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Redundant innate and adaptive sources of IL-17 production drive colon tumorigenesis

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Summary

IL-17-producing Th17 cells, generated through a STAT3-dependent mechanism, have been shown to promote carcinogenesis in many systems, including microbe-driven colon cancer. Additional sources of IL-17, such as γδ T cells, become available under inflammatory conditions, but their contributions to cancer development are unclear. In this study, we modeled Th17-driven colon tumorigenesis by colonizing $Min^{Apc+/-}$ mice with the human gut bacterium, enterotoxigenic *Bacteroides fragilis* (ETBF), to investigate the link between inflammation and colorectal cancer. We found that ablating Th17 cells by knocking out Stat3 in $CD4^+$ T cells delayed tumorigenesis, but failed to suppress the eventual formation of colonic tumors. However, IL-17 blockade significantly attenuated tumor formation, indicating a critical requirement for IL-17 in tumorigenesis, but from a source other than Th17 cells. Notably, genetic ablation of γδ T cells in ETBF-colonized Th17-deficient Min mice prevented the late emergence of colonic tumors. Taken together, these findings support a redundant role for adaptive Th17 cell- and innate γδT17 cell-

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derived IL-17 in bacteria-induced colon carcinogenesis, stressing the importance of therapeutically targeting the cytokine itself rather than its cellular sources.

Keywords

inflammation; gamma delta T cells; Th17; Stat3; enterotoxigenic *Bacteroides fragilis*

Introduction

The immune system plays a major role in cancer development and growth. It can promote or inhibit cancer depending on the specific type of response mediated by both innate and adaptive elements, particularly T cells (1). While T helper type 1 (Th1) CD4 T cells producing IFNγ and CD8 cytotoxic T lymphocytes (CTL) mediate anti-tumor immunity, recent evidence supports the notion that the alternative Th17 response is procarcinogenic, certainly at the level of cancer formation in the setting of chronic and/or recurrent inflammation.

We have studied the role of endogenous IL-17 in *de novo* colon carcinogenesis after colonization with a human colonic bacterium, enterotoxigenic *Bacteroides fragilis* (ETBF) (2) that is a bacterial candidate for induction of colon oncogenesis (3). ETBF is an anaerobe detected in up to 50% of individuals, without clinical symptoms (4, 5). However, ETBF colonization can also cause inflammatory diarrhea in children and adults (6) and is detected in the colon mucosa of ∼90% of human colon cancer patients (4). ETBF induces acute followed by chronic colonic inflammation in mice and promotes tumorigenesis in Min$^{Apc+/-}$ (Min) mice (2, 7). ETBF produces an oncogenic enterotoxin, *B. fragilis* toxin (BFT), which triggers the cleavage of E-cadherin in the colonic epithelium and leads to the activation of Wnt/β-catenin (targeting c-Myc and epithelial proliferation) and NF-κB pathways (targeting pro-inflammatory mediators), all of which are features of human CRC (3). ETBF-induced colitis is characterized by a signal transducer and activator of transcription (STAT)-3/IL-17-driven inflammatory response, associated with rapid DNA damage in epithelial cells and hyperplasia resulting in colonic tumors in Min mice that otherwise typically develop tumors predominantly in the small intestine (2, 8, 9). While our initial findings demonstrated the contribution of an adaptive Th17-driven mucosal immune response to ETBF-promoted colon carcinogenesis, the carcinogenic activity of Th17 cells versus other sources of IL-17 remains to be elucidated.

Th17 cells do not represent an exclusive source of IL-17. γδ T cells and a variety of immune cells also produce IL-17 during autoimmune inflammation and infection, including invariant NK T (iNKT) cells, mucosal-associated invariant T (MAIT) cells, CD3$^-$Thy1^{hi}SCA$^+$ group 3 innate lymphoid cells (ILC3), as well as myeloid cells including mast cells (MC), macrophages (Mφ) and neutrophils (PMN)(10). Some of these cells have been reported in tumor tissues and their production of IL-17 has been associated with tumor outcome, but with very mixed results suggesting that the nature of the cells producing IL-17 may play a critical role in oncogenesis (11). In humans, detection of IL-17-producing mast cells (MC) in esophageal squamous cell carcinoma (ESCC) was associated with a better outcome

whereas IL-17-producing Mφ were associated with the production of the proangiogenic factor, VEGF, and decreased survival in CRC cancers (12, 13). Non-Th17-derived IL-17 is thought to be mainly provided early by innate cells at mucosal and skin surfaces in response to infection and serves to determine the amplitude of the ensuing adaptive Th17 immune response (10, 14). Whereas uncommitted $CD4^+$ Th cells require TCR ligation in the presence of the combined action of IL-6, IL-1β and TGFβ to differentiate into Th17 cells, innate IL-17 producing cells seem to essentially rely on the action of IL-23, IL-1β and/or TLR signaling (14-18). While each of these cell types has been reported to reside within tumors to various degrees, little is known about their role relative to Th17 cells in cancer development.

The role of IL-17-producing γδ T cells (γδT17 cells) in early cancer initiation has not been studied and their role in cancer progression is controversial. γδT17 cells were recently suggested to improve the response to anti-tumor chemotherapy by recruiting immune effector cells (16) or alternatively to be the sole source of IL-17 in promoting tumor growth (19-21). In human colon cancer, γδT17 cells were reported to be the only source of IL-17 that was suggested to promote angiogenesis and tumor growth *via* the recruitment of myeloid-derived suppressor cells (MDSC) (22).

Strikingly, although delayed, the ultimate ETBF-induced colon tumorigenesis in Min mice was not diminished in mice lacking Th17 ($Th17^{null}$ CD4CRExSTAT3FLOX Min mice name hereafter Min-$CD4^{Stat3-/-}$), compared to parental Min mice. This later tumor development was nevertheless IL-17-dependent. ETBF colonization induced initially less robust IL-17 production in the colons of Th17-deficient Min-$CD4^{Stat3-/-}$ mice and the only other significant source of IL-17 in Min-$CD4^{Stat3-/-}$ mice contributing to tumorigenesis was the γδ T cell subset. While γδ T cells were not essential for tumorigenesis in Th17-competent mice, their ablation in Th17-deficient mice completely eliminated tumorigenesis. Whereas IL-17 was recently proposed to protect Min mice from malignant progression of small intestine (SI) adenomas (23), paradoxically, our findings definitively identify ETBF-induced IL-17 in the colon as procarcinogenic with mucosal γδ T cells as an innate source of IL-17 complementing Th17 cells.

Materials and Methods

Mice and bacteria

$Min^{ApcΔ716+/-}$ (Min) mice were provided by Drs. David Huso and Bert Vogelstein (Johns Hopkins University School of Medicine [JHU SOM]). $Villin^{Cre/Cre}$, TCRδ KO ($γδ^-$) and RAG1 KO mice were purchased from The Jackson Laboratory (Bar Harbor, ME). $CD4^{Cre/Cre}$ and $Stat3^{flox/flox}$ mice were provided by Dr Charles Drake (JHU SOM). IL-17A KO mice were obtained from Dr Yoichiro Iwakura (University of Tokyo). Bone marrow (BM) chimera mice were established by tail injection of 10^7 BM cells from donor mice into lethally irradiated (900 rad) recipient Min mice. Animal protocols were approved by the Johns Hopkins University Animal Care and Use Committee. Mice were inoculated with ETBF strain 86-5443-2-2 (ETBF 086)as previously described (2). Orogastric inoculation with antibiotic-resistant ETBF 086 (∼0.1-0.2 ml/bacterial strain or $∼10^8$ CFU/mouse) or the buffer control (Sham) was performed in mice treated for 5-7 days prior inoculation with 5 g /

liter of streptomycin (Sigma) and 100 mg/liter of clindamycin (Pharmacia, Kalamazoo, MI) in their drinking water. Fecal samples were cultured to quantify strain colonization.

