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***Helicobacter* species are potent drivers of colonic T cell responses in homeostasis and inflammation**

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Abstract

Specific gut commensal bacteria improve host health by eliciting mutualistic regulatory T (Treg) cells responses. However, the bacteria that induce effector T (Teff) cells during inflammation are unclear. Here, we addressed this by analyzing bacterial-reactive TCR transgenic cells and TCR

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Author contributions

J.N.C and C.-S.H. conceived the project and designed the experiments; J.N.C, Y.P., S.R., and T.L.A. performed the experiments; J.N.C., Y.P., B.D.S., S.R., and J.S.P. analyzed the data. Z.S., K.A.K., T.T., S.N., K.H., R.D.N., C.O.E., A.L.K., D.A.P., and J.G.F. provided important bacterial isolates, mice, and reagents; T.S.S. assisted in histological evaluation; J.N.C and C.-S.H. wrote the manuscript.

Competing interests

The authors declare that they have no competing interests.

Data and materials availability

TCR sequencing data and 16S rRNA sequencing data are deposited at the European Nucleotide Archive.

repertoires in a murine colitis model. Unexpectedly, we found that mucosal-associated *Helicobacter* species triggered both Treg responses during homeostasis and Teff responses during colitis, as suggested by an increased overlap between the Teff/Treg TCR repertoires with colitis. In fact, 4/6 Treg TCRs tested recognized mucosal-associated *Helicobacter* species *in vitro* and *in vivo*. By contrast, the marked expansion of luminal *Bacteroides* species seen during colitis did not trigger a commensurate Teff response. Unlike other Treg cell-inducing bacteria, *Helicobacter* species are known pathobionts and cause disease in immunodeficient mice. Thus, our study suggests a model in which mucosal bacteria elicit context-dependent Treg or effector cell responses to facilitate intestinal tolerance or inflammation.

Introduction

We cohabitate with trillions of bacteria that reside within our intestines and provide important beneficial metabolic and immunologic functions (1). However, inappropriate immune responses against these bacteria have been implicated in the pathogenesis of inflammatory bowel disease (IBD) (2–4), as germ-free mice are highly resistant to many murine colitis models. Moreover, monocolonization studies of germ-free animals revealed that IBD development may be driven by specific commensal bacterial species (5).

Tolerance to commensal bacteria is thought to depend on CD4⁺ regulatory T (Treg) cells (6–8), which we have recently shown can arise via peripheral Treg (pTreg) cell development from naïve T cells in response to commensal antigens during homeostasis (9). However, intestinal inflammation can result in the exposure of new antigens to the immune system. In murine models, effector T (Teff) cell generation to a commensal bacterial antigen was seen during intestinal inflammation, but not homeostasis (10). Thus, it remains unknown whether effector responses during intestinal inflammation are driven by newly exposed antigens versus the commensal antigens that normally drive Treg cell development during homeostasis.

IBD is associated with marked changes in the gut microbiota, dysbiosis (11). Dysbiosis has been hypothesized to contribute to disease pathogenesis, as it is well established that different commensal bacterial species have distinct effects on the intestinal T cell population. For example, segmented filamentous bacteria (SFB) strongly induces T helper 17 (Th17) cells in the small intestine (12), which has now been confirmed by TCR transgenic studies (13). By contrast, introduction of *Clostridium* clusters XIVa and IV (7, 14) or altered Schaedler flora (ASF) (15) markedly increased the frequency of colonic Foxp3⁺ regulatory T (Treg) cells in germ-free mice. Thus, changes in the microbiome may also affect the intestinal effector vs regulatory T cell population.

Here, we used TCR repertoire analysis coupled with *in vivo* studies of commensal-specific TCRs to address the influence of colitis-mediated dysbiosis on bacteria-specific Treg cell development. We also examined the interaction of T cells with luminal versus mucosal associated antigens during homeostasis and colonic inflammation. Finally, we assessed the impact of commensal-specific T cells during lymphopenic conditions. In summary, our data suggest that the mucosal-associated pathobionts, *Helicobacter* spp., elicit context-dependent T cell responses during homeostasis and colitis.

Results

Overlap of effector and Treg TCR antigen-specificity during colitis

To address the effect of colitis on T cell responses to commensal bacteria, we examined induced models as we wanted to compare mice that started with the same microbiota. We settled on a murine model of inflammatory colitis (Fig. 1A) (16) that incorporates 1% dextran sodium sulfate (DSS) induced mucosal injury along with anti-IL-10R antibody (α IL10R) (15, 17), which blocks an important immune regulatory pathway implicated in human IBD in genome-wide association studies (18). DSS+ α IL10R treatment induced colitis as evidenced by weight loss, increased colon weight/length ratio, and a marked enhancement of Teff (CD44^{hi}CD62L^{lo}) and Treg cells (fig. S1). As the combination showed a greater effect on the microbiota than DSS or α IL10R alone (fig. S1E), we used DSS + α IL10R for subsequent experiments.

Consistent with previous reports, naïve T cells from CT2 and CT6 TCR transgenic (Tg) lines, which express TCRs isolated from colonic Treg cells and recognize commensal antigens (8), showed substantial induction of Foxp3, the canonical Treg cell transcription factor, by 1 week after transfer into control IgG treated mice (Fig. 1A and B) (9). However, in mice undergoing DSS+ α IL-10R mediated colitis, they skewed towards Teff cell generation (Fig. 1B, S2). Teff cell differentiation was primarily to the Th17 phenotype based on IL-17A^{GFP} and IFN γ ^{YFP} reporters (Fig. 1C), consistent with observations in human Crohn's disease (19). In addition, CT2 and CT6 cells underwent extensive proliferation as assessed by Cell Trace Violet dilution and expanded as a fraction of total CD4⁺ cells (Fig. 1D and E). Thus, the normal tolerogenic Treg cell developmental response to commensal bacterial antigens can be re-directed to a Th17 effector response during experimental colitis.

To assess whether the responses of CT2 and CT6 TCR Tg cells were representative of the T cell population, we analyzed the effect of colitis on the TCR repertoire (fig. S3A and B, table S1). Because the great diversity in polyclonal T cells precludes experimental analysis at the individual TCR level, we utilized mice in which TCR diversity is limited by a transgenic fixed TCR β chain (20). Although TCR diversity is diminished, this approach allows high-throughput analysis of the TCR repertoire at the individual TCR level via sequencing of the variable TCR α chains. With colitis, both Treg and Teff repertoires showed increased clonal expansion compared to controls (fig. S3C), consistent with a T cell response. Notably, we saw a marked increase in similarity between the Treg and Teff TCR repertoires within the colon lamina propria (cLP) of individual mice during colitis compared with controls (Fig. 2A, S3D). Examination of the top 25 Teff TCRs per mouse showed that many of these TCRs are also found in the Treg cell subset during colitis (Fig. 2B), consistent with our TCR transgenic studies (Fig. 1). In control mice, the relatively low degree of overlap between the Teff and Treg cell subset within individual mice (Fig. 2A and B, S3D) is consistent with our previous studies (8, 21). As before, we also observed mouse-to-mouse variability of Teff and Treg TCR repertoires between mice (Fig. 2A, S3D and E). To confirm the TCR repertoire analysis, we cloned T7-1 (Fig. 2C), the most abundant TCR found in the Teff subset of colitic mice by mean percentage (fig. S3E). As predicted by the TCR repertoire study, analysis of adoptively transferred peripheral TCR $\alpha\beta$ Tg cells expressing T7-1 showed

that this TCR facilitated Foxp3 induction during homeostasis, but skewed towards Teff cell generation with colitis (Fig. 2D, S4). Thus, these data demonstrate an increased overlap between the Teff and Treg TCRs repertoires during colitis that does not occur at homeostasis.

Our TCR Tg data suggest that the overlap in Treg and Teff TCR usage during colitis is due to enhanced Teff generation by clones that might normally differentiate into Treg cells (Fig. 1). Alternatively, the overlap may arise from increased Treg cell generation by Teff TCRs during colitis. However, transfer of CBir1 Tg cells (22) into DSS+ α IL10R hosts showed increased Teff but not Treg generation (fig. S5), consistent with a previous report in the DSS model (10). Although it remains possible that the CBir1 result in the DSS+ α IL10R model is not generalizable to other model systems, in conjunction with the TCR repertoire analysis, these TCR Tg studies suggest that colitis skews T cell development such that pro-inflammatory Teff cells often use the same TCRs as anti-inflammatory Treg cells.

