonized mice whose CX$_3$CR1$^+$ cells cannot produce IL-10, we found loss of protection by *E. coli* 541–15 colonization with increased weight loss, shorter colon length, and increased pathology characterized by significant inflammatory infiltrate and ulcerations (Figures 3A-3c and S3I). We also found increased immune infiltration with increased Th1 cells, ILC1s, and neutrophils (Figures 3D, 3e, and S3J-S3L). In contrast, we found T cell IL-10 was dispensable as we found complete protection after 541–15 colonization of mice where T cells could not produce IL-10, with improved colon length and histopathology after 541–15 colonization (Figures S3M-S3R).

To identify the cellular target of CX$_3$CR1$^+$ macrophage IL-10 after *E. coli* 541–15 colonization we next analyzed mice in which epithelial cells, CX$_3$CR1$^+$ cells, or T cells lack expression of the IL-10 receptor α (IL-10 Rα). Suppression of colitis by *E. coli* 541–15 was intact when mice lacked IL-10Rα expression in T cells or CX$_3$CR1$^+$ cells (Figures S4A-S4L). In contrast, we found epithelial IL-10Rα is required as 541–15 was no longer protective after DSS treatment in LGR5-CreERT2$^+$ IL-10Rα$^{flox/flox}$ mice as compared to littermates without LGR5-CreERT2, as seen by increased weight loss, shorter colon length, increased histopathology scores, and increased epithelial permeability (Figures 4A-4c, S4M, and S4N). In these mice we found increased immune infiltration with increased Th1 cells (Figures 4D, 4e, and S4O-S4Q). In addition, fecal lipocalin-2 and myeloperoxidase were increased in DSS-treated 541–15 colonized mice when epithelial cells lack IL-10Rα (Figures S4R and S4S). In epithelial cells, IL-10 triggers repair pathways including WNT signaling that leads to downstream expression of the pluripotency gene *nanog* which supports cell proliferation and epithelial barrier repair.[34,35] Confirming the role of IL-10 in epithelial cells, we find that *E. coli*

