Enterobacteriaceae Act in Concert with the Gut Microbiota to Induce Spontaneous and Maternally Transmitted Colitis

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DOI 10.1016/j.chom.2010.08.004

SUMMARY

Disruption of homeostasis between the host immune system and the intestinal microbiota leads to inflammatory bowel disease (IBD). Whether IBD is instigated by individual species or disruptions of entire microbial communities remains controversial. We characterized the fecal microbial communities in the recently described T-bet−/− x Rag2−/− ulcerative colitis (TRUC) model driven by T-bet deficiency in the innate immune system. 16S rRNA-based analysis of TRUC and Rag2−/− mice revealed distinctive communities that correlate with host genotype. The presence of Klebsiella pneumoniae and Proteus mirabilis correlates with colitis in TRUC animals, and these TRUC-derived strains can elicit colitis in Rag2−/− and WT adults but require a maternally transmitted endogenous microbial community for maximal intestinal inflammation. Cross-fostering experiments indicated a role for these organisms in maternal transmission of disease. Our findings illustrate how gut microbial communities work in concert with specific culturable colitogenic agents to cause IBD.

INTRODUCTION

The human intestine is populated with up to 10^{12} microbes per gram of lumenal contents. Coexistence with this microbial community (microbiota) demands a well-regulated homeostasis between the host immune system and the microbiota (Duerkop et al., 2009; Hill and Artis, 2009). Inflammatory bowel disease (IBD) can occur when this homeostasis is disrupted (Sartor, 2009). Whether individual pathogenic species or entire microbial communities instigate inflammation still remains controversial (Frank and Pace, 2008; Hansen et al., 2010). Defining features of the microbiota and host that are associated with or initiate IBD is critical.

In the absence of adaptive immunity, loss of the transcription factor T-bet in conventionally raised T-bet−/− x Rag2−/− knockout mice results in a spontaneous and highly penetrant colitis that shares histologic features with ulcerative colitis in humans. T-bet−/− x Rag2−/− ulcerative colitis (TRUC) is associated with altered colonic barrier function, elevated TNF-α levels, and dysfunctional dendritic cells (DCs) (Garrett et al., 2007, 2009). It is transmissible to WT hosts when they are cross-fostered or cohoused with TRUC mice (Garrett et al., 2007). TRUC mice provide an opportunity to probe the host-microbe relationship in a model that displays both the immunodeficiency and hyperimmunity observed in humans with IBD.

Here, we show that the presence of Proteus mirabilis and Klebsiella pneumoniae correlates with colitis in TRUC mice and that TRUC-derived strains, in conjunction with an endogenous microbial community, incite colitis in WT mice. These studies revealed the utility of using both culture-independent and -dependent approaches to interrogate the contribution of community members to disease pathogenesis. This model also provides a foundation for defining how gut microbial communities work in concert with specific culturable colitogenic agents to cause IBD and creates an opportunity to evaluate preventative or therapeutic measures directed at components of the gut microbiota and/or host.

RESULTS

16S rRNA-Based Time Series Analysis of TRUC versus Rag2−/− Fecal Microbiota

A pilot experiment—using offspring of conventionally raised, specified-pathogen-free (SPF) T-bet−/− x Rag2−/− and Rag2−/− mothers—analyzed fecal samples collected from mothers at a single time point and from their female pups (n = 3/genotype)
At 2 week time intervals from 2 to 10 weeks. A culture-independent survey of their fecal microbiota by multiplex pyrosequencing of V2 region amplicons of bacterial 16S rRNA genes (n = 32 samples; 2348 ± 343 reads/sample) analyzed by principal coordinates analysis (PCoA) plots based on unweighted UniFrac measurements disclosed a correlation between host genotype and community phylogeny at all ages surveyed (Figure 1A). A total of 69 species-level phylogenetic types, belonging to four major bacterial phyla, exhibited significant differences at various ages in the fecal communities of mice of the two genotypes (Table S1). Compared to Rag2−/− controls, TRUC mice had a significantly higher proportional representation of species-level operational taxonomic units (OTUs) belonging to the order Bacteroidales (phylum Bacteroidetes; p = 0.00643) and significantly lower proportional representation of OTUs belonging to the orders Clostridiales (phylum Firmicutes; p = 0.0201) and Deltaproteobacteria (phylum Proteobacteria; p = 0.0299) (p values by Mann-Whitney test with Bonferroni correction) (Figure 1B).

