

Communicable Ulcerative Colitis Induced by T-bet Deficiency in the Innate Immune System

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SUMMARY

Inflammatory bowel disease (IBD) has been attributed to overexuberant host immunity or the emergence of harmful intestinal flora. The transcription factor T-bet orchestrates inflammatory genetic programs in both adaptive and innate immunity. We describe a profound and unexpected function for T-bet in influencing the behavior of host inflammatory activity and commensal bacteria. T-bet deficiency in the innate immune system results in spontaneous and communicable ulcerative colitis in the absence of adaptive immunity and increased susceptibility to colitis in immunologically intact hosts. T-bet controls the response of the mucosal immune system to commensal bacteria by regulating TNF- α production in colonic dendritic cells, critical for colonic epithelial barrier maintenance. Loss of T-bet influences bacterial populations to become colitogenic, and this colitis is communicable to genetically intact hosts. These findings reveal a novel function for T-bet as a peacekeeper of host-commensal relationships and provide new perspectives on the pathophysiology of IBD.

INTRODUCTION

The intestinal lumen is massively colonized by bacteria, and for most metazoans this relationship is beneficial

(Ley et al., 2006). Prokaryotes partner with their eukaryotic hosts to aid in the extraction of energy and nutrients from food, and nonpathogenic bacteria suppress pathogenic species (Backhed et al., 2005). An epithelial cell barrier is essential for this symbiosis as it creates a boundary necessary for coexistence by preventing mucosal inflammation in response to bacterial or other luminal stimuli (Magalhaes et al., 2007). However, in some individuals this balance is upset, resulting in persistent intestinal inflammation that manifests as the two distinct clinical entities of IBD, Crohn's disease (CD) and ulcerative colitis (UC) (Rakoff-Nahoum and Medzhitov, 2006). Determining the factors that regulate these complex host-commensal relationships and promote the development of colitis is of great clinical and scientific interest.

T-bet (Tbx21) is a T-box transcription factor family member that regulates the differentiation and function of immune system cells. Although initially described to direct the development of a major subset of lymphocytes called T helper 1 cells, recent work has firmly established T-bet as a regulator of the type 1 proinflammatory immune response in cells of both the adaptive and innate immune systems (Glimcher, 2007). It has been implicated as a key effector protein in autoimmune, allergic, and neoplastic diseases; nevertheless, its role in balancing immunity and autoimmunity is complex, and it is not well-understood if T-bet protects against or promotes chronic intestinal inflammation (Weigmann and Neurath, 2002).

Here we reveal an unexpected and powerful role for T-bet in host-commensal interactions. Loss of T-bet in mice lacking an adaptive immune system results in a spontaneous, highly penetrant, aggressive, and communicable colitis that resembles human UC. Both the *T-bet*-deficient genetic background and the microbiota its absence

engenders are required for disease initiation; but once established, the microbiota from the afflicted mice is vertically transmissible and causes intestinal inflammation in wild-type (WT) mice. Thus we have identified a novel function for T-bet in moderating host-commensal relationships. This new mouse model of spontaneous UC provides exciting opportunities to further our understanding of the etiology and pathogenesis of UC and interrogate the complex nature of commensal microbial communities and serves as a robust host for the evaluation of new therapeutics.

RESULTS

T-bet Expression Protects against Colitis and *T-bet*^{-/-} × *RAG2*^{-/-} Mice Develop Spontaneous Colitis

The sulfated polysaccharide dextran sodium sulfate (DSS) induces colitis via direct toxicity on mucosal epithelium. *T-bet*^{-/-} mice developed more severe colitis upon DSS administration than WT mice (Figures 1A and 1B), as evidenced by a more extensive and severe inflammatory infiltrate, containing neutrophils, mononuclear cells, and more lymphoid aggregates, more edema, extensive ulceration, and crypt loss (Figure 1B). This observation was unexpected as DSS colitis arises from overabundant type 1 cytokines, IFN γ , IL-12, IL-1, and TNF- α . *T-bet*^{-/-} T cells produce a type 2 cytokine profile that is protective for most T cell-driven colitis models. Indeed, T-bet deficiency is protective against the T cell adoptive transfer SCID colitis model (Neurath et al., 2002). To clarify whether T-bet's protective function resided in innate immunity, we examined *T-bet*^{-/-} mice bred onto the *RAG2*^{-/-} background. A comparative survey of the gastrointestinal (GI) tracts of *T-bet*^{-/-} × *RAG2*^{-/-}, *RAG2*^{-/-}, *T-bet*^{-/-}, and WT mice in our colony revealed that *T-bet*^{-/-} × *RAG2*^{-/-} mice spontaneously developed a highly penetrant and severe colitis (Figures 1C and 1D). This colitis was apparent by 4 weeks of age and increased in severity over time (Figure 1E). Of note, our mice are housed in a barrier facility, documented to be free of known colitogenic pathogens such as *Helicobacter hepaticus*, *bilis*, and *muridarum*.

Spontaneous Colitis in TRUC Mice Phenocopies Human UC and Is Characterized by an Early Colonic Epithelial Barrier Breach

T-bet^{-/-} × *RAG2*^{-/-} (herein referred to as TRUC [*T-bet*^{-/-} × *RAG2*^{-/-} ulcerative colitis]) mice develop colitis that bears a striking resemblance to human UC (Figure 2A), notable for anorectal prolapse (Figure 2A1), from rectal inflammation. TRUC colons demonstrated continuous inflammation of the rectum and left colon by 4 weeks of age (Figure 2A2b), and by 8 weeks, there was marked inflammation and colonic thickening (Figure 2A2d) not seen in *RAG2*^{-/-} controls (Figure 2A2c). Microscopically, TRUC colitis phenocopies many aspects of human UC (Figure 2A4) with a mixed inflammatory infiltrate in the lamina propria containing both mononuclear and polymorphonu-

clear cells, neutrophil infiltration of the crypt and surface epithelium, and epithelial injury with surface denudations and ulcerations associated with crypt loss and epithelial mucodepletion. The stomach and small intestine appeared microscopically normal, similar to UC but in contrast with CD and most spontaneous mouse models of colitis. Examination of mice from one week postnatally revealed no evidence of colitis until 4 weeks (Figure 1E). However, intrarectal instillation of fluorescently labeled dextran, to test the integrity of the colonic epithelial barrier in 3.5-week-old mice (Karhausen et al., 2004), demonstrated significantly increased permeability in TRUC compared to *RAG2*^{-/-} and *T-bet*^{-/-} mice (Figure 2B) with 2-fold higher serum fluorescence ($p = .0002$ TRUC versus *RAG2*^{-/-}). Colonic permeability increased with age, with a 3-fold increase between 4 and 5 weeks and an 8.6-fold increase between 5 and 6 weeks ($p = .00036$) (Figure 2C). Hence, a decrease in colonic epithelial integrity precedes histologically detectable colitis and worsens with progression.

Although abnormal tight junctions may contribute to IBD pathogenesis (Bruewer et al., 2006), EM (electron micrographs) of TRUC and control colonic mucosa from mice aged 2–12 weeks showed intact epithelial tight junctions (Figure 2D: 2 weeks [panel 1], 3 weeks [panel 2], and 4 weeks [panel 3]). However, numerous large epithelial discontinuities were observable on EM by 3.5 weeks of age (Figures 2E1–2E4), which likely accounted for the increased permeability. Further, the degree of cell death was well above the intrinsic rate of epithelial turnover. We quantified colonic epithelial apoptosis by TUNEL (terminal transferase dUTP nick end labeling) in 5-week-old mice when significant colonic permeability exists (Figure 2C). While TUNEL⁺ cells were noted at the top of crypts in all genotypes, TUNEL⁺ cells were distributed throughout the crypt in TRUC mice, and in many cases the entire crypt consisted of TUNEL⁺ cells (Figure 2F). Quantitation of TUNEL⁺ cells/100 crypts for all 4 genotypes showed a 4- to 5-fold increase in TUNEL⁺ cells in TRUC mice (Figure 2G), accentuated if TUNEL⁺ cells/epithelial cells/crypt is plotted (Figure S1B).