Helicobacter spp. are important drivers of peripheral Treg cell development during homeostasis

The observation that certain commensal antigens can activate both Treg cells during homeostasis and Teff cells during colitis suggested that these bacterial antigens are continually presented to the adaptive immune system. Notably, this pattern is different from the CBir1 commensal antigen, which is primarily presented to T cells during colitis (fig. S5) (10). To address this, we assessed the *in vitro* reactivity of these TCRs to fecal antigen preparations presented on CD11c⁺ dendritic cells (DCs) (8). Although TCR activation by fecal antigen from control mice was high with T7-1 and detectable with CT2 and CT6, the degree of enhancement in TCR activation by fecal antigens from colitic mice was limited (Fig. 3A, S6). We therefore asked whether these TCRs show more reactivity to mucosal associated (MA) antigens, which might be predicted to have greater access to the immune system via DC uptake (23), goblet associated passages (24), or outer membrane vesicles (25). Both CT2 and CT6 showed marked enhancement of TCR stimulation with MA compared with luminal antigen (Fig. 3A). We then tested additional Treg TCRs, CT1, CT7 and CT9, which we previously found to react to fecal antigens or commensal isolates *in vitro* (8). Amongst these 6 colonic Treg TCRs, 4 showed high level reactivity *in vitro* to MA compared with luminal antigen, and of these 2 were enhanced by colitis (Fig. 3A).

To confirm that the reactivity in the MA antigen preparation was dependent on the microbiota, we treated specific-pathogen-free (SPF) mice with a broad spectrum antibiotic combination of vancomycin, ampicillin, metronidazole, and neomycin (VAMN), and found that MA antigen no longer stimulated our 4 MA antigen reactive-Treg TCR panel (CT2, CT6, CT9 and T7-1; fig. S7A). This antibiotic cocktail includes vancomycin, which has been reported to decrease Treg cell numbers and *Clostridium* spp. (7). We then tested the individual antibiotics to attempt to identify the bacterial spp. recognized by these TCRs by correlating *in vitro* TCR reactivity to MA antigen with changes in the bacteria composition.

Unexpectedly, these data suggested that *Helicobacter* spp., and not the predicted *Clostridium* spp. (7), were being recognized by the TCR panel. First, of the individual antibiotics, ampicillin and neomycin, but not vancomycin or metronidazole, markedly decreased the

ability of MA antigen to stimulate the TCR panel compared with untreated controls (Fig. 3B). Second, 16S ribosomal RNA (rRNA) gene sequences from the MA antigen preparations revealed that the two most frequent Operational Taxonomic Units (OTUs) lost with ampicillin or neomycin were *Helicobacter typhlonius* (*H. typhlonius*) and *H. apodemus* (Fig. 3C, S7B).

To determine if *Helicobacter* spp. were being directly recognized by these TCRs, we tested cultured isolates that matched the 16S rRNA sequence (Fig. 3D). Notably, there were specific patterns of TCR reactivity, with T7-1 and CT9 recognizing both spp., whereas CT2 reacted only to *H. typhlonius*, and CT6 to *H. apodemus*. Recognition of *Helicobacter* spp. *in vitro* appeared to be TCR specific and not via a superantigen or other antigen-independent mechanism. First, each *Helicobacter* isolate stimulated only some, but not all, TCRs. Second, addition of anti-MHC II blocked TCR activation (fig. S7C). Third, testing of these TCRs against multiple bacterial isolates *in vitro* from different genera including *Clostridium*, *Bifidobacterium*, and *Lactobacillus* failed to activate our TCR panel to the same degree as *Helicobacter* (fig. S8). However, these TCRs could still show cross-reactivity to different bacterial species due to shared epitopes. T7-1 likely recognizes additional bacterial antigens as it reacted to luminal antigens with high efficiency (Fig. 3A). CT6 may also recognize other bacterial species as vancomycin reduced CT6 response to MA antigen without a corresponding decrease in *H. apodemus* frequency by 16S (Fig. 3B and C). We have also shown previously that CT6 reacted to an uncharacterized *Clostridium* spp. (8). While the extent of bacterial cross-reactivity remains to be defined, these data clearly demonstrate that 4 of our 6 colonic Treg TCRs recognize *Helicobacter* spp. *in vitro*.

We then asked whether these *Helicobacter* spp. could induce TCR-specific Treg cell responses *in vivo*. As expected, transfer of CT2/CT6/CT9/T7-1 TCR⁺ cells into ampicillin-treated mice to eliminate pre-existing *Helicobacter* spp. (Fig. 3C) resulted in little proliferation or differentiation (Fig. 4A). However, inoculation of ampicillin-treated mice with a single *Helicobacter* spp. elicited robust expansion and Foxp3 upregulation in appropriate TCR⁺ Tg cells (Fig. 4A). Similarly, *Helicobacter*-free mice obtained from Charles River Laboratories showed little ability to induce TCR-dependent proliferation or Treg cell generation, unless the appropriate *Helicobacter* spp. identified *in vitro* (Fig. 3D) was subsequently inoculated *in vivo* (Fig. 4B, S9). Notably, as CT2 and CT6 Tg cells were co-transferred into the same host in this experiment, it appears that these TCR clones show a high degree of specificity for individual *Helicobacter* strains (Fig. 4B). Thus, these data demonstrate that *Helicobacter* spp. are a major driver of bacterial-specific Treg cell responses during homeostasis.

Marked changes in bacterial composition with colitis

The observation that colitis enhanced the *in vitro* stimulatory ability of MA antigen (Fig. 3A), and increased the proliferation of adoptively transferred CT2 and CT6 TCR Tg cells (Fig. 1B), suggested that there might be alterations in the colonic microbiota. We therefore analyzed the changes in the MA and luminal 16S rRNA profile with colitis. Consistent with previous reports, the bacterial composition in the lumen versus mucosa is quite distinct (Fig. 5A–C) (26, 27). In the MA preparation, we noted a high frequency of *Helicobacter* spp. in

both control and colitic mice (Fig. 5B), consistent with the activation and differentiation of CT2 and CT6 Tg cells *in vivo* under both conditions (Fig. 1). The frequency of *H. typhlonius* was further increased in colitic mice (Fig. 5D and E), which may explain the increased *in vitro* MA antigen reactivity of CT2 and CT9 Treg TCRs with colitis (Fig. 3A). In the lumen, besides the increase in *H. typhlonius* (Fig. 5D and E), we noted that colitis induced a marked expansion of *Bacteroides* (*B.*) spp., particularly *B. vulgatus*, which represented over 20% of 16S reads in some mice (Fig 5D and F). As there was no obvious decrease in bacterial density (fig. S10), these data suggest that DSS+ α IL10R colitis is associated with a major bloom of *Bacteroides* spp. in the lumen.

Lack of T cell responses to the bloom of luminal *Bacteroides* species during colitis

As *Bacteroides* species have been suggested to be important triggers of intestinal inflammation in other murine models (28, 29), we asked whether the dramatic expansion of *Bacteroides* species in DSS+ α IL10R treated mice induced strong anti-microbial effector T cell responses. We assessed the *in vivo* responses of several TCRs that recognize *Bacteroides* strains isolated from DSS+ α IL10R mice *in vitro*, including the *B. vulgatus*-reactive TCR DP1 (Fig. 6A, table S2). These TCRs recognized *Bacteroides* strains through MHC II and did not respond *in vitro* to the *Helicobacter* strains tested (fig. S11A and B). To our surprise, adoptively transferred T cells expressing these *Bacteroides*-reactive TCRs did not show increased proliferation, expansion, or Teff cell development in DSS+ α IL10R treated mice compared with controls (Fig. 6B and C). Consistent with the poor expansion, very few transferred cells were recovered from the colon. In fact, the percentage of the transferred TCR-expressing cells amongst total CD4⁺ T cells was reduced during DSS + α IL10R colitis (Fig. 6C), implying that these antigens are relatively less important during colitis despite their bloom in the lumen. We confirmed *in vitro* that the luminal antigens for DP1 and NT2 increased with colitis (Fig. 6D), as predicted based on 16S rRNA sequencing (Fig. 5F). By contrast, MA antigen showed lower stimulatory ability than luminal antigen for DP1 and NT2 even during colitis (Fig. 6D). We also checked that the *Helicobacter* spp.-reactive TCRs (Fig. 3D) did not respond to these *Bacteroides* strains (fig. S11C). Although it remains possible that these *Bacteroides*-reactive TCR clones do not compete well in the gut or are not representative of polyclonal T cell responses to *Bacteroides*, these data suggest that the marked expansion of luminal *Bacteroides* spp. in this model of colitis does not elicit a corresponding increase in antigen-specific T cell responses.

