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T cell proliferation and colitis is initiated by defined intestinal microbes

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Abstract

Inflammatory Bowel Disease (IBD) has been associated with the dysregulation of T cells specific to antigens derived from the intestinal microbiota. How microbiota-specific T cells are regulated is not completely clear but is believed to be mediated by a combination of Immunglobulin A (IgA), regulatory T cells (T_{regs}) and Type 3 Innate Lymphoid cells (ILC3s). To test the role of these regulatory components on microbiota-specific T cells, we bred CBir1 TCR transgenic mice (CBir1Tg; specific to flagellin from common intestinal bacteria) onto a lymphopenic Rag1^{-/-} background (CBirRag). Surprisingly, CBirRag T cells could not induce colitis and did not differentiate to become effectors under lymphopenic conditions, despite deficits in immunoregulatory factors such as IgA, T_{regs} and ILC3s. In fact, upon transfer of conventional CBir1Tg T cells into lymphopenic mice, the vast majority of proliferating T cells responded to antigens other than CBir1 flagellin, including those found on other bacteria, such as *Helicobacter spp.* Thus, we discovered a caveat in the CBir1 TCR Tg model within our animal facility that illustrates the limitations of using TCR transgenics at mucosal surfaces, where multiple TCR specificities can respond to the plethora of foreign antigens. Our findings also indicate that T cell specificity to the microbiota alone is not sufficient to induce T cell activation and colitis. Instead, other interrelated factors, such as the composition and ecology of the intestinal microbiota and host access to antigen, are paramount in controlling the activation of microbiota-specific T cell clones.

Introduction

The barrier surfaces of mammals are covered in a vast consortium of microorganisms that together comprises the microbiome. Mammals also possess millions of B and T lymphocytes with variable specificities that include responsiveness to antigens present in the microbial flora. Microbiota-specific T cells have been described as part of the healthy T cell repertoire in both mice and humans but are believed to contribute to the development of Inflammatory Bowel Disease (IBD) under certain contexts (1, 2). Many of the genetic polymorphisms associated with IBD have been shown to directly affect T cells or intestinal barrier function

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and therefore access of intestinal antigens to the host immune system (3). Elucidating the mechanisms that maintain homeostasis among microbiota-specific T cells is critical to our understanding of mucosal immunology and the development of IBD (4, 5).

In the T cell mediated model of colitis, transfer of naïve CD4⁺ T cells into lymphopenic mice leads to colitis associated with large numbers of activated helper T cells (IL-17A⁺; IFN γ ⁺) and a relative absence of regulatory T (T_{reg}) cells. This model requires the intestinal microbiota, and many of the colitogenic T cells in these mice are specific to various intestinal bacteria, including *Helicobacter spp.* and CBir1 flagellin-expressing *Clostridia* (6–13). Presumably, presentation of antigens in the lymphopenic lymph node to transferred naïve CD4⁺ T cells in the absence of immunoregulatory factors leads to the development of colitogenic T cell clones. Regulation of microbiota-specific T cells is believed to be controlled by a variety of interrelated factors, including Interleukin 10 (IL-10), regulatory T cells (T_{regs}), Immunoglobulin A (IgA) and Type 3 Innate Lymphoid Cells (ILC3s), though how these factors work together is not clearly understood (14–17). On a lymphoreplete ‘wild-type’ background, CBir1-specific transgenic (CBirWt) mice do not spontaneously develop colitis, and physical breakdown of the intestine by infection (*Toxoplasma gondii*) or chemical irritation (Dextran Sodium sulfate) is necessary to activate CBir1Tg T cells in such hosts (14, 18). This is in contrast to TCR transgenic cells specific to either segmented filamentous bacteria or *Helicobacter spp.*, where colonization is sufficient to induce spontaneous T cell responses, due to the residence of these organisms directly on the epithelial surface (19–21). However, it is not known whether T cell responses in lymphopenic animals are strictly limited to bacteria that live close to the intestinal surface, as it appears to be in lymphoreplete animals, or whether such responses are broadened to other members of the microbiota.

To test the contributions of these various regulatory factors on a genetically lymphopenic background, we bred CBir1Tg mice to a Rag1^{-/-} background (CBirRag). These mice also allowed us to study a population of CBir1-specific T cells that is not contaminated by alternate specificities produced by rearrangements of the endogenous TCR locus. Surprisingly, CBirRag T cells did not become spontaneously activated *in vivo* nor were they capable of inducing colitis upon transfer to either Rag1^{-/-} or Rag2^{-/-} γ c^{-/-} mice, despite the lack of T_{regs}, IgA and ILC3s. The majority of CBirWt T cells that accumulated in Rag1^{-/-} mice post-transfer did not bind a tetramer bearing the relevant peptide from CBir1 flagellin and were responsive to other intestinal bacteria, such as *Helicobacter spp.*, due to the expression of endogenously rearranged non-transgenic TCR α chains. Taken together, our data uncovers a complication of the CBir1 Tg model of T cell mediated colitis and show that antigen specificity alone is not sufficient for the activation of T cells against the microbiota.

Materials and Methods

Mice

C57BL/6 mice were purchased from Taconic. Rag1^{-/-} and Rag2^{-/-} γ c^{-/-} mice were obtained from Jackson Laboratories. CBir1 Tg mice were produced by Dr. Charles Elson (Univ. Alabama-Birmingham) as described, obtained under an MTA and back-crossed to CD45.1 expressing mice, TCR α ^{-/-} mice, or Rag1^{-/-} mice for at least three generations (14). Smarta

TCR transgenic mice were obtained from Dr. Ronald Germain (NIH/NIAID). In some experiments, mice were given autoclaved drinking water supplemented with either vancomycin (0.5mg/mL, Sigma) or a cocktail of metronidazole (1mg/mL, Sigma), ampicillin (1mg/mL, Sigma), neomycin (1mg/mL, Sigma), and vancomycin (0.5 mg/mL, Sigma). Sucralose (Splenda™) (0.8mg/mL) was added to make the antibiotic-containing water more palatable. Antibiotic treatment was started 1 week prior to adoptive T cell transfer or other uses of the mice. Gender-matched and age-matched mice were used and co-housed whenever possible. We used males and females equally in all experiments with the provision that cell transfers were always carried out within one sex group. Donor and recipient mice for cell transfers and analyses were used at 5–10 weeks of age. All mice were maintained at and all experiments were performed in an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility at the University of Pittsburgh and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Mice were housed in specific pathogen-free (SPF) conditions.

Cell isolation, stimulation, and culture

Cells from spleens, mesenteric lymph nodes (mLN), and colonic lamina propria (cLP) were isolated and stimulated for cytokine production with PMA/Ionomycin (Sigma) in the presence of Brefeldin A (ThermoFisher) as previously described (22).