TNF- α Drives Tissue Injury in TRUC Colitis and TNF- α Overproduction Maps to Colonic DCs

We analyzed the TRUC versus *RAG2*^{-/-} colonic cytokine milieu in colon explant cultures. TNF- α was significantly elevated in TRUC explant cultures at 4 weeks and increased over time (Figure 3A). There were no marked differences in the levels of other IBD-associated cytokines, IFN γ , IL-1 α , IL-1 β , IL-6, IL-10, IL-12, IL-13, or IL-23 at 4 weeks (Figure 3B). TNF- α is a key effector cytokine in IBD thought to lead to an increased inflammatory tone of the intestinal epithelium. TNF- α neutralizing Ab therapy is widely used in IBD treatment. Anti-TNF- α Ab therapy suppressed TRUC colitis as evidenced by colon weight (Figure S1A) and colitis score (Figure 3C). The microscopic appearance of TNF- α Ab-treated TRUC colons was indistinguishable from that of control mice. Of interest,

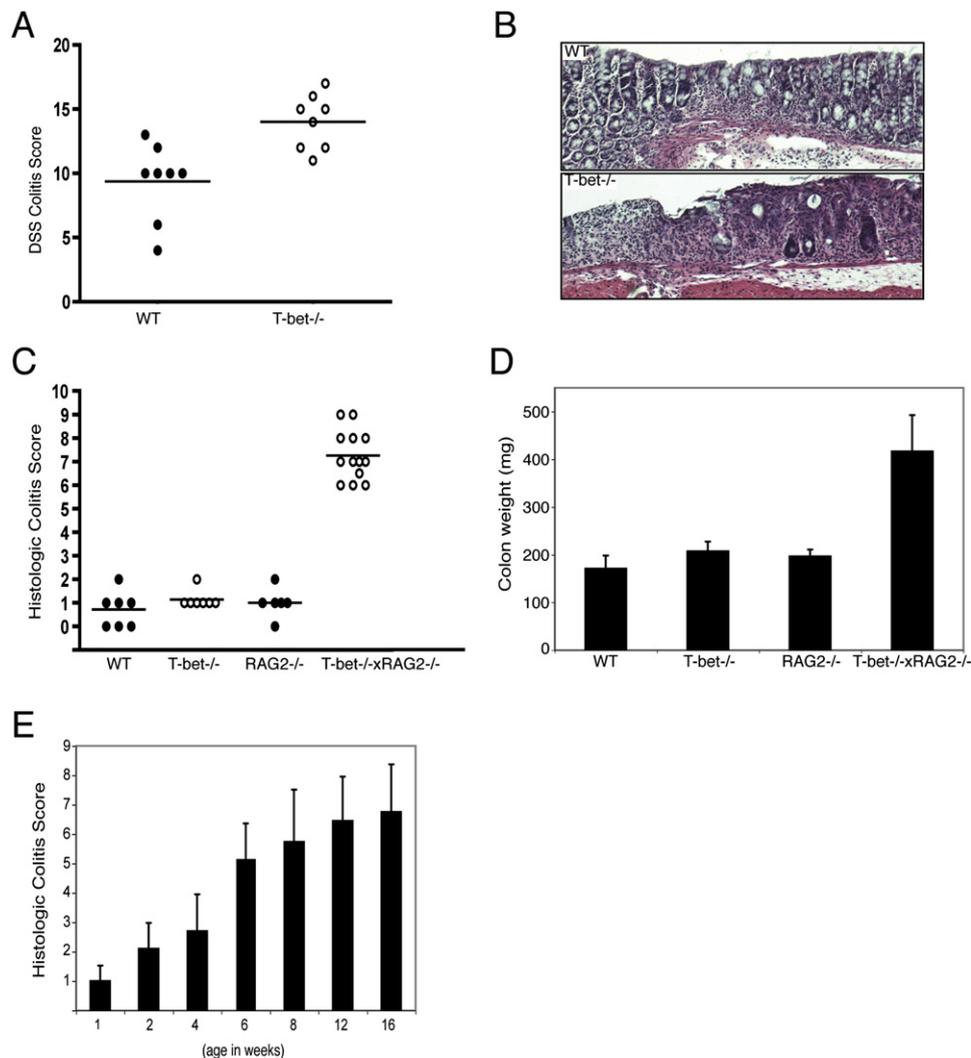


Figure 1. T-bet Expression Protects against Colitis and $T\text{-bet}^{-/-} \times RAG2^{-/-}$ Mice Develop Spontaneous Colitis

(A) WT and $T\text{-bet}^{-/-}$ mice, age 8 weeks, treated with 4% DSS for 8 days. One representative experiment of three, $n = 8$ per group, $p = .0031$.

(B) Photomicrographs of DSS-treated WT (upper panel) and $T\text{-bet}^{-/-}$ (lower panel) colons.

(C) TRUC mice develop a spontaneous, highly penetrant colitis. Colitis score for each mouse denoted by a dot, the mean for each group shown as a horizontal bar. $n = 7$ per group, all 12 weeks old.

(D) Mean colon weights (mg) for the mice in (C). Error bars denote standard deviation (SD).

(E) TRUC colitis severity increases with time. Mean colitis score \pm SD (denoted by error bars), $n = 10$ per group, except for 1-week-old group, $n = 8$.

anti-TNF- α treatment decreased the number of apoptotic epithelial cells to normal levels in mice treated from 4–8 weeks of age (Figure 3D).

TNF- α could modulate either pro-survival or pro-death signaling pathways in cells. We examined the effects of TNF- α on the apoptosis of colonic epithelial cells (CECs), isolated from $RAG2^{-/-}$ and TRUC colons cultured with or without TNF- α . After 6 days, the untreated $RAG2^{-/-}$ and TRUC CECs had quite similar percentages (%) of apoptosis (14.2% versus 14.0%: $RAG2^{-/-}$ versus TRUC); however, TNF- α -treated cells displayed a marked difference in apoptosis (13.7% versus 53.8%: $RAG2^{-/-}$ versus

TRUC) (Figure 3E). Since CECs do not express T-bet, their exposure to the TRUC colonic environment likely sensitized them to the effects of TNF- α . Cell surface TNFR1 does differ between TRUC and $RAG2^{-/-}$ CECs even from 2-week-old mice (data not shown). To discern whether CEC death is a direct or indirect effect of TNF- α , we generated TRUC mice that were also deficient in TNF- α receptor by crossing them onto $TNFR1/p55^{-/-}$ mice. Eight-week-old triple-deficient mice showed no evidence of colitis, suggesting that TNF- α signaling through $TNFR1/p55$ is a central event in disease pathophysiology (Figure 3F). These experiments established the centrality of TNF- α in