Pathogenic potential of *Helicobacter*-reactive TCRs

The differentiation of colonic Treg TCRs into Teff cells during colitis (Fig. 1) suggests that the altered T cell response to bacterial antigens may contribute to colonic inflammation. To test the pathogenic potential of these *Helicobacter* spp.-reactive TCRs, we adoptively transferred naïve CT6 Tg cells into *Rag1*^{-/-} hosts with or without *H. apodemus* inoculation. In contrast with wild-type hosts (Fig. 4), *H. apodemus* drove antigen-specific Teff generation of CT6 Tg cells (Fig. 7A, S12) in *Rag1*^{-/-} hosts likely due to expansion in a lymphopenic environment. In line with a Teff response, we observed colonic inflammation in CT6-transferred hosts only in the presence of *H. apodemus* as evidenced by increased colon weight/length ratio and crypt dropout in histology (Fig. 7B and C). Similarly, Teff response of CT2 Tg cells to *H. typhlonius* lead to colonic inflammation in *Rag1*^{-/-} hosts (fig. S13).

We did not note major weight loss induced by these TCR Tg cells and/or *Helicobacter* spp. during the time frame of this experiment (fig. S13D and E), in contrast with polyclonal T cell transfer models (30). This could be due to limited systemic effects from a single TCR clone or differences in gut microbiota composition. Although *Helicobacter* spp. without T cell transfers have been reported to induce colitis in Rag-deficient mice (31–34), we did not observe inflammation caused by these two strains during the period of the experiment (Fig. 7, S13). Thus, these data demonstrate the potential pathological consequences of antigen-specific Teff expansion against gut bacteria.

Finally, mucosal associated enterohepatic *Helicobacter* spp. in humans have been suggested to be associated with IBD (35). We have also detected the presence of *Helicobacter* spp. in patients with Crohn's disease (CD) or ulcerative colitis (UC) by 16S rRNA sequencing of fecal or colonoscopy samples (fig. S14). However, it remains to be established whether these *Helicobacter* spp. play a similar immunomodulatory role in humans as seen in our mouse studies.

Discussion

Based on this study of T cell responses to commensal antigens during experimental colitis, we make the following observations: First, effector T cell responses during colitis are often to commensal antigens normally presented to the immune system during homeostasis, and not to antigens normally excluded by the mucosal barrier. Second, commensal bacterial species vary widely in their ability to induce T cell responses due in part to their anatomic location relative to the mucosal surfaces. Third, constitutively presented antigens appear to primarily elicit Treg cell responses during homeostasis, implying that tolerance to these antigens is important to preventing colitis. Finally, *Helicobacter* spp. are important inducers of T cell responses during homeostasis and colitis. Thus, these data suggest a model whereby Treg cell-mediated tolerance, and not effector cell mediated immunity, is required against commensal bacteria such as *Helicobacter* spp. that routinely interact with the host immune system.

Our initial goal was to study the T cell response to alterations in gut microbiota structure, a.k.a dysbiosis, that occurs with colitis and inflammatory bowel disease (IBD) and has been proposed to be involved in disease pathogenesis (4, 36, 37). However, rather than observing a dominant effector T cell response to *Bacteroides* spp. that bloom during colitis, the major stimulators were *Helicobacter* spp. that routinely interact with T cells during homeostasis. These data do not exclude the possibility that dysbiosis of *Bacteroides* spp. may exert non-antigen dependent effects on T cell immunity through changes in short chain fatty acids (38–40), polysaccharides (41), and aryl hydrocarbon receptor ligands (42). Furthermore, as our studies were done in specific-pathogen-free mice, variability in gut flora composition as well as strain-level differences could affect the functional outcome of specific bacteria. Nonetheless, these data suggest that dysbiosis during intestinal inflammation may not provide an important antigen-specific trigger of effector responses that contribute to colitis.

The relative inefficiency of *Bacteroides* spp. to induce T cell activation is correlated with their bacterial distribution, as they are preferentially found in the lumen. By contrast,

mucosal associated *Helicobacter* spp. were potent stimulators of T cell response. This is consistent with a recent study of SFB showing that tight attachment to the intestinal epithelium plays an important role in eliciting Th17 cells (43). Thus, understanding the adaptive immune response to commensal bacteria may be facilitated by analysis of mucosal- or epithelial-associated, and not luminal, bacteria.

The recognition that *Helicobacter* spp. are important inducers of antigen-specific Treg cells during homeostasis is consistent with a previous study (44), and suggests that our current notion of Treg inducing bacteria as being “good bacteria” that are protective against colitis may be incomplete (7, 45). In fact, *Helicobacter* spp. are widely prevalent in mice colonies around the world (46), and have often been thought of as pathobionts based on their ability to induce or enhance colitis in lymphopenic mice (32–34) or mice deficient in critical immuno-regulators such as IL-10 (31, 47, 48). To reconcile these different observations regarding *Helicobacter* spp., our data suggest that T cell development is context-dependent, being tolerogenic during homeostasis, and inflammatory during lymphopenia or experimental colitis.

A final implication of this study is that T cell-mediated intestinal inflammation may be driven by bacteria that are always in contact with the adaptive immune system, rather than species that are mostly excluded from being presented to T cells during homeostasis. This contrasts with a previous study of CBir1 TCR Tg cells, which recognize a commensal bacteria-expressed flagellin (49) that is presented to the immune system primarily during inflammation (10). In this case, the CBir1 antigen is normally not seen by the immune system and might be considered a pathogen when seen in the context of inflammation. However, in the DSS+ α IL10R model of colitis, this type of immune response to commensal antigens appears to be less relevant. Thus, our studies bring up an interesting question, that is, to what extent is the human intestinal inflammatory T cell response directed towards commensal antigens that the host is commonly in contact with, i.e. akin to “self” antigens, or commensal antigens that are only presented during injury, i.e. “foreign” antigens.

Materials and Methods

Study design

Animal experiments were performed in a specific-pathogen-free facility in accordance with the guidelines of the Institutional Animal Care and Use Committee at Washington University. Host mice were housed together and interbred to maintain microbial integrity. Gender-matched littermate host mice were used for all comparisons. Cages were randomly assigned into different treatment groups. Both male and female mice were used. All *in vivo* and *in vitro* experiments were performed independently at least two times unless otherwise stated.

Mice

CT2, CT6 (8, 9), and DP1 Tg mice were generated as described (50), and bred to *Rag1*^{-/-} (Jackson Labs (JAX) #002216), *Foxp3*^{IREG-GFP} (JAX #006772) or *Foxp3*^{IREG-Thy1.1} (51), and/or *IL-17A*^{IREG-GFP} (JAX #18472), and *IFN γ* ^{IREG-YFP} (GREAT) (52). TCR β

Foxp3^{ΔRES-Thy1.1} *TCRα*^{+/-} (53) and TClI *TCRαβ* (54) *Foxp3*^{ΔRES-Thy1.1} *Rag1*^{-/-} mice were previously described. *IFNγ*^{ΔRES-YFP} mice were a gift from Richard Locksley (UCSF); *Foxp3*^{ΔRES-Thy1.1} mice were a gift from Alexander Rudensky (MSKCC). Experiments in Fig. 4B used Ly5.1 mice from Charles River Laboratories as hosts; experiments in fig. S6 used Ly5.2 mice inbred in our colony as hosts; all other in vivo experiments used *Foxp3*^{ΔRES-GFP} CD45.1 (JAX #006772) mice. All mice were on a C57BL/6 genetic background.

Reagents, antibodies, and flow cytometry

Anti-IL-10R blocking antibody (BE0050) and isotype IgG1 (BE0088), anti-MHC II antibody (BE0108) were purchased from BioXCell. Fluorescently conjugated antibodies were purchased from Biolegend, eBioscience, and Becton Dickinson. Samples were analyzed using a FACSARIA IIu (Becton Dickinson) and data were processed with FlowJo (Treestar).

Cell isolation from the colonic *lamina propria*

Cells were isolated as described (9). Colonic segments were treated with RPMI medium containing 3% FBS and 20 mM HEPES (HyClone) with DTT (Sigma) and EDTA (Thermo Fisher) for 20 mins at 37°C with constant stirring. Tissue was further digested with 28.3 μg/ml liberase TL (Roche) and 200 μg/ml DNase I (Roche), with continuous stirring at 37°C for 30 min. Digested tissue was forced through a Collector tissue sieve (Bellco Glass) and passed through a 40 μm cell strainer.

T-cell hybridoma stimulation assay

T cell hybridoma cells expressing GFP under NFAT promoter (55) were retrovirally transduced with *TCRα* chains of interest as previously described (8). Hybridoma cells (1.5×10^4) were cultured with flt3-ligand-elicited CD11c⁺ dendritic cells (5×10^4) with and without the indicated antigen preparations (20 μg/well) in flat-bottomed 96-well plates. CD4⁺TCRβ⁺ cells were analyzed for GFP expression after 1.5 days by flow cytometry.