For *in vitro* culture assays, splenic and LN CD4⁺ T cells were isolated by magnetic purification (StemCell) and stained with 1 μ M CFSE (ThermoFisher) as described previously (23). Fecal bacterial antigens were obtained by boiling fecal pellets for 1 min at 100°C and centrifuging at 12,000 \times g for 5 min to collect the supernatant. CD4⁺ T cells were co-cultured for 96 hours with magnetically purified CD11c⁺ splenic dendritic cells from a CD45.2⁺ congenic C57BL/6NTac mouse and either the cleared fecal lysates or 10 ng/mL CBir1 peptide. Proliferation and activation of CD4⁺ T cells were then assessed via flow cytometry.

Antibodies, tetramers, and flow cytometry

All antibodies used for flow cytometry were purchased from either ThermoFisher, BD Biosciences, or BioLegend. The following antibodies were used to discriminate cell surface or intracellular phenotype: TCR β (H57-597), CD3 (500A2), CD90.2 (53-2.1), CD4 (RM4-5), CD8b (H35-17.2), CD45.1 (A20), CD45.2 (104) CD44 (IM7), IFN- γ (XMG1.2), TNF α (MP6-XT22), IL-17 α (eBio17B7), IL-2R α /CD25 (PC61), V α 2 (B20.1) and Foxp3 (FJK-16S). Dead cells were discriminated in all experiments using LIVE/DEAD fixable dead stain (ThermoFisher). All stains were carried out in media containing anti-CD16/32 blocking antibody (clone 93, ThermoFisher). For intracellular cytokine staining, cells were fixed in BD Cytofix buffer (BD Biosciences) and stained in BD PermWash buffer (BD Biosciences). For Foxp3 staining, cells were fixed and permabilized using the Foxp3/Transcription factor staining buffer set according to the manufacturer's directions (ThermoFisher). APC-conjugated CBir1₄₆₄₋₇₂ tetramers (YSNANILSQ) and PE-conjugated HH1713₁₇₂₋₈₆ and HH1713₂₃₀₋₄₄ tetramers (QESPRIAAAYTIKGA and

GNAYISVLAHYGKNG, respectively) were provided by the NIH Tetramer Core Facility (Atlanta, GA). All tetramer stains were performed at room temperature for 45–60 minutes. All flow cytometry was acquired on an LSRFortessa FACS analyzer, and cell sorting was carried out on a FACS Aria (BD Biosciences).

T cell transfer colitis and Histopathologic assessment

Cells from host spleen and lymph nodes were isolated, and CD4⁺ T cells were enriched via magnetic separation (StemCell) and then sort purified via FACs for naïve CD44^{lo}CD45RB^{hi}CD25⁻ CD4⁺ T cells (>99% purity). Of these, 0.5–1.5 × 10⁶ cells were injected i.v. into groups of Rag1^{-/-} or Rag2^{-/-}γc^{-/-} mice. In some experiments, transferred cells were labelled with CFSE or Cell Trace Violet (ThermoFisher), and recipient mice were sacrificed to assess T cell responses in the spleen, mLN, and cLP at 9–13 days post-transfer. In other experiments, unlabeled naïve CD4⁺ T cells were transferred, recipient mice were weighed weekly to assess colitis and sacrificed after 7–10 weeks post-transfer to assess transferred T cell responses in the spleen, mLN, and cLP. Mice were euthanized if their weights drop below 70% of baseline. Sections of colons were fixed in 10% formalin and paraffin embedded. Slides were stained with hematoxylin and eosin by the University of Pittsburgh Pathology Department, and lymphocyte infiltration and villous structural integrity were evaluated via microscopy.

Statistical analyses

Statistical tests used are indicated in the figure legends. All scatter plots show the mean, and all other graphs show the mean ± SD. All statistical analysis was calculated using Prism software (GraphPad). In experiments where cells were transferred to Rag1^{-/-} mice, the resulting number of isolated T cells varied considerably due to the complex nature of the experiments and therefore a Kruskal-Wallis test was used because the data were non-parametric.

Results

To investigate microbiota-specific T cell development without the influence of IgA, B cells, and secondary TCRs, we generated CBirRag mice. We hypothesized that these mice would develop spontaneous colitis because their T cells would express only a single TCR specific to a common intestinal antigen and develop within a lymphopenic environment. In further support of our hypothesis, recent studies show that CBir1 flagellin may be transported by goblet cell-associated antigen passages early in the pre-weaning period of development (24). In light of these findings, we were surprised to discover that CBirRag mice in our facility develop colitis at a rate that is not significantly greater than littermate non-transgenic Rag1^{-/-} mice (CBirRag 1/49; Rag1^{-/-} 2/48). To help explain the lack of disease, we compared CD4⁺ T cells from spleens, mesenteric lymph nodes (mLN), and colonic lamina propria (cLP) of CBirWt, CBirRag, and Smarta TCR Tg (specific to an antigen-derived from Lymphochoriomeningitis virus, which is absent from the standard murine microbiota (25)) mice. In accord with the lack of disease, these studies revealed almost no T cell activation in CBirRag mice, as measured by the expression of CD44 and the production of the cytokines IFNγ, TNFα and IL-17A (Fig. 1A–D and G).

One explanation for the relative quiescence of CBirRag T cells could be that they develop into T_{regs}. Previous studies have shown that TCR transgenic T cells specific to foreign antigens, such as chicken ovalbumin, do not develop into T_{regs} (26). In contrast, microbiota-specific T cells, including CBir1, can differentiate into peripheral T_{regs} (pT_{regs}) (14, 24, 27). Foxp3⁺CD4⁺ T_{regs} were almost undetectable in the secondary lymphoid tissues of CBirRag mice and present at significantly reduced frequencies in the cLP (Fig. 1E and F). Another possible explanation for the lack of T cell activation in CBirRag mice was T regulatory 1 cells that lack FoxP3 expression and produce IL-10. Analysis of colonic T cells from CBirWt, CBirRag and Smarta mice revealed that fewer CBirRag T cells produce IL-10 than CBirWt or Smarta T cells, indicating that this is unlikely to be preventing T cell activation and colitis (Fig. 1G). Thus, T_{regs}, IgA and T cell-derived IL-10 are not necessary to prevent the spontaneous activation and colitogenic potential of CBir1Tg T cells developing on a lymphopenic Rag^{-/-} background.

