

Identifying species of symbiont bacteria from the human gut that, alone, can induce intestinal Th17 cells in mice

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Th17 cells accrue in the intestine in response to particular microbes. In rodents, segmented filamentous bacteria (SFB) induce intestinal Th17 cells, but analogously functioning microbes in humans remain undefined. Here, we identified human symbiont bacterial species, in particular *Bifidobacterium adolescentis*, that could, alone, induce Th17 cells in the murine intestine. Similar to SFB, *B. adolescentis* was closely associated with the gut epithelium and engendered cognate Th17 cells without attendant inflammation. However, *B. adolescentis* elicited a transcriptional program clearly distinct from that of SFB, suggesting an alternative mechanism of promoting Th17 cell accumulation. Inoculation of mice with *B. adolescentis* exacerbated autoimmune arthritis in the K/BxN mouse model. Several off-the-shelf probiotic preparations that include *Bifidobacterium* strains also drove intestinal Th17 cell accumulation.

microbiota | Th17 cells | probiotic | mucosal immunology | intestine

The mammalian gut harbors hundreds of species of symbiont bacteria that play a crucial function in various facets of host physiology, including metabolism, tissue development, and maturation of the immune system (1, 2). Germfree (GF) and antibiotic-treated mice have several defects in T-cell compartments of both their gut-associated and -distal organs, including a paucity of intestinal Th17 and Treg cells and a systemic skewing toward Th2 responses (3, 4). Importantly, specific members or subsets of the microbiota can rescue a dearth of Treg or Th17 cells. Although early reports argued that a consortium of *Clostridium* species from either the murine or human gut is needed to induce Treg cells in the murine colon (5, 6), more recent studies showed that an assortment of individual bacterial species, including *Clostridium* and *Bacteroides* family members, also possess this property (7, 8). Similarly, a single bacterial strain, segmented filamentous bacteria (SFB), is sufficient to drive the accumulation of Th17 cells in the small-intestinal lamina propria (SI-LP) of mice (9, 10); however, Th17-inducing microbes derived from the human gut have not yet been identified. A recent report did document an increase in colonic Th17 cells in GF mice inoculated with fecal material from healthy people and patients with ulcerative colitis, thus showing the existence of Th17-inducing species in the human microbiota (11). However, the microbiota composition differs substantially across both healthy individuals and colitis patients, and the symbionts responsible for Th17 cell induction at steady state remain uncharacterized (11).

In both mice (10, 12) and humans (13, 14), Th17 cells are normally at their highest levels in the SI-LP. They secrete the cytokines IL-17A, IL-17F, and IL-22, which induce the production of antimicrobial peptides and tight junction proteins from intestinal epithelial cells, thereby buttressing gut barrier integrity (15–17). Moreover, IL-17A and IL-17F promote the recruitment of neutrophils via the release of granulocyte colony-stimulating factor, thereby helping to defend the host against infections by fungi and extracellular bacteria (18). Consequently, humans genetically deficient in IL-17 signaling because of mutations in genes such as *STAT3* and *IL17RA* suffer from an increased susceptibility to mucosal infections

by *Candida albicans* and *Staphylococcus aureus* (18, 19). Over-exuberant Th17 responses, however, have been implicated in various inflammatory and autoimmune disorders, including multiple sclerosis, rheumatoid arthritis (RA), and inflammatory bowel disease (IBD) (19, 20). Many of these disorders in both mice and humans are also associated with intestinal dysbiosis (21, 22). An example of the dichotomous effects of symbiont-dependent Th17 cells is provided by SFB, which confers resistance to the enteropathogen *Citrobacter rodentium* in mice but exacerbates disease severity in murine models of multiple sclerosis and RA (10, 23, 24). Hence, fluctuations in the human microbiome are likely to exert important effects on host mucosal defenses and the development of inflammatory conditions, in part via modulation of Th17 responses.

Therefore, we set out to identify bacterial species from the human gut microbiota capable of inducing Th17 cells in the mouse intestine. Focusing on the most robust inducer, *Bifidobacterium adolescentis*, we compared its activities and mechanisms with those of SFB, uncovering distinct modi operandi but similar promotion of autoinflammatory and inflammatory diseases. Several off-the-shelf probiotic preparations—touted to improve human gastrointestinal and metabolic health—promoted SI-LP Th17 cell accumulation in mice, highlighting the potential therapeutic application of Th17-inducing bacteria.

Significance

Th17 cells accumulate in the gut, where they mediate barrier defenses and repair but can also provoke inflammatory disease. In mice, segmented filamentous bacteria (SFB) is sufficient to induce Th17 cells in the gut, but functionally analogous microbes in humans have not been defined. Here, we identified *Bifidobacterium adolescentis* as one of several human symbiont bacterial species that could, alone, induce Th17 cells in the small intestine of mice. *B. adolescentis* and SFB exhibited overlapping but also distinct activities, suggesting multiple routes to intestinal Th17 induction. Like SFB, *B. adolescentis* exacerbated autoimmune arthritis, arguing for its pathological relevance. Our results help to inform the search for therapeutic targets in diseases associated with Th17 responses and mucosal dysfunction.

