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T Cell Proliferation and Colitis Are Initiated by Defined Intestinal Microbes

Pailin Chiaranunt,*,† Justin T. Tometich,*,† Junyi Ji,*,†,‡ and Timothy W. Hand*,†,§

Inflammatory bowel disease has been associated with the dysregulation of T cells specific to Ags 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 IgA, regulatory T cells, and type 3 innate lymphoid cells. To test the role of these regulatory components on microbiota-specific T cells, we bred CBir1 TCR transgenic (CBir1Tg) mice (specific to flagellin from common intestinal bacteria) onto a lymphopenic Rag1$^{-/-}$ background. Surprisingly, T cells from CBir1Tg mice bred onto a Rag1$^{-/-}$ background could not induce colitis and did not differentiate to become effectors under lymphopenic conditions, despite deficits in immunoregulatory factors, such as IgA, regulatory T cells, and type 3 innate lymphoid cells. In fact, upon transfer of conventional CBir1Tg T cells into lymphopenic mice, the vast majority of proliferating T cells responded to Ags other than CBir1 flagellin, including those found on other bacteria, such as *Helicobacter* spp. Thus, we discovered a caveat in the CBir1Tg 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 Ags. 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 Ag, are paramount in controlling the activation of microbiota–specific T cell clones. ***The Journal of Immunology*, 2018, 201: 243–250.**

The barrier surfaces of mammals are covered in a vast consortium of microorganisms that together comprise the microbiome. Mammals also possess millions of B and T lymphocytes with variable specificities that include responsiveness to Ags 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 and therefore affect the access of intestinal Ags 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, the transfer of naive 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 cells (T_{regs}). This model requires 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 Ags 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 IL-10, T_{regs}, IgA, and type 3 innate lymphoid cells (ILC3s), although how these factors work together is not clearly understood (14–17). CBir1 TCR transgenic (Tg) on a wild-type background (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 CBir1 TCR Tg (CBir1Tg) T cells in such hosts (14, 18). This is in contrast to TCR Tg cells specific to either segmented filamentous bacteria (SFB) or *Helicobacter* spp., in which bacterial colonization is sufficient to induce spontaneous T cell responses because of 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 CBir1Wt mice to a Rag1$^{-/-}$ background (CBir1Tg × Rag1$^{-/-}$ [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$^{-/-}$γc$^{-/-}$ mice, despite the lack of T_{regs}, IgA, and ILC3s. The majority of CBirWt T cells that accumulated in Rag1$^{-/-}$ mice posttransfer did not bind a tetramer bearing the

*Richard King Mellon Foundation Institute for Pediatric Research, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh, Pittsburgh, PA 15224; †Department of Pediatrics, University of Pittsburgh Medical School, Pittsburgh, PA 15224; ‡School of Medicine, Tsinghua University, Beijing 100084, China; and §Department of Immunology, University of Pittsburgh Medical School, Pittsburgh, PA 15213

ORCIDs: 0000-0002-5764-198X (J.J.); 0000-0002-2684-9462 (T.W.H.).

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Address correspondence and reprint requests to Dr. Timothy W. Hand, University of Pittsburgh, 4401 Penn Avenue, Rangos Building 8126, Pittsburgh, PA 15224. E-mail address: timothy.hand@chp.edu

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Abbreviations used in this article: CBirRag, CBir1Tg × Rag1$^{-/-}$; CBirTCR, CBir1Tg × TCRα$^{-/-}$; CBir1Tg, CBir1 TCR Tg; CBirWt, CBir1 TCR Tg on a wild-type background; cLP, colonic lamina propria; DC, dendritic cell; IBD, inflammatory bowel disease; ILC3, type 3 innate lymphoid cell; MANV, metronidazole, ampicillin, neomycin, and vancomycin; MHC II, MHC class II; mLN, mesenteric lymph node; SFB, segmented filamentous bacteria; Tg, transgenic; T_{reg}, regulatory T cell.