Antigen preparations

Total colonic lumen contents were collected from longitudinally opened colon and caecum, diluted with PBS (HyClone), vortexed, filtered through a 40 μm strainer, and autoclaved. For MA preparation, lumen contents were removed with forceps and the remaining tissue rinsed with PBS. Mucosal associated particles were released from colonic tissues using PBS with DTT (1 μM/ml) and EDTA (5 μM/ml) for 20 min at 37°C with constant stirring, followed by PBS with EDTA (2 μM/ml) for 3 times. From each of these steps, mucosal associated particles were filtered through a 40 μm strainer. Larger particles such as cells were removed by centrifugation at 1500 rpm for 10 min. The remaining particles in suspension were pelleted at 2500 rpm for 15 min, resuspended in PBS, then autoclaved. For bacterial isolates, *in vitro* cultures were pelleted at 2500 rpm for 15 min, washed twice with PBS, resuspended in PBS, and autoclaved for 45 min.

Adoptive transfer experiments

For experiments with CT2, CT6, or DP1 Tg cells, naïve T cells were FACS purified ($CD4^+ Foxp3^- CD25^- CD44^{lo} CD62L^{hi}$) from peripheral lymph nodes and spleen of $CD45.2 Foxp3^{IRES-GFP} Rag1^{-/-}$ or $Foxp3^{IRES-Thy1.1} IL-17A^{IRES-GFP} IFN\gamma^{IRES-YFP} Rag1^{-/-}$ TCR Tg mice. 10^5 naïve TCR Tg were injected retro-orbitally into congenic $CD45.1 Foxp3^{IRES-GFP}$ mice. In some experiments, cells were first labeled with Cell Trace Violet (Thermo Fisher) before injection. For experiments using TCRs CT7, CT9, NT2, or T7-1, naïve T cells ($CD4^+ Foxp3^- CD25^- CD44^{lo} CD62L^{hi} V\alpha 2^-$) were FACS purified from $CD45.2 Tcli TCR\alpha\beta Foxp3^{IRES-Thy1.1}$ Tg mice ($Rag1^{+/-}$ or $Rag1^{-/-}$), and activated *in vitro* with soluble anti-CD3 (0.1 μ g/ml; 145-2C11; Bio X Cell) and anti-CD28 (1 μ g/ml; 37.51; Bio X Cell) in tissue culture plates coated with rabbit antibody to hamster IgG (127-005-099; Jackson ImmunoResearch) in the presence of anti-cytokine antibodies from Bio X Cell (Cat #): anti-TGF- β (20 μ g/ml, BE0057), anti-IFN- γ (5 μ g/ml, BE0054) anti-IL-4 (5 μ g/ml, BE0045), and anti-IL-12 (5 μ g/ml, BE0052). One day after activation, cells were retrovirally transduced with individual TCR α chain of interest (8). 2×10^5 total cells (both transduced and non-transduced) were transferred into congenic $CD45.1 Foxp3^{IRES-GFP}$ mice.

The colon lamina propria and distal mesenteric lymph node were harvested at indicated times after transfer and analyzed by flow cytometry. Transferred TCR $^+$ cells were identified as $CD4^+ CD45.2^+ CD45.1^- V\beta 6^+ V\alpha 2^+$ (CT2/CT6/CT7/CT9/DP1/NT2) or $CD4^+ CD45.2^+ CD45.1^- V\beta 6^+ GFP^+$ (T7-1).

Antibiotic treatment

Littermates were divided and treated at 3 weeks of age. Vancomycin (0.5 mg/ml), ampicillin (1 mg/ml), and neomycin (1 mg/ml) were administered ad libitum via drinking water continuously for the time indicated. Metronidazole (1 mg/ml) was administered by oral gavage every day for 1 week followed by gavage every other day (56).

Bacterial isolation, culture, and inoculation

Colonic lumen contents from DSS+ α IL10R treated mice were homogenized and serial dilutions were plated on brain-heart-infusion (BHI, BD Difco) agar supplemented with 10% (v/v) defibrinated horse blood (Colorado Serum Co.). Plates were grown for 3 days at 37°C under anaerobic conditions (5% H₂, 20% CO₂, and 75% N₂) in a Coy chamber. 20 colonies were picked and 16S rRNA gene was sequenced. Colonies matched to *B. vulgatus*, *B. acidifaciens*, *B. uniformis* were cultured in BHI medium (EMD) supplemented with 5 g/L yeast extract, 0.5 g/L L-cysteine-HCl, 1 mg/L Vitamin K3, 1.2 mg/L Hematin (all Sigma), and used for T-cell hybridoma assay. *H. typhlonius* (MIT 97-6810) and *H. apodemus* (MIT 03-7007) strains were cultured on Columbia agar (Oxoid) plate supplemented with 7% (v/v) defibrinated horse blood at 37°C under microaerobic conditions in a vented jar (Becton Dickinson) containing N₂, H₂ and CO₂ (75:5:20) for 5 days; bacteria were collected into Brucella broth (BD Difco) and used for T-cell hybridoma assay or mice inoculation. For *Helicobacter* spp. inoculation studies, approximately 2×10^8 CFU bacteria were gavaged into indicated mice every other day for a total of 3 times.

TCR sequencing

Tnaive (CD44^{lo} CD62^{hi}), Treg (Foxp3⁺), and Teff (CD44^{hi} CD62L^{lo}) CD4 T cells were sorted from TCR β *Foxp3*^{ires-Thy1.1} *TCR α* ^{+/-} mice using BD FACSARIA IIu. TCR α cDNA synthesis from purified cells was performed as described (57). A two-step PCR was used to amplify multiple TRAV genes (58) (multiplex PCR, table S1). Amplicons were purified after each PCR reaction using the Agencourt AMPure XP magnetic purification system. The ~200–600 bp amplicons were quantified using Qubit dsDNA BR assay kit (Invitrogen) and pooled in equimolar ratios for 250 bp paired end sequencing using Illumina MiSeq at the Washington University Genome Sequencing Center. Sequences were demultiplexed and analyzed using blastn to identify the TCR alpha variable (TRAV) and TCR alpha joining (TRAJ) gene segments using the IMGT database (59). This information was then used to determine the CDR3 sequence. A unique TCR is identified by its TRAV and CDR3 amino acid sequence. Frequency filtering was applied to keep TCRs >0.1% of raw data in at least one sample. TRAVs with a frequency below 1% of the total population in multiplex PCR were excluded to limit variability from low read numbers due to low primer efficiency. The filtered TRAVs accounted for more than 76.2%±8.8% of the total TRAV repertoire. TRAV_CDR3 species frequencies were then multiplied by a correction factor determined by the ratio of TRAV sequences obtained using multiplex versus template switch PCR (fig. S3B) (60).

16S rRNA gene sequencing

Colonic lumen contents or MA preparations were processed for DNA isolation using the ISOLATE Fecal DNA kit (Bioline). For fig. S1E, terminal pellets were homogenized with PBS and prepared for Bacteria FACS sorting and 16S rRNA sequencing as in (61). The V4 region of 16S rRNA gene was PCR amplified using barcoded primer described previously (62), and sequenced using the Illumina Miseq Platform (2×250bp paired-end reads). OTU picking was performed using UPARSE (usearch v.8.0.162) (63), and taxonomy assigned using the uclust method with the Greengenes 13.8 database (QIIME v1.9 (64)).

Colon histology

Mouse colons were collected, luminal contents removed, cut open longitudinally, and pinned and fixed with 10% (vol/vol) formalin. Fixed samples were paraffin-embedded, cut into 5µm sections, and stained with Hematoxylin and Eosin (H&E) by standard procedures. Crypt height was measured from digital photographs of H&E stained colon sections taken with a Nikon ECLIPSE 50i microscope.

Statistical analysis

GraphPad Prism v7, R v3.3.0, and Qiime v1.9 were used for statistical and graphical analysis. Student's t test, Mann-Whitney U test, one-way ANOVA, and two-way repeated-measures ANOVA were used for between-subjects analyses. Benjamini-Hochberg false discovery rate correction was used on Mann-Whitney U calculations for OTU comparisons. Morisita-Horn statistical test was used for TCR repertoire comparison. TCR Renyi diversity profiles were generated as previously indicated (53), using Renyi entropy values with α /order values ranging from 0 (natural logarithm of species richness) through 2 (natural

logarithm of the inverse Simpson index). This includes $\alpha = 1$, which represents the commonly used Shannon entropy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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One-sentence summary

Helicobacter in the intestine induce regulatory T cells during homeostasis and effector T cells during colonic inflammation.

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Context is Critical in IBD

The intestine hosts trillions of commensal microbes; however, exactly how these microbes contribute to a balanced immune response in the intestine is still being explored. Now, Chai *et al.* report that mucosal-associated *Helicobacter* species can trigger either regulatory T cell (T_{reg}) or effector T cell (T_{eff}) activation in mouse intestine, depending on context. T cells specific to the bacteria activated T_{regs} in homeostatic conditions. In contrast, in a mouse model of colitis, *Helicobacter* species induced T_{effs}. These data suggest that a pathobiont such as *Helicobacter* species may induce immune tolerance in homeostatic conditions, but switch to contribute to pathogenesis in the presence of inflammation.