Figure 1. 16S rRNA-Based Time Series Analysis of TRUC versus Rag2−/− Fecal Microbiota

(A) Principal coordinates analysis (PCoA) of unweighted UniFrac distances from 2- to 10-week-old TRUC (n = 3) and Rag2−/− (n = 3) mice and their mothers. Host genotype influences microbial community structure. Abbreviations: A, B, C, individual pups colored by genotype, followed over time (A.2, A.4, A.6, A.8, and A.10 refer to mouse A sampled at 2, 4, 6, and 10 weeks of age), (B) Distribution of order-level phylotypes in TRUC and Rag2−/− fecal microbial communities. Relative abundance (%) is plotted for each age group.

K. pneumoniae and P. mirabilis Correlate with the Presence of Colitis in TRUC Mice

We also performed quantitative cultures to obtain independent verification of differences in bacterial burden for defined species and to have culturable isolates available to test the specific effects of individual strains on disease initiation and progression. A total of 57 bacterial species were recovered and identified from fecal pellets obtained from three TRUC and three Rag2−/− mice surveyed at 2, 4, 6, 8, and 10 weeks of age and from their mothers (Figure 2A and Table S2). Experiments administering oral antibiotics (Abx) helped further refine potential classes of colitogenic commensal organisms. Gentamicin (gent) or metronidazole (metro) but not vancomycin (vanco) were highly effective in ameliorating TRUC colitis and resulted in clinically and statistically significant changes in colitis scores (mean colitis score 0.5 ± 0.52, p < 0.0001 compared to water control) (Figure 2B). This result pointed us to a role for Gram-negative facultative organisms in TRUC.

The in vitro Abx resistance profiles of the commensal strains selectively recovered from TRUC but not Rag2−/− fecal samples (Figures 2A and 2B, lower panel) corresponded to the in vivo Abx sensitivity of the colitis (Figure 2B, upper panel) since K. pneumoniae and P. mirabilis, both facultative enterics, were sensitive to gent but resistant to vanco (Figure 2B, lower panel).

A more extensive, culture-based survey of a larger number of TRUC and Rag2−/− mice to determine if these bacteria were present in afflicted but absent from healthy mice revealed that K. pneumoniae and P. mirabilis were culturable in all TRUC mice tested (n = 126) at every time point (Figure 2C). In contrast, both species were below our limit of detection (<4.4 log10 cfu/g fecal material) in Rag2−/− mice at each time point (Figure 2C). We treated 4-week-old TRUC mice with Abx using the protocol shown previously to ameliorate TRUC colitis and cultured feces obtained 1 day before and 1 day after Abx administration. K. pneumoniae and P. mirabilis fecal levels fell below our.
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limit of detection when mice were treated with gent or metro, but treatment with vanco neither abolished colitis nor reduced levels of these bacteria (Figure 2D).

To test the hypothesis that *K. pneumoniae* and *P. mirabilis* play a role in TRUC pathogenesis, we determined the physical location of these species using 16S and 23S rDNA fluorescence in situ hybridization (FISH) oligonucleotide probes on whole colonic sections. We focused on the degree of colonization in the lumen, the mucus layer over the epithelium, and the mucosa. As has been reported in IBD patients using a universal bacterial 16S rRNA FISH probe (Swidsinski et al., 2005), we observed that the colonic mucus of colitic TRUC mice harbored numerous bacteria and that there was a consistent loss of a “bacterial-free zone” adjacent to the colonic epithelium (Figure 2E). Healthy
(noncolitic) Rag2−/− mice did not exhibit any of these phenotypes (Figure 2E). Using a set of probeBase consortium 23S and 16S rDNA probes to detect K. pneumoniae and P. mirabilis (Loy et al., 2007), we visualized a small number of organisms adjacent to the epithelium (Figures 2F and 2G). Hence, K. pneumoniae and P. mirabilis may have invasive potential, or the proximity of their bacterial products to the apical epithelial surface may trigger inflammatory responses without frank invasion. Either could explain their role in TRUC colitis, as access to the mucosa would increase the opportunity for eliciting a host proinflammatory response.