Tumor counting and Histopathology

Macroscopic tumors were counted in formalin-fixed, methylene blue-stained colons blindly by two investigators (SW and CLS). For histologic studies including microadenoma quantification, formalin-fixed tissue was paraffin-embedded (FFPE), sectioned (5μm) and stained with hematoxylin and eosin (H&E staining). Inflammation scoring was performed blinded to identifiers by a board-certified veterinary pathologist (D. Huso), using a previously described scale (2, 9).

Tumor processing and flow cytometry

Colons were collected, flushed and enzymatically processed as previously described (2). Briefly, minced distal colons were washed 3 times for 20 min at 37^{o}C in 2mM EDTA, 10% FCS, 25mM Hepes, HBSS buffer and subsequently digested using a Liberase/DNAse 1 (Sigma-Aldrich, St Louis, MO). Mononuclear cells were isolated by Percoll gradient separation (GE Healthcare Life Science, Pittsburgh, PA). Splenocytes were isolated from enzymatically dissociated spleen using Lymphoprep density gradient (Accurate Chemical & Scientific Corporation, Westbury, NY). For cytokine intracellular staining (ICS), cells were stimulated for 4.5 hours with phorbol 12-myristate 13-acetate (PMA) (30 nM), ionomycin (1 μM) in presence of monensin (GolgiStop, BD Biosciences, San Jose, CA). Antibodies used for staining are listed in Supplementary Table S1. A live/dead (L/D) dye (Life Technologies, Carlsbad, CA) was used to exclude dead cells. FACS data were acquired using a LSRII cytometer (BD Biosciences) and analyzed with DIVA software (BD Biosciences). In some experiments, populations were cell sorted using Aria II (BD Bioscience). To block IL-17, mice were injected intra-peritoneally with 150ug of anti-IL17a mAb (Rat IgG1κ, clone TC11-18H10.1, Biolegend, San Diego, CA) or isotype control mAb twice weekly as described in each figure.

Real time PCR

Sorted cells were collected in lysis buffer of RNeasy purification kit, to perform RNA extraction following the manufacturer's instructions (Qiagen, Valancia, CA). RNA was converted to cDNA (*High Capacity RNA-to-cDNA Kit* , Life Technologies). A pre-amplification step was performed using pre-amplification Master Mix kit (Life Technologies) and a pool of the primers used for the Taqman PCR. The pre-amplified material is tested for the expression of target genes, and normalized by *Gapdh* expression using Taqman-based technology (Life Technologies). Results were expressed as $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$.

Human studies and sample processing

Tumor tissues were collected at Johns Hopkins Hospital and University of Malaya Medical Center (UMMC, Kuala Lumpur, Malaysia) from patients with primary sporadic CRC and free of prior chemotherapy. Demographic and pathologic status are detailed in Tables S2-3. This study was approved by the JHU Institutional Review Board and UMMC Medical Ethics

Committee. All samples were obtained in accordance with the Health Insurance and Accountability Act. Tumor specimens were collected and dissociated using an enzymatic cocktail as previously described (24). Antibodies for staining and FACS analysis are listed in Table S1.

Statistics

Comparison of means was done by unpaired, two-tailed Mann-Whitney U testing. A p value of < 0.05 designates a significant difference. For analysis of the tumor size distribution between ETBF-infected mice strains, we used Chi-squared test. A p value of < 0.05 designates a significant difference.

Results

Ablation of Stat3 in the CD4 compartment abrogates Th17 development but not IL-17 production in the colon

ETBF colonization of C57BL/6 mice leads to rapid and persistent activation of Stat3 selectively in the colonic mucosa (2, 9). Stat3 phosphorylation in myeloid and lymphoid cells is first detected 6 hours after ETBF colonization, followed by Stat3 activation in colonic epithelial cells (CEC). We previously reported that ETBF colonization is long lasting, generally persisting for up to one year (9). Since Stat3 is a key transcription factor in Th17-driven inflammation-associated cancer and Th17 cells were critical to ETBF-triggered IL-17 colitis and tumorigenesis as we previously reported (2), we sought to further assess the contribution of Stat3 signaling selectively in the CD4 compartment. To do so, we generated CD4-CreXStat3-Flox ($CD4^{Stat3-/-}$) mice, and compared colonic inflammation with wild type (WT) C57BL/6 mice after ETBF colonization. Because Stat3 is a critical transcription factor for *Il17a* gene expression, Th17 development in $CD4^{Stat3-/-}$ mice is completely inhibited following ETBF colonization (Fig. 1a) (25). Obliteration of IL-17 production from $CD4^+$ T cells was found in all tissues tested including SI and colon lamina propria (LP), spleen and lymph nodes (25). In ETBF colonized WT ($CD4^{Stat3+/+}$) mice, IL-17-producing γδ T cells and $CD4^+$ T cells represent 12.5+/-3.9 % and 55+/-6.5% of the total IL-17-producing cells in LP, respectively (Fig.S1). Upon colonization with ETBF, $CD4^{Stat3-/-}$ mice develop colitis that is histologically indistinguishable from ETBF colitis in WT mice (Table S3). γδT17 cells, which do not express CD4, were conserved during ETBF-triggered colitis in $CD4^{Stat3-/-}$ mice, and their numbers remained unchanged relative to WT mice (Fig.1b). We did not detect a significant difference in the total number of IL-17-producing cells in the colons of $CD4^{Stat3-/-}$ mice developing ETBF-triggered acute colitis (day 7) compared to WT mice (Fig.1c), likely, in part, due to higher numbers of T cells recovered from inflamed colons in these mice compared to WT mice (Fig.S2). Total numbers of IFN-γ^+ cells including IFN-γ^+ $CD4^+$ T (Th1) cells ($p=0.01$), as well as IFN-γ^+ γδT cells ($p=0.07$) were increased in $CD4^{Stat3-/-}$ mice (Fig.1a,b) underscoring the inhibitory effect of Stat3 signaling on Th1 differentiation (25). Western blot analysis, performed on CD4-enriched splenocytes of ETBF-colonized WT and $CD4^{Stat3-/-}$ mice, confirmed the absence of phosphorylated Stat3 (pStat3) in $CD4^+$ T cells, proving the efficiency of the CD4-Cre system (Fig.1d). Immunohistochemistry (IHC) demonstrated that pStat3 was detected in the colonic epithelium and stroma of ETBF-colonized $CD4^{Stat3-/-}$ mice, although

modestly decreased (Figs.1e and S3). This result suggests selective Stat3 inactivation in the CD4 compartment may diminish, at early time points, CEC Stat3 activation in ETBF-colonized WT mice. This result is consistent with our previous report that, in ETBF-colonized WT mice, colon immune cell Stat3 activation precedes CEC Stat3 activation and that BFT, the key virulence protein of ETBF, does not directly activate CEC Stat3 (9).

Although the number of IL-17-producing cells was not significantly decreased (Fig.1c), we found that expression of Th17-associated genes including *Il17a* (p=0.03; Fig.1f), *Il17f* and *Il21* (p=0.05 and 0.0085, respectively; Fig.1g&S4) as well as *Il23r* (p=0.18; Fig.1g&Fig.S4) were lower in colon tissues of CD4$^{Stat3-/-}$ mice compared to WT mice. Of note, the abrogation of Stat3 signaling in CD4^{+} cells also led to the decreased expression of Treg-associated genes (Fig.1g) including *Foxp3*, *Il10* and *Ctla4* (p=0.02, 0.04 and 0.01, respectively; Fig.S4). The ablation of Stat3 signaling in CD4 cells likely hinders Treg functions (i.e decreased IL-10 production) and therefore may account for the higher cellularity in the inflamed colons of ETBF-colonized CD4$^{Stat3-/-}$ compared to WT mice (Fig.S2). The decreased *Il17a* expression 7 days after ETBF colonization emphasizes that the absence of Th17 differentiation in CD4$^{Stat3-/-}$ mice globally diminishes the ETBF-induced initial IL-17 burst. Further, the difference between IL-17^{+} cell counts and RNA expression (Fig.1c,f) suggests that, overall, alternate sources of IL-17 that accumulate in the colon of ETBF-colonized CD4$^{Stat3-/-}$ mice, produce less IL-17 per cell compared to Th17 cells, resulting overall in lower IL-17 in the distal colon. However, γδT cells whose numbers in the mucosa did not increase in ETBF-colonized CD4$^{Stat3-/-}$ compared to WT mice (Fig. 1b), demonstrate higher production of IL-17 than Th17 cells (mean fluorescence intensity [MFI]=59,000 versus 34,000, respectively; Fig.S5). In contrast, the remaining non-γδ non-CD4^{+} T cells exhibit only a low MFI (17,000) for IL-17 ICS (Fig.S5).

