Regulatory T-cell Response to Enterotoxigenic Bacteroides fragilis Colonization Triggers IL17-Dependent Colon Carcinogenesis

Abby L. Geis¹, Hongni Fan¹, Xinqun Wu², Shaoguang Wu², David L. Huso³, Jaime L. Wolfe⁴, Cynthia L. Sears¹,²,⁵, Drew M. Pardoll¹,², and Franck Housseau¹
ABSTRACT

Many epithelial cancers are associated with chronic inflammation. However, the features of inflammation that are procarcinogenic are not fully understood. Regulatory T cells (Treg) typically restrain overt inflammatory responses and maintain intestinal immune homeostasis. Their immune-suppressive activity can inhibit inflammation-associated cancers. Paradoxically, we show that colonic Tregs initiate IL17-mediated carcinogenesis in multiple intestinal neoplasia mice colonized with the human symbiote enterotoxigenic Bacteroides fragilis (ETBF). Depletion of Tregs in ETBF-colonized C57BL/6 FOXP3<sup>−/−</sup> mice enhanced colitis but diminished tumorigenesis associated with shifting of mucosal cytokine profile from IL17 to IFNγ. Inhibition of ETBF-induced colon tumorigenesis was dependent on reduced IL17 inflammation and was independent of IFNγ. Treg enhancement of IL17 production is cell-extrinsic. IL2 blockade restored Th17 responses and tumor formation in Treg-depleted animals. Our findings demonstrate that Tregs limit the availability of IL2 in the local microenvironment, allowing the Th17 development necessary to promote ETBF-triggered neoplasia, and thus unveil a new mechanism whereby Treg responses to intestinal bacterial infection can promote tumorigenesis.

SIGNIFICANCE: Tregs promote an oncogenic immune response to a common human symbiote associated with inflammatory bowel disease and colorectal cancer. Our data define mechanisms by which mucosal Tregs, despite suppressing excessive inflammation, promote the earliest stages of immune precarcinogenesis via enhancement of IL17 production at the expense of IFNγ production. Cancer Discov; 5(10); 1–12. ©2015 AACR.

See related commentary by Irrazabal and Martin, p. 1021.

INTRODUCTION

Colorectal cancer remains the third most common cancer and third leading cause of cancer-related death in the United States (1). Chronic intestinal inflammation represents one of the main environmental risk factors associated with development of colorectal cancer, a feature that is strongly supported by the much higher incidence of colorectal cancer in patients suffering from inflammatory bowel disease (IBD), including both Crohn disease and ulcerative colitis (2–4). A role for the IL23/Th17 inflammatory response in IBD patients is supported by genome-wide association studies demonstrating an extremely high association of Crohn disease with a specific polymorphism of the IL23 receptor (IL23R; ref. 5). Multiple murine models of immune-mediated carcinogenesis establish the IL17 response as critical, particularly in the intestine (6–9). Even in colorectal cancer that is not overtly colitis-associated, inflammation represents a critical component of the tumor microenvironment (TME) and has a critical impact on the clinical outcome (10). Identification of a Th17 immune signature, defined by the increased expression of genes IL17a, RORC, and IL23r, has been linked with a poor prognosis in colorectal cancer patients (11). Because of these emerging insights into specific immune pathways that can promote both early and later stages of cancer progression, particularly in the gut, inflammatory triggers and molecular mechanisms initiating pathogenic Th17 immune responses are sought as therapeutic targets for intestinal inflammatory disorders and colon tumorigenesis.

Microbial symbionts are key determinants of intestinal inflammation. In mice, development of Th17 cells in intestinal lamina propria is dictated by the microbiota, and, in one model, colonization of germ-free mice by segmented filamentous bacteria (SFB) is required for mucosal Th17 differentiation (12). It was further shown that the balance between intestinal Th1 and Th17 responses could be determined by the nature of the bacterium recognized (e.g., Listeria monocytogenes vs. SFB, respectively; ref. 13). Although SFB has been identified as a trigger for mucosal Th17 differentiation and is sufficient to promote autoimmunity in murine models (14), no microbial promoter of IL17 has yet been formally associated with IBD or colorectal cancer in humans. However, there are now emerging data linking enterotoxigenic Bacteroides fragilis (ETBF), a human colonic bacterium associated worldwide with inflammatory diarrheal diseases (15, 16), to IBD (17, 18) and colorectal cancer in humans (19, 20). Furthermore, we recently showed that ETBF triggers chronic STAT3/IL17-driven colitis in C57BL/6 mice and promotes rapid colon tumorigenesis in multiple intestinal neoplasia (Min) mice (heterozygous for the adenomatous polyposis coli...
Characterized by the accumulation of Treg cells, as well as IL17-producing cells, characteristic Th17 colitis associated with ETBF colonization. We show that Tregs promote acute IL17-driven colitis via local consumption of IL2, which inhibits Th17 polarization while enhancing expansion of Th1 cells. Although mucosal Tregs are initially required to promote Th17 polarization, they do not participate in the stabilization of the IL17 response at later stages of ETBF colitis. Thus, we identify an unexpected role for Tregs in promoting the early stages of colon carcinogenesis.