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broth (CTRL) and 3 days later 2% DSS was provided. At day 7 of DSS treatment, samples were collected. (c) Representative H&E staining of distal colons. Scale bars represent 50 µm. (d) Histopathology scores. (e) Colon lengths. Colon lamina propria cells were isolated. (f) Number of immune cells. (g) Frequencies of Th1 and Tregs. Each replicate is a biologically independent sample. Individual dots represent samples from individual mice. Data are shown as individual values and mean, compared by two-tailed unpaired t-test or two-way ANOVA with Fisher's LSD post hoc test. The results are representative of at least two independent experiments. *P < .05 was considered statistically significant; **P <.01; ***P < .001; ****P < .0001. See also Figure S2.
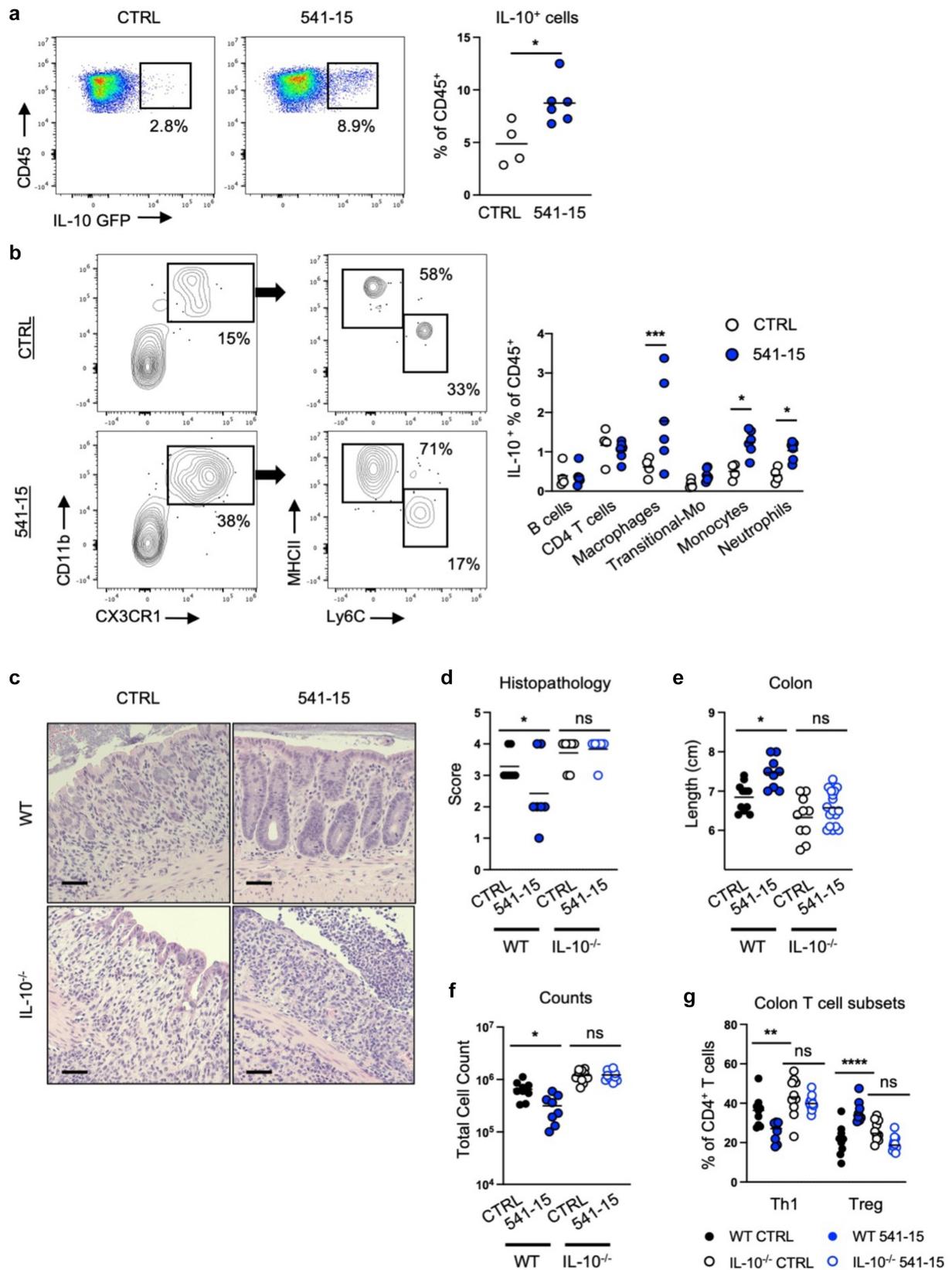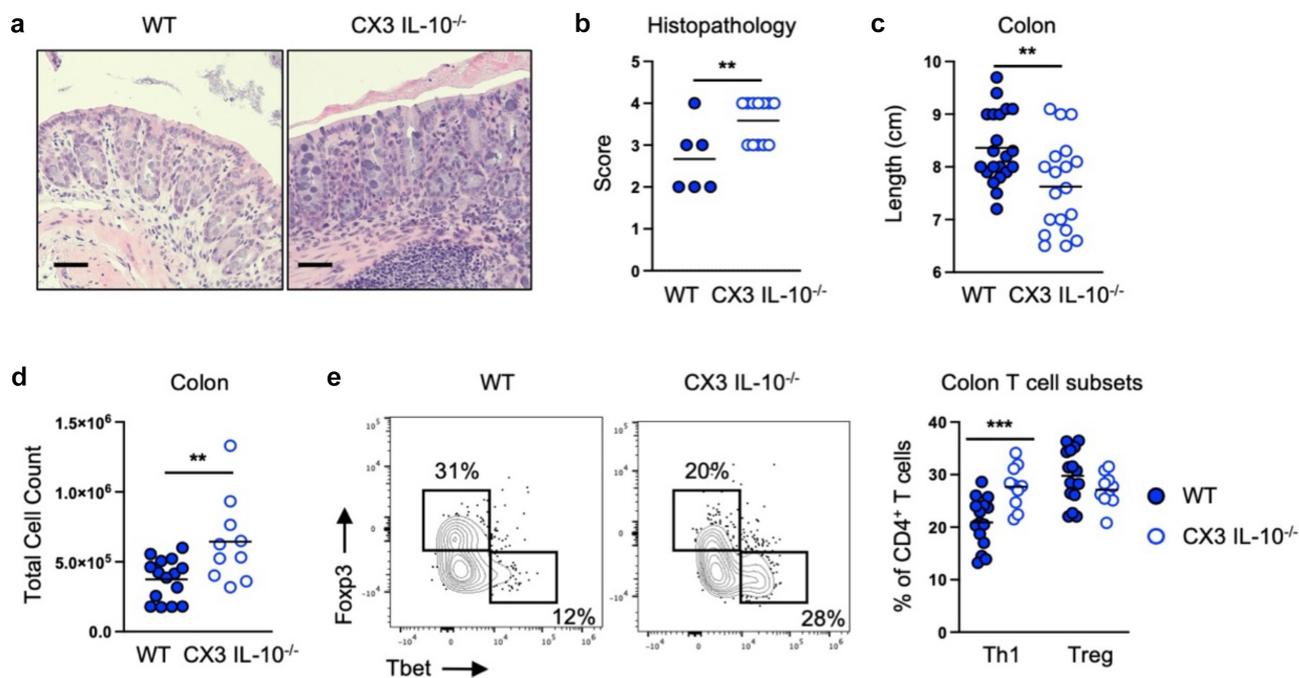
Figure 3. Intestinal colonization with *E. coli* 541–15 prevents colitis by inducing IL-10 production by CX3CR1+ macrophages. IL-10flox/- (CX3 IL-10-/-) and IL-10flox/+ (WT) littermate CX3CR1-CreERT2 mice were colonized with *E. coli* 541–15 and 3 days later 2% DSS was provided. At day 7 of DSS treatment, samples were collected. 3 days before colonization and every 2 days throughout the experiment 4OHT was injected to both groups. (a) Representative H&E staining of distal colons. Scale bars represent 50 μm. (b) Histopathology scores. (c) Colon lengths. Colon lamina propria cells were isolated. (d) Number of immune cells. (e) Flow cytometry and frequencies of Th1 and Tregs. Each replicate is a biologically independent sample. Individual dots represent samples from individual mice. Data are shown as individual values and mean, compared by two-tailed unpaired t-test or two-way ANOVA with Fisher's LSD post hoc test. The results are representative of at least two independent experiments. *P < .05 was considered statistically significant; **P <.01; ***P < .001. See also Figure S3.