It remained possible that CD4⁺ T cells in CBirRag are being prevented from spontaneous activation by either the small population of T_{regs} found in CBirRag mice or other immunoregulatory cells, such as CD8⁺ regulatory T cells (Supplemental Fig. 1A) (28). Thus, we sought to determine if the transfer of purified CD4⁺ CBirRag T cells to lymphopenic hosts will induce disease, as shown previously for CBirWt T cells (8, 13). We transferred FACs-purified naïve CBirWt, CBirRag or Smarta T cells to Rag1^{-/-} mice and monitored colitis development via weight loss. In accordance with previous studies, Rag1^{-/-} mice containing CBirWt T cells started losing weight 3 weeks post-transfer, and histological analysis of the recipient colons showed significant hyperplasia and pathology (Fig. 2A and B). In contrast, Rag1^{-/-} recipients of CBirRag T cells gained weight and showed no obvious pathology (Fig. 2A and B). CBirWt T cells accumulated in >10-fold larger quantities in Rag1^{-/-} recipients and displayed significantly higher expression of IFN γ , TNF α and IL-17A in the cLP compared to CBirRag T cells (Fig. 2C–E). The lack of accumulation among CBirRag T cells was not due to increased conversion into T_{regs} since they were almost undetectable among transferred CBirRag T cells (Fig. 2F). Therefore, despite the fact that both CBirRag and CBirWt T cells express the same TCR, only CBirWt T cells are capable of inducing colitis, implying that responsiveness to CBir1 flagellin alone is not sufficient for colitogenic T cell activation in lymphopenic hosts.

It was possible that CBir1 specific T cells are not becoming spontaneously activated because CBir1 flagellin-bearing bacteria were lost from the microbiota of our mouse colony. To confirm the presence of CBir1 flagellin in our mice, we co-cultured the supernatants from boiled fecal samples from various mouse strains (CBirWt, CBirRag, CBir1Tg x TCR α ^{-/-} (CBirTCR), Rag1^{-/-}) with CBirWt T cells and dendritic cells (DCs). All samples tested were capable of inducing T cell proliferation, confirming the presence of CBir1-bearing bacteria in our colony (Fig. 3A). Furthermore, CBirWt T cells did not proliferate in response to fecal antigens mice treated with vancomycin, which depletes Gram-positive anaerobes (including CBir1-bearing *Clostridia*) (Fig. 3B). A previous study had shown that TCR transgenic T cells on a Rag1^{-/-} background do not proliferate as well as those on a conventional background (29). However, CBirRag T cells proliferate just as well as CBirWt cells and CBirTCR cells in response to CBir1 peptide *in vitro*, indicating that these cells do not have any cell-intrinsic proliferative defects and do not require a second TCR specificity

to respond to CBir1 antigen (Fig. 3C). Therefore, neither the loss of CBir1 flagellin nor proliferative potential amongst CBirRag CD4⁺ T cells could explain their inability to cause colitis.

Another possibility that could explain the selective inability of CBirRag T cells to cause colitis was interactions with Innate Lymphoid cells type 3 (ILC3s). ILC3s have been shown to control the accumulation of CBirWt T cells and are present in Rag1^{-/-} hosts. The lack of a possible secondary TCR α chain may also render CBirRag T cells more susceptible to deletion by ILC3s (15, 16). To test the importance of ILC3s, we transferred CBirRag or CBirWt T cells to ILC3-deficient Rag2^{-/-} γ c^{-/-} mice. Activated CBirRag T cells did not accumulate in significant numbers in the GI tract of Rag2^{-/-} γ c^{-/-} mice nor did they cause colitis as measured by weight loss and histology (Fig. 4A–C). Thus, while ILC3s may delete activated microbiota-specific T cells, they are not the primary regulatory element that prevents CBir1-specific T cell activation in lymphopenic settings.

It remained unclear why CBirWt, but not CBirRag, T cells became activated and induced colitis upon transfer to Rag1^{-/-} mice. One explanation is that re-arrangement of endogenous TCR α chains in CBirWt mice can lead to TCR transgenic T cells that express multiple functional TCRs and that these secondary specificities confer additional functionality. Analysis of CBirWt T cells showed that while all cells express V β 8, associated with the CBir1 TCR, some cells (~4%) co-expressed a secondary V α 2 chain (the CBir1 TCR transgene expresses V α 6), indicating that they exhibit multiple specificities (Supplemental Fig. 1B) (14). Therefore, we sought to measure the TCR expression of CBirWt and CBirRag T cells (with non-transgenic C57BL/6 as controls) using MHC class II tetramers bearing the relevant peptide from CBir1 flagellin. These experiments confirmed that nearly all CBirRag T cells bind the CBir1 tetramer, as re-arrangement of endogenous TCR α loci is not possible in CBirRag mice (Fig. 5A). However, surprisingly, a significant fraction of T cells in CBirWt mice do not express sufficient levels of transgenic CBir1 TCR to bind the tetramer (Fig. 5A and B). Strikingly, splenic CBir1 tetramer-positive cells in CBirWt and CBirRag mice were phenotypically indistinguishable in that they almost completely lacked the expression of V α 2 (as a surrogate for the ability to re-arrange endogenous TCR α chains), high level expression of CD44 and differentiation to FoxP3⁺ T_{regs} (Fig. 5A, C and D). Conversely, the expression of CD44, V α 2 and IL-2R α /FoxP3 was almost solely attributable to CBir1 tetramer negative T cells from CBirWt mice (Fig. 5C and D). Thus, perhaps the difference in the ability to induce colitis was not due to phenotypic differences in CBir1-specific T cells from CBirWt and CBirRag mice but rather due to CBirWt T cells that have re-arranged a second TCR α chain, leading to the potential for novel specificities.

To test the hypothesis that the ability of CBirWt T cells to induce colitis was associated with non-transgenic specificities, we measured the TCR expression of proliferating CBir1Tg T cells in lymphopenic hosts. We transferred CFSE-labeled naïve CBirWt or CBirRag CD4⁺ T cells to Rag1^{-/-} recipients and analyzed the transferred CBir1Tg cells ten days later. Analysis of mLN and spleens (cLPs of CBirRag transferred mice contained no transferred cells) indicated that most CBirWt T cells showed rapid proliferation (complete loss of CFSE), and the majority (~60%) of rapidly proliferating T cells lacked the ability to bind CBir1 tetramer (Fig. 5E and F). This is in contrast to transferred CBirRag T cells, which