**RESULTS**

**Simultaneous Expansion of Mucosal Tregs and IL17-Producing Cells Precedes Colon Tumorigenesis in ETBF-Colonized Min Mice**

ETBF colonization of 4- to 5-week-old C57BL/6 mice (Apc(−/−)) elicits acute, self-limited (3–4 days) inflammatory diarrhea, associated with a robust mucosal IL17 response. ETBF colonization then persists, leading to a chronic (≥1 year), asymptomatic Th17-mediated colitis (21, 22) that promotes distal colon tumorigenesis in Min mice (ref. 6; Fig. 1A). Because IL17A is required for ETBF-triggered tumorigenesis, we sought to understand the regulation of inflammation and colon tumorigenesis in the context of ETBF colonization. Because mucosal Tregs are instrumental in tuning the inflammatory response in the colon (27), we hypothesized that depleting Tregs would diminish the magnitude of the IL17 response and thereby inhibit tumorigenesis. Indeed, depletion of Tregs with anti-FOXP3 or anti-IFNγ treatment (6, 21, 22) reduced the number of tumors and prevented the development of the characteristic Th17 colitis associated with ETBF colonization. We show that Tregs promote acute IL17-driven colitis via local consumption of IL2, which inhibits Th17 polarization while enhancing expansion of Th1 cells. Although mucosal Tregs are initially required to promote Th17 polarization, they do not participate in the stabilization of the IL17 response at later stages of ETBF colitis. Thus, we identify an unexpected role for Tregs in promoting the early stages of colon carcinogenesis.
components (24, 28), we looked for a possible Treg response to ETBF. We found that soon after ETBF colonization (day 7), IL17+ and FOXP3+ T-cell density (cells per gram of tissue) and percentage of lymphocytes concurrently increased in the tumor-prone distal colon compared with uninfected mice (Fig. 1B and C). The synchronized expansion of ETBF-producing and Treg cells following ETBF colonization suggests that Treg responses may play a role in modulating IL17-driven immune procarcinogenesis in the colon.

**Tregs Promote Colonic Neoplasia in ETBF-Colonized Min Mice**

We previously showed that ETBF-induced colon tumorigenesis is IL17-dependent because injection of an anti-IL17 blocking antibody inhibited tumor formation (6). FOXP3+ Treg function in intestinal mucosa is typically thought to suppress excessive effector immune responses to microbiota and protect the integrity of the intestinal barrier (29–31). Thus, we initially hypothesized that Tregs are necessary to limit ETBF-induced IL17-mediated tumorigenesis. To assess the impact of Tregs on ETBF tumorigenesis, we crossed Min mice to FOXP3DTR-GFP mice that express the diphtheria toxin receptor (DTR) on FOXP3+ cells (32). Intraperitoneal administration of DT, therefore, selectively depletes FOXP3+ cells. Because autoimmunity develops when Tregs are systemically depleted for more than 2 weeks (32), we tested the impact of Treg depletion on early microadenoma induction by ETBF in Min mice, which develop microadenomas in the distal colon beginning 1 week after ETBF colonization. This early time window (day 13) allowed us to assess the effect of FOXP3+ cells on early ETBF tumorigenesis prior to any systemic effects of Treg depletion. DT or PBS was administered to Min × FOXP3DTR mice starting 1 day prior to ETBF inoculation and every other day thereafter until harvest at day 13. Not surprisingly, Treg deletion significantly increased colonic inflammation (Fig. 2A and B), emphasizing that the immunosuppressive function of colonic Tregs remains intact. Surprisingly, however, microadenoma formation was significantly reduced in Treg-depleted mice compared with Treg-sufficient controls (Fig. 2A and C).

During the acute colitis stage at 1 week, inflammation upon Treg depletion was similar to that of Treg-competent mice in sham (0.0 ± 0.0, mean ± SD in sham and sham + DT mice, P > 0.999, N = 7–8 mice per group) or ETBF-colonized mice (1.78 ± 0.67 vs. 2.1 ± 0.88, ETBF vs. ETBF + DT mice, P = 0.5, N = 9–10 mice per group; Fig. 2D and E). Of note, 1-week ETBF-colonized mice with or without Treg depletion displayed marked colitis compared with sham mice (P < 0.0001 for both comparisons, Fig. 2E).

**Tregs Provide Help for Th17 Responses to ETBF Colonization via a Cell-Extrinsic Mechanism**

Because IL17 induction is an absolute requirement for ETBF-induced colon tumorigenesis (6) and microadenoma formation was significantly reduced in ETBF-colonized Treg-depleted mice, we asked whether Tregs in ETBF-colonized mice modified the mucosal Th1/Th17 balance in favor of protumoral Th17 effectors, potentially contributing to the development of distal colon tumorigenesis. To determine the impact of Tregs on the Th1/Th17 balance in ETBF-colonized mice, lamina propria lymphocytes (LPL) were isolated after 1 week from the colons of sham or ETBF-colonized FOXP3DTR-GFP mice treated or not with DT and analyzed for cytokine production by intracellular staining (ICS). Indeed, in accordance with the unexpected finding that Treg depletion diminished early in situ tumor formation, depletion of Tregs in ETBF-induced colitis dramatically reduced the proportion of Th17 cells (5% ± 2%, N = 5) in the colonic lamina propria compared with ETBF-colonized Treg-sufficient mice (27% ± 8%, N = 5, P < 0.0001; Fig. 3A and B). Notably, the effect of Treg depletion was not limited to Th17 cells, as IL17 production by other cell types (CD4+) was also mitigated (Supplementary Fig. S1). Conversely, FOXP3+ Treg depletion in ETBF-colonized mice strongly enhanced the Th1 response with a marked increase in the proportion of IFNγ+ CD4+ T cells. Because IFNγ+ CD4+ T cells also increased in sham mice after Treg depletion, our data further support the concept that mucosal regulation of Th1 by Tregs is key to intestinal immune homeostasis (Fig. 3A).