K. pneumoniae and P. mirabilis Elicit Colitis but Require a Maternally Transmitted Endogenous Microbial Community for Maximal Intestinal Inflammation

Following postnatal exposure to a TRUC dam, WT and Rag2−/− mice develop histopathologic features of colitis (penetrance of phenotype: 94% at 8 weeks of age) (Garrett et al., 2007). We asked if this maternally transmitted disease had a pattern of Abs sensitivity similar to spontaneous TRUC colitis. We cross fostered TRUC, Rag2−/−, and WT pups on TRUC mothers who received water, gent, metro, or vanco from preconception through weaning. Gent and metro markedly improved the colitis score for all mice in a statistically significant fashion, while vanco did not, similar to what we observed in spontaneous TRUC colitis (n = 2 foster mothers/genotype; 2–4 pups/litter surveyed) (Figure 3A).

We cultured fecal samples from WT and Rag2−/− mice that developed transmissible colitis from cross-fostering (Figure 3B), K. pneumoniae and P. mirabilis were detected in all fecal samples obtained from 8-week-old TRUC-fostered Rag2−/− and WT pups at levels comparable to age-matched TRUC-fostered TRUC mice. In contrast, neither organism was detected in any control Rag2−/−-fostered Rag2−/− or WT-fostered WT animals (n = 2 foster mothers/genotype; 2–3 pups/litter surveyed) (Figure 3B). Neither TRUC mice fostered on Rag2−/− nor WT mothers had histologic evidence of colitis or K. pneumoniae or P. mirabilis at 8 weeks of age (Figures S2 and 3B).

The presence of K. pneumoniae and P. mirabilis in colitic TRUC mice and TRUC fostered Rag2−/− and WT mice and the lack of detectable bacteria in the fecal microbiota of healthy Rag2−/− WT, and WT or Rag2−/−-fostered TRUC provided additional evidence for an association between the presence of these bacteria and colitis.

One possibility is that the presence of K. pneumoniae and P. mirabilis is a consequence rather than a cause of inflammation. Intestinal inflammation caused by Citrobacter rodentium may drive blooms of Enterobacteriaceae, although this result is controversial (Hoffmann et al., 2009; Lupp et al., 2007). To investigate the effects of inflammation on intestinal colonization by K. pneumoniae and P. mirabilis, we treated 8-week-old WT and Rag2−/− mice with the mucosal toxin dextran sulfate sodium to induce colitis (n = 8 mice/genotype). We did not detect culturable K. pneumoniae or P. mirabilis in the fecal microbiota of any of these mice during our period of surveillance (n = 8 mice/genotype; samples collected before and 1 day after completion of a 1 week treatment) (Figure S1), arguing against an inflammatory response causing expansion of K. pneumoniae and P. mirabilis in the TRUC gut microbiota.