Min-CD4$^{Stat3-/-}$ mice develop colon tumors upon ETBF colonization

While C57Bl/6 CD4$^{Stat3-/-}$ mice exhibited an acute colitis similar to that observed in WT mouse upon ETBF colonization (Table S4), Min-CD4$^{Stat3-/-}$ are characterized by significantly decreased macroscopic colon tumor numbers 8 weeks after ETBF colonization compared to parental Min mice, validating our earlier report of the importance of Th17 responses for colon tumorigenesis within this time period after ETBF colonization (2). However, unexpectedly, tumor numbers in Min-CD4$^{Stat3-/-}$ surged between 2 and 3 months following ETBF colonization. The tumor numbers were significantly higher at 3 months compared to 2 months after ETBF colonization of Min-CD4$^{Stat3-/-}$ mice (23.4±3.6 tumors per colon at 12 weeks versus 6.8±1.8 at 8 weeks post-colonization, mean±SEM, p<0.004) demonstrating a late acceleration of tumor development and/or growth in ETBF-colonized Min-CD4$^{Stat3-/-}$ mice. In contrast, tumor numbers in parental ETBF-colonized Min mice stabilized between 2 and 3 month-colonization (15.8±1.9 tumors per colon at 3 month versus 14.9±2.7 at 2 months, mean±SEM, p=0.867). These findings show that, although delayed, ETBF-triggered tumorigenesis persisted in Min-CD4$^{Stat3-/-}$ mice. When comparing micro-adenoma numbers in Min *vs.* Min-CD4$^{Stat3-/-}$ mice 4, 8 and 12 weeks after ETBF colonization, both strains of mice displayed a similar number of microadenomas (Fig.2b-c) suggesting that, in Min-CD4$^{Stat3-/-}$ mice, the diminished early IL-17 burst (Fig 1f) normally provided by Th17 cells in parental Min mice, impacted tumor growth rate rather than the

ETBF-dependent initiation events. Consistent with this hypothesis, comparison of tumor size in 3 month ETBF-colonized mice revealed significantly higher numbers of smaller tumors in Min-CD4$^{Stat3-/-}$ mice compared to Min-CD4$^{Stat3+/+}$ mice (Fig.S6). These findings underline a likely combined impact of IL-17 on ETBF-mediated tumor initiation and tumor growth (2). It also follows that IL-17-secreting non-Th17 cells can contribute to the initiation of the carcinogenesis.

Colon tumorigenesis in ETBF-colonized Min-CD4$^{Stat3-/-}$ mice remains IL-17-dependent

To determine the nature of the protumoral factors in Min-CD4$^{Stat3-/-}$ mice, we compared the inflammatory environment in colon tumors from 3 month ETBF-colonized parental Min and Min-CD4$^{Stat3-/-}$ mice. We assessed expression of inflammation-related genes using qRT-PCR on whole tumor tissue. Surprisingly, we found that in CD4$^{Stat3-/-}$, similar to parental Min mice, *Il17a* gene expression was highly increased in ETBF tumors compared to surrounding normal colonic tissue (Fig.3a). In two independent experiments, CD3$^+$CD4$^+$ T cells sorted from the Min-CD4$^{Stat3-/-}$ colon tumors consistently expressed a higher level of Th1-(increased *Tbx21, Stat1*), Th2-(*Gata3 and Il4*) associated genes as well as T cell effector function (*Pdcd1, 4-1BB, Ox40, Klrg1, Ccr7, Itgae and Il10ra*) compared to those sorted from tumors from parental Min mice but were characterized by lower Th17-associated genes including *Rorc*, *Il17a, IL17f, Il22, Il21, Il23r and Ccr6* (Fig.3b). This result confirms the absence of Th17 differentiation in Min-CD4$^{Stat3-/-}$ 3 months after ETBF colonization. When testing the ability of tumor-infiltrating lymphocytes (TIL) to produce IL-17 using ICS and flow cytometry analysis, we detected residual IL-17$^+$ cells including substantial numbers of γδ$^+$ T cells in colon tumors derived from Min-CD4$^{Stat3-/-}$ mice (Fig. 3c). Functional blockade of IL-17 using anti-IL-17 mAb injection twice weekly between 6 and 12 weeks after ETBF colonization significantly mitigated ETBF colon tumorigenesis in Min-CD4$^{Stat3-/-}$ mice compared to mice injected with the isotype control mAb (Fig.3d) indicating that sole blockade of IL-17 is sufficient to impede ETBF-triggered tumorigenesis and importantly reinforces the direct contribution of IL-17 to colon tumorigenesis. Interestingly, blockade of IL-17 in ETBF-colonized parental Min mice between 6 and 12 weeks did not modify ETBF colon tumorigenesis (Fig.S7), suggesting the critical role of IL-17 in promoting tumor initiation and growth early during ETBF tumorigenesis in the presence of Th17 as we previously reported (2). Altogether, these findings demonstrate that IL-17 but not Th17 cells *per se* is a critical requirement for ETBF tumorigenesis. In the absence of an initial Th17 response to ETBF colonization, the initial lower (Fig 1f) and then seemingly slower accumulation of IL-17 hinders but does not abrogate ETBF carcinogenesis.

In the absence of Th17 cells, mucosal γδT17 cells are the critical source of IL-17 promoting colon tumor growth

We next sought to identify the IL-17-producing cells promoting ETBF-mediated tumorigenesis in the absence of Th17 cells. Flow cytometry analysis of enzymatically digested tumor tissue from 3 month ETBF-colonized Min-CD4$^{Stat3-/-}$ mice confirmed that only CD3$^+$γδ$^+$ T cells as well as undefined CD4$^-$γδ$^-$ CD3$^+$ T cells secreted IL-17 (Fig.3c). We cell-sorted multiple leukocyte populations from colon tumors in ETBF-colonized Min-CD4$^{Stat3-/-}$ mice and tested each of them for *Il17a* expression (Fig.4a). These populations

included granulocytic MDSC (PMN-MDSC; $CD11b^{hi}GR1^{hi}$ cells), monocytic MDSC (MO-MDSC; $CD11b^{hi}GR1^{lo}$ cells), and mature myeloid cells ($CD11b^{+}GR1^{neg}$, including Mϕ and dendritic cells [DC]), together with $CD11b^{neg}CD3^{neg}$ cells (including ILC3) and $CD3^{+}CD4^{+}\gamma\delta^{-}$. *Il17a* expression was not detectable in $CD11b^{+}Gr1^{neg}$ or $CD11b^{neg}CD3^{neg}$ populations (Fig 4a). Whereas ICS did not identify MDSC as IL-17 producers (data not shown), qRT-PCR identified low level expression of the *Il17a* gene only in the MO-MDSC subset (Fig 4a). Although MDSCs represented 80 to 90% of the infiltrating leukocytes in the tumor microenvironment (TME) and γδT17 cells only 0.1% (Fig S8), we found that γδ T cells expressed on average nearly 2,000 times more IL-17 per cell than MO-MDSC (Fig 4a). These findings established that, whereas MO-MDSC and γδT cells produce IL-17 in the TME, γδT cells represent overwhelmingly the primary provider of IL-17 in 3 month ETBF tumors when Th17 are missing. To confirm the role of γδ T cells as the principal alternate source of IL-17 in the absence of Th17, we first studied colitis in ETBF-colonized $\gamma\delta^{-}$-$CD4^{Stat3-/-}$ mice, lacking γδ and Th17 cells. We found that $\gamma\delta^{-}$ $CD4^{Stat3-/-}$ mice expressed much less *Il17a* than WT and $CD4^{Stat3-/-}$ C57BL/6 mice (Fig.4b). $\gamma\delta^{-}$-$CD4^{Stat3-/-}$ mice also exhibited a reduced expression of MDSC-associated genes including *Arg1 and Nos2* (70 and 9 times less respectively in Fig.4c; p=0.11 and 0.13 respectively in Fig.S4B), an immune profile that reflects overall diminished protumoral inflammation in $\gamma\delta^{-}$-$CD4^{Stat3-/-}$ mice compared to $Th17^{null}$-$CD4^{Stat3-/-}$ mice 7 days after ETBF colonization, and supports the role of IL-17 in recruiting MDSC in the TME (22).