Because STAT1/IFNγ signaling is well established as an inhibitor of the STAT3/Th17 pathway (33), we explored the possibility that loss of IL17 production upon Treg depletion was indirectly due to inhibition by the increased Th1 response. We crossed FOXP3DTR mice to IFNγ−/− mice and demonstrated that, even though T cell–generated IL17 did trend slightly higher in IFNγ−/− mice compared with IFNγ+/− mice after Treg depletion, in the absence of IFNγ, Treg depletion still mitigated the proportion of Th17 cells in LPLs (Fig. 3B and C). This result demonstrated that decreased IL17 production in Treg-depleted mice is mostly not due to increases in IFNγ. Because Tregs are viewed as components of the TME that suppress antitumor immunity and promote tumor growth (34), it is possible that Treg depletion impaired ETBF tumorigenesis by unleashing a robust antitumoral IFNγ response. Thus, we asked whether increased IFNγ-driven inflammation in the absence of Tregs might promote potent antitumor immunity. Depletion of Tregs in Min × FOXP3DTR × IFNγ−/− mice reduced microadenoma numbers similar to those observed in Treg-depleted Min × FOXP3DTR mice, establishing that decreased Treg-mediated IL17 production, and not increased IFNγ, is most likely responsible for reduced neoplasia (Fig. 3D).

Although these results suggest that Tregs are providing cell-extrinsic “help” for the differentiation of naïve CD4+ LPLs to Th17 cells, it is possible that the effects of FOXP3+ cell depletion could be cell-intrinsic, i.e., via depletion of FOXP3+ precursors to the colonic Th17 cells. Indeed, there is evidence that Th17 cells and peripherally induced Tregs may differentiate from a common FOXP3+ROSY+ precursor, or Th17 cells may result from “trans-differentiation” of Tregs (35, 36). Consistent with a potential intrinsic mechanism of FOXP3 in cells differentiating into Th17 cells, we indeed observed the presence of IL17+ FOXP3+ CD4+ T cells in colon tumors (Supplementary Fig. S2).

To determine whether FOXP3 marked precursors to Th17 cells (intrinsic mechanism) or if FOXP3+ Treg help was necessary for Th17 differentiation (extrinsic mechanism) in ETBF colitis, we transferred both CD45.2+ FOXP3DTR and CD45.1+ FOXP3WT bone marrow (BM) at a 1:1 ratio into sublethally irradiated RAG1−/− recipients, establishing mixed BM chimera mice. This approach allowed us to selectively deplete CD45.2+ FOXP3DTR Tregs following DT injection, and it enabled us to monitor the origin (CD45.1+ or CD45.2+ BM) of
Figure 2. Treg depletion reduces microadenoma formation in Min mice. A, B6.FOXP3<sup>ΔTR</sup> × Min mice were inoculated with ETBF on day 0. Either 150 µL purified sterile H<sub>2</sub>O or 50 ng/g DT was administered i.p. on days −1, 0, 1, 3, and every other day until sacrifice and harvest. Colons were harvested on day 13, cleaned, rolled, and fixed in 10% formalin for histology and scoring. Images are of distal colon. Microadenoma is encircled. B, each symbol represents total inflammation score per Min mouse from A. Data shown include 2 separate experiments with 5 to 12 mice per group per experiment. Bars indicate mean ± SD. C, microadenomas counted per colon in B. D, B6.FOXP3<sup>ΔTR</sup> mice were inoculated with sham or ETBF on day 0. Either 150 µL purified sterile H<sub>2</sub>O or 50 ng/g DT was administered i.p. on days −2, −1, 0, 1, 3, and 5 until sacrifice and harvest. Colons were harvested on day 7, cleaned, rolled, fixed in 10% formalin, paraffin embedded, and hematoxylin and eosin stained for histology and scoring. Images are of distal colon. E, each symbol represents total inflammation score per mouse from D. Data shown include 2 separate experiments with 2 to 6 mice per group per experiment. Bars, mean ± SD.