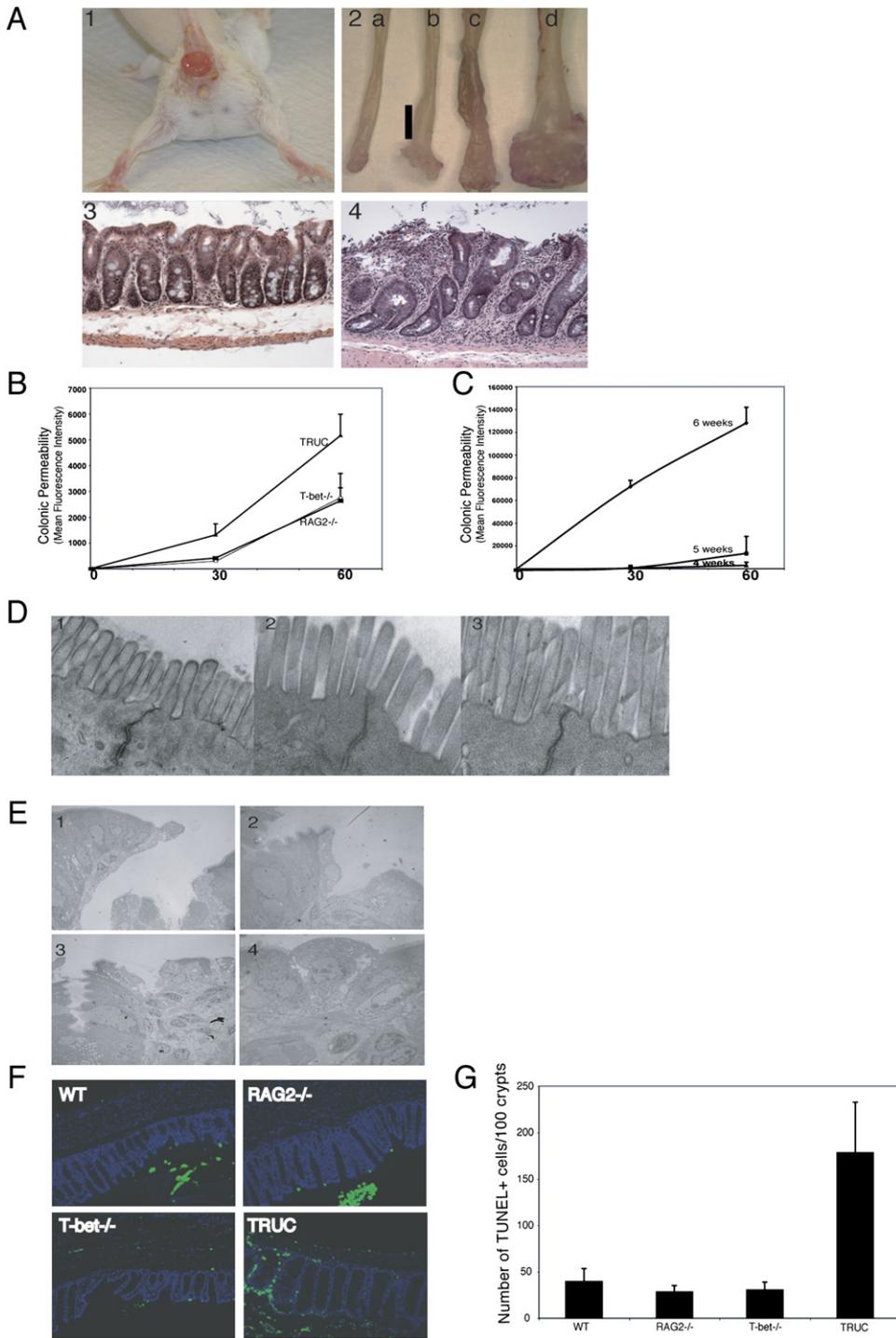


Figure 2. Spontaneous Colitis in TRUC Mice Phenocopies Human UC and Is Characterized by a Colonic Epithelial Barrier Breach (A) (1) TRUC mouse (8 weeks) with anorectal prolapse. (2) Photograph of distal colons of RAG2^{-/-} mice at 4 weeks (a) and 8 weeks (c) and of TRUC distal colons at 4 weeks (b) and 8 weeks (d) (anorectal junction at bottom). Vertical bar delineates the inflamed, thickened colonic wall. Colonic wall thickening with prolapsed rectal mucosa in 8-week-old TRUC mice. (3) Normal colonic mucosa in 6-week-old RAG2^{-/-} mouse, 100 \times . (4) Representative disease in 6-week-old TRUC mice. Note mucosal thickening, surface ulceration, crypt distortion and hyperplasia, and dense mixed inflammatory cell infiltrate in the lamina propria (compare to panel 3), 100 \times . (B) Intrarectal FITC-dextran was administered to 3.5 week T-bet^{-/-}, RAG2^{-/-}, and TRUC. Serum fluorescence measured at the indicated time points. One representative experiment of three, n = 4–6 per group (p = .0002, 60 min TRUC versus RAG2^{-/-}). (C) Intrarectal FITC-dextran was administered to 3.5 week TRUC mice. Serum fluorescence measured at the indicated time points. One representative experiment of three, n = 4–6 per group (p = .0002, 60 min TRUC versus 4 weeks TRUC). (D) Electron micrographs of the colonic epithelium. (E) Immunofluorescence images of the colonic mucosa. (F) Immunofluorescence images for WT, RAG2^{-/-}, T-bet^{-/-}, and TRUC mice. (G) Bar graph showing the number of TUNEL+ cells per 100 crypts.

this model and led to the question of what cell type(s) was responsible for TNF- α production in the colon.

We performed intracellular cytokine analysis on cells isolated from TRUC and *RAG2*^{-/-} colons. Leukocyte (CD45⁺) populations had higher staining for TNF- α than epithelial and other nonleukocyte cells (Figure 3G). Among the leukocyte subsets, there was no observable staining for NK cells by flow cytometry (FC) or IHC in TRUC colons (data not shown). TNF- α in macrophages, granulocytes, and neutrophils could not account for the levels of TNF- α observed (Figure 3G). However, there was significant accumulation of TNF- α in TRUC colonic DCs (Figure 3G), suggesting that colonic DCs are the principal TNF- α -producing cell type. A time course of TNF- α production of TRUC and *RAG2*^{-/-} colonic DCs from mice aged 2–8 weeks (Figure 3H) showed increased TNF- α as early as 2 weeks of age, prior to any observed epithelial discontinuities by microscopy. Thus overproduction of TNF- α by colonic DCs precedes the development of epithelial barrier abnormalities and colitis.

T-bet Regulates Production of TNF- α in DCs

To determine if T-bet was expressed in colonic DCs as in DCs in the peripheral lymphoid system, *RAG2*^{-/-} colons were stained with CD11c to identify DCs and adjacent serial sections were stained for T-bet (Figures 4A1 and 4A2). While the required serial sectioning prevents precise colocalization, there were many T-bet⁺ cells in areas of dense CD11c staining. We confirmed this finding by staining FACS-isolated colonic DCs from *RAG2*^{-/-} mice with α -T-bet Ab and DAPI. T-bet staining (red) is evident, and there is colocalization with the DAPI (blue) stain in the nucleus (Figure 4A3).

We next examined T-bet expression in human colonic biopsies. Specimens were stained with S100 (DCs red) and T-bet (brown). A representative DC with T-bet staining is shown (Figure 5A4). Demonstration of T-bet expression in human colonic DCs is a novel observation as T-bet expression had only previously been demonstrated in human colonic T cells (Neurath et al., 2002). There was no T-bet expression in CECs (Figure 4A2) by IHC nor was T-bet been detectable in CEC lysates (data not shown). T-bet is expressed in both mouse and human colonic DCs.

To explore T-bet's role in DC TNF- α production, we used mouse bone marrow DCs (BMDCs) as colonic DC yields are insufficient for biochemical evaluation. LPS-matured *T-bet*^{-/-} BMDCs, similar to colonic DCs, overproduce TNF- α compared to WT BMDCs (Figure 4B). The observations that loss of T-bet expression in colonic

and BMDCs results in increased TNF- α production suggested that T-bet may directly regulate TNF- α in DCs.

Chromatin immunoprecipitation (ChIP) and quantitative real time PCR (qPCR) (Figure 4C) assays revealed that T-bet bound the mouse *TNF- α* promoter at two T-box consensus sites at approximately -1200 and -500 relative to the transcription start site (TSS) but not at regions examined upstream of -1200 (data not shown), with *T-bet*^{-/-} BMDCs and preimmune sera as controls. We also performed ChIP assays in human myeloid DCs. Although there is little sequence homology between the 5' UTR of the mouse and human *TNF- α* promoters and T-box consensus sites can be rather degenerate, T-bet bound the human *TNF- α* promoter as demonstrated by ChIP and qPCR (Figure 4D) at approximately -400 relative to the TSS and did not bind more upstream.

To investigate whether T-bet is a negative regulator of *TNF- α* transcription as would be predicted from the TNF- α overproduction phenotype of *T-bet*^{-/-} DCs, RAW cells were cotransfected with a *TNF- α* promoter luciferase construct, a constitutively active I κ B β mutant to activate *TNF- α* transcription, and T-bet. Transfection with *T-bet* reduced NF κ B-driven *TNF- α* promoter activity in RAW cells in a dose-dependent fashion, suggesting that T-bet functions as a negative regulator of *TNF- α* transcription (Figure 4E).

CD4⁺CD25⁺ T-Regulatory Cells Control TRUC Colitis

T-bet^{-/-} immunosufficient mice do not develop spontaneous colitis although they display increased susceptibility to DSS and have been shown to have increased susceptibility to oxazolone-induced colitis, a Th2 model of colitis (Neurath et al., 2002). *T-bet*^{-/-} and WT mice possess an adaptive immune system as do most IBD patients. To test the hypothesis that a component of the adaptive immune system might act as a repressor of colitis in immunosufficient hosts, we performed adoptive transfer (AT) experiments in TRUC. As evidenced by colitis scores, the protection we observed could not be attributed to conventional T helper or B cells because transfer of naive CD4 T cells (*T-bet*^{-/-} or WT) or B cells did not protect against disease (Figure 5A). The CD4 T-regulatory cell (T-reg) is a potent repressor of effector T cell function that might have prevented the development of colitis in the *T-bet*^{-/-} host. AT of T-regs reverses intestinal inflammation in *H. hepaticus*-infected *RAG2*^{-/-} mice, an innate immune-driven colitis model (Maloy et al., 2005). AT of either WT or *T-bet*^{-/-} T-regs controlled TRUC colitis (Figure 5B). Colons from mice infused with T-regs were free of any signs

(C) Intrarectal FITC-dextran administered to TRUC at 4, 5, and 6 weeks. Serum fluorescence at the indicated time points ($p = .0036$, 60 min 6 versus 5 weeks). One representative experiment of three, $n = 5$ per group. Error bars denote SD.