maintained CBir1 tetramer binding and exhibited antigen-independent 'slow' proliferation associated with the increased availability of homeostatic cytokines such as IL-7 (Fig. 5E and F) (30). It is possible that the lack of the CBir TCR surface expression is due to cognate T cell activation (with CBir flagellin) and receptor endocytosis. However, overnight culture of CFSE⁻ CBirWt T cells (sorted from Rag1^{-/-} recipient mice) without antigen did not result in restoration of the ability to bind CBir1 tetramer, making cognate antigen-driven TCR down-regulation an unlikely explanation (Supplemental Fig. 2). We tested for downregulation of the CBir1-specific TCR directly by transferring either FACS-sorted tetramer positive or negative CBirWt naïve CD4⁺ T cells to Rag1^{-/-} recipients (Supplemental Fig. 3A). In accord with our *in vitro* experiments, analysis of transferred cells 13 days later revealed minimal re-expression of the CBir1-specific TCRs on cells sorted as tetramer negative (Supplemental Fig. 3B). Further, sorted CBir1 tetramer negative T cells proliferated and accumulated in much greater numbers than their tetramer positive counterparts adding further evidence that these cells dominate the response in lymphopenic hosts. (Supplemental Fig. 3C). Interestingly, in contrast to CBirRag T cells, recipients of CBir1 tetramer positive CBirWt T cells came to be dominated by tetramer negative T cells, indicating that lymphopenic conditions may select for the outgrowth of cells expressing alternative TCR specificities and that such expression may exclude the transgenic V α chain (Supplemental Fig. 3B and C). It should be noted that those CBirWt T cells that maintained expression of the transgenic TCR are much more likely to exhibit 'slow' proliferation further indicating that the expression of a second TCR specificity may favor rapid proliferation (Supplemental Fig. 3C). Surface staining of CFSE⁻ CBir1Wt T cells from Rag1^{-/-} mice revealed no significant expression of TCR β variable regions, aside from the transgenic V β 8.3 (expressed on all transferred cells), negating the possibility that tetramer negative CBirWt T cells are escaping β exclusion to produce complete non-transgenic TCRs or downregulating the TCR altogether (Fig. 5G). Taken together our results support the hypothesis that the dominant proliferating population of CBirWt T cells transferred to Rag1^{-/-} hosts do not respond to CBir1 antigen and instead carry endogenously re-arranged TCR α chains that provide alternate specificities.

To further test if CBir1 flagellin-bearing bacteria were required for the activation of the tetramer negative CBirWt cells, we transferred CBirWt T cells to Rag1^{-/-} mice that had been treated with either vancomycin (previously shown in Figure 3B to deplete CBir1 flagellin⁺ bacteria) or a broad-spectrum collection of antibiotics (metronidazole, ampicillin, neomycin and vancomycin; MANV), which has been shown to reduce intestinal bacteria >100-fold and largely prevent 'rapid' microbiota-driven T cell proliferation and the development of T cell-induced transfer colitis (8, 30, 31). CBirWt T cells isolated from untreated and vancomycin-treated Rag1^{-/-} mice were dominated by rapidly proliferating (CellTrace⁻) T cells in their mLN and colon (Fig. 6A). In contrast, CBirWt T cells transferred to Rag1^{-/-} mice treated with MANV showed significantly fewer rapidly proliferating T cells in their mLN, indicating that the rapid response requires an intact microbiota but not CBir1 flagellin (Fig. 6A) (8, 30). CBirWt T cells that accumulated in the cLP of MANV-treated mice had mostly diluted their CellTrace dye, indicating rapid proliferation, but the reduced numbers (~8–20X) of transferred cells in the cLP compared to both untreated and vancomycin-treated controls indicates the importance of the microbiota

for the accumulation of CD4 T cells (Supplemental Fig. 4). Furthermore, our data show that CBirWt T cell rapid proliferation and accumulation in the colon post-transfer into lymphopenic hosts is driven by the microbiota but does not require the presence of the CBir1 flagellin. In fact, tetramer-positive (CBir1-responsive) CBirWt T cells and CBirRag T cells that can only express the CBir1-specific TCR were at a significant *disadvantage* compared to tetramer-negative cells with regard to proliferation and accumulation in the colon, as is evident from the frequency of CBir tetramer binding cells in the spleen of CBirWt mice compared to those cells only 10 days post-transfer to a Rag1^{-/-} host (Fig. 5A and E). Thus, while specificity against the microbiota may be necessary for colitogenic T cells to induce disease, it is not a sufficient property.

It was still not clear whether the actively proliferating CBirWt T cells were specific to microbiota-derived antigens or responding to microbiota-dependent inflammation, as previous studies have indicated the importance of both (8, 30). Therefore, we looked into whether CBir1 tetramer-negative T cells were specific to antigens derived from other members of the bacterial microbiota. Multiple publications suggest T cells specific to *Helicobacter spp.* may dominate the repertoire of colonic resident microbiota-specific T cells, and *Helicobacter*-specific T cells are sufficient to induce disease in multiple models of colitis (10, 17, 20). Therefore, we stained CBirWt T cells transferred to Rag1^{-/-} mice with a recently described MHC II tetramer specific to *Helicobacter spp.* (32). Despite the limited repertoire of CBirWt T cells, a modest proportion of cLP CBirWt T cells were specific to peptides derived from *Helicobacter spp.* (Fig. 6B). This is similar to observations made with OT-II (ovalbumin-specific) Tg T cells that respond to Segmented Filamentous bacteria (SFB) in the small intestine (33). Depletion of Gram-positive anaerobes with vancomycin increased *Helicobacter*-specific responses, indicating that these tetramers are not cross-reactive to CBir1 flagellin and also that removal of Gram positive anaerobes may increase the availability of antigens derived from *Helicobacter* (Fig. 6B). These results are consistent with a growing body of studies indicating that the most immunogenic members of the microbiota are those capable of making direct contact with the host intestinal epithelium, such as SFB in the ileum and *Helicobacter* in the colon (19, 20). Further, they underscore the finding that specificity for CBir1 flagellin is not the driving force behind the proliferation and accumulation of CBir1Wt T cells and that instead these are provided via other TCR specificities associated with re-arrangement of the endogenous TCR α locus.

Discussion

In aggregate, our data show that specificity towards a common intestinal antigen, CBir1 flagellin, is not sufficient for the spontaneous activation of T cells nor for the development of colitis. This is in line with our previous study, which showed that activation of CBir1 Tg T cells required the breakdown of the intestinal lining, which presumably allowed greater access of the antigen to antigen presenting cells and T cells (18). In these experiments, despite the activation of microbiota-specific T cells after gastrointestinal infection, spontaneous colitis never developed, which was presumably due to immunoregulatory mechanisms (18). In contrast, CBir1 Tg T cells have been used commonly by multiple laboratories to look at immune responses against the microbiota under lymphopenic conditions where the barrier integrity is not obviously compromised (8, 13, 15, 16, 34, 35).