Irradiated Min mice reconstituted with a $RAG1^{-/-}$ BM ([$Rag1^{-/-} \rightarrow Il17a^{-/-}$-Min]), and colonized with ETBF 6 weeks after engraftment developed no tumors 12 weeks after ETBF colonization (Fig.4d) indicating that any IL-17 provided *de novo* by myeloid, NK or ILC3 (Fig.4a and Fig.S9) cells was insufficient to sustain colon tumorigenesis. Colon tumorigenesis in ETBF-colonized [$\gamma\delta^{-}$-$CD4^{Stat3-/-} \rightarrow$ $IL\text{-}17^{-/-}$-Min] BM chimera mice (no Th17 and γδ T cells) was markedly reduced compared to [WT $\rightarrow$ $Il17a^{-/-}$-Min] BM chimeras, a result validating the tumorigenic potential of γδT17 cells (Fig.4d,e). In contrast, ETBF-colonized Min-$\gamma\delta^{-}$ mice, showed no significant difference in tumor numbers compared to parental Min mice (Fig.4f), demonstrating that, when Th17 are present, γδT17 cells are dispensable for tumor formation.

Both Th17 and γδT17 cells are present in human colon cancer

In contrast to murine transplantable tumor models and colonic epithelial barrier disruptive models of colonic inflammatory tumorigenesis (e.g. AOM/DSS), the ETBF model is induced by a human colonic bacterium linked to colitis and colon cancer (2). We sought to determine the distribution of IL-17-producing cells in human colon cancer. Figure 5 and Tables 1&2 suggest that human colon cancer (JHU cohort, n=12; Malaysian cohort, n=11) has a similar distribution of Th17 and γδT17 cells as the ETBF model, that is, both Th17 and γδT17 cells are found in the majority of colon cancers (which have a wide range of overall IL-17-producing TIL), with Th17 representing the majority of IL-17 producers. This result contrasts with a recent publication proposing that γδT17 cells are overwhelmingly the primary source of IL-17 in human colon cancer. This discrepancy does not appear to be due to the source of tumors (Asian vs American populations), since we observe similar Th17 predominance in a cohort of patients from Malaysia (Table 2).

Discussion

Our ETBF Min mouse model mirrors features of human CRC including altered APC/β-catenin signaling, a predominant distal location of colon tumors with validation of the proposed pathogenic role of protracted asymptomatic mucosal Th17 responses in human CRC (26, 27). We tackle herein the role and cellular origin of endogenous IL-17 (induced by a single human colonic bacterium introduced to the microbiota as opposed to non-physiologic chemical-induced models) in promoting tumor development and to distinguish the pro-carcinogenic role of adaptive Th17 cell-derived IL-17 from alternative non-Th17 cell sources (primarily innate IL-17). Inactivation of Stat3 in CD4 cells using CD4$^{Stat3-/-}$ mice (28) led to ablation of *Il17a* transcription in CD4 cells as well as decreased expression of Th17-associated genes (*Il17a*, *Il17f*, *Il21*, *Il22* and *Il23r*) during acute ETBF colitis (Day 7). However, though macroscopic colon tumors were decreased 2 months after ETBF colonization, colon tumorigenesis rebounded and *Il17a* was highly expressed in tumor tissues suggesting that non-CD4^{+} T cells (therefore not Th17, Treg or LTi) infiltrating colon tumors were producing IL-17 (29). The unaltered early microadenoma numbers combined with the reduced tumor size in Th17^{null} Min-CD4$^{Stat3-/-}$ compared to parental Min mice suggest that the lower levels of IL-17 did not interfere with the ETBF-initiation step but likely impeded tumor growth. These findings emphasize that adaptive (Th17) and innate (non-Th17) IL-17 sources differ in their kinetics but are redundant in their capacity to drive colonic tumor initiation and growth. *Il17a* transcription in the colon of ETBF-colonized CD4$^{Stat3-/-}$ mice was associated with increased *Arg1* and *Nos2* expression (Fig.4C) that play an essential role in the pro-tumoral function of MDSC a feature that emphasizes the persistence of a protumoral microenvironment in the colon of ETBF-colonized Min-CD4$^{Stat3-/-}$ mice, although Th17 cells are absent.

Although access of microbiota to immune cells during acute ETBF colitis likely impacts the mucosal immune response to ETBF colonization, ETBF colon tumorigenesis requires molecular events triggered by the action of BFT on CECs that, in part, account for IL-17-dependent tumor initiation and growth (3, 30). Indeed, in this model, Th17 detection in mice persists long after resolution of acute colitis and healing of colon barrier function (9). Importantly, these features reinforce the clear contrast in Min mice between spontaneous polyposis (mainly in SI) for which IL-17 was reported, in one publication, to protect from cancer progression (23) and ETBF-induced tumorigenesis (strictly in the colon) for which IL-17 is required to promote colon tumor (2). In another study, Min mouse SI tumorigenesis was also reported to be promoted by IL-17 (31). A multitude of alternative IL-17-producing cell subsets including γδ-T cells, NK cells, ILC3 and myeloid-derived cells such as PMN, MΦ and MDSC exist (19, 20, 32-36). These innate cells are rapidly recruited to inflamed colon tissues, do not require antigen recognition but respond to stimulation of pattern recognition receptors (PRR) or the action of inflammatory mediators such as IL-23 and IL-1β (10). Herein, the ablation of colon tumorigenesis in BM chimera Min mice reconstituted with RAG1$^{-/-}$ or γδ^{-}-CD4$^{Stat3-/-}$ BM provided confirmation of the protumoral role of γδT17 cells in the absence of Th17 cells while the other innate cells (ILC3, NK and myeloid cells) although present in BM chimera mice are not sufficient to provide IL-17 in amounts able to sustain ETBF tumorigenesis.