(D) TRUC colonic tight junctions surveyed by EM: representative images prior to 2 weeks (1), 3 weeks (2), and 4 weeks (3), 25,000 \times .

(E) Colonic epithelial discontinuities present in 3.5 week TRUC. Representative EM images, (1) 800 \times , (2) 1500 \times , (3) 1000 \times , (4) 3000 \times .

(F) Increased apoptosis in TRUC colonic epithelium. WT, *T-bet*^{-/-}, *RAG2*^{-/-}, and TRUC colonic epithelium at 5 weeks stained with DAPI (blue) and TUNEL (green), 200 \times .

(G) Epithelial crypt and TUNEL⁺ cell counts. Five slides generated from each genotype group (2–3 mice per genotype). Five hundred crypts per genotype were scored. Where shown, error bars denote SD.

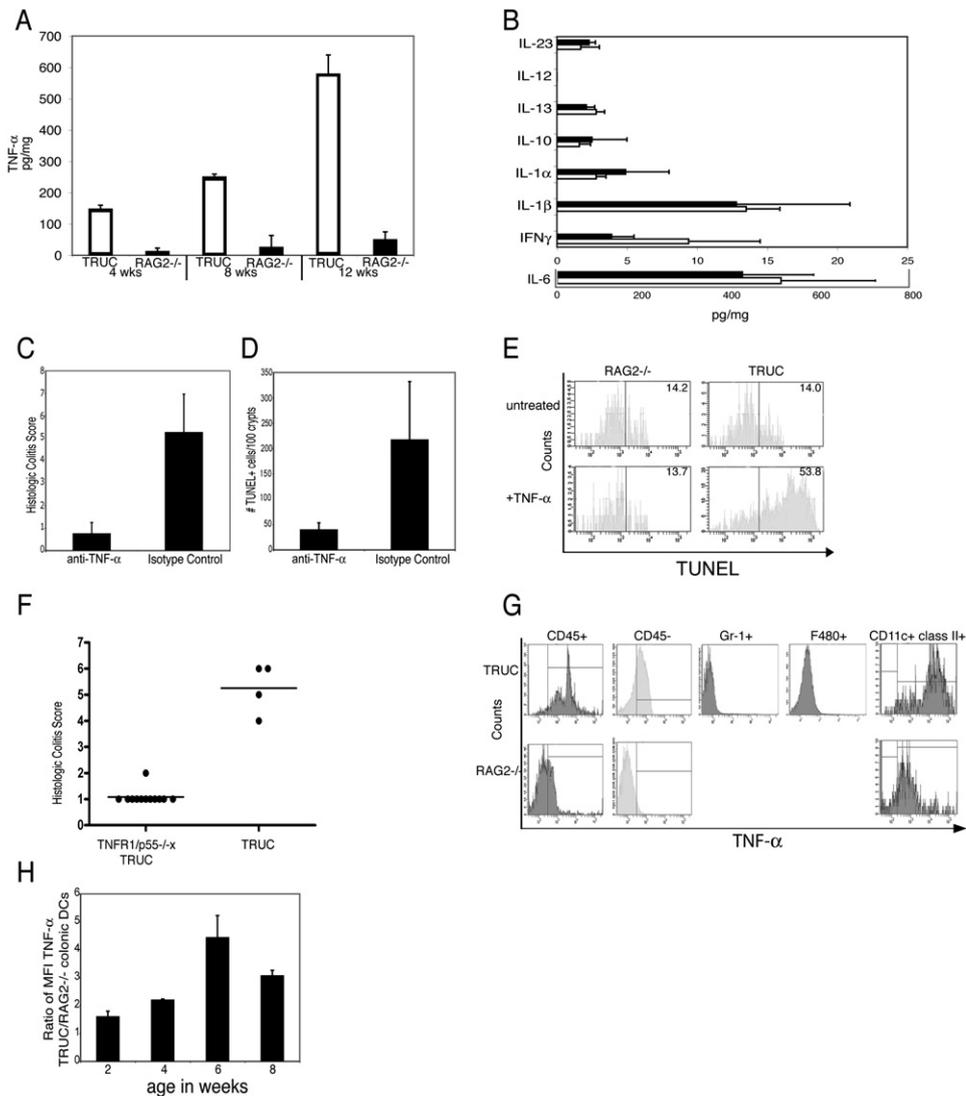


Figure 3. TNF- α Drives Tissue Injury in TRUC Colitis and TNF- α Overproduction Maps to Colonic DCs

(A) TNF- α ELISAs on colon explant cultures from 4-, 8-, and 12-week-old TRUC (open) and *RAG2*^{-/-} (shaded). One representative experiment of three is shown; samples pooled, n = 4 per group.

(B) IL-23, IL-13, IL-12p40, IL-10, IL-6, IL-1 α , IL-1 β , and IFN γ levels of colon explant cultures from TRUC (open) and *RAG2*^{-/-} (shaded); samples pooled, n = 4 per group. Data represent the mean of three independent experiments, error bars denote SD.

(C) 4 week TRUC were treated with anti-TNF- α Ab or an isotype control for 4 weeks. Colitis scores for treated versus control, p = .0023. One representative experiment of three is shown, n = 4 per group.

(D) Apoptotic (TUNEL⁺) epithelial cells counts; anti-TNF- α versus control, p = .0016.

(E) TUNEL staining of cultured TRUC colonic epithelial cells (CD45⁻ cytokeratin 5/8⁺) treated with TNF- α compared to TNF- α treated *RAG2*^{-/-} colonic epithelial cells (53.8% versus 13.7%). One representative experiment of four is shown.

(F) Colitis scores for 8-week-old TRUC *TNFR1/p55*^{-/-} and TRUC mice, p < .0001. Colonic cell suspensions were stained with Abs directed against cell-surface markers and permeabilized to allow for detection of intracytoplasmic TNF- α by flow cytometry.

(G) TNF- α production by immunocytes (CD45⁺). *RAG2*^{-/-} cells as controls. Abs against Gr-1, F4/80, and CD11c paired with class II were used to identify the immunocytes with the highest TNF- α production.

(H) Colonic DC production of TNF- α during the disease course, 2–8 weeks. The mean and SD of three independent experiments are plotted. Vertical bars denote the right-sided tail of the isotype control staining. Data are representative of three independent experiments; samples were pooled, n = 10–20 per group. Where shown, error bars denote SD.

of colitis. Imaging TRUC colons to document that the transferred cells had trafficked to the colon using CD3 staining revealed that the CD3⁺ cells were in close contact

with DCs as shown by costaining with CD11c (Figure 5C), suggesting that crosstalk between T-regs and DCs may suppress the *T-bet*-deficient DC proinflammatory