Here we show under both genetically lymphopenic conditions (CBirRag) and adoptive transfer into Rag1^{-/-} mice that CBir1-specific T cells do not become activated and differentiate to either effector or regulatory states nor do they traffic or accumulate significantly in the colon. Instead, CBir1-specific T cells appear to mostly undergo 'lymphopenia-induced' proliferation, which is antigen independent and driven by the increased availability of IL-7 in lymphopenic mice (30). We also show that upon transfer to lymphopenic mice, proliferating T cells are dominated by clones that lack the surface expression of the CBir1 TCR, and at least some of the proliferating T cells are specific to other bacteria, such as *Helicobacter spp.*

Interestingly, all T cells in CBirWt mice express the V β 8.3 chain associated with the transgenic TCR, indicating that those cells lacking high avidity binding to the CBir1 tetramer have not lost the ability to express the transgene. This implies that the endogenously rearranged TCR α chain is favored and potentially provides the cell with a dominant second specificity. The expression of secondary TCRs responsive to environmental antigens presumably is a possibility for all TCR transgenic mice on conventional backgrounds. However, it is perhaps most critical when trying to use them to interrogate responses at mucosal sites populated with various environmental antigens and particularly microbiota-specific TCRs, which respond to organisms that typically do not invade and proliferate in the host and thus provide low concentrations of their antigens. In that respect, it is somewhat surprising that Smarta transgenic mice on a wild-type background do not induce colitis over the time frame examined. There are a number of possible explanations for this discrepancy, but tetramer staining (I-A^b:LCMVgp₆₆₋₇₇) of Smarta transgenic T cells reveals a significantly higher frequency of tetramer positive cells (85+%) compared to CBir1Wt (35–80%), indicating that the repertoire of 'alternative' TCR specificities may be smaller. Our work also underscores the importance of confirming the results of experiments using TCR transgenics with tetramer-based measurements of the response of the endogenous repertoire to the same antigen (18, 21, 36).

The differences between what we have observed for CBir1 specific T cells and what has been widely observed for SFB and *Helicobacter spp.* highlight the importance of understanding the ecology of intestinal bacteria (20, 21, 32). Recent studies suggest that spontaneous microbiota-specific T cell activation is largely made against organisms that live in close apposition to the intestinal epithelium, as this allows for a steady traffic of antigens from mucosa spanning antigen-presenting cells without the need for intestinal breach (37, 38). Indeed, we cannot exclude the possibility that in other mouse colonies, CBir1 flagellin may be expressed by intestinal bacteria that live in or on the mucosa, allowing for spontaneous T cell activation and CBir1-specific T cell colitis. However, our results using CBirWt T cells are consistent with previous studies in regard to the incidence and timing of disease, so it is reasonable to presume that our findings are not unique to the microbiota within our facility and are broadly applicable (8, 13, 16). The observation that not all microbiota-derived antigens induce spontaneous T cell activation is seemingly at odds with studies indicating broad specificity to microbiota-derived antigens (including CBir1 flagellin) among activated T and B cells in human subjects (1, 2). However, the ecological behavior of most bacteria within the intestine remains unknown, and human lifestyles and infectious histories may lead to bacterial translocation events that are not modeled in murine

systems within carefully controlled environments. We hypothesize that responses to the dominant immunogenic organisms of the GI tract are required to initiate immune responses that later spread to include other specificities, such as CBir1, as damage to the intestine intensifies. Therefore, the frequency of a given T cell clone in clinical IBD may not correlate to their etiological importance. Thus, we posit that the organisms most critical to the initiation of IBD either secrete products that directly interact with the host (such as toxins) or live within the mucosal barrier itself and therefore access to intestinal antigens precedes the necessity for more active forms of T cell regulation (T_{reg}s, IgA, ILCs). For example, Adherent-Invasive *E. coli* are commonly associated with IBD, and mouse experiments indicate that their association with the intestinal epithelium is required for the induction of inflammation (39, 40). We assert that future studies on the role of the microbiota in IBD should focus on the interaction between the immune system and those organisms whose ecological behavior initiates spontaneous T cell responses and that particular attention should be addressed to those organisms living on the surface of the intestine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

CBirWt	CBir1 TCR transgenic
CBirTCR	CBir1 x TCR $\alpha^{-/-}$
CBirRag	CBir1 x Rag1 $^{-/-}$
cLP	colonic lamina propria
DC	dendritic cell
IBD	inflammatory bowel disease
IgA	immunoglobulin A
ILC3	innate lymphoid cell type 3
MANV	metronidazole, ampicillin, neomycin, vancomycin
mLN	mesenteric lymph node
SFB	segmented filamentous bacteria

T_g	transgenic
T_{reg}	regulatory T cell

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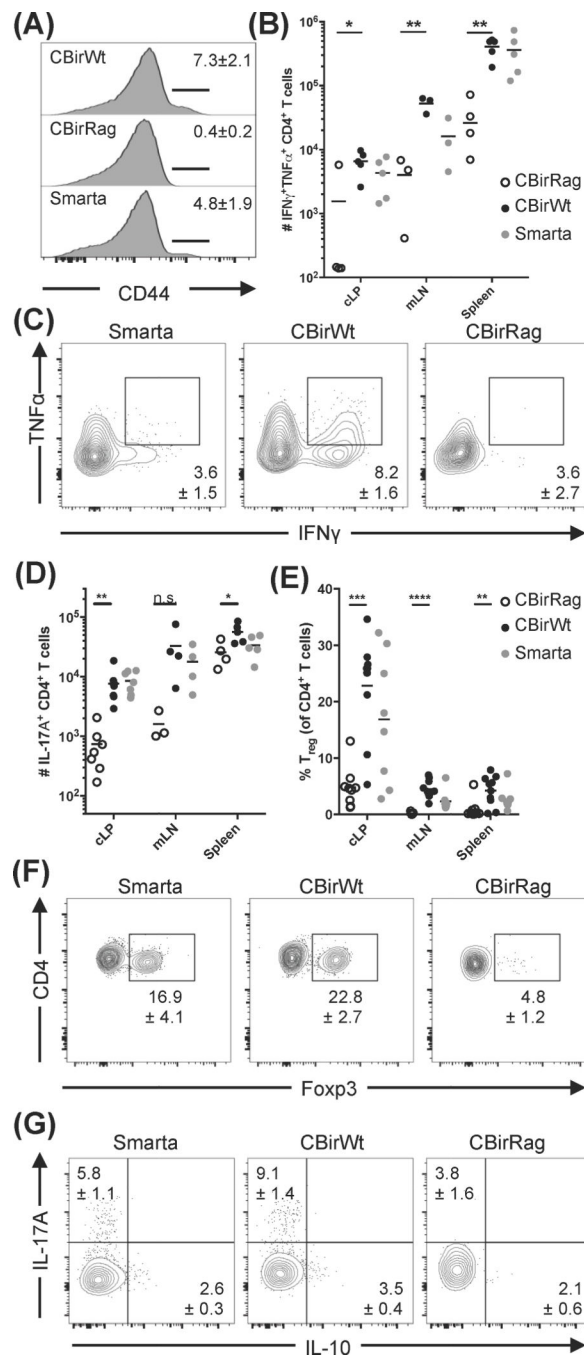