541–15 colonization increased *nanog* expression in the colon of DSS-treated wild-type mice (Figure S4T).

Intestinal colonization with E. coli 541-15 protects from colitis-associated colorectal cancer

As intestinal inflammation is a risk for CAC development and dysbiosis characterized by enriched mucosa adherent bacteria such as *E. coli* is seen during inflammatory conditions in human IBD patients, in mouse IBD models, and in colon cancer,[10,11] we asked if colonization with *E. coli* 541–15 modulated an inflammation-driven mouse model of CAC. 6-week-old *E. coli*-free SPF B6 mice were subjected to a chemically induced colitis associated carcinoma model. In this colon tumor model, mice are treated with azoxymethane (AOM), a mutagen, followed by three cycles of 2% DSS.[36] Under the AOM/DSS model, mice develop tumors in the distal colon as early as 1 week after the second cycle of DSS.[36] We

colonized mice with *E. coli* 541–15 before the first DSS administration and assessed tumor formation 4 weeks after the last DSS cycle (Figure S5A). As predicted from its anti-inflammatory effect,[16] we found *E. coli* 541–15 was protective with reduced number and size of tubular adenomas (Figures 5A and 5b). Spleen size was also reduced indicating reduced systemic inflammation (Figure S5B).

We further assessed how 541–15 colonization affected the immune microenvironment within the tumors (tumor microenvironment, TME) and surrounding tissue. Using flow cytometry, we found decreased infiltration of myeloid cells including tumor associated macrophages (TAMs), mononuclear myeloid-derived suppressor cells (M-MDSCs), and polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) in tumors of mice colonized with 541–15 (Figures 5C and S5C). TAMs both inhibit anti-tumor T cell responses and directly promote tumor growth and angiogenesis.[37] M-MDSCs resemble monocytes while PMN-
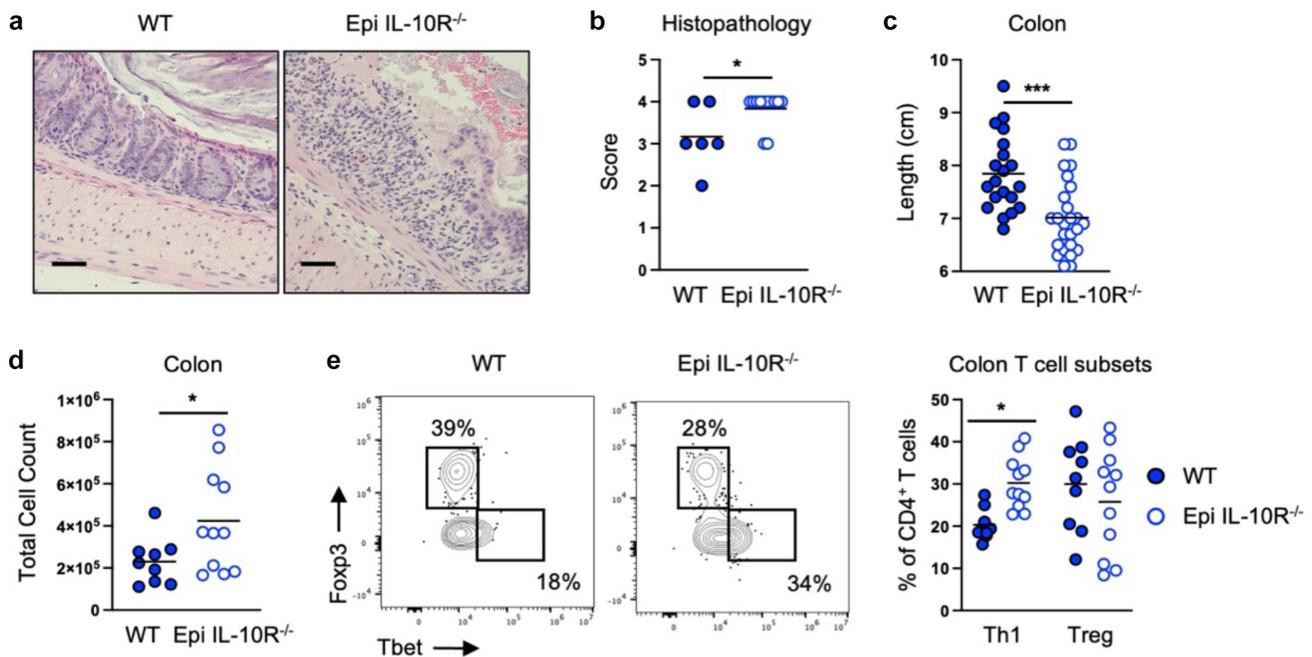
Figure 4. Intestinal colonization with *E. coli* 541–15 prevents colitis by modulating IL-10 signaling to intestinal epithelium. LGR5-CreERT2+ (Epi IL-10R-/-) and LGR5- CreERT2- (WT) littermate IL-10Raflox/flox mice were colonized with *E. coli* 541–15 and 3 days later 2% DSS was provided. At day 7 of DSS treatment, samples were collected. 7 days before colonization 4OHT was injected on two consecutive days to both groups. (a) Representative H&E staining of distal colons. Scale bars represent 50 μm. (b) Histopathology scores. (c) Colon lengths. Colon lamina propria cells were isolated. (d) Number of immune cells. (e) Flow cytometry and frequencies of Th1 and Tregs. Each replicate is a biologically independent sample. Individual dots represent samples from individual mice. Data are shown as individual values and mean, compared by two-tailed unpaired t-test or two-way ANOVA with Fisher's LSD post hoc test. The results are representative of at least two independent experiments. *P < .05 was considered statistically significant; ***P < .001. See also Figure S4.