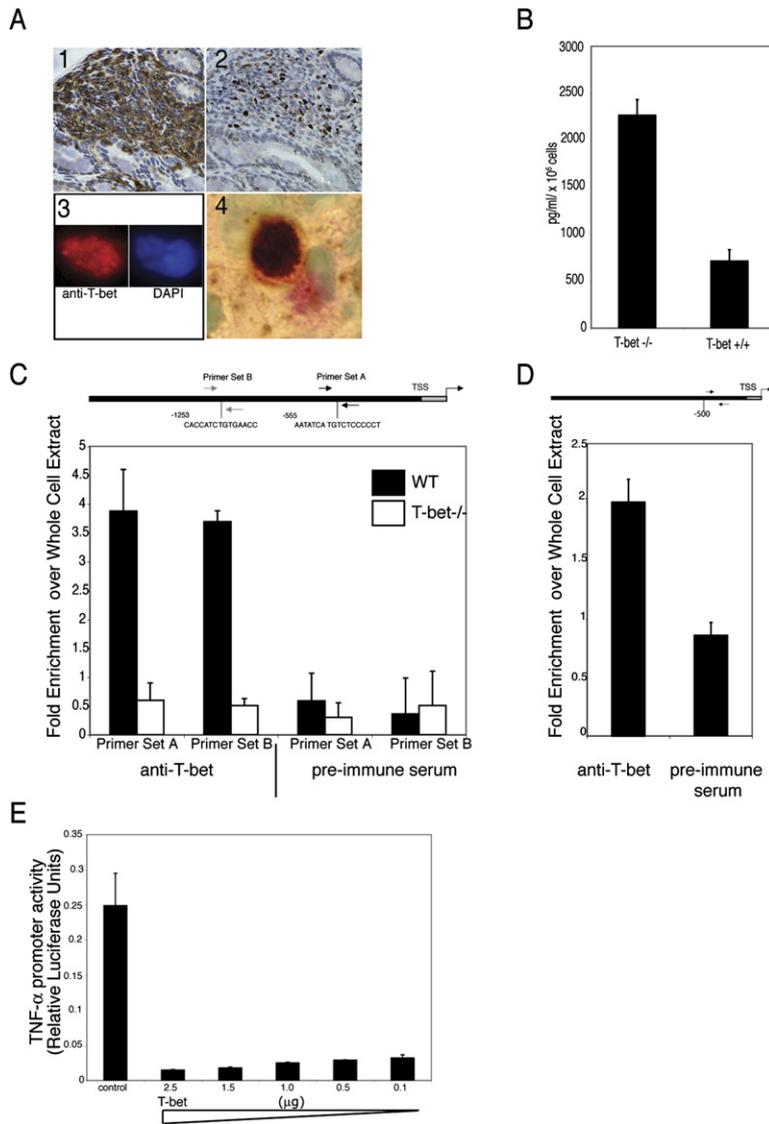


Figure 4. T-bet Regulates Production of TNF- α in DCs

(A) T-bet is expressed in colonic DCs. (1) CD11c staining of *RAG2*^{-/-} colonic mucosa, 200×. (2) Serial section of (1) stained with anti-T-bet Ab, 4B10, 200×. (3) Sorted colonic mouse DC stained with anti-T-bet Ab (red) and DAPI (blue), 1000×. (4) Human colonic DC, S100 (pink), anti-T-bet (brown), 1000×. (B) Loss of T-bet expression in BMDCs results in increased production of TNF- α . T-bet binds the *TNF*- α promoter. (C) qPCR of mouse bone BMDCs ChIP samples. Primer sets A and B, approximately 500 and 1200 bp upstream of the TSS. A schema of the promoter showing T-box consensus sites and the location of primers used. Data are the mean of three experiments. (D) qPCR of human myeloid DC ChIP samples, one representative experiment of three. (E) T-bet negatively regulates *TNF*- α gene transcription. RAW cells transiently cotransfected with a 3 kb *TNF*- α promoter luciferase construct, a constitutively active *I κ B β* mutant construct to activate *TNF*- α transcription, a Renilla construct for normalization of transfection efficiency, and varying concentrations of a full-length *T-bet* cDNA construct or control plasmid. *TNF*- α promoter activity is shown as a function of relative luciferase activity. Data are representative of three independent experiments. Where shown, error bars denote SD.

phenotype. Colon explant cultures from TRUC mice injected with T-regs 4 weeks earlier showed markedly reduced TNF- α levels similar to control *RAG*^{-/-} colonic explants (Figure 5D). We hypothesized that colonic DC T-bet deficiency might confer a genetic susceptibility to aberrant TNF- α inflammatory responses to the microbial environment.

The TRUC Colonic Environment Is a Niche Supporting a Colitogenic Microbial Community Whose Suppression Prevents Colitis across Generations

The colonic luminal contents are abundant in microbiota (Hooper and Gordon, 2001), and intestinal DCs constantly sample these microbes (Chieppa et al., 2006; Niess and Reinecker, 2006). Most mouse models of colitis are microbe dependent, and patients with IBD often have favorable if not durable responses to antibiotic therapy. To

determine if TRUC colitis was driven by the gut microbiota, mice were treated with broad spectrum antibiotics: vancomycin (V), metronidazole (M), neomycin (N), and ampicillin (A)—a combination previously shown to deplete enteric microbial communities (Fagarasan et al., 2002; Rakoff-Nahoum et al., 2004). Treatment with the VMNA combination cured the mice of their colitis, and selective treatment with metronidazole alone cured the colitis as well, as demonstrated by colitis scores (Figure 6A). Conventional culturing of fecal pellets from VMNA antibiotic-treated and control mice demonstrated relatively unchanged aerobic colony counts (10^{10.11} versus 10^{10.16}), but anaerobic colony counts were dramatically decreased (10^{9.74} versus <10^{4.01}; log₁₀CFU/gram dry weight of stool). Treatment for several months induced not only a 100,000-fold decrease in the culturable fecal anaerobes but also remission in all treated TRUC mice. These two observations drew attention to the potential pathogenicity of TRUC anaerobic commensals.

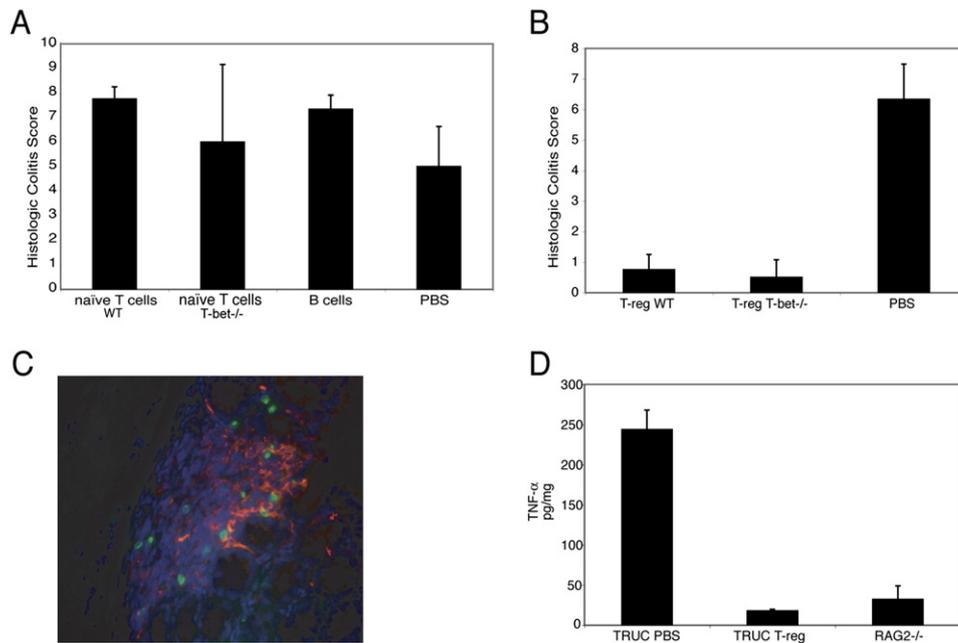


Figure 5. CD4⁺CD25⁺ T-Regulatory Cells Control TRUC

(A) Four week TRUC injected with PBS, 1×10^6 B cells, or 1×10^6 naive T cells, WT or *T-bet*^{-/-}. Colitis scores at 2 weeks post-injection. Representative data from one of three experiments, n = 4 per group.

(B) Four week TRUC mice injected with 75,000 T-regs (CD4⁺CD25⁺CD62L⁺), WT or *T-bet*^{-/-}, or PBS. Colitis scores at 4 weeks post-injection. One representative experiment of three is shown, n = 6–8 per group.

(C) Photomicrograph of TRUC colon (T-reg treated). T-regs (CD3, green) and DCs (CD11c, red), 400 \times .

(D) Adoptive transfer of T-regs normalizes TRUC TNF- α levels. Four week TRUC injected with 75,000 T-regs (CD4⁺CD25⁺CD62L⁺) or PBS. TNF- α levels from colon explant cultures 4 weeks post-injection, *RAG2*^{-/-} colon explants as a control. Samples pooled, n = 4–5 per group. Where shown, error bars denote SD.

We questioned the origin of the pathogenic TRUC microbial community. Did pathogenic anaerobic commensals expand anew at each generation in response to the developing TRUC colonic niche, or were colitogenic microbes propagated transgenerationally from founder TRUC mice? Genetic susceptibilities are heritable and microbial communities are also “heritable,” as they are passed from parent to offspring (Tannock, 2006). We asked whether the TRUC colitogenic microbial community was vertically transmissible. TRUC colitis is highly penetrant and develops at a young age. TRUC progeny had colitis, raising the issue of whether the progeny of antibiotic-treated (Abx) TRUC mice would develop colitis. We bred Abx mice and examined their progeny, who had no exposure to antibiotics after weaning, after they matured to adulthood. The progeny had no evidence of colitis (Figure 6B). Thus, in TRUC mice, such treatment cures them of disease and prevents their progeny from developing colitis into adulthood. These experiments interested us in the communicability of TRUC colitis.