γδT cells are detected at virtually all mucosal surfaces and skin where they participate in tumor immunosurveillance (16, 19, 22, 37). In microbial infection, γδT17 cells are critical to orchestrate the recruitment of monocytes and neutrophils (19, 38) and to amplify the ensuing adaptive Th17 response (17). That MO- and PMN-MDSC were highly recruited to colon tumors of Min-CD4$^{Stat3-/-}$ mice in response to the BFT-mediated oncogenic alteration of CEC and IL-17 production is compatible with the notion that γδT17 cells contribute to the production of protumoral IL-17 as well as shape the TME which includes the accumulation of MDSC (19, 21, 22). As a matter of fact, Wu *et al.* recently reported that virtually all the IL-17-producing cells in human colon cancers were γδT cells and their detection was associated with the recruitment and survival of protumoral PMN-MDSC (22). Notably, we find a very similar distribution of Th17 and γδT17 cells subsets between our ETBF tumorigenesis model and human colon cancers that have high IL-17 in their TME; namely, γδT17 cells represent a smaller proportion of the IL-17-producing cell population than do Th17 cells (Fig. 5 and Tables 1-2). It is unlikely that this marked discrepancy is due to fundamental differences in the CRC TME in Chinese vs American patients as we identified a similar distribution of Th17 and γδT17 cells between a cohort of US CRC (Table 1) and Malaysian (that included Chinese patients; Table 2) samples. Our finding of Th17 and γδT17 redundancy in our model of *de novo* tumorigenesis driven by a human colonic bacterium also contrasts with the findings of Ma *et al* in a transplantable murine hepatocellular carcinoma model where conventional Th17 cells played little role in promoting tumor growth while γδT17 cells (expressing Vγ4) were the predominant source of tumor growth-promoting IL-17 (19). Coffelt et al. found in a KEP-based model of spontaneous breast cancer metastasis, that although both γδ and CD4+ T cells produced IL-17 only depletion of γδ T cells led to a decreased level of IL-17 in serum, and number of pulmonary metastasis (21). Therefore, whether γδT17 cells are pro-tumorigenic or not appears to be highly context and model-dependent. However, the contribution of γδT17 cells to ETBF tumorigenesis when Th17 are present remains uncertain. ETBF infection of Min-γδ^{-} mice resulted in similar tumorigenesis as in parental Min mice suggesting limited impact of γδT17 cells in the presence of Th17 cells. Early γδT17 cells have been shown to be important for triggering and amplifying the ensuing adaptive Th17 immune response (17). Combined, these pathways are novel and likely highly relevant to the pathogenesis of human CRC. Our model of microbial-induced *de novo* carcinogenesis has direct translational implications as it suggests that therapeutic approaches to limit pathogenic Th17 will likely be insufficient to impact colon carcinogenesis if alternative sources of IL-17 (i.e., γδT cells and myeloid-derived cells) do not respond to the same regulatory mechanisms. Finally, we propose that our results herein, in combination with our previous demonstration that early inhibition of IL-17, using anti-IL17 mAb injection (2) or Treg depletion (39) in ETBF-colonized Min mice impedes microadenoma formation, support a novel paradigm that IL-17, independent of its origin, has carcinogenic potential, promoting not solely tumor growth but also tumor initiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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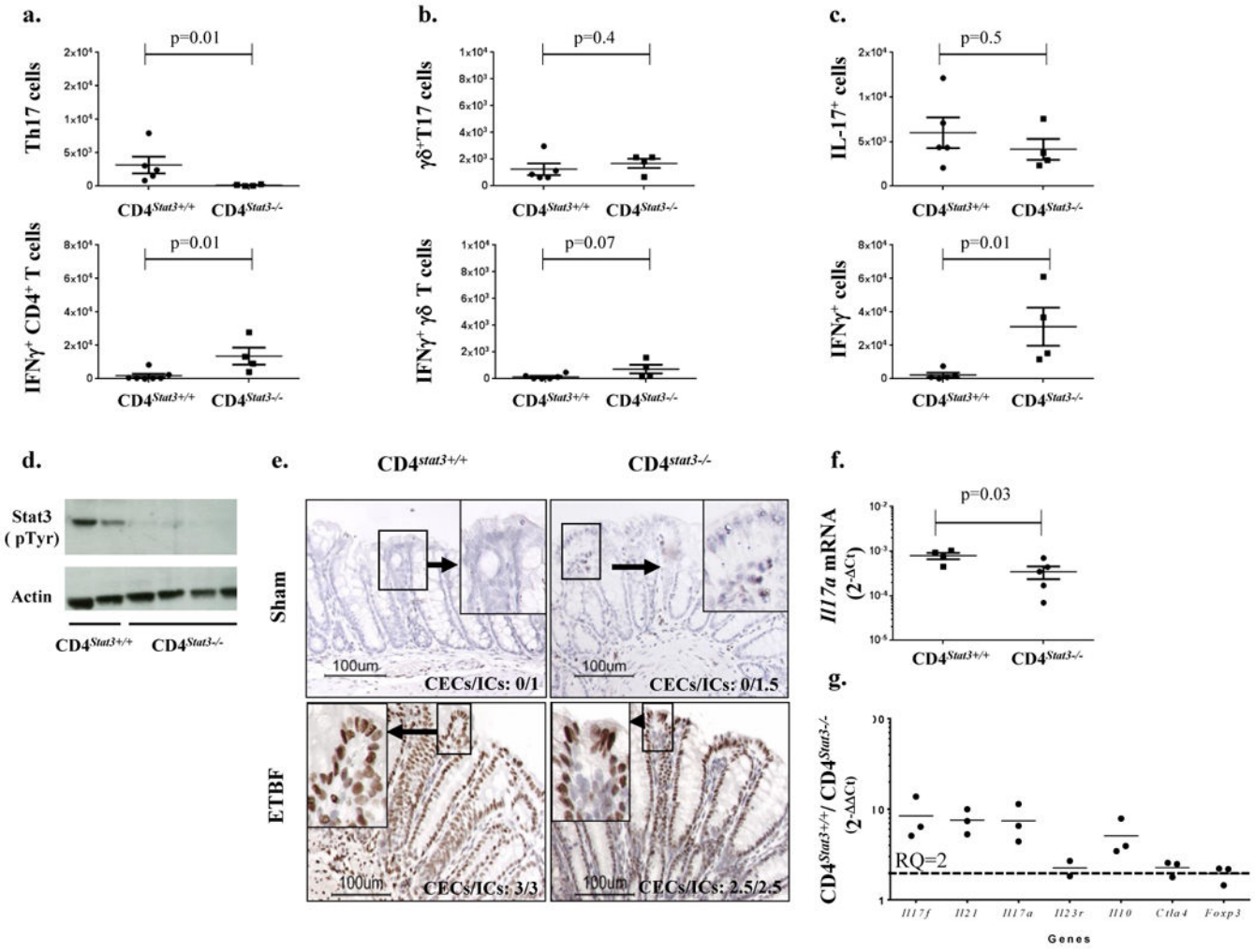

Figure 1. ETBF triggers IL-17-mediated colitis in Th17^{null} CD4$^{Stat3-/-}$ mice

Absolute numbers of IL-17- (upper) and IFNγ- (lower) producing, CD4$^+$ (**a**), γδ$^+$ (**b**) total (**c**) cells per distal colon of wild-type (WT) C57BL/6 (CD4$^{Stat3+/+}$) and CD4$^{Stat3-/-}$ C57BL/6 mice 7 days after ETBF colonization. Mean+/-SEM. p value, Mann Whitney U test. **d**, pStat3 (anti-pTyr) Western blot of CD4-enriched splenocytes of ETBF-colonized WT and CD4$^{Stat3-/-}$ C57BL/6 mice. **e**, pStat3 immunohistochemistry performed on FFPE colon tissue in CD4$^{Stat3+/+}$ WT (Left) versus CD4$^{Stat3-/-}$ (right) C57BL/6 mice colonized (lower) or not (Sham, upper) with ETBF. pSTAT3 scores for epithelial cells (CEC) and immune cells (IC) are indicated on each panel. Scale bars, 100μm. **f**, *Il17a* expression in ETBF-colonized WT (CD4$^{Stat3+/+}$) and CD4$^{Stat3-/-}$ C57BL/6 mice distal colon (whole tissue). The graph shows $2^{-\Delta Ct}$ where ΔCt represent normalized Ct_{Il17a} values. Mean+/-SEM. p value, Mann Whitney U test. **g**, Inflammation-related genes overexpressed in distal colon of C57BL/6 CD4$^{Stat3+/+}$ compared to CD4$^{Stat3-/-}$ mice 7 days after ETBF colonization. Each dot represents the ratio RQ (CD4$^{Stat3+/+}$ / CD4$^{Stat3-/-}$) =$2^{-\Delta\Delta Ct}$. The mean of three experiments is shown. Statistical analysis is shown in Fig.S4A.