Figure 1. CBir1 flagellin-specific T cells do not become activated during development in lymphopenic hosts (A–D)

Lymphocytes from spleen, mesenteric lymph nodes (mLN) and colonic lamina propria (cLP) of various mouse strains were analyzed by flow cytometry. **(A)** CD44 expression on splenic CD4⁺TCRβ⁺ cells; numbers represent mean percentage ± SEM. **(B–D)** Cells were stimulated with PMA/ionomycin and analyzed for **(B and C)** TNF α /IFN γ co-expression or **(D)** IL-17A expression. Numbers on flow cytometry plots **(C)** represent mean positive events in cLP ± SEM. **(E and F)** Percent of cells from various tissues **(E)** expressing Foxp3;

the flow cytometry plots (**F**) represent cells from the cLP. Numbers on flow cytometry plots show the mean percent of Foxp3⁺ cells in the cLP \pm SEM. (**G**) Percent of CD4⁺ T cells from the cLP expressing IL-10 and IL-17A. Numbers on flow cytometry plots represent mean positive events in cLP \pm SEM. Flow cytometry plots were gated on Live CD90.2⁺TCRb⁺CD4⁺CD8⁻ cells (and additional Foxp3⁻ for **A–D**). Data are representative of 3 independent experiments, $n=8–10$ mice/group. Statistical test: one way ANOVA with Tukey's test for multiple comparisons; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

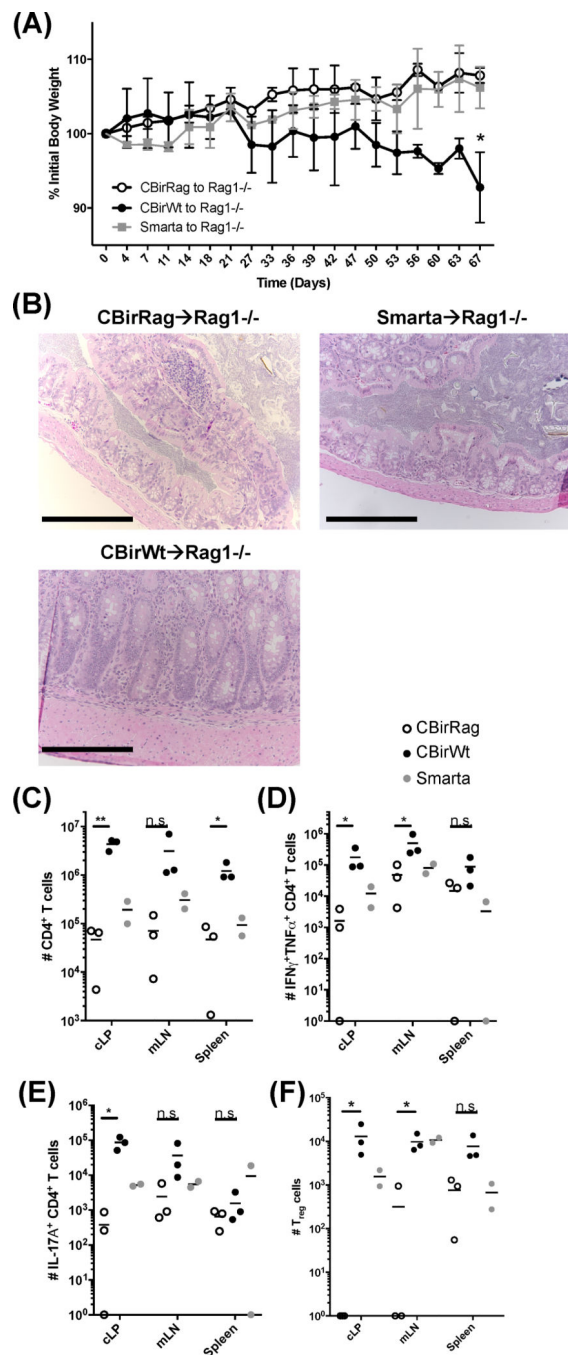


Figure 2. Transfer of CBir1 flagellin-specific T cells on a Rag1^{-/-} background to lymphopenic hosts does not result in the development of colitis (A–F)
 5×10^5 FACs-sorted (CD4⁺CD44^{lo}CD45RB^{hi}CD25⁻) CD4⁺ T cells were transferred to gender-matched Rag1^{-/-} recipients ($n=3$). (A) Weight change was tracked, and (B) colons were isolated for H&E histological staining (scale bars = 250 μm). (C–F) Lymphocytes were isolated from Rag1^{-/-} recipient mice in (A), stimulated with PMA and ionomycin, and quantified via flow cytometry for (C) total donor CD4⁺ T cells, (D) IFN γ ⁺TNF α ⁺ CD4⁺ T cells and (E) IL-17a⁺ CD4⁺ T cells. (F) Number Foxp3⁺ T_{reg} cells isolated from different