The Th17 cells during ETBF colitis (Fig. 4A). Upon CD45.2<sup>+</sup> FOXP3<sup>ΔTR</sup> depletion, a cell-intrinsic mechanism would diminish Th17 responses among CD45.2<sup>+</sup> T cells, whereas a cell-extrinsic mechanism would not diminish Th17 responses among either CD45.1<sup>+</sup> or CD45.2<sup>+</sup> T cells. When CD45.2<sup>+</sup> FOXP3<sup>ΔTR</sup> cells were depleted in the mixed BM chimeras, we found that neither CD45.1<sup>+</sup> (i.e., CD45.2<sup>−</sup>) nor CD45.2<sup>+</sup> Th17 cells were decreased compared with Treg-sufficient mixed BM chimeras (Fig. 4B). In these experiments, CD45.2<sup>+</sup> Tregs were fully depleted, but CD45.1<sup>+</sup> Tregs were unaffected (Fig. 4B). As a positive control for the action of DT, we depleted total Tregs from ETBF-colonized CD45.2<sup>+</sup> FOXP3<sup>ΔTR</sup> BM chimeras. As expected, this resulted in a Th17 proportion in LPLs similar to the sham mixed BM chimera mice (Fig. 4B).
Tregs Initiate IL17-Mediated Colorectal Cancer

RESEARCH ARTICLE

Figure 3. Treg depletion mitigates the Th17 response to ETBF in favor of a Th1 response. A, colonic LPLs from 1 to 2 B6.FOXP3ΔTREG mice per group were harvested on day 7 following inoculation with ETBF or Sham on day 0. Either 150 μL purified sterile H2O or 50 ng/g DT was administered i.p. on days −2, −1, 1, 3, and 5. Cells were stimulated ex vivo, followed by ICS. Plots show viable CD3+CD4+FOXP3− LPLs and are a representation of 4 to 5 separate experiments. B, aggregate data from combined experiments showing percentage of viable CD3+CD4+FOXP3− LPLs that are IL17A+. Each symbol represents 1 to 2 Treg-depleted B6.FOXP3DTR mice (+, DT) or Treg-sufficient mice (○, no DT), and error bars represent 1 SD from the mean in each direction. The Holm-Sidak method for multiple comparisons was used to compare no DT versus + DT groups. C, B6.FOXP3ΔTREG and B6.FOXP3ΔTREG×IFNγ−/− mice were inoculated with ETBF on day 0. Either 150 μL purified sterile H2O or 50 ng/g DT was administered i.p. on days −2, −1, 1, 3, and 5. Colonic LPLs from 1 to 2 mice per group were harvested on day 7. Cells were stimulated ex vivo, followed by ICS. Dot plots show viable CD3+CD4+FOXP3− LPLs and are representative of 4 to 5 experiments. D, B6.FOXP3ΔTREG×IFNγ−/−×Min mice were inoculated with ETBF on day 0. Either 150 μL purified sterile H2O or 50 ng/g DT was administered i.p. on days −1, 0, 1, 3, and every other day until sacrifice and harvest. Colons were harvested on day 13, cleaned, rolled, and fixed in 10% formalin, paraffin embedded and hematoxylin and eosin stained for microadenoma counting per colon. Each symbol represents 1 colon, and error bars represent 1 SD from the mean in each direction. Two to 5 mice per group per experiment. Combined data from 2 experiments. IFNγ+/−×Min microadenoma counts from Fig. 16B are also shown for easier side-by-side comparison.

Tregs Promote Th17 Development in the Colon via Consumption of IL2

Tregs, which express high levels of IL2 receptors, particularly when activated, do not produce endogenous IL2. Thus, they are extremely dependent on exogenous sources of IL2 for their survival. Treg proliferation and activation upon ETBF colonization may therefore deprive the local inflammatory environment of IL2. Because IL2 downregulates IL17 production via STAT5 signaling, Tregs may promote Th17 differentiation by limiting the amount of local IL2 available to uncommitted T cells (37–39). In contrast, IL2 may promote proliferation of Th1 cells (40). To determine whether Treg...
Figure 4. Mixed chimeras reveal a cell-extrinsic requirement for FOXP3 in Th17 differentiation. A, schematic of the experiment designed to determine whether FOXP3 expression is a cell-extrinsic or cell-intrinsic requirement for Th17 differentiation. 10^7 total BM cells from B6.FOXP3DTR CD45.2 mice only (DT control), or mixed 1:1 with BM cells from B6.FOXP3WT CD45.1 mice, were transferred retro-orbitally into RAG1−/− recipients that had received 300 rads irradiation 5 to 6 hours prior. Following 6 weeks for hematopoietic cell reconstitution, mice were inoculated with ETBF or sham on day 0, and either 150 μL purified sterile H2O or 50 ng/g DT was administered i.p. on days −2, −1, 1, 3, and 5. Colonic LPLs from 1 to 2 mice were harvested on day 7 following ETBF or Sham inoculation. Cells were stimulated ex vivo followed by ICS. Dot plots show viable CD3+CD4+LPLs and are representative of 2 experiments.

B, colon LPLs from 1 B6.FOXP3DTR mouse per group were harvested on day 7 following inoculation with ETBF or sham on day 0. For no depletion and early depletion, purified sterile H2O or DT, respectively, was administered i.p. on days −2, −1, 1, 3, and 5. Cells were stimulated ex vivo, followed by ICS. Plots show viable CD3+CD4+FOXP3− LPLs and are representative of 2 mice per group.

C, no DT, early DT, late DT. Cells were stimulated ex vivo, followed by ICS. Plots show viable CD3+CD4+FOXP3− LPLs and are representative of 2 mice per group.