TRUC Colitis Is Communicable to *T-bet*-Sufficient Mice

We performed crossfostering experiments to determine if TRUC intestines possessed a microbial community that was colitogenic in *T-bet* sufficient mice. We provided

a mother different from the birth mother at the day of birth to *RAG2*^{-/-} and WT mice and assessed the development of colitis in these crossfostered (X-F) progeny. Adult *RAG2*^{-/-} and WT mice X-F by TRUC mothers developed colitis that was histologically similar to TRUC (Figures 6C and 6D). Colitis scores from conventionally reared *RAG2*^{-/-} and WT mice are shown for comparison. The colitis of X-F *RAG2*^{-/-} mice was more severe than that of X-F WT. Hence, genetically “resistant” strains can vertically acquire colitis from association with the pathogenic microbiota of affected mice.

The vertical transmission of TRUC colitis raised the issue of whether TRUC colitis is horizontally transmissible. Perhaps colitis transmission could only occur in the neonatal period at a time when the immune system is immature, or perhaps transmission required maternally transmitted factors such as breast milk in addition to commensal microbiota. To test these possibilities, adult *RAG2*^{-/-} mice were co-housed at a 1:3 ratio with age- and sex-matched adult TRUC mice for 8 weeks. Remarkably, both *RAG2*^{-/-} and WT co-housed mice had evidence of colitis with elevated colitis scores, demonstrating that TRUC colitis is horizontally transmissible. These results suggest that not only is TRUC colitis communicable but also that the *T-bet*-deficient mucosal innate immune system creates a niche for a colitogenic microbial community. The

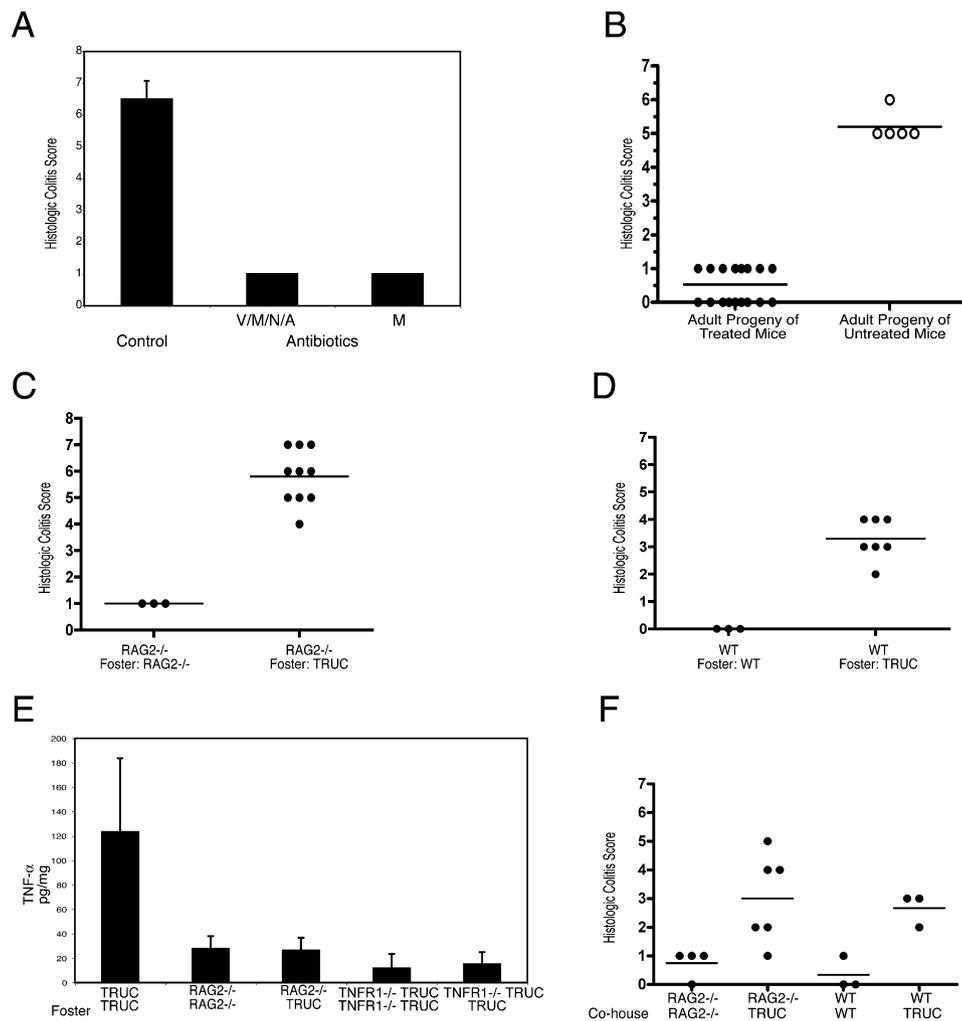


Figure 6. The TRUC Niche Generates a Colitogenic Microbial Community that Is Transmissible to *T-bet*-Sufficient Mice

(A) Colitis scores for 6-week-old TRUC mice treated with vancomycin (V), metronidazole (M), neomycin (N), and ampicillin (A) or metronidazole (M) alone for 6 weeks. One representative experiment of three, $n = 4$ per group.

(B) Colitis scores for adult progeny of antibiotic-treated mice. Three separate litters are shown for antibiotic-treated breeders and 1 litter is shown from untreated breeders, $p < .0001$.

Communicability of TRUC colitis. Colitis scores for crossfostered *RAG2*^{-/-} mice (C) and WT mice (D). Mice are from three experiments (TRUC and *RAG2*^{-/-}) and two experiments (TRUC and WT). *RAG2*^{-/-} reared by *RAG2*^{-/-} female and WT reared by WT female shown for comparison.

(E) TNF- α ELISA on colon explant cultures: *RAG2*^{-/-} crossfostered by TRUC, TRUC reared by TRUC, *RAG2*^{-/-} reared by *RAG2*^{-/-}, TRUC *TNFR1*^{-/-} fostered by TRUC, and TRUC *TNFR1*^{-/-} reared by TRUC *TNFR1*^{-/-}. Samples were pooled, $n = 4$ –8 per group.

(F) Colitis scores for *RAG2*^{-/-} and WT co-housed with TRUC for 8 weeks. *RAG2*^{-/-} and WT co-housed with genotype-identical mice shown for comparison. Horizontal bars represent the mean. Where shown, error bars denote SD.

following question remained: Was the colitis contracted by *T-bet*-sufficient mice characterized by the high levels of TNF- α that are central to the pathophysiology of TRUC colitis and originate from *T-bet* deficiency in DCs. Perhaps the transmitted colitogenic microbial community acted via a different mechanism to induce colitis in *T-bet*-sufficient hosts. *RAG2*^{-/-} and TRUC *TNFR1*^{-/-} mice were fostered by TRUC mice and then explant cultures were performed on X-F and control mice. As expected, the *RAG2*^{-/-} X-F mice displayed evidence of colitis. TNF- α levels were not elevated in TRUC X-F *RAG2*^{-/-} or

TRUC *TNFR1*^{-/-} explant cultures (Figure 6F). TRUC mice harbor microbiota that drive a TNF- α -mediated colitis in the absence of *T-bet* yet induce an etiologically distinct colitis in *T-bet*-sufficient mice.

DISCUSSION

Our results reveal an unexpected role for the transcription factor *T-bet* in maintaining host-commensal relationships in the GI tract. *T-bet* fosters harmony between colonic eukaryotes and prokaryotes by establishing in the immune

system genetic programs that affect the behavior of the colonic epithelium. T-bet functions in DCs to avoid proinflammatory responses to commensal microorganisms by regulating TNF- α production to ensure the integrity of the epithelial barrier. From the perspective of the microbial communities inhabiting the colon, the TRUC colonic environment facilitates the growth of a collection of colitogenic bacteria. Once generated, this community of bacteria drives intestinal inflammation even in immunosufficient mice that express T-bet.

The pathophysiology of IBD has been attributed to mucosal immune system dysregulation or gut microbiota and/or abnormal epithelial barrier function (Strober et al., 2007). Deletion of IKK β or NEMO, solely in intestinal epithelium, for example, results in spontaneous colitis (Nenci et al., 2007; Zaph et al., 2007). The characteristics of TRUC colitis described here suggest a link between these two seemingly disparate mechanisms since T-bet expression impacts both the host's mucosal immune system and barrier function as well as the commensal microbial community.