MDSCs are similar to neutrophils and both inhibit T cell recruitment and suppress T cell responses within the tumor.[38,39] We also found changes in recruitment of lymphoid cells to tumors in 541–15 colonized mice. As compared to control mice, mice colonized with *E. coli* 541–15 had increased T helper 1 (Th1) cells, cytotoxic T lymphocytes (CTLs), and type 1 innate lymphoid cells (ILC1s), and decreased regulatory T cells (Tregs) within tumors (Figures S5D and S5E). This shift in immune infiltration supports that 541–15 is restricting tumor development as smaller, better controlled tumors have increased T effector cells as compared to Tregs and MDSCs.[37] In contrast to tumor tissue, in tumor adjacent colonic tissue from 541 to 15 colonized mice we found the opposite effects with decreased Th1 cells and increased Tregs as compared to uncolonized mice (Figure S5F). This indicated 541–15 induced tumor-specific changes in infiltrating immune populations. Interestingly, as in DSS-induced colitis, *E. coli* 541–15 colonization did not

alter microbiota composition in mice under the CAC model (Figures S6A and S6B). This indicated that immune changes leading to protection against CAC were specific for 541–15 and is not attributed to changes in other microbial populations.

E. coli 541–15 modulates tumorigenesis through intestinal epithelium responses to IL-10 production from CX₃CR1⁺ macrophages