Promotion of Th17 differentiation upon ETBF colonization occurs via limiting local IL2, ETBF-colonized Treg-depleted FOXP3DTR mice were injected with a blocking antibody against IL2 (S4B6-1), and LPLs were isolated after 7 days of ETBF colonization. Figure 5 shows that anti-IL2 treatment indeed restored the Th17 response to ETBF in the absence of Treg cells. In contrast, when Treg cells were present, anti-IL2 treatment minimally increased the proportion of Th17 cells (Fig. 5A and B). In keeping with the notion that IL2 expands Th1 cells, anti-IL2 treatment partially mitigated the increase in IFNγ-producing cells upon Treg depletion (Fig. 5A). When Tregs were depleted, the expression of total IL17 mRNA was...
Figure 5. Anti-IL2 restores the Th17 response to ETBF in the absence of FOXP3 Tregs. A, four to nine B6.FOXP3DTR mice per group were inoculated with ETBF. Either 150 μL purified sterile H2O or 50 ng/g DT was administered i.p. on days −2, −1, 1, 3, and 5, and either rat anti-mouse IL2 or rat IgG2a isotype (JES3-19F) was delivered i.p. daily (day −2 through day 6). Colonic LPLs from each mouse were harvested on day 7. Cells were stimulated ex vivo, followed by ICS. Representative dot plots show viable CD3+ FOXP3− CD4+ LPLs from 1 mouse per group, showing percentage of viable CD3+ FOXP3− LPLs that are IL17A+.

Each symbol represents one B6.FOXP3DTR mouse, and error bars represent 1 SD from the mean in each direction. Isotype-treated animals were not different from untreated animals, so these two animal groups were combined.

C, tissue for RNA isolation was taken from the middle colon from each mouse in B, and Taqman qRT-PCR was performed for IL17A. ΔCt was calculated by subtracting Ct of G6PD from Ct of IL17A and averaging 2 technical replicates. Each symbol represents one mouse, and error bars represent 1 SD from the mean in each direction. Isotype-treated animals were not different from untreated animals, so these two animal groups were combined.

D, inflammation scores per colon in E. Six to 12 B6.FOXP3DTR mice per group were inoculated with ETBF on day 0. Either 150 μL purified sterile H2O or 50 ng/g DT was administered i.p. on days −2, −1, 1, 3, and 5. Colonic LPLs from 1 to 3 mice per group were harvested on day 7 for fluorescence-associating cell sorting. Effector T cells (CD11b−, FOXP3−, CD4+) were sorted from Treg-depleted mice (•, no DT) and from Treg-sufficient mice (○, + DT), or CD8+ T cells were sorted from Treg-sufficient mice (□, +/+ ETBF), and Taqman qRT-PCR was performed for indicated genes. G6PD was used as housekeeping control for total RNA quantity (ΔCt), and average of 2 technical replicates was used for each sample. CD4+ FOXP3+ Tregs were sorted from Treg-sufficient mice as the reference sample for calculating ΔΔCt. Fold change = 2ΔΔCt (ΔCt). Each symbol represents one mouse (or pooled group), and error bars represent 1 SD from the mean in each direction. The Holm-Sidak method for multiple comparisons was used to compare no DT versus + DT groups.

E, microadenoma counts from Fig. 3D are also shown for easier side-by-side comparison. F, inflammation scores per colon. Published OnlineFirst July 22, 2015; DOI: 10.1158/2159-8290.CD-15-0447
similarly restored when anti-IL2 monoclonal antibody (mAb) was administered (Fig. 5C). The decreased availability of IL2 likely resulted from cytokine consumption by Tregs (“sink effect”) and not inhibition of IL2 production by effector cells, as IL2 mRNA expression was indeed similar between effector T cells (CD11b+ FOXP3+ CD4+ or CD8+) sorted from LPLs of ETBF-colonized Treg-sufficient and Treg-deficient mice (Fig. 5D). As expected, expression of FOXP3 was low in both groups of effector T cells, and IL17 mRNA was decreased in effector T cells sorted from Treg-depleted mice compared with effector T cells from Treg-sufficient mice (Fig. 5D).

Importantly, when Treg-depleted Min × FOXP3DTR mice were treated with anti-mouse IL2 mAb, microadenoma formation was restored (Fig. 5E), demonstrating that the IL17-dependent protumoral activity of mucosal Tregs in the ETBF-driven TME requires IL2 deprivation. Despite the restoration of microadenomas upon anti-IL2 treatment of FOXP3-depleted mice, colonic inflammation was reduced compared with Treg-depleted isotype-treated Min × FOXP3DTR mice (Fig. 5F). The enhanced colitis of Treg depletion was also reduced in Treg-depleted IFNγ−/− mice (Fig. 5F). These results dissociate colitis from tumorigenesis in that Th1-mediated colitis upon Treg depletion (Fig. 3A) does not induce microadenomas as does Th17-mediated colitis (Fig. 2C). Thus, we conclude that Treg cells facilitate Th17 differentiation in the inflammatory microenvironment of the ETBF-colonized colon by limiting excess IL2 that can otherwise prevent the development of a procarcinogenic Th17-mediated immune response.