T-bet and the Cytokine Milieu

An altered mucosal cytokine milieu drives inflammation in both UC and CD (Bouma and Strober, 2003). We show that TNF- α is selectively elevated prior to and at the onset of pathologically diagnosable disease, and treatment starting at 2 weeks of age, prior to observable disease, and treatment at 8 weeks of age, in the setting of fulminant disease, are both therapeutic (Figures S1B and S1C). Taken together, our studies have firmly established TNF- α as a key initiating cytokine in our model.

Neutralizing TNF- α Ab may act by binding TNF- α in the serum, sequestering it and preventing it from binding its receptor, or by binding cell-surface-associated TNF- α to induce signaling events resulting in apoptosis of TNF- α -producing cells (Shen et al., 2006). Colonic DCs appear to produce the bulk of TNF- α in the TRUC colon, but intestinal epithelial cells and colonic myofibroblasts also produce TNF- α (Lan et al., 2005; Rogler et al., 2001). Analysis of small intestinal biopsies from CD patients suggests that an imbalance in intestinal DC populations may initiate and perpetuate inflammation (Silva et al., 2004). How mucosal DCs decode environmental signals to mount appropriate immune responses is of great interest as its dysregulation is clearly a driving force in TRUC and in human IBD (Iwasaki, 2007).

Anti-TNF-treated TRUC colons were indistinguishable from controls, with no evident DC accumulation consistent with the notion that anti-TNF- α therapy induces apoptosis of TNF- α -producing cells in colitis. Our experiments also address a causality dilemma in the IBD field: that is, whether the epithelial barrier breach or the aberrant inflammation is the initial pathologic event. Elevated TNF- α in TRUC colonic DCs preceded remarkable epithelial discontinuities, and antibiotic treatment reduced TNF- α concentrations in colon explant cultures. Thus, we favor the idea that bacterially driven production of TNF- α from

colonic DCs is an early, initiating event that triggers breakdown of the intestinal epithelium with consequent ongoing influx of pathogenic microbiota resulting in chronic inflammation. TNF- α blockade is an important therapeutic modality to treat IBD but, as with many biologics, has limiting toxicities. Local augmentation of T-bet activity in intestinal mucosal DCs may be an alternative approach to regulating TNF- α production.

TRUC Transmissibility

TRUC colitis is both vertically and horizontally transmissible to T-bet-sufficient mice, although the penetrance and severity of horizontally transmitted TRUC colitis was reduced relative to vertically transmitted colitis. Cellular, cytokine, or growth factor differences in TRUC milk may contribute to TRUC colitis, but additional factors, aside from the absence of potentially inflammatory breast milk, may be involved. Bacterial colonization of mice and humans starts in the birth canal and continues after parturition.

The adult mice used in the co-housing experiments have well-established microbial communities that may hinder the influx of colitogenic microbiota. Also, effective horizontal transmission depends on the coprophagic and grooming behaviors of individual mice and can vary greatly. The transmissibility experiments suggest that TRUC colitis is not only a microbially driven disease but also that colitogenic microbes have the ability to drive distinct colitides dependent upon different immunological mechanisms based on host genotype.

T-bet and the Microbial Flora

TRUC colitis is microbe dependent and transmissible. Conventional culture data from antibiotic-treated versus untreated TRUC mice suggest that a set of anaerobic microbes may be pathogenic. We were unable to detect *Helicobacter hepaticus*, *bilis*, or *muridarum* by PCR-based methods, and there were no marked differences in the culturable anaerobes, including *Enterococcus* and *Prevotella* species, or aerobes from fecal pellets from WT, T-bet^{-/-}, RAG2^{-/-}, and TRUC mice (data not shown); however, standard culturing techniques are capable of identifying only a small fraction of the known anaerobic microbiota. Genomic approaches using 16S rDNA enumerations are underway to identify this novel collection of colitogenic bacteria.

A genomic survey of mouse intestinal microbiota showed an anaerobic expansion of the microbiota in RAG2^{-/-} mice (Suzuki et al., 2004). Deficiency of both T-bet and RAG2 may result in microbial population shifts such that a host is exposed to an excessive dose of proinflammatory commensals and/or an insufficient dose of protective commensals. The crossfostering experiments suggest that T-bet deficiency influences the nature of microbial communities resulting in a niche favorable for a colitogenic microbial population. These colitogenic bacteria can be transmitted regardless of the genotype of the recipient. Hence, T-bet-sufficient pups fostered by a TRUC mother develop the disease. We envision the

colitogenic process as instigated by T-bet deficiency but, once established, perpetuated independently of T-bet. The molecular link between T-bet genetic programs enforced in the gut and the generation of colitogenic bacteria remains unclear. Intriguingly, vertically transmitted colitis is not characterized by high levels of TNF- α , suggesting that other colitogenic mediators, perhaps IL-23 or IL-17, can be evoked by the transfer of colitogenic bacteria. Identification of the colitogenic bacteria present in these *T-bet*-sufficient fostered pups should provide insight into microbial origins of mechanistically distinct colitides.

Control of T-bet-Initiated Colitis by T-Regulatory Cells

The difference in severity of TRUC X-F WT and *RAG2*^{-/-} colitis mice coupled with the observation that immunosufficient *T-bet*^{-/-} mice do not develop spontaneous colitis led to experiments demonstrating that T-regs prevented the development of colitis in *T-bet*^{-/-} mice. The biology of T-regs and their contribution to promoting tolerance and preventing autoimmunity has burgeoned recently (Fontenot and Rudensky, 2005) with demonstrations that T-regs can downregulate innate immunity-driven inflammation in both the *H. hepaticus* *RAG2* infection model and the SCID T cell adoptive transfer model (Maloy et al., 2005; Uhlig et al., 2006). We found that infusion of T-regs was an effective treatment for TRUC colitis. Following T-reg transfer, we observed DCs in close contact with T-regs in the colon as described in NOD pancreatic LN (Tang et al., 2006). Clinical trials involving T-reg transfer for IBD patients are underway. Here we provide compelling evidence for an important role of T-regs in a novel model of innate-immune system-driven UC.

TRUC colitis mirrors much of the complexity of human UC. This colitis involves mucosal innate immune cell dysregulation, cytokine-driven inflammation, epithelial barrier breach, and pathogenic commensal microbial communities and invokes a role of T-regs in the maintenance of innate immune cell homeostasis in the enteric immune system. Our experiments suggest that there may be a window of vulnerability in early life that confers susceptibility to development of colitis. The TRUC model provides opportunities to study the pathogenesis of this inflammatory disease and more broadly to understand the biological programs that balance host-commensal relations at mucosal surfaces. T-bet is not only a key regulator of type 1 immune responses but also a peacekeeper at the prokaryotic-eukaryotic interface.

EXPERIMENTAL PROCEDURES

Animal Husbandry

Mice were housed in microisolator cages in a barrier facility and studied according to institutional and NIH guidelines. All mice used were specific pathogen free and negative for *Helicobacter hepaticus*, *bilis*, and *muridarum*. We thank Drs. K. Malloy (Oxford University) and T. Yatsunenکو (Washington University) for carrying out the Hepaticus screening. *TNFR1/p55*^{-/-} mice were a gift of Dr. R. Levy (University of Miami).

For crossfostering, on the day of birth the mother was removed from the birthing cage and placed in a clean cage with the crossfoster litter. Pups were weaned between days 21 and 28.

Co-housing of female, 8-week-old *RAG2*^{-/-} or WT mice with TRUC mice was at a ratio of 1:3 for 8 weeks. By 12 weeks of age, many TRUC mice required euthanasia and were replaced with 8-week TRUC mice for the duration of the co-housing.

For studies of the progeny of antibiotic-treated mice, pups were weaned at day 21 and examined 6 weeks after weaning. There was no exposure to antibiotics after weaning.

Histology

Colons were cleaned with PBS or HBSS prior to fixation followed by routine paraffin embedding, sectioning, and staining with H&E.

Sections were examined and the degree of colitis scored by one of the authors (J.N.G.), who was blinded to the genotype and experimental protocols used. Each of 4 histologic parameters were scored as absent (0), mild (1), moderate (2), or severe (3): mononuclear cell infiltration, polymorphonuclear cell infiltration, epithelial hyperplasia, and epithelial injury (Neurath et al., 2002). Colons from DSS-treated mice were scored on a scale of 0–4 based on % of colon involved by inflammation, % of crypt loss, presence of lymphoid follicles, edema, erosions, and density of inflammatory cells. The scores for the parameters were summed for a total severity score.