relevant peptide from CBir1 flagellin and were responsive to other intestinal bacteria, such as *Helicobacter* spp., because of the expression of endogenously rearranged non-Tg TCRα-chains. Taken together, our data uncover a complication of the CBir1Tg model of T cell–mediated colitis and show that Ag 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$^{-/-}$γc$^{-/-}$ mice were obtained from Jackson Laboratory. CBir1Tg mice were produced by Dr. C. Elson (University of Alabama at Birmingham, Birmingham, AL) as described, obtained under a material transfer agreement, and back-crossed to CD45.1-expressing mice, TCRα$^{-/-}$ mice, or Rag1$^{-/-}$ mice for at least three generations (14). Smarta TCR Tg mice were obtained from Dr. R. Germain (National Institutes of Health/National Institute of Allergy and Infectious Diseases). In some experiments, mice were given autoclaved drinking water supplemented with either vancomycin (0.5 mg/ml; Sigma-Aldrich) or a mixture of metronidazole (1 mg/ml; Sigma-Aldrich), ampicillin (1 mg/ml; Sigma-Aldrich), neomycin (1 mg/ml; Sigma-Aldrich), and vancomycin (0.5 mg/ml; Sigma-Aldrich). Sucralose (Splenda) (0.8 mg/ml) was added to make the antibiotic-containing water more palatable. Antibiotic treatment was started 1 wk prior to adoptive T cell transfer or other uses of the mice. Gender-matched and age-matched mice were used and cohoused 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 wk 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 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-Aldrich) in the presence of Brefeldin A (Thermo Fisher) as previously described (22).

For in vitro culture assays, splenic and lymph node CD4$^+$ T cells were isolated by magnetic purification (STEMCELL Technologies) and stained with 1 μM CFSE (Thermo Fisher) as described previously (23). Fecal bacterial Ags were obtained by boiling fecal pellets for 1 min at 100˚C and centrifuging at 12,000 × *g* for 5 min to collect the supernatant. CD4$^+$ T cells were cocultured for 96 h with magnetically purified CD11c$^+$ splenic dendritic cells (DCs) 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.

Abs, tetramers, and flow cytometry

All Abs used for flow cytometry were purchased from either Thermo Fisher, BD Biosciences, or BioLegend. The following Abs were used to discriminate cell surface or intracellular phenotype: TCRβ (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-17α (eBio17B7), IL-2Rα/CD25 (PC61), Vα2 (B20.1), CD45RB (C363-16A), and Foxp3 (FJK-16S). Dead cells were discriminated in all experiments using LIVE/DEAD fixable dead stain (Thermo Fisher). All stains were carried out in media containing anti-CD16/32–blocking Ab (clone 93; Thermo Fisher). 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 permeabilized using the Foxp3/Transcription Factor Staining Buffer Set according to the manufacturer's directions (Thermo Fisher). APC-conjugated CBir1$_{464–72}$ tetramers (YSNANILSQ) and PE-conjugated HH1713$_{172–86}$ and HH1713$_{230–44}$ tetramers (QESPRIAAAYTIKGA and GNAYISVLAHYGKNG, respectively) were provided by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). All tetramer stains were performed at room temperature for 45–60 min. 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 Technologies) and then sort-purified via FACs for naïve CD44^{lo}CD45RB^{hi}CD25$^-$ CD4$^+$ T cells (>99% purity). Of these, 0.5–1.5 × 10^6 cells were injected i.v. into groups of Rag1$^{-/-}$ or Rag2$^{-/-}$γc$^{-/-}$ mice. In some experiments, transferred cells were labeled with CFSE or Cell Trace Violet (Thermo Fisher), and recipient mice were sacrificed to assess T cell responses in the spleen, mLN, and cLP at 9–13 d posttransfer. In other experiments, unlabeled naïve CD4$^+$ T cells were transferred, recipient mice were weighed weekly to assess colitis, and were sacrificed after 7–10 wk posttransfer to assess transferred T cell responses in the spleen, mLN, and cLP. Mice were euthanized if their weights dropped below 70% of baseline. Sections of colons were fixed in 10% formalin and paraffin embedded. Slides were stained with H&E 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 in which cells were transferred to Rag1$^{-/-}$ mice, the resulting number of isolated T cells varied considerably because of the complex nature of the experiments, and therefore a Kruskal–Wallis test was used because the data were nonparametric.

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 Ag and develop within a lymphopenic environment. In further support of this hypothesis, recent studies show that CBir1 flagellin may be transported by goblet cell–associated Ag passages early in the preweaning 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-Tg Rag1$^{-/-}$ mice (CBirRag 1/49; Rag1$^{-/-}$ 2/48). To help explain the lack of disease, we compared CD4$^+$ T cells from spleens, mLNs, and cLP of CBirWt, CBirRag, and Smarta TCR Tg [specific to an Ag derived from Lymphochoriomeningitis virus, which is absent from the standard murine microbiota (25)] mice. In accordance 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 IFN-γ, TNF-α, and IL-17A (Fig. 1A–D, 1G).