DISCUSSION

In an effort to further understand the mechanisms of ETBF-driven procarcinogenic colitis, we investigated the impact of mucosal Treg cells on the inflammatory response to ETBF murine colonization. These studies led to the surprising conclusion that Tregs are critical to initiate the Th17 colitis necessary for tumor induction. Through consumption of IL2, Tregs inhibit the development of Th1 colitis, which is not procarcinogenic, and shift T-cell differentiation to Th17 in the lamina propria of ETBF-colonized mice. This helper role of Tregs for Th17 development occurs only at the very initial stages of ETBF-mediated colitis.

Recent reports showed that mucosal Tregs, which commonly orchestrate mucosal immune homeostasis by restraining inflammatory responses to microbiota in the gut, can cooperate with immune effector cells to protect against and eradicate infections (27, 38). Th17 cells, by their production of IL17, are predicted to be important to the mucosal defense against ETBF, resulting in the recruitment of bac- tericidal polymorphonuclear and phagocytic mononuclear cells. Consistent with this idea, IL17+ mice colonized with ETBF exhibit increased morbidity and mortality compared with wild-type mice (C. Deja and C. Sears; unpublished data). However, the mucosal Th17-mediated defense against ETBF is not antiseptic and thus do not prevent chronic colitis associated with the persistence of both ETBF and mucosal IL17 production (22). In conjunction with a host genetic predisposition, such as the Apc mutation in Min mice, ETBF-induced mucosal IL17 is instrumental in promoting marked distal colon tumorigenesis (6). Importantly, a predominant IL17 response is associated with worse survival in human colorectal cancer (11). ETBF represents the first common human commensal identified as a potent trigger for murine colon tumorigenesis (6, 19). Surprisingly, ETBF triggers its carcinogenic IL17 response, in part, through the accumulation of mucosal Tregs. This is in stark contrast with the mucosal immune homeostasis proposed as resulting from nontoxic B. fragilis–mediated Treg induction (41, 42). Our results show that ETBF-mediated colitis drives the accumulation of FOXP3+ Tregs that, despite their suppression of Th1 mucosal inflammation, consume IL2, resulting in Th17 polarization that is critical to ETBF tumorigenesis. IL2 is a potent inhibitor of IL17 production via competition between STAT5 (IL2 signaling) and STAT3 (IL17 signaling) for binding to the IL17 promoter (43). Further, Tregs are incapable of producing IL2 and are highly dependent on exogenous sources of IL2, indispensable to their survival (44, 45). Thus, Tregs capture IL2 in their environment via their elevated constitutive expression of the high-affinity IL2 receptor CD25. By modulating levels of exogenous IL2, Tregs release the STAT3-inhibitory effect on Th17 differentiation of uncommitted CD4+ T cells or suppress already-committed effector T cells by IL2 deprivation (45, 46). Although Tregs have also been shown to inhibit IL2 production by effector T cells (endogenous IL2; refs. 45, 47), we have shown herein that ETBF-induced Tregs do not alter IL2 transcription by lamina propria effector T cells. Importantly, our results establish that Tregs strictly intervened at the initiation stage of Th17 differentiation, but they are not required for the stabilization of the colonic Th17 immune signature because late Treg depletion does not impair the Th17 response to ETBF infection (Fig. 4C). Our findings described here may be related to those of Pandiyan and colleagues, who showed in a murine model of oral Candida albicans that poorly suppressive Tregs were important to initiate the antifungal Th17 response, but they eventually resumed their suppressive function and inhibited pathogenic Th17 effectors (38).

Because Treg and Th17 cells demonstrate functional plasticity, we experimentally ruled out the possibility that ablation of FOXP3 limited Th17 cells generated via elimination of FOXP3+ progenitors. Tregs have been reported to convert into IL17- or IFNγ-producing cells (48–50), and Th17 cells have been reported to upregulate TBET and produce IFNγ (51, 52) or upregulate FOXP3 and acquire suppressive function (36, 53). Xu and colleagues demonstrated that, in the presence of IL6, FOXP3+ Tregs can be induced to become Th17 cells (48). Moreover, IL17+ Tregs have been detected in mouse and human cancer and have been shown to be highly pathogenic (54). Even though a small proportion of IL17+FOXP3+ T cells was detected in the colon of ETBF-colonized mice (Supplementary Fig. S2), our use of mixed BM chimeras demonstrated that ablation of CD45.2+ BM-derived FOXP3+ Tregs did not impair the generation of CD45.1+ Th17 cells when CD45.1+ Tregs were present. This indicated Tregs act to promote Th17 differentiation via an extrinsic mechanism in the ETBF murine model.