We next assessed if colonization with *E. coli* 541–15 prevented CAC through the same pathways through which it protected from intestinal inflammation. IL-10$^{flox/+}$ and IL-10$^{flox/-}$ CX₃CR1-CreERT2 mice under the AOM/DSS model were colonized with 541–15 and concomitantly treated with tamoxifen (Figure S6C). Loss of CX₃CR1⁺ cell IL-10 during DSS treatment in the presence of *E. coli* 541–15 led to increased tumor number and size likely due to increased inflammation as described above (Figure S6E). No change in
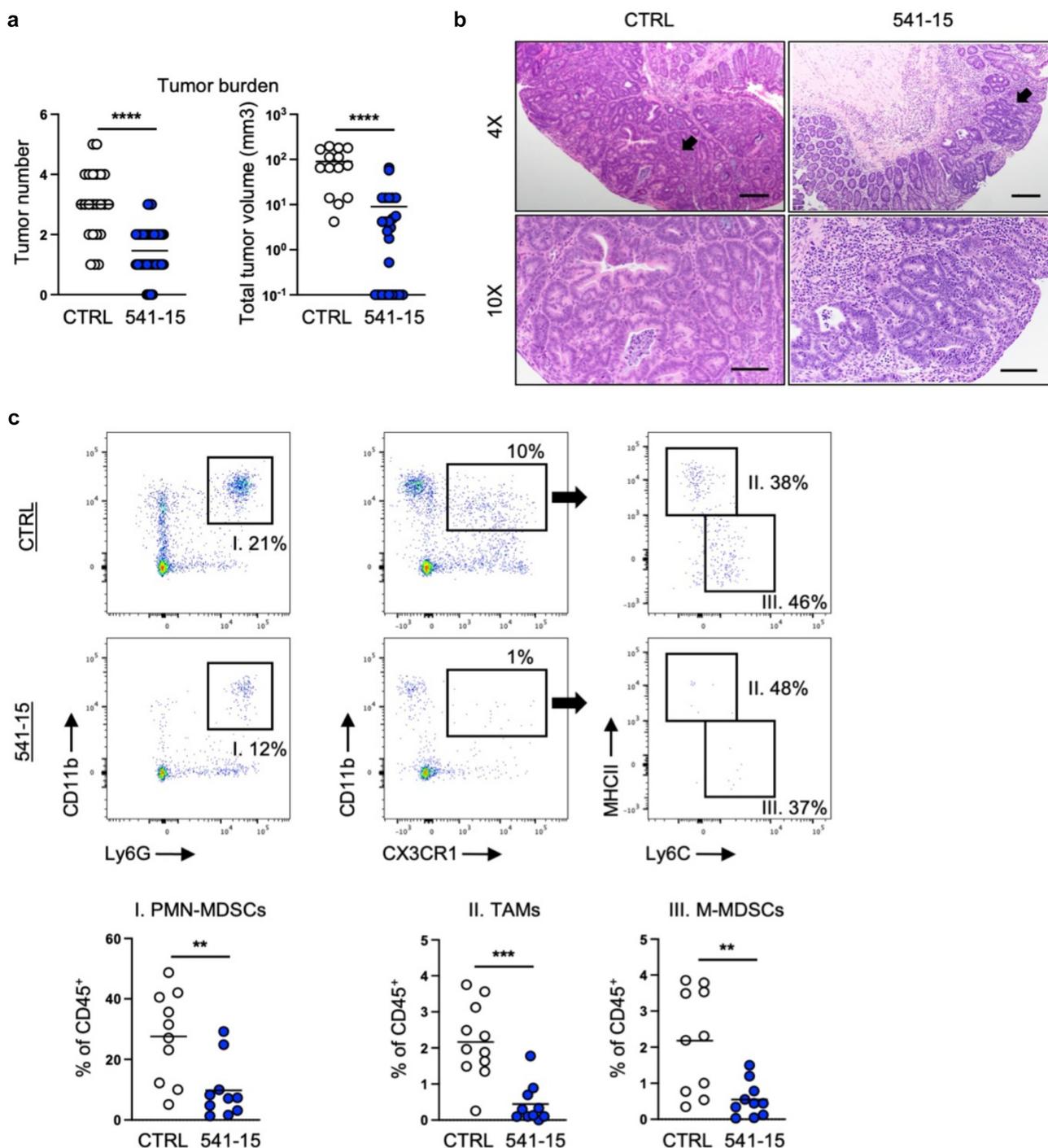
Figure 5. Intestinal colonization with *E. coli* 541–15 protects from colitis-associated colorectal cancer. Wildtype mice were administered AOM followed by 3 cycles of 2% DSS. Before administration of DSS mice were colonized with *E. coli* 541–15 or gavaged with LB broth (CTRL) and samples were collected as indicated in Figure S1A. (a) Number and size of colonic tumors. (b) Representative H&E staining of distal colons. Black arrow shows a tubular adenoma. Scale bars represent 4x = 200 μm and 10x = 100 μm. Tumors were individually collected, digested, and the tumor microenvironment was analyzed by flow cytometry. (c) Flow cytometry and frequencies of PMN-MDSCs, TAMs, and MMDSCs. Each replicate is a biologically independent sample. Individual dots represent samples from individual mice. Data are shown as individual values and mean, compared by two-tailed unpaired t-test. The results are representative of at least two independent experiments. *P < .05 was considered statistically significant; **P <.01; ***P < .001; ****P < .0001. See also Figures S5 and S6.

tumor number or size was observed in mice lacking IL-10 production by T cells colonized with 541–15 (Figure S6E).

To assess the role of epithelial IL-10 signaling in *E. coli* 541–15 modulation of tumorigenesis, we used mice in which epithelial cells are deficient for IL-10Rα after treatment with tamoxifen. IL-10Rα$^{flox/flox}$ mice with and without LGR5-CreERT2 were colonized with 541–15 and simultaneously treated with tamoxifen followed by treatment with AOM/DSS (Figure S6D). Similar to mice in which CX$_3$CR1$^+$ cells do not produce IL-10, 541–15 colonized mice lacking epithelial IL-10Rα had more frequent, larger tumors (Figure S6E). We saw no change in tumor number or size when CX$_3$CR1$^+$ or T cells lacked IL-10Rα (Figure S6E).

These results identify that microbiota modulation of macrophage anti-inflammatory function has direct outcomes on inflammation and CAC development. IL-10 produced primarily by CX$_3$CR1$^+$ macrophages is sensed by epithelial cells potentially triggering epithelial repair processes helping in the resolution of inflammation and protecting from tumor development[34] (Figure S7A).

Discussion

Understanding the role of tissue inflammation is crucial to preventing and treating IBD, a chronic disease linked with changes in the gut microbial landscape that are thought to drive pathogenesis.[5–9] Here, we find that colonization with a specific intestinal microbe restricts inflammation in a macrophage-dependent manner.