One explanation for the relative quiescence of CBirRag T cells could be that they develop into T$_{regs}$. Previous studies have shown that TCR Tg T cells specific to foreign Ags, such as chicken OVA, do not develop into T$_{regs}$ (26). In contrast, microbiota-specific T cells, including CBir1Tg T cells, can differentiate into peripheral T$_{regs}$ (14, 24, 27). Foxp3$^+$CD4$^+$ T$_{regs}$ were almost undetectable in the secondary lymphoid tissues of CBirRag mice and were present at significantly reduced frequencies in the cLP (Fig. 1E, 1F). Another possible explanation for the lack of T cell activation in CBirRag mice was IL-10 production from CD4 T cells that lack Foxp3 expression. 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.

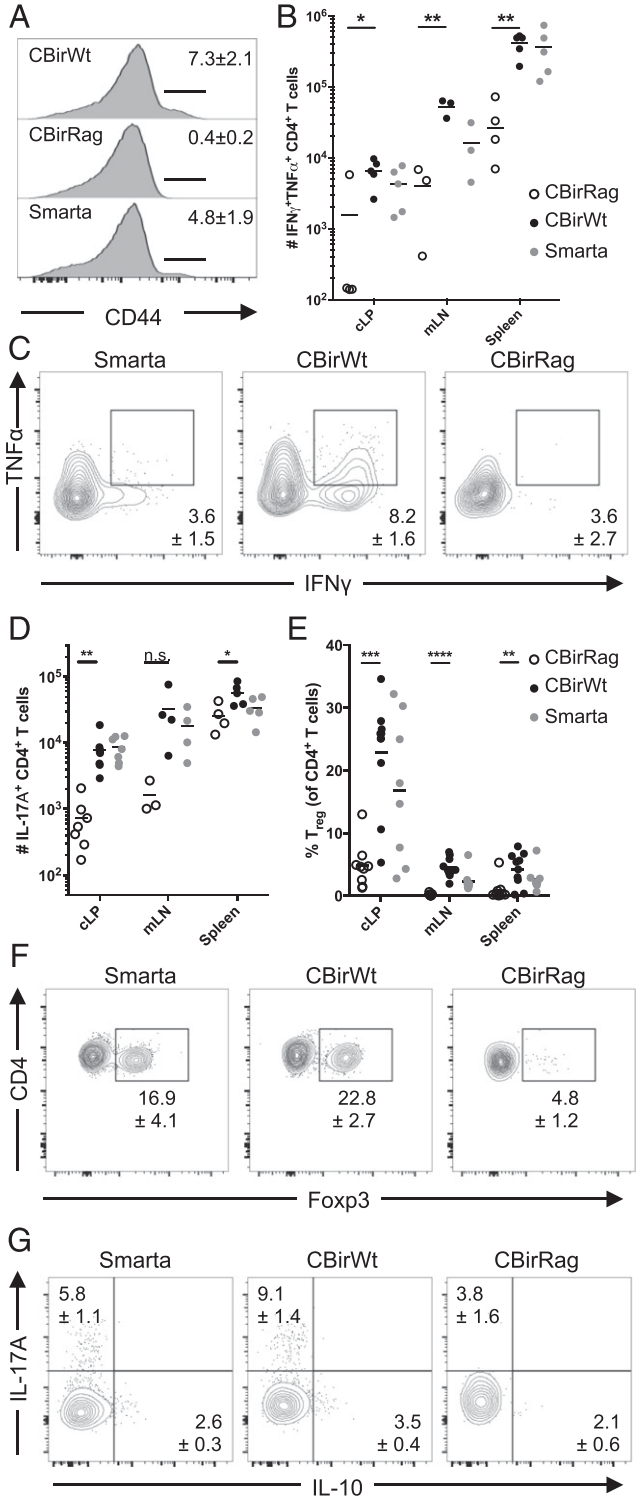

FIGURE 1. CBir1 flagellin-specific T cells do not become activated during development in lymphopenic hosts. (**A**–**D**) Lymphocytes from spleen, mLNs, and cLP of various mouse strains were analyzed by flow cytometry. (A) CD44 expression on splenic $CD4^+TCR\beta^+$ cells; numbers represent mean percentage ± SEM. (B–D) Cells were stimulated with PMA/ionomycin and analyzed for (B and C) TNF-α/IFN-γ coexpression or (D) IL-17A expression. Numbers on flow cytometry plots (C) represent mean positive events in cLP ± SEM. (**E** and **F**) Percentage 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 ± SEM. (**G**) Percentage of $CD4^+$ T cells from the cLP expressing IL-10 and IL-17A. Numbers on flow cytometry plots represent mean positive events in *(Figure legend continues)*