Our study showed that, in the absence of Tregs, ETBF colonization induced a strong inflammatory response associated with increased IFNγ production. Although commonly associated with antitumor host defenses, we demonstrated that the IFNγ/Th1–type immune response was not responsible for
Tregs Initiate IL17-Mediated Colorectal Cancer

Reduced colon neoplasia in Treg-depleted ETBF-colonized Min mice. This result highlights the impact of IL17 in tumor initiation. The increased IFNγ response to Treg depletion is not surprising, especially in the gut where there is constant exposure to inflammatory stimuli such as pathogen-associated molecular patterns on commensal bacteria. In fact, Treg cell depletion has recently been shown to increase the inflammatory cytokines IFNγ and IL17 in the intestines (24), and adoptive transfer of Treg cells can cure experimental models of IBD (26, 55). These data emphasize that Tregs exercise strong immunosuppressive function on effector T cells in other settings. However, in ETBF colonization, mucosal Tregs polarize a robust IL17 mucosal immunity while suppressing Th1 immunity. We noted that the inflammation score 1 week following ETBF colonization, in contrast with inflammation following 13 days of ETBF, remained similar with or without Tregs; however, the character of this inflammation differed, with Tregs promoting IL17 while repressing IFNγ.

Our findings allow a better understanding of the Th17 polarization induced by ETBF colonization and its contribution to colon tumorigenesis. However, the cellular and molecular mechanisms by which ETBF selectively increases Tregs in the distal murine colon remain unknown. As BFT, which profoundly alters the biology of colonic epithelial cells (23), is required for ETBF-driven oncogenic IL17 production, we postulate that epithelial-derived signals, following the rapid cleavage of E-cadherin induced by BFT (56), contribute to the recruitment of effector immune cells, including Tregs. It has recently been shown that diminished epithelial barrier function induced the production of alarmins that directly recruit and activate Tregs (57). For example, IL33 stimulated Tregs and opposed the restraining effect of proinflammatory IL23 on Tregs in microbiota-induced chronic colitis. Additional inflammatory signals, including those potentially provided by BFT-altered epithelial cells, may play a critical role in the Treg response to ETBF and promotion of protumoral IL17 production. Furthermore, although the precise mechanism by which IL17 contributes to ETBF-mediated tumorigenesis is still unclear, IL17 has been shown to directly influence colon epithelial cell signaling, survival, and proliferation (58). The elucidation of IL17-induced epithelial-derived signaling pathways will contribute to a better understanding of the regional distribution of ETBF-induced colon tumors and may eventually provide therapeutic targets to control deleterious mucosal IL17 and Treg responses.

METHODS

Mice and Reagents

C57BL/6 FOXP3Δ716Δ716 (Min) mice were provided by A. Rudensky (Memorial Sloan Kettering Cancer Center, New York, New York). C57BL/6 Rag1−/−, CD45.1, and IFNγ−/− mice were purchased from Jackson Laboratories. ArsΔ3 of Min mice were obtained from Dr. David Huso and Bert Vogelstein (Johns Hopkins University, Baltimore, MD). IFNγ−/− × FOXP3Δ716Δ716 mice were obtained in our facility by crossing C57BL/6 FOXP3Δ716Δ716 mice with Min mice. In some experiments, BM chimera mice were established by retro-orbital injection of 10⁷ BM cells from donor mice into sublethally irradiated (300 rad) recipient C57BL/6 Rag1−/− mice. Irradiated mice were rested 6 hours before BM cell injection. Reconstituted mice were maintained on prophylactic antibiotic treatment (Sulfatrim) prior to ETBF inoculation (see below). In accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care International, mice were maintained under specific pathogen-free conditions and studied according to protocols approved by the Johns Hopkins University Animal Care and Use Committee. Aquacell viability dye was purchased from Life Technologies. Flow cytometry antibodies against CD3, CD4, CD45.2, IFNγ, IL17A, and IFNγ were purchased from ImmunoTech/eBioscience. ICS was performed with the eBioscience FoxP3 staining kit according to the manufacturer’s instructions. Anti-IL2 mAb (clone S4B6) and isotype control (rat IgG2a) were purified from hybridomas purchased at the ATCC.

ETBF Mouse Models

ETBF strain 86-5443-2.2 was used in this study (6, 21). B. fragilis strains were grown anaerobically on brain-heart infusion (BHI) medium plates containing 37 g of brain heart infusion base (Difco Laboratories) per liter along with 5 g of yeast extract (Difco) per liter, 0.1 mg of vitamin K per liter, 0.5 mg of hemin per liter, 50 mg of L-cysteine, and 6 μg of clindamycin per liter (all from Sigma). A single colony was inoculated into BHI broth and grown anaerobically overnight at 37°C. Pelleted, washed bacteria were resuspended in 0.1 N sodium bicarbonate buffer and adjusted to an optical density corresponding to approximately 10⁹ colony-forming units (CFU)/mL for mouse inoculations. Three- to 4-week-old mice were treated with clindamycin and streptomycin (0.1 g/L and 5 g/L, respectively, in water bottles) 5 days prior to ETBF inoculation by gavage (~10⁸ bacteria in PBS). BM chimera mice were inoculated with ETBF 6 weeks after sublethal irradiation. All strains are resistant to these antibiotic treatments. Mice were sacrificed 7 days after colonization, unless otherwise noted.