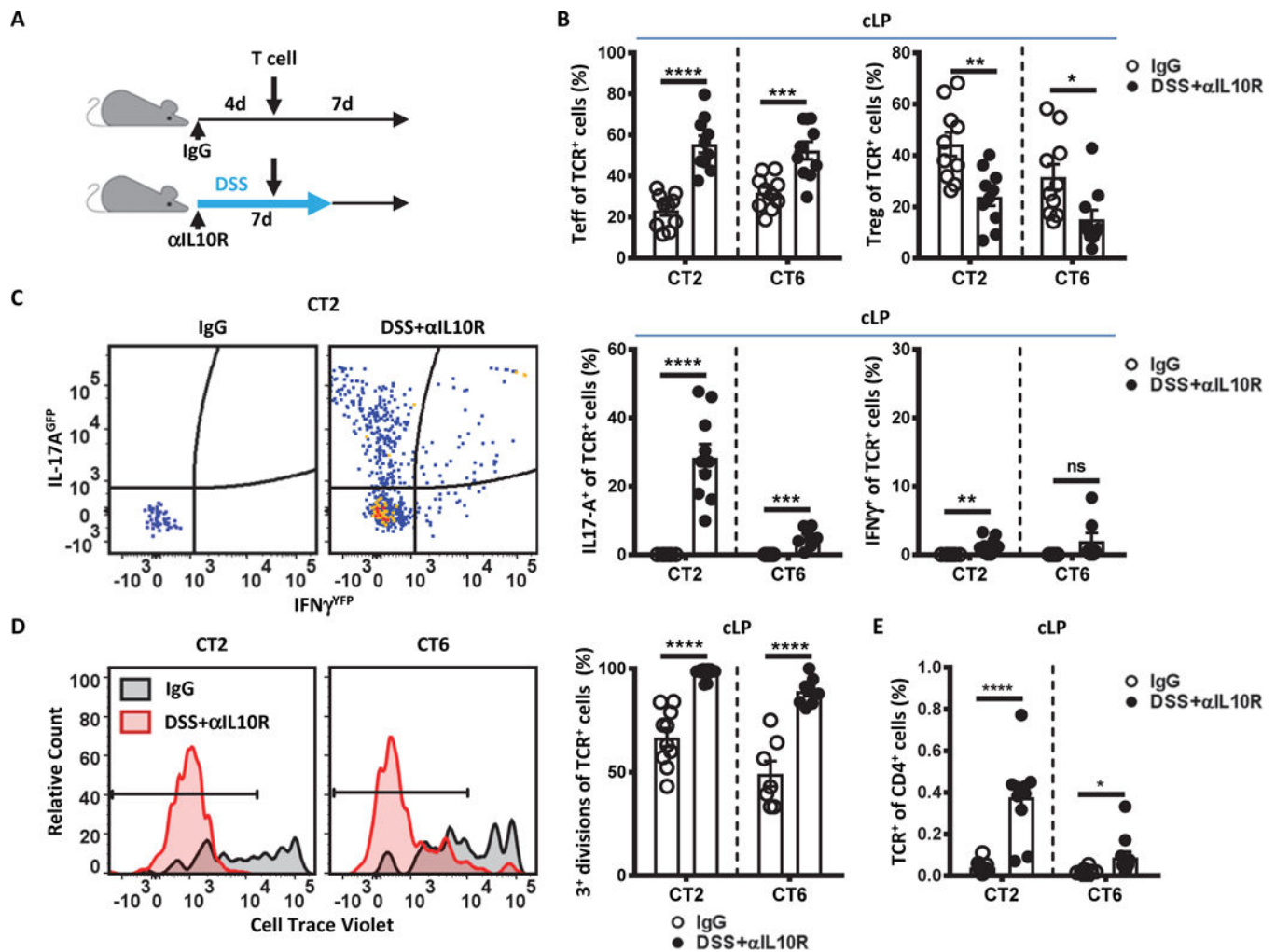


Fig. 1. Colonic Treg TCRs (CT2 and CT6) drive effector T cell development in inflammation
 (A) Experimental model. CD45.1 4–5 week old SPF mice were administered α IL10R (1 mg/mouse) on day 0 and kept on 1% DSS water for 7 days to initiate colitis. Control mice were given isotype IgG (1 mg/mouse) on day 0. Four days after initiation of colitis, congenically marked naïve CT2/CT6 Tg cells (10^5) were intravenously transferred and analyzed 7 days later. (B to D) Effector cell induction and expansion with colitis. Transferred TCR Tg cells from the colon lamina propria (cLP) were analyzed by flow cytometry for (B) development of Teff (CD44^{hi} CD62L^{lo} Foxp3⁻) and Treg (Foxp3⁺) makers ($p=0.000003$; 0.0007 ; 0.0024 ; 0.0157 ; $n=10$; Student's t-test); (C) upregulation of cytokines using IL-17A^{GFP} and IFN γ ^{YFP} ($p=0.000001$; 0.0007 ; 0.0029 ; $n=10$ for CT2; $n=7$ for CT6; Student's t-test); (D) proliferation indicated by Cell Trace Violet (CTV) dilution ($p=0.000002$; 0.00002 ; $n=10$ for CT2; $n=7, 8$ for CT6; Student's t-test); and (E) expansion indicated by the *in vivo* frequency amongst the host CD4⁺ T cells ($p=0.00005$; 0.0275 ; $n=10$ for CT2; $n=10, 11$ for CT6; Student's t-test). Bars indicate mean \pm SEM. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

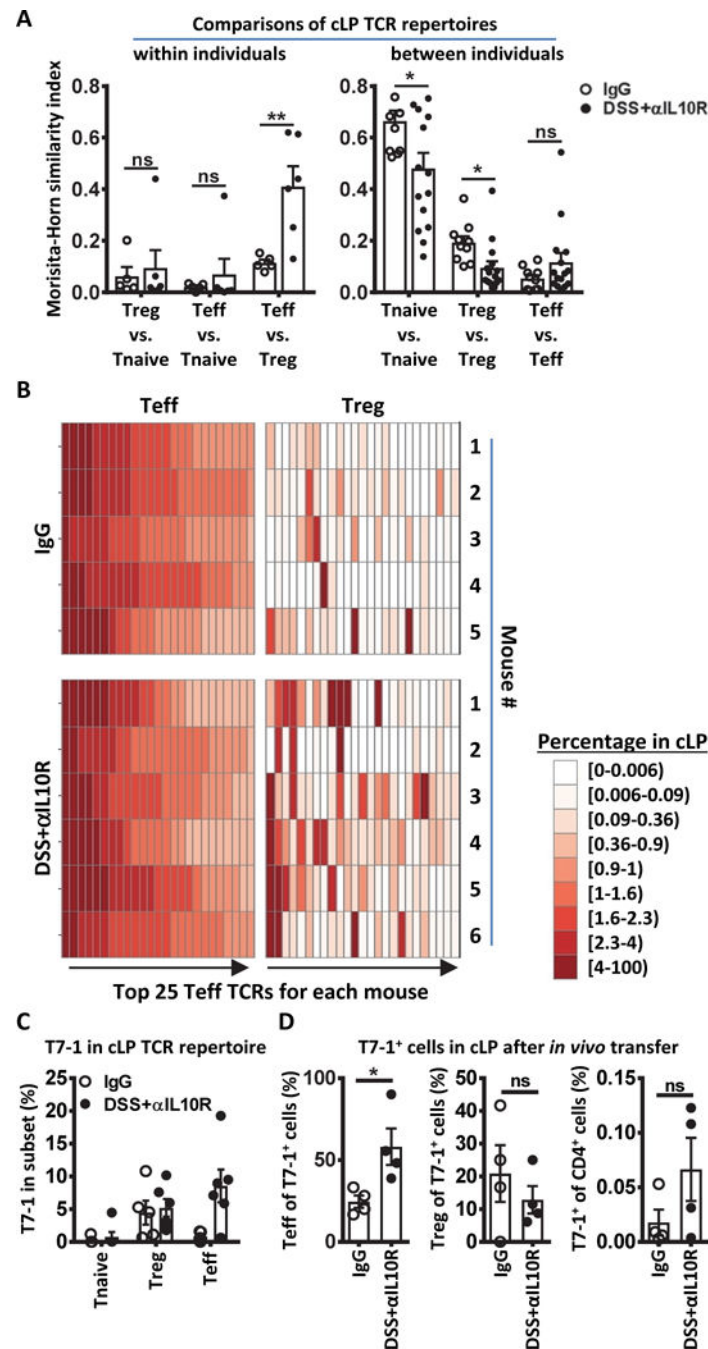


Fig. 2. Treg and Teff subsets show increased TCR overlap during colitis
(A to C) Analysis of TCRα repertoires from Tnaive (CD44^{lo} CD62^{hi}), Treg (Foxp3⁺), and Teff (CD44^{hi} CD62L^{lo}) cells in the cLP of TCRβ *Foxp3*^{ΔRES-Thy1.1} *Tcrα*^{+/-} mice 2 weeks after initiation of IgG or DSS+αIL10R. n=5, 6. (A) Morisita-Horn similarity comparison between two different T cell subsets within each mouse (left) or between different mice within each T cell subset (right). An index value of 1 indicates that the two samples are completely similar and an index value of 0 means they are completely dissimilar (left: p=0.009; n=5,6; right: p=0.033; 0.014; n=10,15; Student's t-test). (B) Heatmap