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 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 wk posttransfer, and histological analysis of the recipient colons showed significant hyperplasia and pathology (Fig. 2A, 2B). In contrast, $Rag1^{-/-}$ recipients of CBirRag T cells gained weight and showed no obvious pathology (Fig. 2A, 2B). 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 with CBirRag T cells (Fig. 2C–E). The lack of accumulation among CBirRag T cells was not due to increased conversion into T_{regs} because 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 were 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 cocultured the supernatants from boiled fecal samples from various mouse strains (CBirWt, CBirRag, CBir1Tg × $TCR\alpha^{-/-}$ [CBirTCR], $Rag1^{-/-}$) with CBirWt T cells and 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 Ags in mice treated with vancomycin, which depletes Gram-positive anaerobes (including CBir1-bearing *Clostridia*) (Fig. 3B). A previous study had shown that TCR Tg 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 Ag (Fig. 3C). Therefore, neither the loss of CBir1 flagellin nor proliferative potential among 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 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 TCRα-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 $Rag2^{-/-}\gamma c^{-/-}$ mice. Activated CBirRag T cells did not accumulate in significant numbers in the gastrointestinal tract of $Rag2^{-/-}\gamma c^{-/-}$ mice nor did they cause colitis as measured by weight loss and

cLP ± 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 per group. Statistical test: one-way ANOVA with Tukey test for multiple comparisons. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