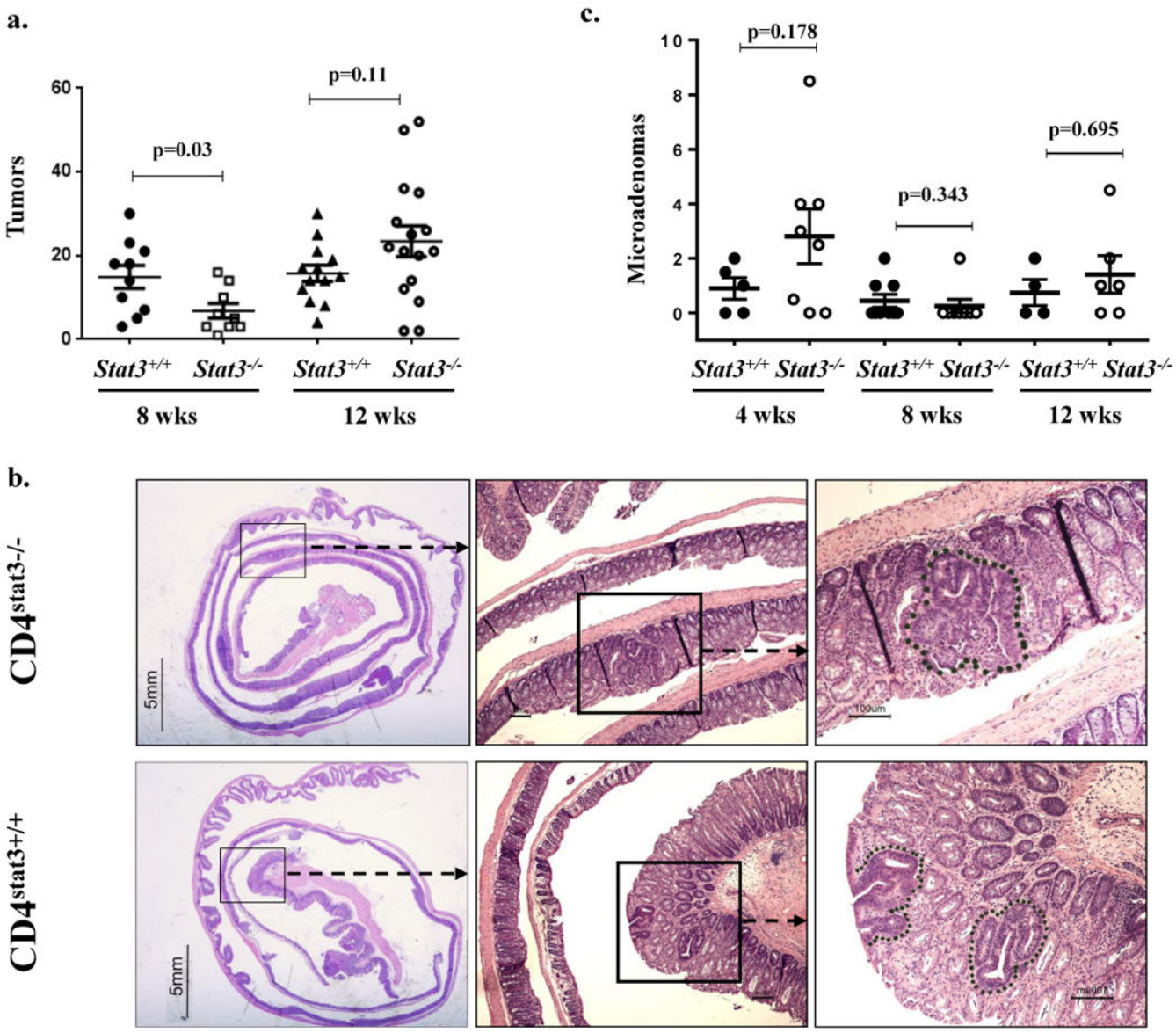

Figure 2. ETBF triggers colon tumorigenesis in Th17^{null} Min-CD4$^{Stat3-/-}$ mice

a, colon tumors in parental (Stat3$^{+/+}$) and CD4$^{Stat3-/-}$ (Stat3$^{-/-}$) Min mice 8 and 12 weeks after ETBF colonization; Mean+/-SEM. **b**, representative FFPE sections of rolled colons (left panel) from parental (Stat3$^{+/+}$; lower panel) and CD4$^{Stat3-/-}$ (Stat3$^{-/-}$; upper panel) Min mice 3 month after ETBF colonization and micrographic characterization of microadenomas (middle and right panels) in CD4$^{Stat3+/+}$ (lower panel) and CD4$^{Stat3-/-}$ (upper panel) Min mice 4 weeks after ETBF colonization. **c,** colonic microadenoma numbers 4, 8 and 12 weeks after ETBF colonization median+/-IQR. p values, non-parametric Mann Whitney U t test.

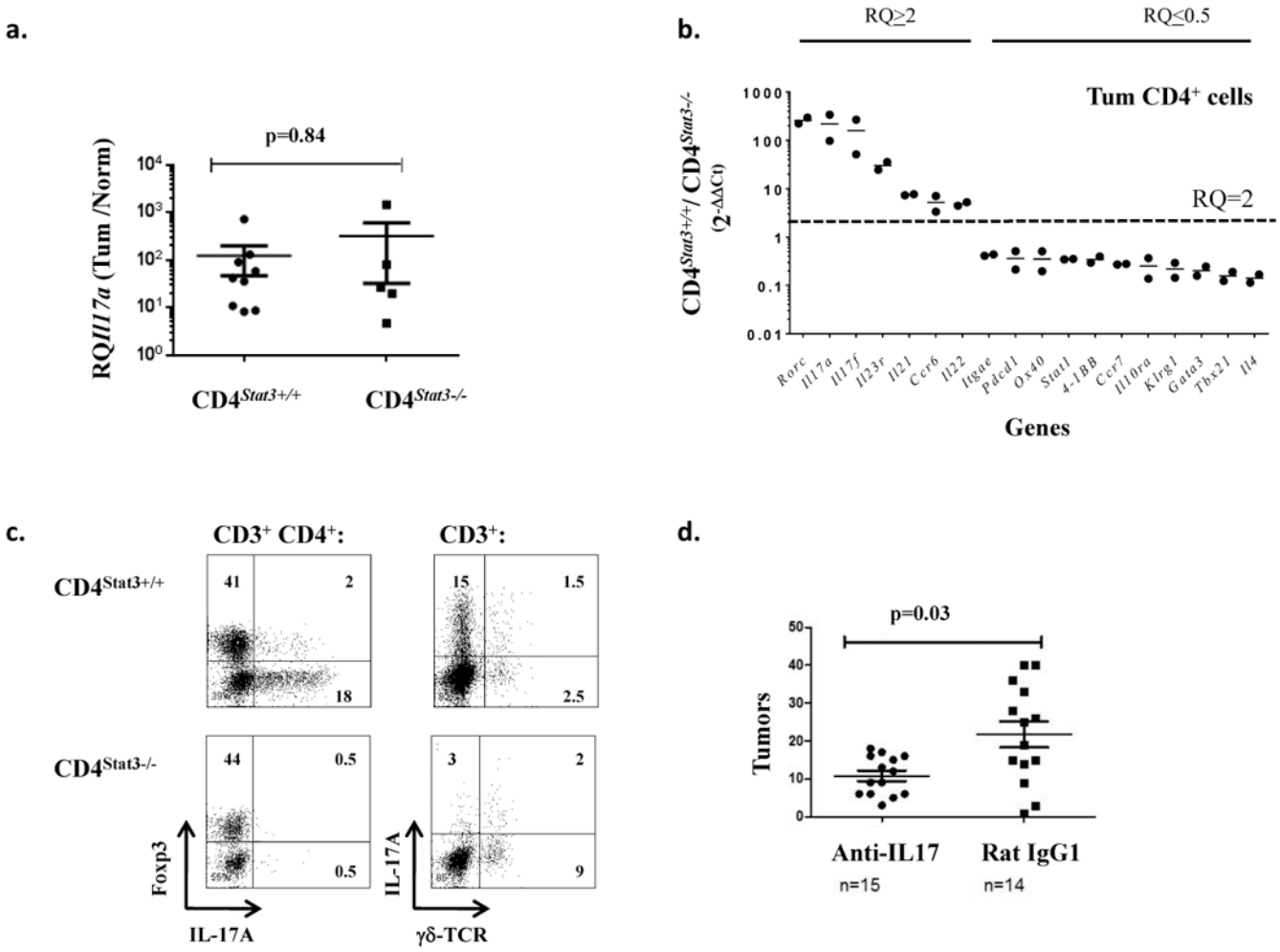

Figure 3. ETBF-mediated colon tumorigenesis is IL-17-dependent in Th17^{null} Min-CD4$^{Stat3-/-}$ mice