To directly test the colitogenic potential of K. pneumoniae and P. mirabilis, we rederived conventionally raised TRUC mice as germ-free and cocolonized the mice with these two Enterobacteriaceae at 8 weeks of age for 8 weeks (n = 5 mice). Both organisms established themselves in the guts of all recipients (mean value 1011.29 ± 0.46 cfu/microbial species/g dry weight of feces; assayed 48 hr and weekly after the initial gavage) (Figure S2). Colonic inflammation did not develop in these cocolonized gnotobiotic TRUC mice, suggesting that interactions among K. pneumoniae, P. mirabilis, and other members of a gut microbial community are required to ignite the immunoinflammatory cascade that leads to colitis. To evaluate this possibility, we colonized 2 week SPF WT and Rag2−/− mice with K. pneumoniae, P. mirabilis, or a combination of K. pneumoniae and P. mirabilis (recovered from feces from the female TRUC mother in Figure 2A and administered by direct oral instillation of 104 cfu and by addition of 105 cfu to the drinking water every other day for 8 weeks; n = 5–18 mice/treatment group). Control groups of mice received a TRUC-derived E. coli strain. Both K. pneumoniae and P. mirabilis established themselves in the gut microbiota of both Rag2−/− and WT (as defined by cfu assays of feces obtained 2 days after the completion of 8 weeks of treatment [Figure 3C]). Feces from WT and Rag2−/− hosts contain E. coli, but we did not have the tools to distinguish these indigenous strains from the exogenously administered TRUC-associated E. coli strain. While no colonic inflammation was observed after inoculation with E. coli (Figure 3D), treatment with P. mirabilis, K. pneumoniae, or a combination of the two organisms induced inflammation in both WT and Rag2−/− mice, with colitis severity being significantly greater in Rag2−/− mice exposed to both species compared to P. mirabilis alone (Figure 3D). We conclude that two Enterobacteriaceae, in concert with members of the microbiota, are able to elicit colitis, even in mice not genetically predisposed to developing immunopathologic responses.

The penetrance and severity of colitis observed in the cocolization experiments with K. pneumoniae and P. mirabilis were decreased compared to that observed in the spontaneous TRUC model (e.g., Figure 2C) and in neonatal cross-fostering experiments (TRUC-fostered Rag2−/− mean colitis score 5.6 ± 1.14 and TRUC-fostered WT 3.17 ± 0.75) (Figure 3A). Instead, it resembled experiments where adult TRUC mice were co-housed with adult Rag2−/− or WT mice (Garrett et al., 2007), speaking to a possible role of maternal/foster bacterial and nonbacterial factors in structuring microbial communities in the neonate. Consistent with this, we found that TRUC milk has a proinflammatory cytokine profile (Figure S5) and that the microbiota of 2-week-old TRUC mice clusters in a distinct group, as judged by PCoA plots of UniFrac measurements of 16S rRNA-defined communities (Figure 1A).

K. pneumoniae and P. mirabilis Colonization Patterns Change in Response to Immunotherapy, and Both Strains Induce TNF-α Production in T-bet−/− Rag2−/− MyD88−/− Bone Marrow-Derived DCs

We next asked whether K. pneumoniae and P. mirabilis colonization patterns might change in response to two immunotherapeutic interventions previously shown to cure TRUC colitis, i.e., TNF-α neutralization and T-regulatory cell (T-reg) transfer (Garrett et al., 2007). We used quantitative culture-based...
Figure 3. *K. pneumoniae* and *P. mirabilis* Elicit Colitis but Require a Maternally Transmitted Endogenous Microbial Community for Maximal Intestinal Inflammation

(A) The Abx sensitivities of colitis transmitted via TRUC cross-fostering are the same as spontaneous TRUC colitis. Abx-treated pregnant TRUC females were used as foster mothers and treated with Abx in their water until weaning. Histologic colitis scores are shown for the fostered mice at 8 weeks of age.

(B) *K. pneumoniae* (squares) and *P. mirabilis* (circles) are detected in the fecal microbiota of TRUC cross-fostered Rag2−/− and WT mice at 8 weeks of age but not in 8-week-old TRUC mice fostered by Rag2−/− or WT mice. TRUC-fostered TRUC, Rag2−/−-fostered Rag2−/−, and WT-fostered WT are shown as controls. Limits of detection: 10^4.4 cfu/g dry weight of feces. Each symbol represents a fecal sample from a different mouse.