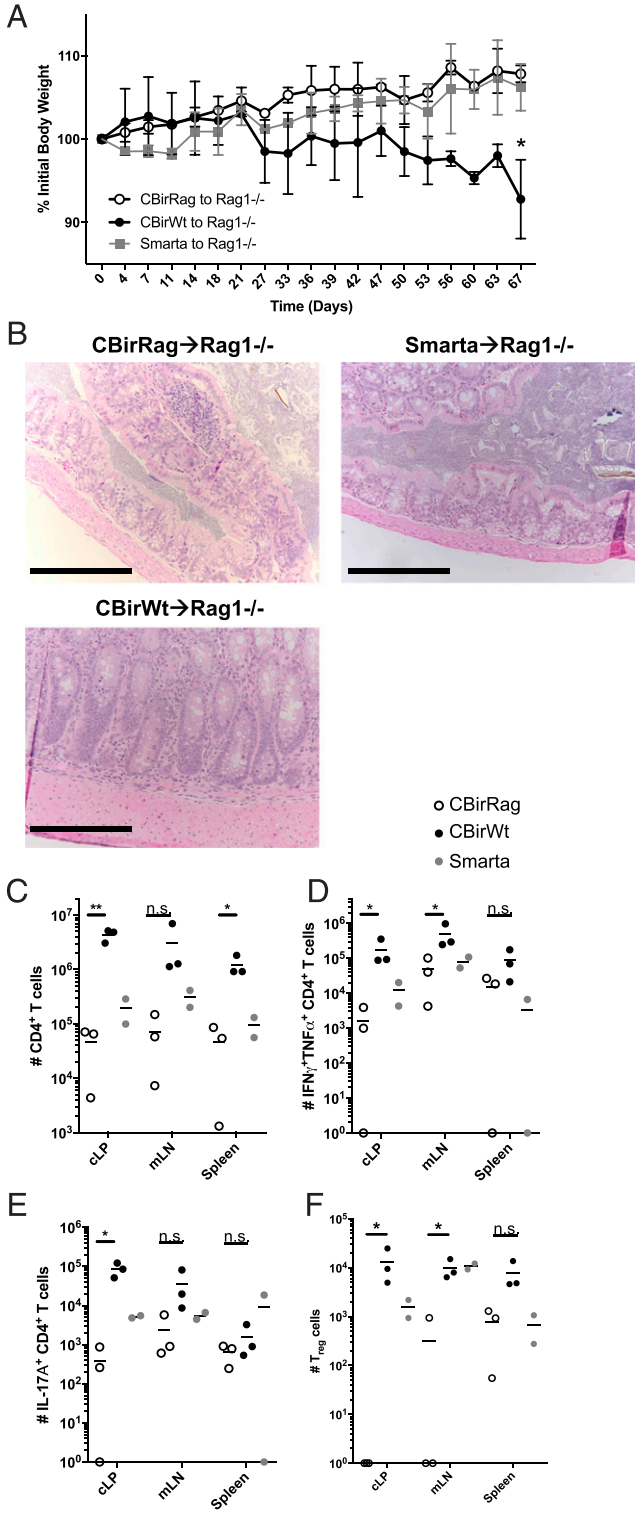

FIGURE 2. Transfer of CBir1 flagellin-specific T cells on a Rag1$^{-/-}$ background to lymphopenic hosts does not result in the development of colitis. (**A**–**F**) 5 × 10^5 FACS-sorted (CD4$^+$CD44^{lo}CD45RB^{hi}CD25$^-$) CD4$^+$ T cells were transferred to gender-matched Rag1$^{-/-}$ recipients (n = 3). (A) Weight change was tracked, and (B) colons were isolated for H&E histological staining (scale bar, 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 of Foxp3$^+$ T$_{regs}$ isolated from different tissues, as indicated. Statistical test: Kruskal–Wallis for multiple comparisons. *$p < 0.05$, **$p < 0.01$.

histology (Fig. 4). Thus, although 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 rearrangement of endogenous TCRα-chains in CBirWt mice can lead to TCR Tg T cells that express multiple functional TCRs and that these secondary specificities confer additional functionality. Analysis of CBirWt T cells showed that, whereas all cells express Vβ8, associated with the CBir1 TCR, some cells (~4%) coexpressed a secondary Vα2 chain (the CBir1 TCR transgene expresses Vα6), 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-Tg C57BL/6 as controls) using MHC class II (MHC II) tetramers bearing the relevant peptide from CBir1 flagellin. These experiments confirmed that nearly all CBirRag T cells bind the CBir1 tetramer, as rearrangement of endogenous TCRα 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 Tg CBir1 TCR to bind the tetramer (Fig. 5A, 5B). Strikingly, splenic CBir1 tetramer-positive cells in CBirWt and CBirRag mice were phenotypically indistinguishable in that they almost completely lacked the expression of Vα2 (as a surrogate for the ability to rearrange endogenous TCRα-chains), high-level expression of CD44, and differentiation to Foxp3$^+$ T$_{regs}$ (Fig. 5A, 5C, 5D). Conversely, the expression of CD44, Vα2, and IL-2Rα/Foxp3 was almost solely attributable to CBir1 tetramer-negative T cells from CBirWt mice (Fig. 5C, 5D). 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 was rather due to CBirWt T cells that have rearranged a second TCRα-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-Tg 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 10 d 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 the CBir1 tetramer (Fig. 5E, 5F). This is in contrast to transferred CBirRag T cells, which maintained CBir1 tetramer binding and exhibited Ag-independent slow proliferation associated with the increased availability of homeostatic cytokines such as IL-7 (Fig. 5E, 5F) (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 Ag did not result in restoration of the ability to bind the CBir1 tetramer, making cognate Ag-driven TCR downregulation 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 accordance with our in vitro experiments, analysis of transferred cells 13 d later revealed minimal re-expression of the CBir1-specific TCRs on cells sorted as tetramer negative (Supplemental Fig. 3B). Furthermore, sorted CBir1 tetramer-negative T cells proliferated and accumulated in much greater numbers than their tetramer-