We and others previously demonstrated that the intestinal microbiota is a key regulator of intestinal macrophage inflammatory potential with the microbiota required for IL-10 production by intestinal macrophages.[16] Particularly, we demonstrated that colonization with *E. coli* 541–15 was sufficient to restore CX$_3$CR1$^+$ macrophage IL-10 production in microbiota depleted mice.[16] Here, we find that 541–15 reduces intestinal pathology after DSS treatment. 541–15 colonization followed by DSS treatment results in enhanced macrophage IL-10 in the presence of the normal microbiota. Macrophage IL-10 production is required for protection as 541–15 is unable to protect from pathology in IL-10 deficient mice or in mice lacking IL-10

production by CX$_3$CR1$^+$ macrophages. This confirms a critical role for microbial regulation of macrophage IL-10 in controlling intestinal inflammation.

We also find that intestinal epithelial cell response to IL-10 was required downstream of 541–15 colonization. In epithelial cells, IL-10 triggers repair pathways including WNT signaling pathways that support epithelial barrier repair further limiting tissue inflammation.[34,35] Together, colonization with 541–15 protected from colitis only when intestinal epithelial cells were able to sense IL-10, highlighting the importance of crosstalk between macrophages and epithelial cells to support recovery from epithelial injury.

We next asked how this shift toward barrier support by 541–15 colonization regulated tumor development in a CAC model. In CRC, evidence supports that increased intestinal inflammation enhances intestinal tumors,[40,41] likely driven by environmental and lifestyle changes that can interfere with proper host–microbiota interactions, leading to unresolved damage and unchecked inflammation that promotes CRC.[13] In inflammatory disease, changes in microbiota composition can lead to lost regulatory capacity including decreased IL-10 production, that promotes or sustains inflammation.[16,42,43] Recent studies identify select microbes as increased in CRC.[10,11] One bacterium associated with worse CRC outcome is the attaching bacterium *Fusobacterium nucleatum*,[10] which induces cell proliferation and modulation of the tumor microenvironment without altering inflammation.[44] We find that enrichment of *E. coli* 541–15, an adherent *E. coli* isolated from the intestine of an IBD patient,[45,46] also affects CRC. However, contrary to *F. nucleatum*, 541–15 limits tumorigenesis by engaging IL-10 signaling pathways that suppress inflammation. Macrophages are known to produce additional factors that can promote tumor development. This includes transforming growth factor β1 (TGF-β1), insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor α (VEGF-α), which promote cellular proliferation and blood-vessel development.[47] It remains to be seen if 541–15 activates these pathways in addition to IL-10. As predicted by its ability to promote macrophage IL-10, colonization with 541–15 before induction of colitis in the mouse model of CAC led to reduced tumor incidence with fewer, smaller tumors in colonized mice. As in colitis, decreased tumorigenesis depended on

intestinal macrophage IL-10 production and IL-10 sensing by the intestinal epithelium.[34,35]

Our findings suggest that modulating the function of CX_3CR1^+ macrophages represents a therapeutic avenue to decrease inflammation that can lead to CAC in IBD patients.[48,49] Although targeting specific microbes may offer therapeutic advantages, much work remains to be done to better characterize IBD-associated microbes and the downstream pathways they activate. Further studies should assess if using probiotics with immunomodulatory properties have therapeutic potential that provides protection against IBD and IBD-related cancer.

Materials and methods

Mice

All mice were bred in house at the animal facility of Baylor College of Medicine or Memorial Sloan Kettering Cancer Center and maintained as *E. coli* free. The colony is routinely tested by qPCR and immunophenotyping to confirm lack of *E. coli* colonization. C57BL/6 J (Jax #000664), IL10-GFP (Vert-X) (JAX #014530), IL-10KO (JAX #003968), CD11c-Cre (JAX #008068), LRG5-CreERT2 (JAX# 008875), CX_3CR1-CreERT2 (JAX #021160),[33] and CD4-Cre (JAX #017336), mice were originally purchased from Jackson laboratory before being bred in house. CX3CR1-STOP-DTR (JAX #025629),[32] CX_3CR1-DTR,[30] IL-10 flox,[50] and IL-10Rα flox[51] were previously described. See also Table S1. All mice were crossed at least 12 generations to the C57BL/6 J background. All mouse experiments were performed with mice between 6–20 weeks of age with males and females at similar ratios, unless otherwise specified. Littermate controls were used for each experiment, and mice were randomly assigned to experimental groups. All experiments were performed in accordance with approved protocols by the Institutional Animal Care and Usage Committee at Baylor College of Medicine and Memorial Sloan Kettering Cancer Center.

Induction of colitis and colonic tumors

Induction of colitis was performed by administering dextran sulfate sodium salt (DSS, mol wt 40 kDa; Alfa Aesar).[22] Mice were administered 2% DSS in their drinking water for 7 days. Mice were weighed daily and analyzed after 7 days of DSS. For induction of colonic tumors, mice received a single injection of azoxymethane (AOM, Sigma) at 10 mg/kg body weight intraperitoneally. One week later, mice were treated with 3 cycles of 2% DSS (7 days) followed by 7 days of regular water. 4–5 weeks after the last cycle of DSS mice were analyzed.[36]