a, *Il17a* expression in tumor compared to normal colon tissue of parental Min (CD4$^{Stat3+/+}$) and Min-CD4$^{Stat3-/-}$ (CD4$^{Stat3-/-}$) mice 3 months after ETBF colonization. The plot shows $2^{-\Delta\Delta Ct}$ where $\Delta\Delta C_t$ represents ΔCt_{tum}-ΔCt_{norm} . Each symbol represents one mouse. Mean +/- SEM. p values, non-parametric Mann Whitney U t test. **b**, Gene expression profile in CD4$^+$ cells sorted from pooled distal colon tumors (3 to 5 tumors) in parental (CD4$^{Stat3+/+}$) and CD4$^{Stat3-/-}$ (CD4$^{Stat3-/-}$) Min mice 3 months after ETBF colonization. RQ (CD4$^{Stat3+/+}$ / CD4$^{Stat3-/-}$) = $2^{-\Delta\Delta Ct}$ where ΔCt of genes of interest in ETBF Min-CD4$^{Stat3+/+}$ is compared to ETBF Min-CD4$^{Stat3-/-}$. Genes overexpressed in parental Min-CD4$^{Stat3+/+}$ mouse (RQ>2) and genes overexpressed in Min-CD4$^{Stat3-/-}$ mouse (RQ<0.5) are indicated. The mean of expression from two independent experiments is shown. **c**, Representative flow cytometry analysis of enzymatically digested tumor tissue obtained from Min (CD4$^{Stat3+/+}$, upper panels) and Min-CD4$^{Stat3-/-}$ (CD4$^{Stat3-/-}$, lower panels) tumors 3 month after ETBF colonization. **d**, ETBF-colonized Min-CD4$^{Stat3-/-}$ were treated with a blocking anti-IL-17 or isotype control Rat IgG1 mAbs twice weekly between week 6 and 12 following ETBF colonization. Mice were sacrificed 12 weeks after colonization and tumors were counted.

Each symbol represents one mouse. Mean +/- SEM. p values, non-parametric Mann Whitney U t test.

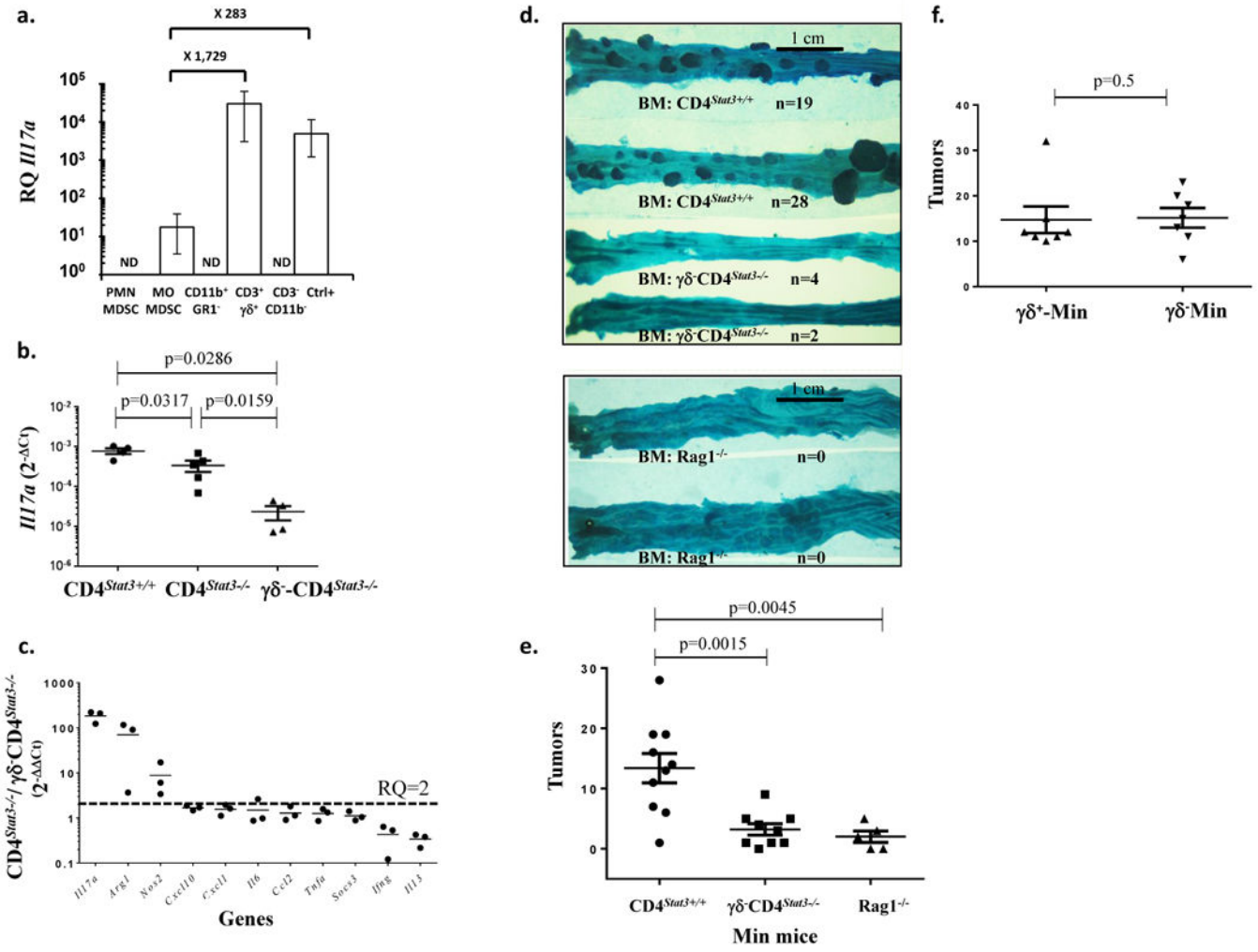

Figure 4. Late ETBF-driven colon tumorigenesis in Th17^{null} Min-CD4$^{Stat3-/-}$ is γδT17-dependent

a, *Il17a* gene expression in tumor-infiltrating cell populations (PMN-MDSC, MO-MDSC, CD11b^{+}GR-1^{-}, γδ^{+}CD3^{+}, CD3^{-}CD11b^{-}) sorted from 3 month colon tumors of ETBF Min-CD4$^{Stat3-/-}$ mice. Positive control population (ctrl+) represents CD3^{+}CD4^{+} cells sorted from colon tumors of ETBF Min-CD4$^{Stat3+/+}$ mice. Bars represent RQ IL-17=2$^{-\Delta\Delta Ct}$ where ΔΔCt represents the difference between normalized Ct_{Il17a} (ΔCt_{Il17a}) in the population of interest and ΔCt_{Il17a} in CD3^{+}CD4^{+} cells of ETBF Min-CD4$^{Stat3-/-}$ mice. ND=not detectable. Fold increases in *Il17a* expression between populations are displayed on the top. **b**, *Il17a* expression in distal colon of WT CD4$^{Stat3+/+}$, CD4$^{Stat3-/-}$ and γδ^{-}-CD4$^{Stat3-/-}$ C57BL/6 mice 7 days after ETBF colonization. Graph represents 2$^{-\Delta Ct}$ where ΔCt is normalized Ct_{Il17a}. Each symbol represents one mouse. Mean+/-SEM, p values, non-parametric Mann Whitney U t test **c**, Comparison of inflammation gene expression in the distal colon of CD4$^{Stat3-/-}$ and γδ^{-}-CD4$^{Stat3-/-}$ C57BL/6 mice 7 days after ETBF colonzation. Each dot represents the ratio RQ (CD4$^{Stat3-/-}$ / γδ^{-}-CD4$^{Stat3-/-}$) =2$^{-\Delta\Delta Ct}$. The mean of three experiments is shown. A ratio RQ >2 indicates genes overexpressed in CD4$^{Stat3-/-}$ compared to γδ^{-}-CD4$^{Stat3-/-}$ mice. Statistical analysis is shown in Fig.S4B. **d**, colon tumorigenesis in BM chimera Min mice