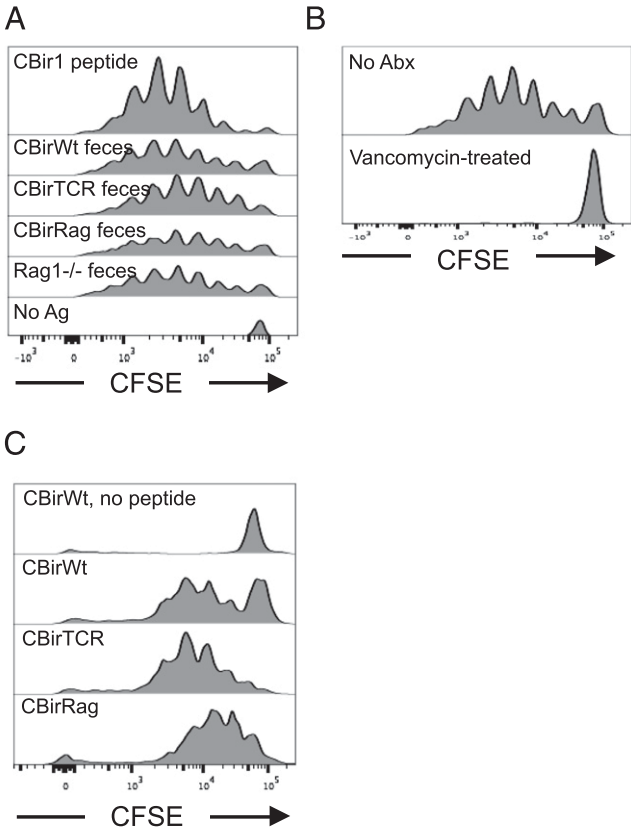

FIGURE 3. CBirRag T cells proliferate normally and respond specifically to CBir1 flagellin, which is found in all mouse strains used in this study. (**A** and **B**) CFSE-labeled CBirWt splenocytes were cultured with splenic CD11c$^+$ DCs for 4 d and stimulated with either (A) 10 ng/ml CBir1 peptide 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 d with splenic CD11c$^+$ DCs and 10 ng/ml CBir1 peptide. Shown is one representative experiment of three independent experiments.

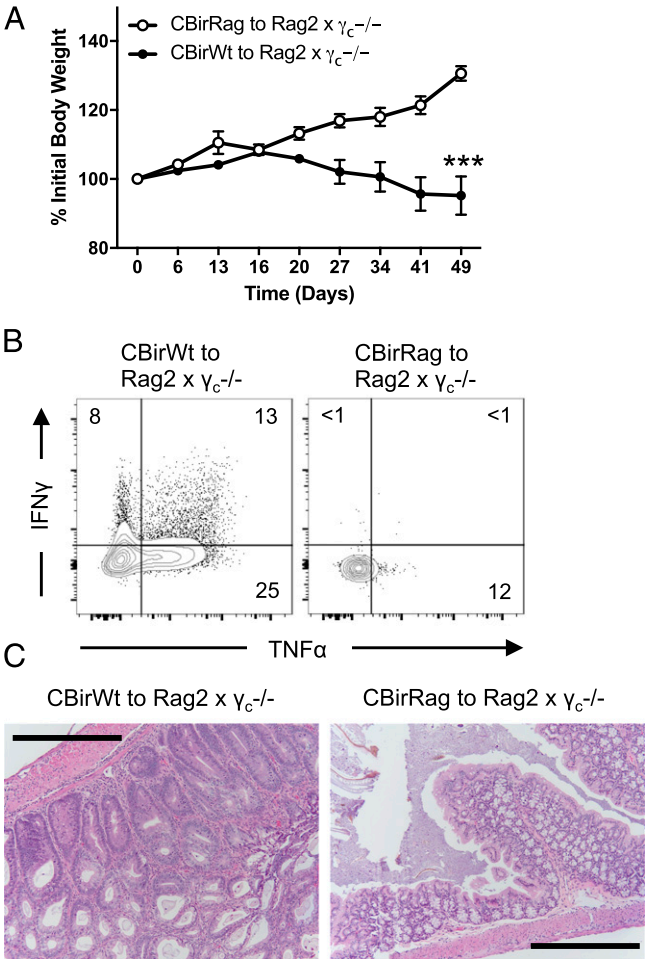

FIGURE 4. ILCs are not the primary regulatory element that prevents CBir1-specific T cell activation in lymphopenic settings. (**A**–**C**) 5 × 10^5 FACS-sorted naïve CD4$^+$ T cells were transferred to Rag2$^{-/-}$γc$^{-/-}$ mice (n = 6). (A) Weight change was measured. (B) Posttransfer cLP lymphocytes were stimulated with PMA/ionomycin and analyzed for TNF-α/IFN-γ coexpression. (C) Colons were isolated for H&E histological staining (scale bar, 250 μm). Numbers on flow cytometry plots represent mean positive events in cLP. Flow cytometry plots were gated on Live CD45.1$^+$CD90.2$^+$TCRβ$^+$CD4$^+$CD8$^-$ cells. Graph shows mean ± SD. Data are representative of two independent experiments; n = 6 mice per group. ***$p < 0.001$, unpaired Student t test.