Colonization of mice with E. coli

E. coli (EC) 541–15 was cultured overnight in LB media. Mice with a SPF microbiota were colonized with a single gavage of 10^8 CFU of mouse commensal *E. coli*. Colonization was confirmed by qPCR with *E. coli*-specific primers using 16S (for fecal pellets) as housekeeping gene. Primers used were: 16S F: CGGTGAATACGTYCGG, 16S R: GGWTACCTTGTTACGACTT,[52] *E. coli* F: GGTAGAGCACTGTTTTGGCA, *E. coli* R: TGTCTCCCGTGATAACTTTCT.[53]

Diphtheria toxin (DT) and 4-hydroxytamoxifen (4OHT) administration

Mice were injected every other day with 200ng diphtheria toxin (DT, Sigma) in PBS. (Z)-4-Hydroxytamoxifen, 98% Z isomer (4OHT, Sigma) was resuspended to 20 mg/ml in ethanol by heating to 37°C. 4OHT was then diluted in corn oil (Sigma) to 2 mg/ml and mice were injected every 3 days with 100ul. All injections were intraperitoneal.

In vivo gut permeability assay with Fluorescein isothiocyanate (FITC)-dextran

Mice were weighed and fasted for 3 hours prior to oral FITC-dextran (4 kDa, Sigma) administration. A total concentration of 250 mg/kg body weight was administered through gavage. 3 hours after administration, blood samples were collected through retro-orbital bleeding and 50 ul of serum was placed in a fluorescence plate reader to determine the concentration by fluorescence excitation at 495 nm/519 nm reference.

DNA isolation and real-Time quantitative PCR (qPCR) analysis

For bacterial DNA identification, fecal pellets were collected and stored at −80°C. DNA was isolated using the DNeasy PowerSoil Kit according to the manufacturer's protocol (Qiagen). Real-time qPCR was performed using SYBR Green Supermix (Bio-Rad Laboratories) in the Bio-Rad thermocycler 384 well plates. Thermocycling program was 40 cycles at 95°C for 15s, 60°C for 30s, and 72°C for 30s, with an initial cycle of 95°C for 120s. Primers used were described above. Relative expression of target gene was determined using a standard curve to calculate the number of nucleotides per sample and the ΔΔCT method.

Cell isolation

Intestinal lamina propria cells were isolated as previously described.[16,32,54] Briefly, mouse large intestines were washed in PBS, once with freshly prepared 30 mM EDTA and 1 mM DTT and once with 30 mM EDTA (both at 37°C), and then digested at 37°C in collagenase 8 (100 U/ml, Sigma) and DNase-containing (150 μg/ml, Sigma) media with 10% fetal bovine serum. Digested material was separated on a 40%/80% Percoll (GE Health) gradient. Tumors were digested as above, washed in PBS, and passed through a 40um strainer.

Antibodies, cell staining, and flow cytometry

Cells isolated from colon lamina propria and tumors were blocked with CD16/32 (Fc receptor) to prevent nonspecific antibody binding. Then, cells were surface-stained in FACS buffer (PBS, 2 mM EDTA, 2% fetal bovine serum) for 30 minutes in the dark at 4°C with fluorescently conjugated antibodies from BD, eBiosciences, or BioLegend specific to CD45 (30-F11), TCR β (H57-597), CD11b (M1/70), CD11c (N418), MHCII (M5/114.15.2), Ly6C (AL-21), CX3CR1 (SA011 F11), Ly6G (1A8), B220 (RA3-6B2), CD3 (145–2C11), CD90.2 (53–2.1), CD4 (GK1.5), and CD8 (53–6.7). For T cell subsets identification, after staining for surface antigens, cells were fixed and permeabilized using a Fixation/Permeabilization Solution

Kit (ThermoFisher) overnight at 4°C and stained with antibodies specific to mouse FOXP3 (FJK-16s) or T-bet (4B10) for 45 minutes at room temperature. To assess cytokine production after ex vivo restimulation, single-cell suspensions from mesenteric lymph nodes were incubated for 4 hours at 37°C with 5% CO_2 in the presence of 50 ng/mL PMA and 500 ng/mL ionomycin with 2 μM monensin (BD Biosciences). Flow cytometric analysis was performed on an LSR II (BD Biosciences) or Aurora (Cytek) and analyzed using FlowJo software (Tree Star Inc.). DAPI or live/dead fixable blue dead cell stain kit (ThermoFisher) was used to exclude dead cells. Total cell counts were determined with Precision Count Beads (BioLegend). Gating strategy is shown in Figure S5C. PMN-MDSCs were CD11b$^+$Ly6G$^+$, TAMs were CD11b$^+$CX$_3$CR1$^+$MHCII$^+$Ly6C$^-$, M-MDSCs were CD11b$^+$CX$_3$CR1$^+$MHCII$^-$Ly6C$^+$, Th1 cells were CD3$^+$TCRβ$^+$CD4$^+$CD8$^-$Tbet$^+$, CTLs were CD3$^+$TCRβ$^+$CD4$^-$CD8$^+$Tbet$^+$, ILC1s were CD3$^-$TCRβ$^-$CD90.2$^+$Tbet$^+$, and Tregs were CD3$^+$TCRβ$^+$CD4$^+$CD8$^-$Foxp3$^+$. All antibody information is listed below:

Antibody, Fluorochrome, Clone	Source	Catalog number
Anti-mouse MHCII (I-A/I-E), Pacific blue, clone M5/114.15.2	BioLegend	Cat# 107,620
Anti-mouse CD11c, PEcy7, clone N418	BioLegend	Cat# 117,317
Anti-mouse/human CD11b, APC/Cy7, clone M1/70	BioLegend	Cat# 101,226
Anti-mouse CX₃CR1, FITC, clone SA011 F11	BioLegend	Cat# 149,020
Anti-mouse Ly6C, Alexa Fluor 700, clone AL-21	BD	Cat# 561,237
Anti-mouse Ly6G, PE, clone 1A8	BioLegend	Cat# 127,608
Anti-mouse CD4, Brilliant Violet 510, clone GK1.5	BioLegend	Cat# 100,449
Anti-human/mouse T-bet, PEcy7, clone 4B10	eBiosciences	Cat# 25–5825-82
Anti-mouse CD3, APC/Cy7, clone 145–2C11	BioLegend	Cat# 100,330
Anti-mouse TCRβ, Percp/Cy5.5, clone H57-597	BioLegend	Cat# 109,228
Anti-mouse/rat Foxp3, eFluor660, clone FJK-16s	eBiosciences	Cat# 50–5773-82
Anti-mouse CD45, Brilliant Violet 785, clone 30-F11	BioLegend	Cat# 103,149
Anti-human/mouse B220, Brilliant Violet 650, clone RA3-6B2	BioLegend	Cat# 103,241
Anti-mouse CD90.2, BUV395, clone 53–2.1	BD	Cat# 565,257
Anti-mouse CD8, APC, clone 53–6.7	BioLegend	Cat# 100,712
Anti-mouse IFNg, FITC, clone XMG1.2	BioLegend	Cat# 505,806
Anti-mouse IL-17A, BV421, clone TC11-18H10.1	BioLegend	Cat# 506,926

Enzyme-linked immunosorbent assays (ELISAs)

Fecal lipocalin-2 (Lcn-2) and myeloperoxidase (MPO) were determined by ELISA (R&D Systems). Fecal samples were solubilized in PBS with protease inhibitors at 10 mg/100 µl using a Mixer Mill.

Histology

Distal colons were fixed in 10% neutral buffered formalin, routinely processed, sectioned at 6 mm, and stained with hematoxylin and eosin (H&E) for light microscopic examination. Images were taken at 40X or 100X magnification using a ZEISS Axio Observer 2. Samples were assessed in a blinded fashion and scored 0 to 4 based on the criteria previously described:[55] (grade 0) Minimal chronic inflammatory infiltrate; (grade 1) Chronic inflammatory infiltrate; (grade 2) Few or rare neutrophils in lamina propria or epithelium; (grade 3) Multiple clusters of neutrophils in lamina propria and/or epithelium; (grade 4) Ulceration.

16S rDNA high-Throughput sequencing

Mouse fecal samples were collected sterilely and stored at −80 C. DNA was extracted utilizing Promega Max Prep & Promega Maxwell RSC 48 Instrument. The V4 and V5 regions of the 16S rDNA were PCR amplified, normalized, pooled, and sequenced using the Illumina MiSeq platform with 2×300 bp paired end reads. Analysis of 16S rDNA sequencing reads was performed using the software USEARCH (version 11.0.667) assigning taxonomy using the RDP 16S training set (version 16) as reference database. Merge reads with an expected error number over 1.0 were filtered. Filtered operational taxonomic units (OTUs) were rarefied to a depth of 17,481 reads per sample. Extraction, sequencing, and data processing were performed by the Microbiome Core Lab of Weill Cornell Medicine.

Statistics and reproducibility

Statistical analysis was performed using GraphPad Prism. All data are presented as individual values and mean. Two-tailed unpaired Student's t-test using a 95% confidence interval was used to evaluate the difference between two groups. For more than two groups, One-way ANOVA was used. For more than two groups under different conditions Two-way ANOVA was used. A probability value of $p < .05$ was considered significant. Statistical significance is indicated in each figure. Each experiment was repeated independently at least 2 times with similar results. Representative flow plots and micrographs were selected from the pool of biological replicates indicated in their respective quantifications.

Disclosure statement

The authors declare no conflict of interests.

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Contributions

D.F. Zegarra-Ruiz, R.S. Longman, and G.E. Diehl designed the experiments and analyzed data. D.F. Zegarra-Ruiz and G.E. Diehl wrote the manuscript with input from all co-authors. D. F. Zegarra-Ruiz, D.V. Kim, K. Norwood, F.B. Saldana-Morales, M. Kim, C. Ng, R. Callaghan, M. Uddin, L. Chang, and G.E. Diehl conducted experiments and analyzed data.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials. 16S sequencing data are openly available in NCBI under BioProject ID PRJNA845002.

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