tissues, as indicated. Statistical test: Kruskal-Wallis for multiple comparisons; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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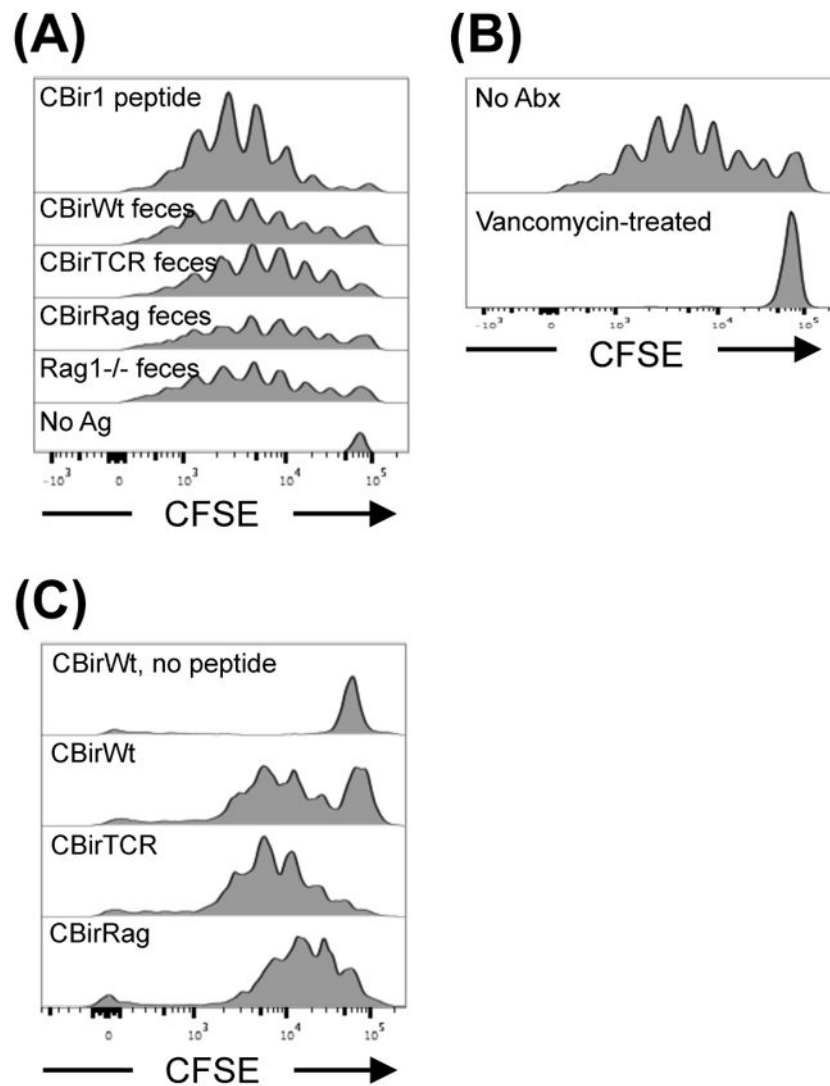


Figure 3. CBirRag T cells proliferate normally and respond specifically to CBir1 flagellin, which is found in all mouse strains used in this study (A and B)

CFSE-labeled CBirWt splenocytes were cultured with splenic CD11c⁺ dendritic cells for 4 days and stimulated with either (A) 10ng/mL CBir1 peptide or supernatant from boiled fecal pellets of indicated mouse strains or (B) fecal pellets from Rag1^{-/-} mice orally treated with vancomycin. (C) Splenocytes from CBirWt, CBirTCR and CBirRag mice were labeled with CFSE and cultured for 4 days with splenic CD11c⁺ dendritic cells and 10ng/mL CBir1 peptide. Shown is one representative experiment of three independent experiments

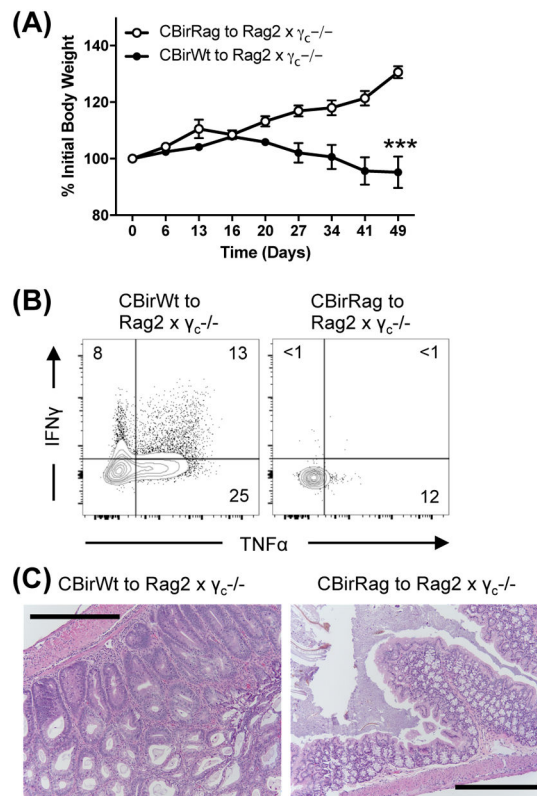


Figure 4. ILCs are not the primary regulatory element that prevents CBir1-specific T cell activation in lymphopenic settings (A–C)

5×10^5 FACS-sorted naïve CD4⁺ T cells were transferred to Rag2^{-/-} $\gamma_c^{-/-}$ mice ($n=6$). (A) Weight change was measured. (B) Post-transfer cLP lymphocytes were stimulated with PMA/ionomycin and analyzed for TNF α /IFN γ co-expression. (C) Colons were isolated for H&E histological staining (scale bars = 250 μ m). Numbers on flow cytometry plots represent mean positive events in cLP. Flow cytometry plots were gated on Live CD45.1⁺CD90.2⁺TCR β ⁺CD4⁺CD8⁻ cells. Graph shows mean \pm SD. Data are representative of 2 independent experiments, $n=6$ mice/group.