(C) Left panel: Fecal bacterial counts for cocolonized gnotobiotic TRUC mice. Mean values ± 1 SD are shown for *K. pneumoniae* (squares) and *P. mirabilis* (circles) (n = 5 mice). Right panel: Histologic colitis scores of germ-free TRUC and germ-free TRUC mice cocolonized with *K. pneumoniae* and *P. mirabilis* from the TRUC mother in Figure 1.
methods to assay *K. pneumoniae* and *P. mirabilis* levels in feces prior to treatment of 4-week-old TRUC mice with anti-TNF-α, during weekly treatment for 4 weeks, and for 6 weeks after the last dose (Figure 4A) (n = 10 mice, anti-TNF-α; n = 10, isotype control) (histologic colitis scores in Figure S3). Significant differences in fecal *K. pneumoniae* levels between the TNF-α neutralization and isotype control groups were observed after 7 weeks of treatment (age 11 weeks; p = 0.0172; Mann-Whitney test) and for *P. mirabilis* after a shorter period of treatment (p = 0.008, p = 0.0012, p = 0.0004, and p = 0.0403 at 7, 8, 9, and 10 weeks of age). Two-way ANOVA revealed that anti-TNF-α neutralization accounted for 10.7% of the total variance observed in fecal *P. mirabilis* levels (adjusting for matching: F = 22.83, DFn = 1, DFd = 18, p = 0.0002). TNF-α did not directly affect the growth kinetics of either *K. pneumoniae* or *P. mirabilis* under in vitro monoculture conditions (Figure S4).

We performed a similar analysis in TRUC mice that had received 75,000 purified WT T-reg cells at 4 weeks of age (histologic colitis scores at 12 weeks of age in Figure S3). Surprisingly, while T-reg infusion ameliorated this colitis (Garrett et al., 2007), it did not affect fecal levels of either of these two bacterial species (Figure 4B). These results demonstrate that *K. pneumoniae* and *P. mirabilis* levels are not simply associated with inflammation, as both these modalities reduced host inflammation but did not uniformly alter Enterobacteriaceae representation. Our results illustrate that certain host-directed treatments may exert their effects not only by altering host inflammatory pathways but also by directly impacting the microbiota.

To begin to identify cell-based mechanisms by which TRUC-derived Enterobacteriaceae elicit a host immune response, TNF-α production was measured in *T-bet*−/− *Rag2*−/− *MyD88*−/− bone marrow-derived DCs cocultured with the *K. pneumoniae* and *P. mirabilis* TRUC strains, as DCs and TNF-α production are key features of the immunopathogenesis in TRUC mice, and the TRUC inflammatory response is independent of *MyD88* (Garrett et al., 2007, 2009). Both live and heat-killed bacteria stimulated TNF-α production from *T-bet*−/− *Rag2*−/−, and *MyD88*-deficient DCs (Figure 4C). These latter findings set the stage for future bacterial cell fractionation experiments where the microbial molecular determinants of host responses can be characterized using in vitro systems composed of genetically manipulated immune cells.

**DISCUSSION**

Defining microbial features that are associated with or initiate IBD is complicated by host genetics, inflammatory state, and diet (Peterson et al., 2008). Designing prospective studies in human IBD to identify microbial communities that instigate inflammation has not been feasible, even in genetically susceptible populations. Thus, we performed a time series screen in a mouse model of IBD that shares several pathophysiological features of human IBD, including immunodeficiency, compromised host barrier function, and hyperimmunity, to characterize a colitogenic microbiota. We established that host genotype influenced the global structure of the associated microbial community detected in feces and observed a number of significant order- and species-level differences. Combined with culture-dependent methods, we were able to identify bacterial species whose role we could test in the development of disease. Our experiments demonstrate that *K. pneumoniae* and *P. mirabilis*, together with other members of the endogenous microbiota, can elicit colitis even in WT mice. It will be important to determine if significant associations are noted between these Enterobacteriaceae species and ulcerative colitis or Crohn’s disease in ongoing (e.g., Qin et al., 2010) and future metagenomic studies of gut microbial ecology in various populations of patients with IBD.

*K. pneumoniae* and *P. mirabilis* can colonize mouse and human intestine (Lau et al., 2008). Notably, we only recovered these microbes from TRUC mice in our colony, not in *Rag2*−/− or WT animals. While there was individual variation in bacterial counts, the colonization pattern of these species across the TRUC population over time was not significantly different and did not vary as colitis worsened with age. Inciting inflammation with dextran sulfate sodium in WT mice in the colony did not result in an emergence of these bacteria. In contrast, Abx treatment had a dramatic effect on the degree of host colonization with *K. pneumoniae* and *P. mirabilis*, as expected. The increased counts observed in response to vancomycin suggest that in the untreated host, members of the Gram-positive flora affect the degree of colonization by members of the Enterobacteriaceae.