showing the top 25 Teff TCRs in one mouse per row and their corresponding percentage in the Treg subset. Note that each column does not represent one TCR across all mice. (C) Percentage of T7-1 TCR in repertoire of Tnaive, Treg, and Teff subsets. Each dot represents data from an individual mouse. (D) *In vivo* analysis of T7-1 TCR. T7-1 was retrovirally transduced into *in vitro* activated naïve TClβ *Rag1*^{-/-} Tg cells and 2×10⁵ cells were transferred into IgG or DSS+αIL10R hosts as indicated in Fig. 1A. Seven days post-transfer, cells from the cLP were analyzed by flow cytometry for Teff and Treg cells, and the *in vivo* frequency amongst the host CD4⁺ T cells ($p=0.029$; $n=4$; Student's t-test). Bars indicate mean ± SEM. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

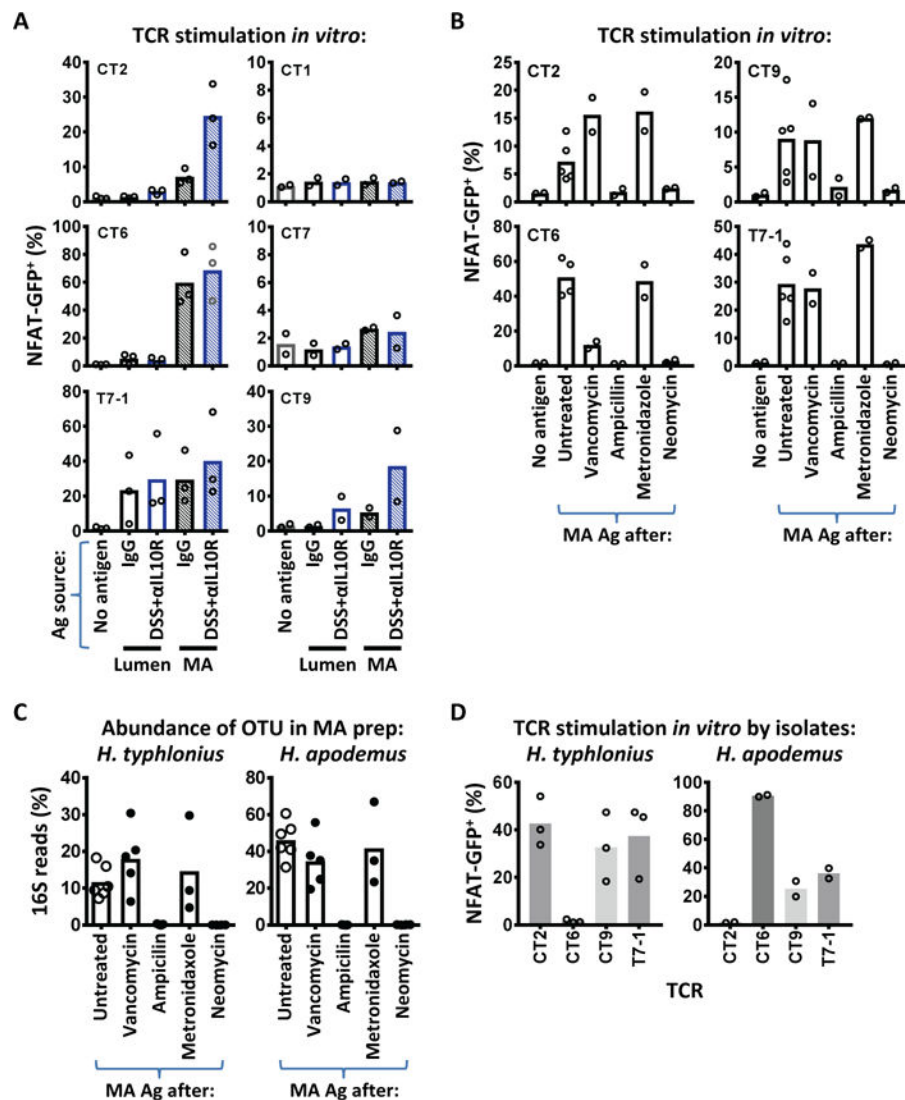


Fig. 3. Colonic Treg TCRs react to mucosal-associated *Helicobacter* species

(A) Treg TCRs preferentially react to mucosal-associated antigens (Ag). Hybridoma cells expressing different TCRs were cultured with CD11c⁺ dendritic cells and the indicated Ag obtained 2 weeks after initiation of colitis. NFAT-GFP upregulation was assessed by flow cytometry 1.5 days later. (B) Selective elimination of MA Ag using individual antibiotics. TCRs from (A) that react to MA Ag were stimulated with colonic MA Ags isolated from antibiotic-treated or untreated mice as per (A). (C) Changes in *H. typhlonius* and *H. apodemus* OTUs correlate with *in vitro* reactivity to MA Ag in (B). Data shown are the percentage of 16S OTUs from the MA preparations of individual antibiotic-treated or untreated mice. (D) *In vitro* recognition of *H. typhlonius* or *apodemus*. Cultured isolates were tested for TCR reactivity *in vitro* as per (A). 2–3 independent experiments. Bars indicate mean.

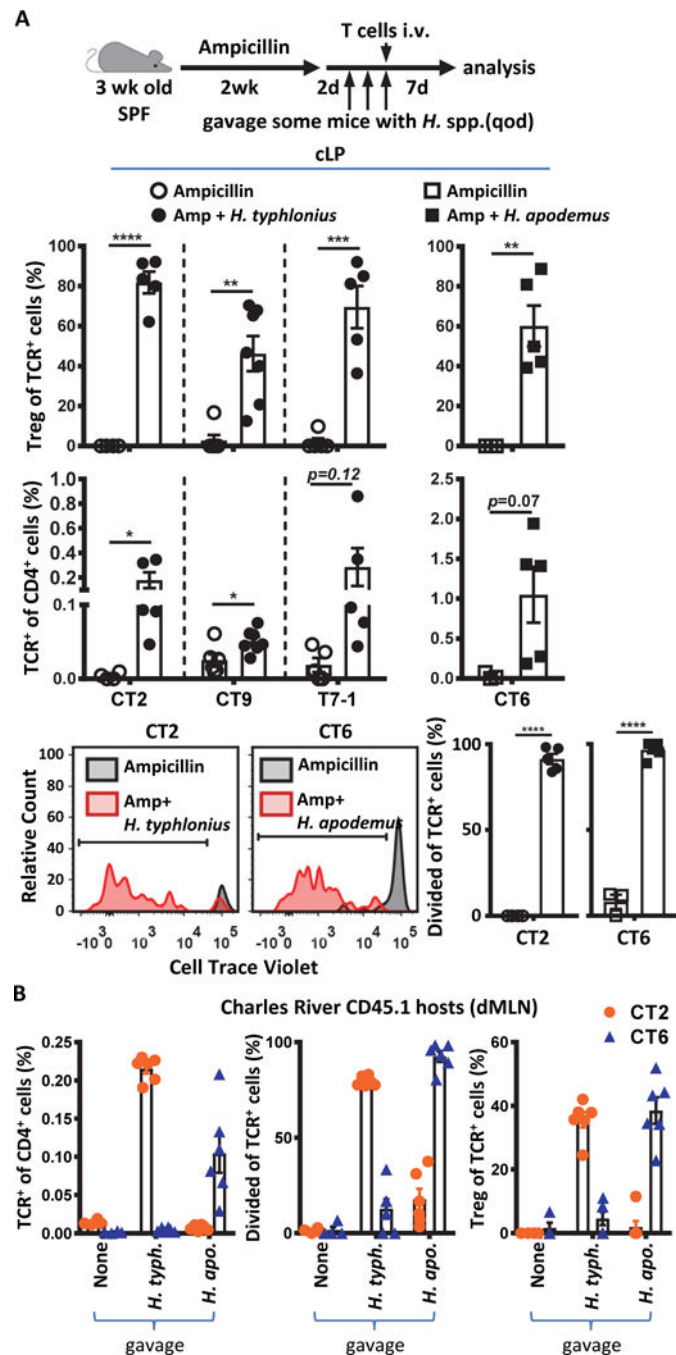


Fig. 4.
***Helicobacter* species induce peripheral Treg cell differentiation during homeostasis.** (A) *In vivo* validation of TCR reactivity to *Helicobacter* species. 3 week old SPF mice were treated with ampicillin (amp) for 2 weeks via drinking water. Two days after the last treatment, *H. typhlonius* or *H. apodemus* were gavaged 3 times total every other day. With the last gavage, congenically marked naïve CT2/CT6 Tg cells or retrovirally expressed CT9/T7-1 cells were transferred. Seven days post transfer, cLP cells were analyzed by flow cytometry for (top) development of Treg (Foxp3⁺) cells (p=0.000003; 0.001; 0.0002; 0.0046;