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 Tg Vα-chain (Supplemental Fig. 3B, 3C). It should be noted that those CBirWt T cells that maintained expression of the Tg 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 TCRβ variable regions, aside from the Tg Vβ8.3 (expressed on all transferred cells), negating the possibility that tetramer-negative CBirWt T cells are escaping β exclusion to produce complete non-Tg 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 Ag and instead carry endogenously rearranged TCRα-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 Fig. 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 the 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 (~8–20×) of transferred cells in the cLP compared with 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

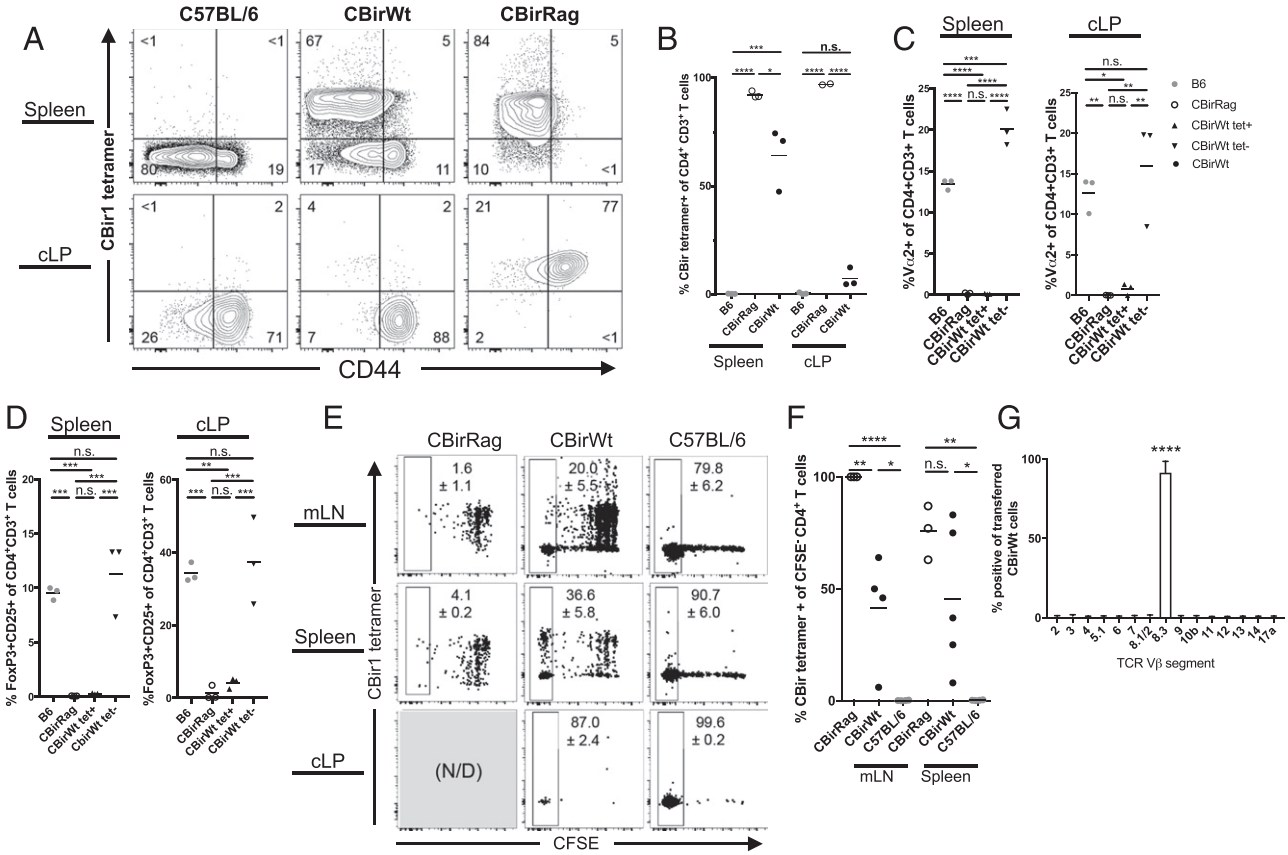

FIGURE 5. Endogenous rearrangement of the TCRα-chain in CBir1Tg allows for multiple TCR specificities and consequent T cell activation. (**A**–**D**) Lymphocytes from spleen and cLP from various mouse strains analyzed by flow cytometry. (A and B) CD44 expression and binding to CBir1 tetramer by $CD4^+$ $CD3^+$ cells; numbers represent percentage of cells in each gate. (C) Vα2 expression and (D) Foxp3 and CD25 coexpression in $CD4^+CD3^+$ T cells. (**E**–**G**) 10^6 FACS-sorted ($CD44^{lo}CD45RB^{hi}CD25^-$ $CD4^+$), CFSE-labeled naïve $CD4^+$ T cells were transferred to gender-matched $Rag1^{-/-}$ recipients. Ten days posttransfer, lymphocytes were analyzed via flow cytometry to determine (E) the percentage of $CFSE^-$ cells and tetramer expression. Numbers on flow cytometry plots represent mean positive events ± SEM. (F) CBir1 MHC II tetramer specificity among $CFSE^-$ $CD4^+$ T cells, and (G) Vβ expression among transferred CBirWt $CD4^+$ T cells from the spleen of $Rag1^{-/-}$ recipients (mean ± SD). Flow cytometry plots were gated on Live $CD45.1^+CD90.2^+CD4^+$ $CD8^-$ cells. Data are representative of two to three independent experiments; $n = 3$–8 mice per group. Statistical test: one-way ANOVA with Tukey test for multiple comparisons. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. B6, C57BL/6 mouse.