A key feature of the colonic inflammation in TRUC mice is elevated TNF-α. While both neutralizing antibodies and T-reg infusion reduce mucosal TNF-α levels, these interventions had disparate effects on *K. pneumoniae* and *P. mirabilis* fecal counts. Cytokines may interact with bacteria, and TNF-α has been shown to affect *Salmonella typhimurium* replication in vivo (Romanova et al., 2002). While TNF-α did not appear to affect the proliferation of TRUC-derived *K. pneumoniae* and *P. mirabilis* in vitro, in vivo there were significant effects in response to TNF-neutralizing antibodies. Neutralizing antibodies and infusion of T-regs both lower TNF-α levels in TRUC mice, but through different mechanisms. T-regs also produce both IL-10 and TGF-β (Izcue et al., 2009). T-regs and neutralizing antibodies may have direct but distinct effects on microbial populations or indirect effects through their differential effects on colonic DCs.

Opportunistic infection with *K. pneumoniae* and *P. mirabilis* is well recognized in the respiratory and urinary tracts. However, *Klebsiella oxytoca* but not *K. pneumoniae* has been tied to intestinal pathology (Högener et al., 2006). *Klebsiella* and Proteus species are observed more frequently in the stool of ulcerative colitis patients than healthy controls (Dorofeyev et al., 2009; Kanareykina et al., 1987), and elevated titers of Enterobacteriaceae antibodies have been reported in IBD patients (Cooper et al., 1988; Ibbotson et al., 1987; Tiwana et al., 1998). Genome sequencing of these isolates and comparisons to other sequenced isolates obtained from other body and environmental habitats could yield...
testable hypotheses about genetic determinants that may underlie their ability to drive development of an IBD phenotype. Future studies can take advantage of the fact that both heat-killed and live K. pneumoniae and P. mirabilis induce TNF-α in T-bet−/− Rag2−/− MyD88−/− bone marrow-derived DCs to identify the responsible bacterial molecules and their host receptors. However, it is important to also emphasize that an endogenous microbial community is required for K. pneumoniae and P. mirabilis to exert their colitogenic effects.

In addition, interactions between K. pneumoniae and P. mirabilis and microbial community members may result in the acquisition of traits by these two Enterobacteriaceae (e.g., invasion) or by other community members that elicit intestinal inflammation. Convergence of host genetic susceptibility and microbial community features could also affect the behavior of these Enterobacteriaceae and the immune response to them, as we have observed in the TRUC model.
Elevated TNF-α and beneficial responses to TNF-α-neutralizing antibodies are common to both human IBD and several experimental colitis models. Host factors, like elevated TNF-α, may have virulence-promoting effects on these microbes. This notion is not without precedent, as the *Pseudomonas aeruginosa* protein OprF binds the proinflammatory cytokine IFN-γ, resulting in expression of PA-I lectin, a quorum-sensing-dependent virulence determinant (Wu et al., 2005).

In summary, future studies need to be directed at defining the genomic features of TRUC-associated *P. pneumoniae* and *P. mirabilis* strains, identifying co-occurring cultivable members of the microbiota that contribute to disease pathogenesis in conventionally raised and gnotobiotic mouse models, characterizing host factors that drive these microbes to become colitogenic, and determining the microbial-associated molecular patterns and pattern recognition receptors involved in spontaneous and transmitted TRUC colitis. Together, these efforts may provide mechanistic insights about how gut microbial communities, working in concert with specific colitogenic agents, contribute to initiation and perpetuation of IBD in susceptible human hosts and provide the foundation for proof-of-concept tests of preventative or therapeutic measures. An additional benefit may be to help elucidate the association between IBD and increased risk for tumorigenesis, since the majority of TRUC mice spontaneously develop colonic dysplasia and rectal adenocarcinoma (Garrett et al., 2009).