n=4, 5 for CT2; n=6, 7 for CT9; n=5 for T7-1; n=3, 5 for CT6; Student's t-test); (middle) frequency of transferred TCR-expressing cells amongst the host CD4⁺ T cells (p=0.0442; 0.021; 0.0046;0.0705; n=4, 5 for CT2; n=6, 7 for CT9; n=5 for T7-1; n=3, 5 for CT6; Student's t-test); or (bottom) CTV dilution (p=0.00000002; 0.0000007; n=4, 5 for CT2; n=3, 5 for CT6; Student's t-test). (B) T cell response to *Helicobacter in vivo* is species-specific. Three week old SPF mice obtained from Charles River Laboratories were gavaged with *H. typhlonius* or *H. apodemus* (3 times total every other day). With the last gavage, congenically marked naïve CT2 and CT6 Tg cells (10⁵ each) were co-transferred. One week post-transfer, cells from the dMLN were analyzed by flow cytometry for the frequency of transferred TCR-expressing cells amongst the host CD4⁺ T cells (left); CTV dilution (middle); or development of Treg (Foxp3⁺) cells (right) (n=4, 6, 6). Bars indicate mean ± SEM. **p* < .05, ***p* < .01, ****p* < .001, *****p* < .0001.

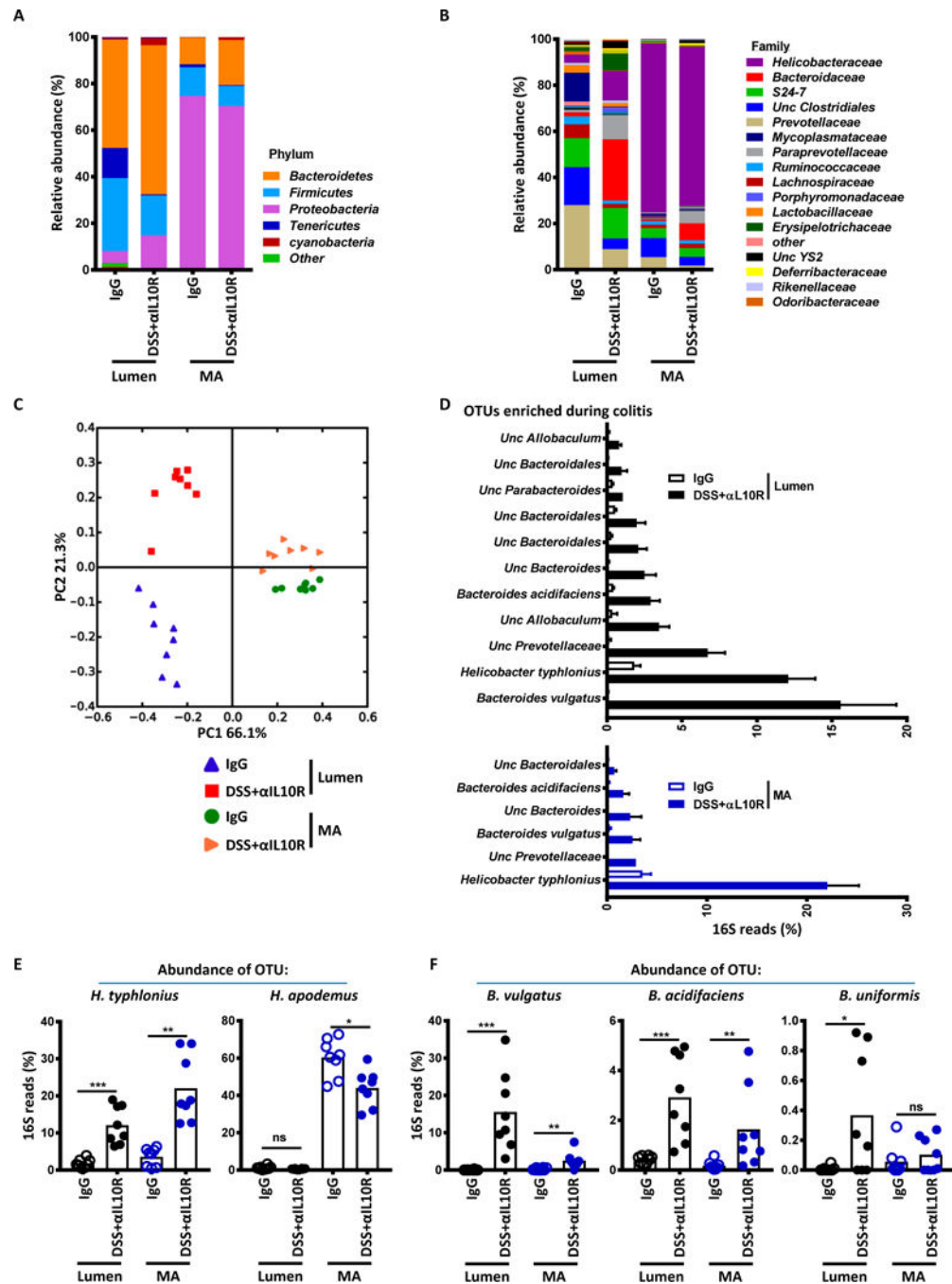


Fig. 5. DSS+αIL10R colitis is associated with differential changes of bacterial composition in the lumen and mucosa

(A to D) 16S rRNA sequencing of colonic lumen contents or MA preparations 2 weeks after initiation of IgG or DSS+αIL10R. $n=8$. Data shown are mean bacterial changes at the phyla (A) or family (B) level, and principal coordinates analysis on unweighted UniFrac distances (C). (D) Bacteria enriched during colitis. Shown are OTUs enriched in DSS+αIL10R mice with average percentage >1% and Benjamini-Hochberg adjusted p value <0.05 (Mann-Whitney U) from lumen contents or MA preparations. (E) Increase of *H. typhlonius* in the mucosa with colitis. Percentages of *H. typhlonius* and *H. apodemus* OTUs are shown

(Benjamini-Hochberg adjusted p value: $p=0.0009$; 0.0023 ; 0.0277 ; $n=8$; Mann-Whitney U test). (F) Marked expansion of *Bacteroides* species in the lumen with colitis. Percentages of *B. vulgatus*, *B. acidifaciens*, and *B. uniformis* OTUs are shown (Benjamini-Hochberg $p=0.0009$; 0.0086 ; 0.0009 ; 0.0086 ; 0.0266 ; $n=8$; Mann-Whitney U test). Bars indicate mean \pm SEM. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