Barrier Function Assay

Mice were sedated with ketamine and xylazine. Fluorescein-dextran (MW 3000, Invitrogen, 0.6 mg/gram mouse weight) was delivered per rectum using a 3.5 Fr catheter. Serum fluorescence was measured, and preinjection fluorescence was used as the blank. n = 4–6 per group.

Preparation of Samples for EM

Samples were prepared for Epon embedding as per standard protocols. Blocks were sectioned and stained with toluidine blue for light microscopy. Thin sections were picked up on uncoated grids, stained with saturated aqueous uranyl acetate and acetone followed by lead citrate, then examined with a JEOL 1200 electron microscope.

Abs

Abs were obtained from BD PharMingen unless otherwise noted: α -B220/CD45R (RA3-6B2), α -CD11c (HL3), α -CD11b (M1/70), α -MHC class II (AF6-120.1 and 39-10-8), α -CD45 (30-F11), α -Gr-1 (eBiosciences), α -F4/80 (BM8, eBiosciences), α -CD8 (53-6.7), α -CD49b (DX5), α -CD4 (RM4-5), α -CD3 (DAKO # AO452), α -S100 (Dako Inc.), α -CD62L (MEL-14), α -cytokeratin 5/8 (RCK102, Santa Cruz), α -TNFR1 (H-5, Santa Cruz), and α -CD25 (PC61).

Immunohistochemistry

Immunostaining for T-bet was performed on paraffin-embedded tissue sections as described (Dorfman et al., 2003). For double staining of human colonic biopsies, sections were stained with T-bet and S100 (Dako Inc). T-bet staining was as above with α -S100 using an ABC amplification kit (Vector Laboratories). For CD11c IHC, biotinylated α -CD11c and an amplification protocol were used.

For IF staining of colonic leukocytes, sections were stained with α -CD3 and biotin α -CD11c and amplification protocols were used. Sections were counterstained with DAPI (Sigma), viewed with an Olympus B40 microscope, and digitally photographed. Composites were assembled in Adobe Photoshop (Adobe Systems Inc.).

TUNEL staining on the designated paraffin-embedded tissues was performed using the fluorescein in situ cell death detection kit (Roche).

Anticytokine Therapy

α -TNF- α (clone TN3-19.12), a hamster α -mouse TNF- α neutralizing IgG1, and control Ab, hamster α -GST IgG, were generous gifts of Dr. R. Schreiber. TN3-19.12 and control hamster IgG were purchased

from Leinco Technologies, Inc. TN3-19.12 or control were given intraperitoneally at a dose of 15 $\mu\text{g}/\text{gm}$ mouse weight every 7 days for 4 weeks.

Colon Explant Culture

Explant cultures were carried out following a modification of described procedures (Rakoff-Nahoum et al., 2004). Media were collected after 4 hr and centrifuged, and the supernatant was aliquoted and stored at -80°C .

Measurement of Cytokines

Cytokines were measured in culture supernatants utilizing SearchLight high dynamic range (HDR) imaging and analysis. For TNF- α , the mouse BD OptEIA ELISA kit was used. Total supernatant protein concentration was measured and used to calculate the cytokine concentration in pg/mg.

Colonic Epithelial Cell Culture

Epithelial cell culture and TUNEL staining was performed as described with the following modifications (Baumgart et al., 1998). After 6 days, cells were harvested and stained with α -CD45, cytokeratin 5/8, and TUNEL reagent to label the apoptotic cells. After event acquisition using a LSRII, CD45 $^{-}$ cytokeratin 5/8 $^{+}$ cells were gated and the % of TUNEL cells was quantitated using FACSDiva software.

Isolation of Colonic DCs for Flow Cytometry and Intracytoplasmic Cytokine Staining

Mononuclear cells were isolated from the colon as described with modifications as noted (Camerini et al., 1993). Cell suspensions were layered onto Percoll gradients, and the 30%/70% interface, enriched for mononuclear cells, was harvested.

Methods for FC and intracytoplasmic cytokine staining of colonic leukocytes are as described with modifications noted (Rigby et al., 2005). Cells were stained with PE-conjugated Abs directed against TNF- α and the appropriate PE-conjugated isotype controls in parallel. For FC, samples were acquired using a Becton Dickinson LSRII and data were analyzed using FACS Diva software. For colonic DCs, acquisition gates were constructed such that an equal number of CD11c $^{+}$ class II $^{+}$ cells were collected from RAG2 $^{-/-}$ and TRUC samples. Cell sorting was performed using a FACS Aria II at the BWH Center for Neurological Diseases Cell Sorting Core Facility.

Bone Marrow DC Culture

BMDCs were generated as described with the following modification: MACS bead depletion for class II, CD8, CD4, and B220 was employed and cells were cultured in the presence of GM-CSF (Inaba et al., 1992).

Human Myeloid DCs

CD14 $^{+}$ cells were isolated from pooled human buffy coats and cultured in GM-CSF and IL-4 as has been previously described (Sallusto and Lanzavecchia, 1994).

ChIP and qPCR

20×10^6 DCs, harvested on day 6 and treated with LPS 100 ng/ml for 24–30 hr and IFN γ 20 ng/ml, were used per IP. Cells were fixed for 30 min at room temperature (RT) for IP with the T-bet polyclonal Ab, 9856. ChIPs were performed as described (Ansel et al., 2004). 1/20 vol of the ChIP sample was used per qPCR rxn. qPCR products were submitted to agarose gel electrophoresis using SyBR gold dye (Invitrogen) to verify amplification of products of the correct size. Primers sequences for qPCR are available upon request. qPCR reactions were carried out in an ABI Prism 7700 Sequencer Detector using SYBR green reagents.

Luciferase Assay

Constructs are as follows: 3 kb mouse TNF- α promoter luciferase construct (generously provided by Dr. A. Goldfeld, Harvard University), a constitutively active *J $\kappa\kappa\beta$* mutant construct, a Renilla construct for

normalization of transfection efficiency, and full-length *T-bet* cDNA construct, or control plasmid. Luciferase and Renilla activity were assayed using the Dual-Luciferase Reporter Assay System (Promega) and a Monolight 2010 luminometer.

Broad Spectrum Antibiotic Treatment of TRUC Colitis

Mice were treated as described (Fagarasan et al., 2002; Rakoff-Nahoum et al., 2004). Alterations are as noted: ampicillin (1 g/l; Roche), vancomycin (1 gm/l; Henry Schein, Inc. [Hospira, Inc.]), neomycin sulfate (1 g/l; Sigma), and metronidazole (1 g/l; Sigma) were dissolved in autoclaved drinking water and fluid intake was monitored. Treatment was for 6 weeks.

Bacterial Culture

Bacteria culture methods were performed as described (Rakoff-Nahoum et al., 2004). After 48 hr for aerobes or 72 hr for anaerobes at 37°C , colonies were counted. Anaerobic cultures were grown in anaerobic chambers.

Adoptive Transfer Experiments

Peripheral LNs were harvested and cell suspension generated. Immunodepletion of CD8 $^{+}$, CD11c $^{+}$, B220 $^{+}$, Ter-119 $^{+}$, DX5 $^{+}$, class II $^{+}$ populations was performed with MACS beads depletion. The enriched cell population was stained with α -CD4 (clone RM4-5), anti-CD62L (clone MEL-14), and anti-CD25 (clone PC61) and subjected to FACS. CD4 $^{+}$, CD62L hi, and CD25 $^{+}$ and negative populations were collected and resuspended in PBS, and 75,000 were injected per mouse. 1×10^6 CD4 $^{+}$ CD62L $^{+}$ CD25 $^{-}$ cells were injected per mouse as noted. For B cell AT, splenic B cells were purified using MACS-positive selection and 1×10^6 CD19 $^{+}$ resuspended in PBS were injected per mouse. After 4 weeks, mice were sacrificed except for mice receiving the naive T cells or B cells, which were sacrificed after 2 weeks due to morbidity. PBS was used for sham injection (error bars denote SD).

Statistical Analysis

All experiments were repeated three times unless otherwise noted. Statistical analysis was performed using the unpaired Student's *t* test. Error bars represent standard deviations.

Supplemental Data

Supplemental Data include one figure and can be found with this article online at <http://www.cell.com/cgi/content/full/131/1/33/DC1/>.

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