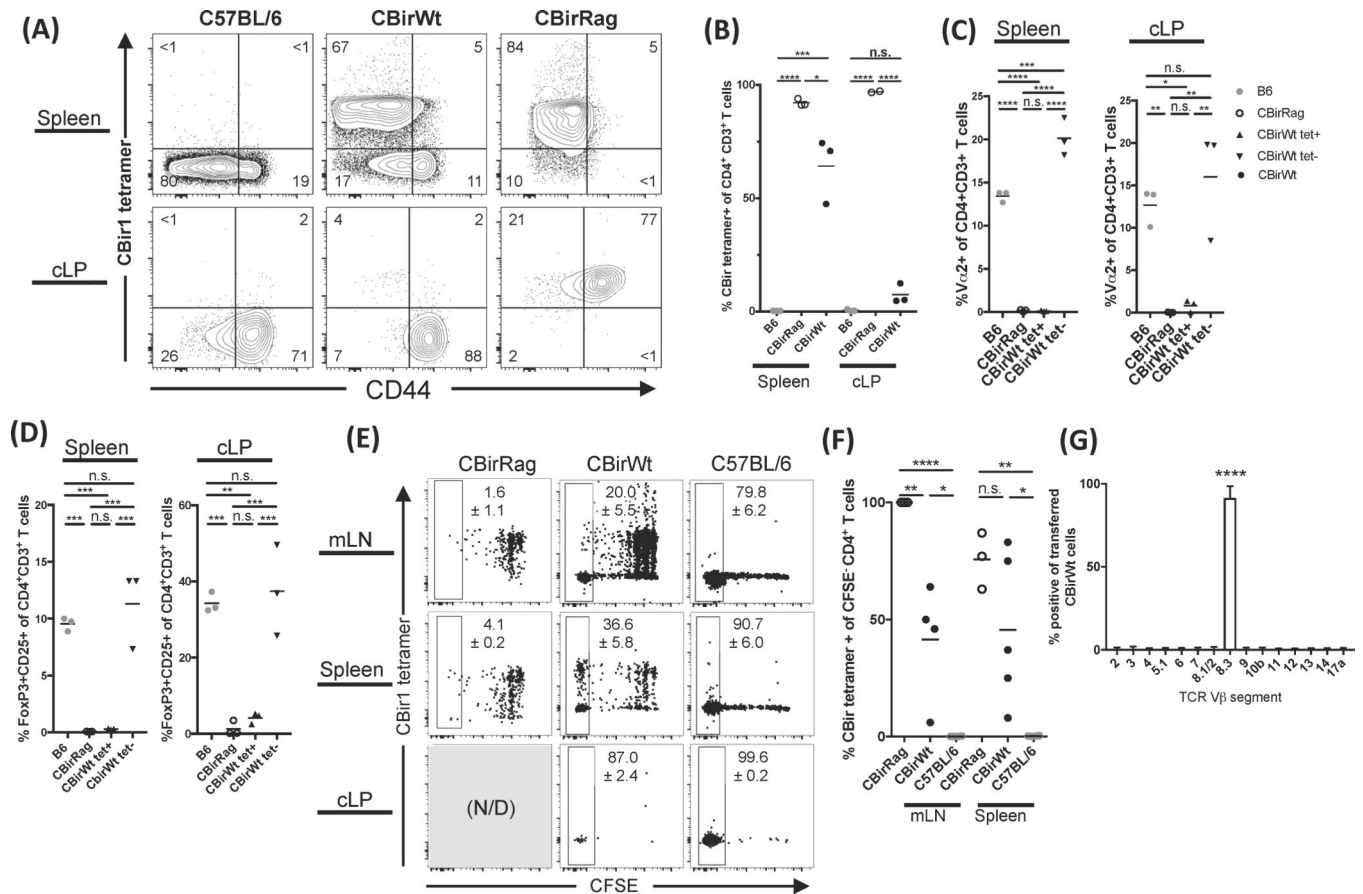


Figure 5. Endogenous rearrangement of the TCR α chain in CBir1Tg mice allows for multiple TCR specificities and consequent T cell activation and colitis induction (A–D) Lymphocytes from spleen and cLP from various mouse strains analyzed by flow cytometry. **(A and B)** CD44 expression and binding to CBir1 tetramer by CD4⁺ CD3⁺ cells; numbers represent percent of cells in each gate. **(C)** V α 2 expression and **(D)** Foxp3 and CD25 co-expression in CD4⁺ CD3⁺ T cells. **(E–G)** 10⁶ FACs-sorted (CD44^{lo}CD45RB^{hi}CD25⁻ CD4⁺), CFSE-labelled naïve CD4⁺ T cells were transferred to gender-matched Rag1^{-/-} recipients. Ten days post-transfer, lymphocytes were analyzed via flow cytometry to determine **(E)** percent of CFSE⁻ cells and tetramer expression, Numbers on flow cytometry plots represent mean positive events \pm SEM. **(F)** CBir1 MHCII tetramer specificity among CFSE⁻ CD4⁺ T cells, and **(G)** V β expression among transferred CBirWt CD4⁺ T cells from the spleen of Rag1^{-/-} recipients (mean \pm SD). Flow cytometry plots were gated on Live CD45.1⁺CD90.2⁺CD4⁺CD8⁻ cells. Data are representative of 2–3 independent experiments, $n=3-8$ mice/group. Statistical test: one way ANOVA with Tukey's test for multiple comparisons; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

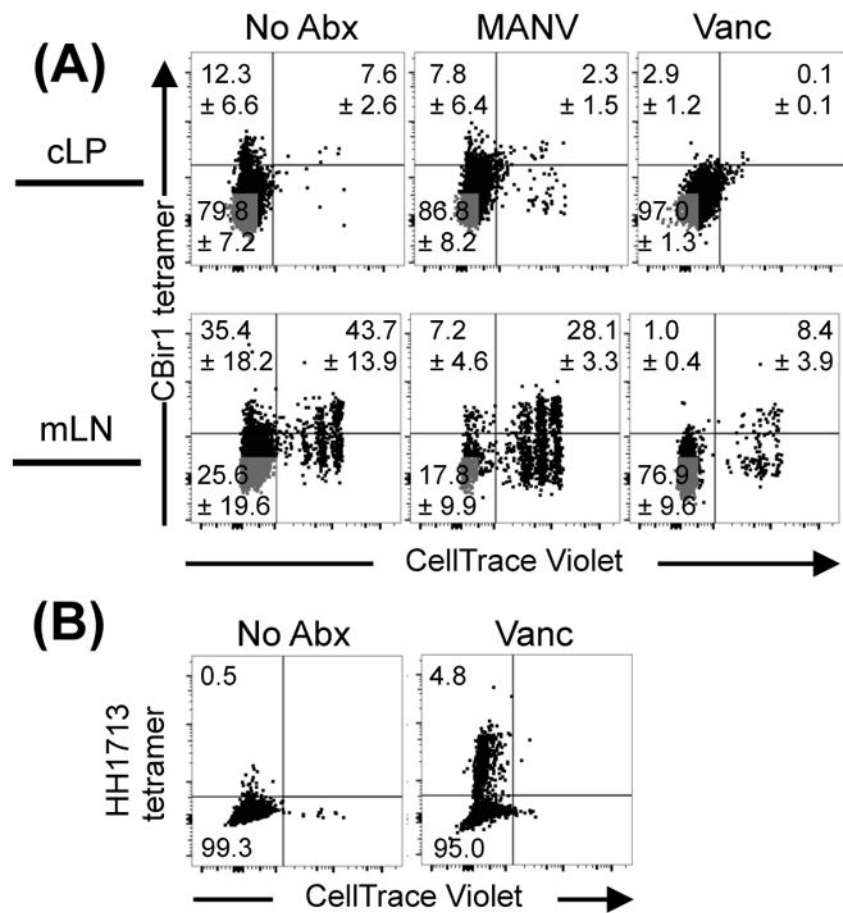


Figure 6. CBir1 flagellin is not the main antigenic driver of proliferation of CBir1-specific T cells in lymphopenic hosts (A and B)

Rag1^{-/-} mice were treated with antibiotics as indicated for 1 week and then injected with 10⁶ FACS-sorted, CellTrace Violet-stained naïve CD4⁺ T cells. Ten days later, transferred cells were analyzed for dilution of CellTrace dye and binding to either (A) CBir1 tetramer or (B) *Helicobacter hepaticus* MHCII tetramer. Numbers on flow cytometry plots show mean percentage of gated cells ± SEM. Flow cytometry plots were gated on Live CD45.1⁺CD90.2⁺CD4⁺CD8⁻ cells. Data are representative of 2 independent experiments; n=3 mice/group.