**EXPERIMENTAL PROCEDURES**

**Husbandry of Conventionally Rased Mice**

*Rag2* null, *T-bet* null, and *MyD88* null mice were provided by The Jackson Laboratory (Bar Harbor, ME). *Rag2*−/−, *T-bet*−/−, and *MyD88*−/− mice and their genotyping have been described (Garrett et al., 2009). Mice were housed in microisolator cages in a barrier facility at the Harvard School of Public Health under a 12 hr light cycle.

**16S rRNA-Based Analyses of Fecal Microbial Communities**

**Community DNA Preparation**

Fecal samples were flash frozen on collection and stored at −80°C before processing. DNA was extracted by bead-beating as described (Turbaugh et al., 2009).

**Sequencing and Analysis of 16S rRNA Gene Amplicons**

The V2 region (primers 8F-338R) of bacterial 16S rRNA genes was targeted for amplification and multiplex pyrosequencing with error-correcting barcodes (Hamady et al., 2008). A total of 75,145 high-quality reads were generated from 32 samples (2348 ± 343 reads per sample). See also Supplemental Experimental Procedures.

**Culture-Based Studies of Fecal Microbial Community Structure**

**Stool Collection**

A minimum of three fecal pellets was collected from each mouse in a laminar flow hood. Each mouse (three females/genotype; TRUC and *Rag2*−/−) was sampled every 2 weeks at the same time of day from 2 to 10 weeks of age. Mothers were sampled only when their pups were 2 weeks old.

**Culture**

Fecal pellets were collected into tubes of PBS with 0.05% cysteine HCl.

Serial 10-fold dilutions were made and plated on nonselective and selective media. Anaerobes were incubated at 37°C in a Coy Anaerobic chamber for a minimum of 5 days. Aerobes were incubated for 24–48 hr at 37°C.

**Fecal Collection and Culture of Gram-Negative Aerobes**

Mice were singly placed in autoclaved plastic cages. Four to six pellets were collected/ mouse/ time point. *Rag2*−/− mice in Figure 2F were sampled twice over a 3-day period for each weekly time point. Pellets were resuspended in sterile PBS; 10-fold serial dilutions were generated, plated on MacConkey’s medium, and incubated in ambient air at 37°C overnight. The lower limit of detection for these studies was 10^4.4 cfu/gram fecal dry weight.

**Histology**

Colons were harvested and prepared for histology as described (Garrett et al., 2007). See Supplemental Experimental Procedures for more detail.

**Antibiotic Treatment**

Mice were treated with the following 4txs dissolved in their autoclaved drinking water as indicated: ampicillin (1 g/l; Roche), vancomycin (500 mg/l; Sigma), neomycin sulfate (1 g/l; Sigma), metronidazole (1 g/l; Sigma; solubilized with 15 ml of 0.1 N acetic acid/1), and gentamicin (2 g/l; Cell Gro). Fluid intake was monitored.

**Fluorescence In Situ Hybridization**

Colons harvested from 16 *Rag2*−/− and 15 TRUC (3- to 8-week-old) mice were fixed in Carnoy’s solution overnight and embedded in paraffin, and 5 μm thick sections prepared (Swidsinski et al., 2005). The sequences of the following FISH probes were obtained from probeBase (http://www.microbial-ecology.net/probebase/) (Loy et al., 2007): the “universal” bacterial probe–EBU338 (p8-00159), Enterobacteriaceae targeted probe (p8-00914), *K. pneumoniae*–directed probe (p8-00352), and *P. mirabilis* probe (p8-02110).

**Cross-Fostering**

On the day of birth, the mother was removed from the birthing cage and placed in a clean cage. A litter of pups with the designated genotype was then put into the cage. Pups were weaned on postnatal day 21 (Garrett et al., 2007).