obtained by engrafting WT (top), $\gamma\delta^{-}$-CD4$^{Stat3-/-}$ (middle) or Rag1$^{-/-}$ (bottom) BM into lethally irradiated IL-17$^{-/-}$ Min mice. BM chimera mice were colonized with ETBF 6 weeks after engraftment and colon tumor counts determined 12 weeks after colonization. Tumor numbers are indicated. **e**, Tumor numbers in BM chimera Min mice that received either C57BL/6 CD4$^{Stat3+/+}$ (n=10; circles) or $\gamma\delta^{-}$-CD4$^{Stat3-/-}$ (n=9; squares) BM and colonized 3 months with ETBF. BM chimera Min mice that received Rag1$^{-/-}$ BM (n=2) did not differ from Rag1$^{-/-}$ Min mice (n=3) upon ETBF colonization, so the groups were combined (Rag1$^{-/-}$, n=5; triangles). Each symbol represents one mouse. Mean +/- SEM. p values, non-parametric Mann Whitney U t test. **f**, tumor numbers in parental ($\gamma\delta^{+/+}$-Min) and $\gamma\delta^{-/-}$-Min mice 8 weeks after ETBF colonization. Each symbol represents one mouse. Mean +/- SEM. p values, non-parametric Mann Whitney U t test.

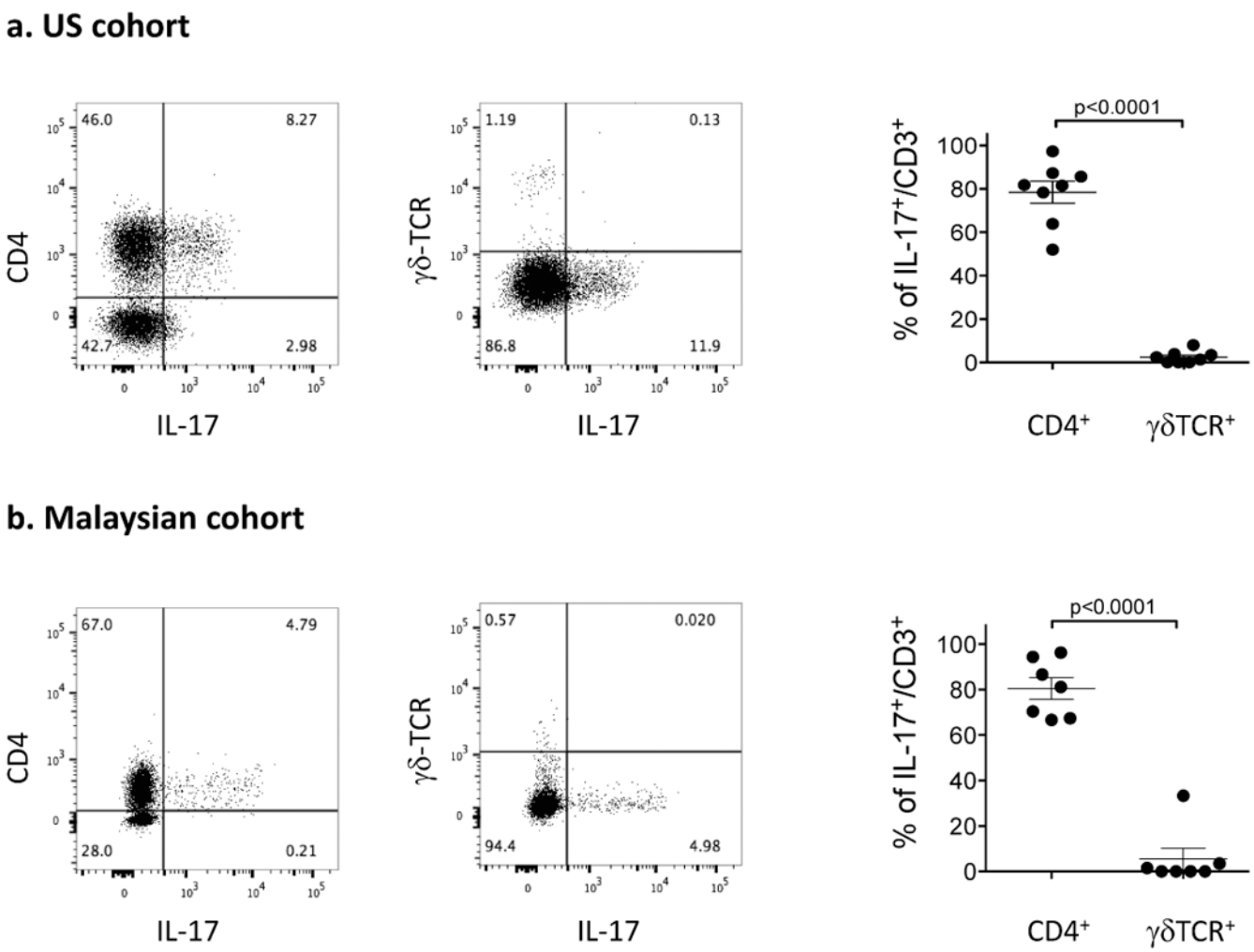

Figure 5. IL-17-producing cells in human CRC samples

Intracellular cytokine staining and flow cytometry analysis were performed on tumor-infiltrating lymphocytes isolated from CRC patients recruited at Johns Hopkins Hospital (a, US cohort) and UMMC (b, Malaysian cohort). Representative dot plot showing IL-17 staining in CD4 (left) or γδ (right) T cells is shown. The statistical analysis of the $CD4^+$ and $\gamma\delta TCR^+$ IL-17-producing T cells is shown for patients with detectable IL-17 (>0.2%) in scatter plots on the right using unpaired, two-tailed Mann-Whitney U testing. Each symbol represents one human sample. $p<0.05$ is significant. US cohort, n=8 out of 13 patients tested; Malaysian cohort, n=7 out of 11 patients tested.

Table 1

Flow cytometry analysis of IL-17-producing cells in tumors collected from US CRC patients

Patient ID*	IL-17+ % leukocytes	IL-17+ % CD3+
3989	1.8	2.6
4033	0.4	0.6
4044	0.3	0.3
4047	0.2	1.1
4049	0.9	1.3
4054	0.2	0.4
4057	0.3	1.5
4069	0.2	0.3
4074	4.7	6.5
4083	0.8	1.4
4084	0.3	0.3
4086	0.2	0.1

* 12/13 patients tested (Table S1) had detectable IL-17

Table 2

Flow cytometry analysis of IL-17-producing cells in tumors collected from Malaysian CRC patients

Patient ID	IL-17+ % leukocytes	IL-17+ % CD3+
S010	0.7	1.4
S039	0.6	0.4
S041	3.4	1.9
S044	0.2	0.1
S056	1.9	3.2
S058	1.4	2.3
S059	0.2	0.3
S065	0.1	0.1
S068	0.1	0.1
S090	1.9	4.2
S095	0.5	0.7