accumulation in the colon posttransfer into lymphopenic hosts are driven by the microbiota but do 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 with 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 with those same cells in the colon only 10 d posttransfer to a $Rag1^{-/-}$ host (Fig. 5A, 5E). Thus, although 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 Ags 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 Ags 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 (OVA-specific) Tg T cells that respond to 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 Ags 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). Furthermore, 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 rearrangement of the endogenous TCRα locus.

Discussion

In aggregate, our data show that specificity toward a common intestinal Ag, CBir1 flagellin, is not sufficient for the spontaneous

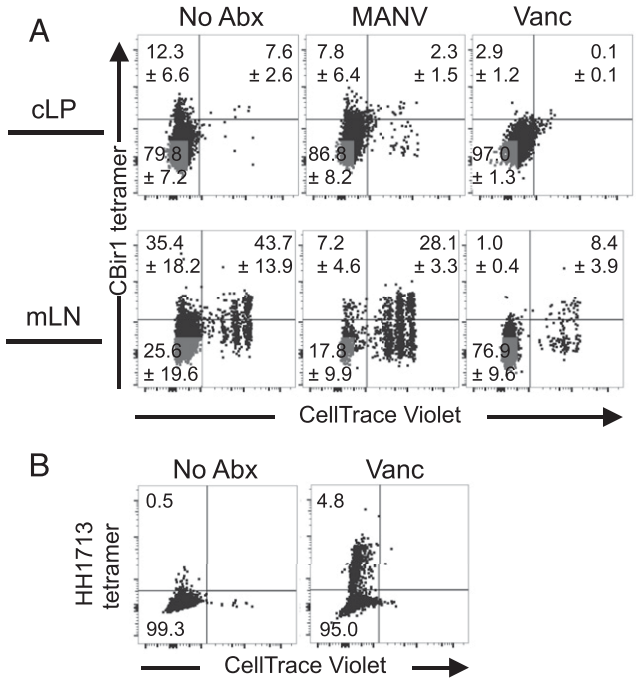

FIGURE 6. CBir1 flagellin is not the main antigenic driver of proliferation of CBir1-specific T cells in lymphopenic hosts. (**A** and **B**) Rag1$^{-/-}$ mice were treated with antibiotics as indicated for 1 wk and then injected with 10^6 FACS-sorted, CellTrace Violet–stained naïve CD4$^+$ T cells. Ten days later, transferred cells were analyzed for dilution of CellTrace dye and binding to either (A) CBir1 tetramer or (B) *Helicobacter hepaticus* MHC II 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 two independent experiments; n = 3 mice per group.

activation of T cells nor for the development of colitis. This is in line with our previous study, which showed that activation of CBir1Tg T cells required the breakdown of the intestinal lining, which presumably allowed greater access of the Ag to APCs 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 because of immunoregulatory mechanisms and re-formation of the epithelium (18). In contrast, CBir1Tg T cells have been used commonly by multiple laboratories to look at immune responses against the microbiota under lymphopenic conditions in which the barrier integrity is not obviously compromised (8, 13, 15, 16, 34, 35). In this article, 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 Ag 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 Vβ8.3 chain associated with the Tg 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 TCRα-chain is favored and potentially provides the cell with a dominant second specificity. The expression of secondary TCRs responsive to environmental Ags presumably is a possibility for all TCR Tg 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 Ags 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 Ags. In that respect, it is somewhat surprising that Smarta Tg 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:LCMVgp$_{66\text{-}77}$) of Smarta Tg T cells reveals a significantly higher frequency of tetramer-positive cells (85+%) compared with 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 Ag (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 Ags from mucosa spanning APCs 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 Ags induce spontaneous T cell activation is seemingly at odds with studies indicating broad specificity to microbiota-derived Ags (including CBir1 flagellin) among activated T cells 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 gastrointestinal 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 Ags precedes the necessity for more active forms of T cell regulation (T_{regs}, IgA, ILCs). For example, adherent-invasive *Escherichia 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.

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Disclosures

The authors have no financial conflicts of interest.

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