**Gnotobiotic Mouse Experiments**

All protocols related to the generation and husbandry of germ-free mice were approved by the Washington University (Wash U) Animal Studies Committee. Conventionally raised SPF *T-bet*−/−× *Rag2*−/− mice were rederived as germ-free in the gnotobiotic facility at Wash U. Subsequent experiments were carried out at the Harvard Digestive Disease Center (HDDC) gnotobiotic facility. Five mice were maintained germ-free, and another five mice (3 female and 2 male) were co colonized by introducing 4.8 × 10^9 cfu of *K. pneumoniae* and 9.2 × 10^9 cfu of *P. mirabilis* into their oral cavity and simultaneously spreading an equivalent amount of organisms on their fur and anus. See Supplemental Experimental Procedures for more detail.

**Invasion Experiments**

*K. pneumoniae*, *P. mirabilis*, *E. coli*, or both *K. pneumoniae* and *P. mirabilis* (2 × 10^10 cfu each; all isolated from the TRUC mother in Figure 1) were instilled into the oral cavity of each mouse using a sterile pipette tip, and 1 × 10^8 cfu was placed into a new container of their drinking water every other day.

**Anti-TNF-α Treatment**

Mice were injected with anti-TNF-α (clone TN3-19.12), a hamster anti-mouse TNF-α-neutralizing IgG1 antibody, and control Ab (hamster anti-GST IgG1) (Leinco Technologies, St. Louis) (15 mg/kg) once a week for 4 weeks (Garrett et al., 2007).

**Adaptive Transfer of T-Regulatory Cells**

FACS-sorted lymph node CD4+ CD62Lhi CD25+ cells (T-reg, 75,000 cells) or FACS-sorted lymph node CD4+ CD62Lhi CD25+ cells (T-reg, 75,000 cells) or PBS were injected per mouse at 4 weeks of age (n = 10 for T-reg; n = 9 for PBS) (Garrett et al., 2007). This experiment was terminated by euthanasia at 12 weeks because two control group mice became moribund from colitis.

**Coculturing Bone Marrow-Derived Dendritic Cells and Bacterial Strains**

Mouse bone marrow–derived DCs were generated as described (Garrett et al., 2007) and purified using anti-mouse CD11c-coupled magnetic beads. *K. pneumoniae* or *P. mirabilis* was cocultured with DCs at a ratio of 1 cfu/DC for 4 hr at 37°C in a cell culture incubator at 5% CO2. Gent (50 μg/ml) was then added to the media for 1 hr, and cells were collected, washed, and incubated with medium containing gent (20 μg/ml) for an additional 16 hr. Bacteria were also heat-killed (incubation at 100°C for 3 min followed by plating to
Cells were cocultured for 20 hr. TNF-α (confirm killing) and added to cultures of DCs at ratios of 1:1, 10:1, and 100:1. OptEIA ELISA kit (BD Biosciences) and expressed as ng/ml/10^6 DCs.

**Statistical Analysis**
The Prism graphing and analysis program was used for calculation of statistical measures including mean values, standard deviations, p values (Mann-Whitney test), and two-way ANOVA.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, two tables, and four figures and can be found with this article online at doi:10.1016/j.chom.2010.08.004.

**ACKNOWLEDGMENTS**
We thank J. Braun (UCLA), D. Relman (Stanford U.), A. Swidsinski (Charite Humboldt U.), G. Hansson (U. of Gothenburg), and members of the Gordon and Glimcher labs for helpful discussions. We thank J. Ramirez for care of our SPF mice and S. Wagoner, V. Yesliseyev, and C. Belzer for care of germ-free TRUC. This work was supported by the NIH (CA112663 to L.H.G.), Danone Research (L.H.G.), and the Crohn’s and Colitis Foundation of America (J.I.G.), plus career development awards from the Burroughs Wellcome Fund and the NIH (K08AI078942) to W.S.G. The HDCC germ-free core is supported by P30-DK03485. 454 pyrosequencing reads have been deposited in the NCBI Short Read Archive. L.H.G. declares that she is a member of the Board of Directors of the Bristol-Myers Squibb Corporation.

Received: February 9, 2010
Revised: May 25, 2010
Accepted: July 22, 2010
Published: September 15, 2010

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Received: February 9, 2010
Revised: May 25, 2010
Accepted: July 22, 2010
Published: September 15, 2010

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