Treg Depletion and IL2 Neutralization

Mice received 50-ng i.p. injections of DT (Sigma-Aldrich) per gram body weight on days 1 and 2 prior to ETBF inoculation (D-2 and D-1) and on days 1, 3, and 5 after ETBF inoculation (D1, D3, D5). Colonos were harvested from mice 7 days after ETBF inoculation. In some experiments, IL2 was neutralized by i.p. injection of 0.5 mg/mouse of anti-IL2 mAb (clone S4B6) or isotype control (rat IgG2a) 2 days prior to and every day after ETBF inoculation until mouse sacrifice. For tumor experiments, mice received 50 ng DT per g body weight on D1, D0, D1, D3, and D5, and every other day until sacrifice and harvest on D13.

LPL Isolation

Dissected colons were flushed with 20 mL Ca²⁺, Mg²⁺ free PBS 1X and cut longitudinally. Tissues were cut in ≤0.5 mm pieces and washed 3 times for 20 minutes in 37°C in 2 mmol/L EDTA, 10% FCS, 25 mmol/L Hepes, and HBSS buffer. Tissue pieces were subsequently digested 30 minutes in 5% FCS RPMI in the presence of 400 unit/mL Liberase (Roche Diagnostic) and 0.2 mg/mL DNAse 1 (Roche Diagnostic). Mononuclear cells were isolated by 20/40/80 Percoll gradient separation (GE Healthcare Life Science).

Flow Cytometry and FACS

Colons from 1 to 2 mice per group were processed to obtain LPLs as previously described (59). Mononuclear cells collected by Percoll gradient separation were cultured 4 hours in Iscove’s Modified Dulbecco’s Medium (IMDM) with 5% FCS and in the presence of Cell Stimulation Cocktail (plus protein transport inhibitors; eBioscience). Cells were then washed and stained for cell surface markers followed by fixation and permeabilization (FoxP3 fixation buffer; eBioscience). ICS was performed for IFNγ, IL17A, and FoxP3. Flow cytometry acquisition was performed on an LSR II cytometer (BD
Biosciences), and data were analyzed using FACSDiva 6.1.3 software. In some experiments, FACS was performed on FACSARia II (BD Biosciences).

**Quantitative RT-PCR**

Total RNA was isolated from sorted cells or whole tissue using TRIzol reagent from Life Technologies (Grand Island) according to the manufacturer’s instructions. RNA (1 μg) was reverse transcribed using the high-capacity RNA-to-cDNA Kit (Applied Biosystems). Forty cycles of TaqMan Gene Expression qRT-PCR was performed on 1 ng RNA per sample for indicated genes. ΔCt was calculated by subtracting Ct of Gapdh from Ct of target gene and averaging 2 technical replicates.

**Histology and Microadenoma Counts**

Colons were dissected and preserved in 10% buffered formalin. Histologic examination was performed after hematoxylin and eosin (H&E) staining of 5-μm sections. To facilitate longitudinal examination of the full-length colon, colons were “Swiss-rolled” prior to embedding and sectioning. Total colon inflammation was scored as previously described (21). Microadenoma counts on formalin-fixed paraffin-embedded H&E colon tissue sections from 2-week ETBF-colonized Min mice, and all histopathology scoring, were performed by a pathologist (David L. Huso).

**Statistical Analysis**

Comparison of mean was done by unpaired, two-tailed Mann–Whitney U testing, unless otherwise indicated. A P value of ≤0.05 was considered to designate a significant difference.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: A.L. Geis, C.L. Sears, F. Housseau

Development of methodology: A.L. Geis, S. Wu, C.L. Sears, F. Housseau

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.L. Geis, X. Wu, D.L. Huso, J.L. Wolfe, D.M. Pardoll

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.L. Geis, D.L. Huso, C.L. Sears, F. Housseau

Writing, review, and/or revision of the manuscript: A.L. Geis, D.L. Huso, J.L. Wolfe, C.L. Sears, D.M. Pardoll, F. Housseau

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Fan, S. Wu, F. Housseau

Study supervision: C.L. Sears, F. Housseau

**Acknowledgments**

The authors greatly appreciate the support of the SKCCC Flow Core, particularly Ada Tam and Richard L. Blosser.

**Grant Support**

This work was funded by the NIH (RO1CA151325, to C.L. Sears and D.M. Pardoll; R01DK080817, to C.L. Sears; P30DK0889502 GI Core; P30CA006973 SKCCC Core; T32AI007247, to A.L. Geis).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 16, 2015; revised July 15, 2015; accepted July 16, 2015; published OnlineFirst July 22, 2015.

**REFERENCES**


Tregs Initiate IL17-Mediated Colorectal Cancer


50. Feng T, Cao AT, Elson CO, Cong Y. Interleukin-12 converts Foxp3+ regulatory T cells to interferon-gamma-producing Foxp3+ T cells that inhibit colitis. Gastroenterology 2011;140:2031–43.


Regulatory T-cell Response to Enterotoxigenic *Bacteroides fragilis* Colonization Triggers IL17-Dependent Colon Carcinogenesis

Abby L. Geis, Hongni Fan, Xinqun Wu, et al.

*Cancer Discovery* Published OnlineFirst July 22, 2015.

Updated version Access the most recent version of this article at: doi:10.1158/2159-8290.CD-15-0447

Supplementary Material Access the most recent supplemental material at: http://cancerdiscovery.aacrjournals.org/content/suppl/2015/07/22/2159-8290.CD-15-0447.DC1

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.