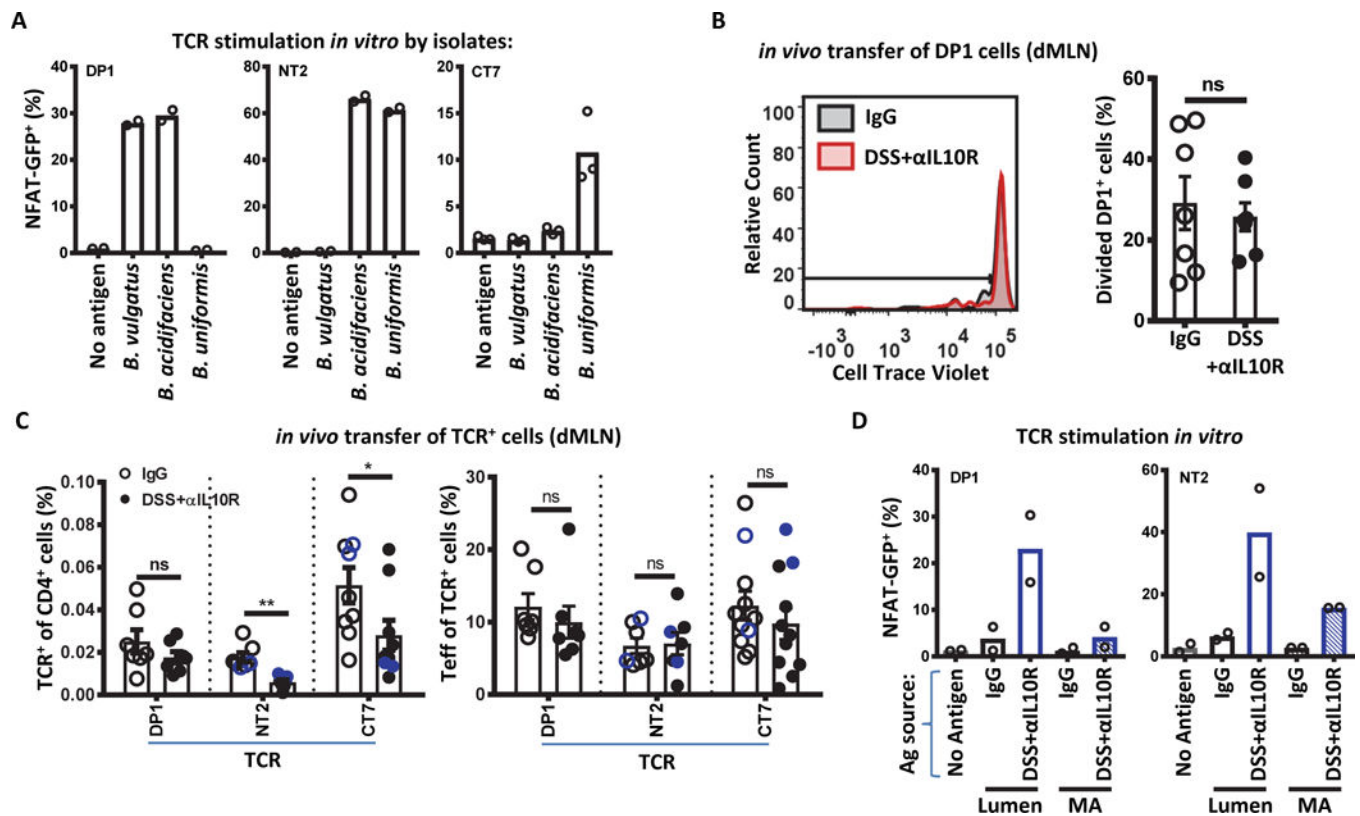


Fig. 6. Expansion of *Bacteroides* species during colitis does not enhance TCR-specific T cell responses

(A) Antigenic reactivity of *Bacteroides*-reactive TCRs used. *In vitro* stimulation by the *Bacteroides* isolates are shown as per Fig. 3A. 2–3 independent experiments. (B and C) *In vivo* expansion and effector cell development of *Bacteroides*-reactive T cells. Congenically marked naïve DP1 Tg cells (10^5) were transferred into CD45.1 hosts as indicated in Fig. 1A, and analyzed 7 days post-transfer by flow cytometry for (B) CTV dilution in the dMLN ($n=7$), (C, left) frequency amongst the host CD4⁺ T cells ($p=0.0005$; 0.047 ; $n=7$ for DP1 and NT2; $n=9$ for CT7; Student's t-test), and (C, right) development of Teff (CD44^{hi} CD62L^{lo} Foxp3⁻) ($n=7$ for DP1 and NT2; $n=11$ for CT7; Student's t-test). NT2 and CT7 (C) were retrovirally transduced into *in vitro* activated naïve V α 2⁻ TCR β Tg cells from Rag1^{+/-} (black) or Rag1^{-/-} (blue) mice before transfer of 2×10^5 cells. (D) *In vitro* reactivity to *in vivo* Ag preparations is consistent with *Bacteroides* expansion by 16S rRNA analysis. Colonic lumen contents or MA preparations from IgG or DSS+ α IL10R mice were tested as per Fig. 3A. 2 independent experiments. Bars indicate mean \pm SEM. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

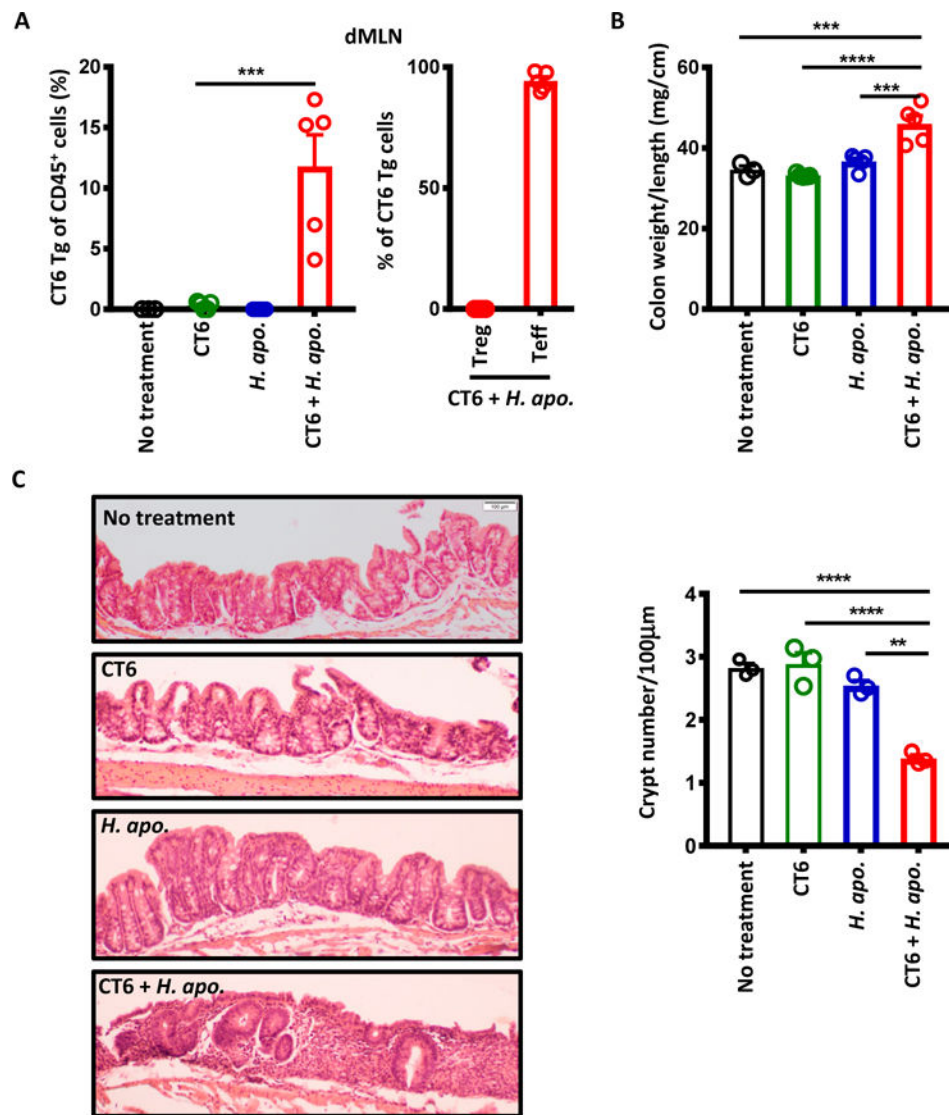


Fig. 7. Pathogenic potential of naive *Helicobacter*-reactive CT6 cells in lymphopenic mice
Six week old *Rag1*^{-/-} mice were given *H. apo.* with or without naive CT6 T cell transfer. *H. apo.* was gavaged 3 times total every other day. Naive CT6 cells (10⁵) was transferred at the time of last gavage. (A) Flow cytometry analysis for the frequency of Tg cells amongst the host CD45⁺ cells (one-way ANOVA, Turkey's post-hoc test: $p=0.0002$ CT6 vs CT6+*H. apo.*; $n=3, 5, 5, 5$), and development of Treg (Foxp3⁺) and Teff (CD44^{hi} CD62L^{lo} Foxp3⁻) markers ($n=5$) in dMLN at 6 weeks. (B) Colon weight/length ratio at 6 weeks (one-way ANOVA with Turkey's post-hoc test: $p=0.0003$ No treatment vs CT6+*H. apo.*; 0.00002 CT6 vs CT6+*H. apo.*; 0.0005 *H. apo.* vs CT6+*H. apo.*; $n=3, 5, 5, 5$). (C) Representative haematoxylin/eosin-stained section of the ascending colon at 6 weeks (original magnification $\times 10$), and quantification of crypt number (one-way ANOVA with Tukey's post-hoc test: $p=0.00007$ No treatment vs CT6+*H. apo.*; 0.00005 CT6 vs CT6+*H. apo.*; 0.0003 *H. apo.* vs CT6+*H. apo.*; $n=3$). The number of crypts observed per 100μm of ascending colon was averaged from five fields. Decreased crypt number reflect crypt

dropout due to inflammation. Bars indicate mean \pm SEM. $*p < .05$, $**p < .01$, $***p < .001$, $****p < .0001$.

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