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Results

***B. adolescentis* Is a Human Gut Symbiont That Strongly Induces Intestinal Th17 Cells in Mice.** To identify human gut symbionts capable of influencing host immunity, we screened a large set of

microbes by monoclonizing GF mice and evaluating a variety of immunologic parameters 2 wk later. The screen revealed a few phylogenetically diverse species that elicited SI-LP Th17 populations as large as those induced by SFB (Fig. 1A). Of the

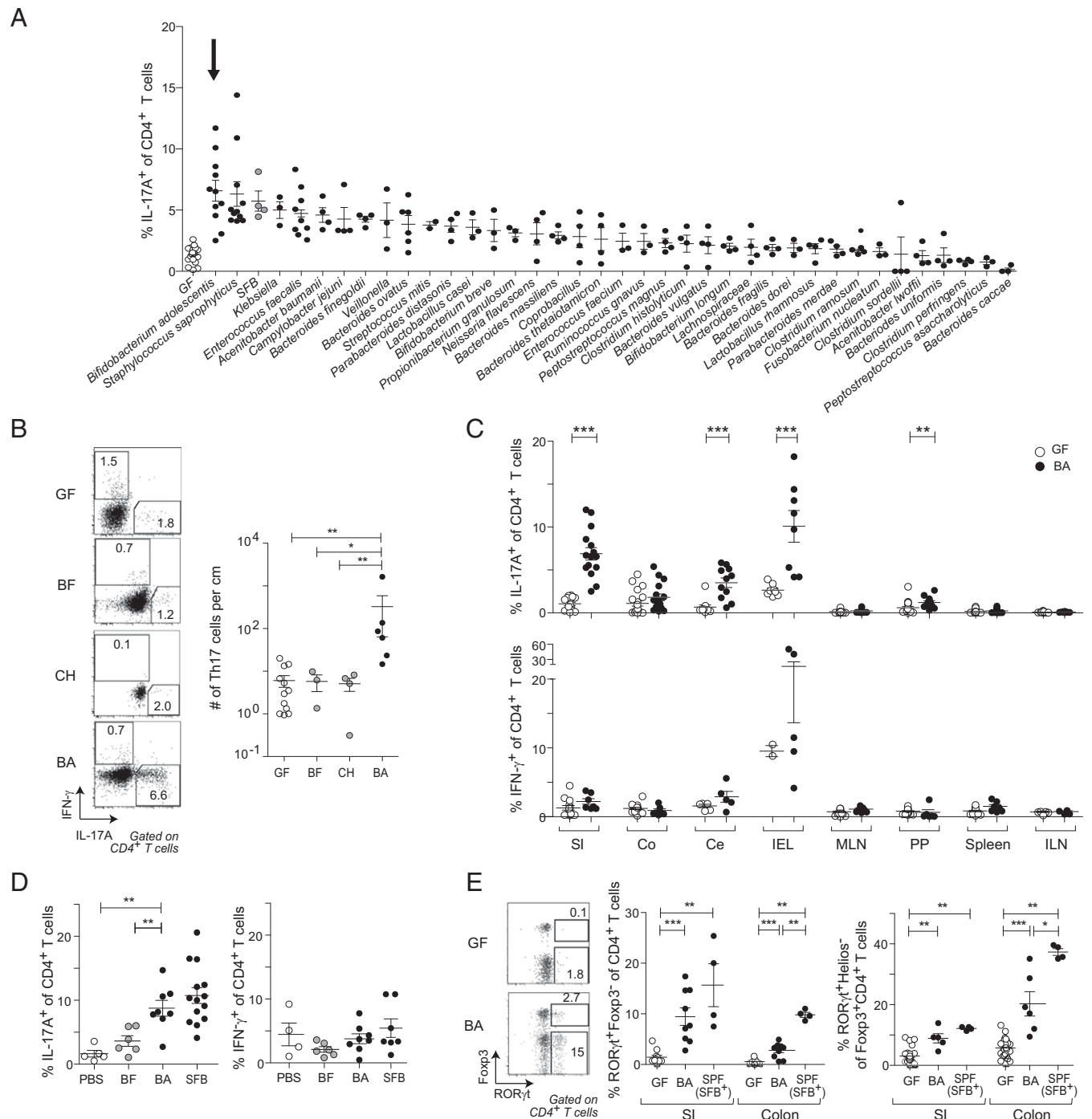


Fig. 1. *B. adolescentis* (BA) induces a robust intestinal Th17 population. (A) Frequency of SI-LP Th17 cells in GF mice monoclonized with individual symbiont bacteria as described in *Materials and Methods*. White and gray symbols represent GF and SFB-monoclonized mice, respectively. Arrow indicates BA-monoclonized mice. (B) Inflammatory cytokine production by SI-LP CD4⁺ T cells in mice colonized as indicated. (Left) Representative flow cytometric dot plot. (Right) Summary data. (C) Frequencies of (Upper) Th17 and (Lower) Th1 cells in various tissues of mice colonized as indicated. (D) Frequencies of (Left) Th17 and (Right) Th1 cells in the SI-LP of SPF mice gavage as described in *Materials and Methods* with the indicated microbes. SFB⁺ SPF mice were bred at Harvard Medical School and naturally colonized with SFB. (E) Induction of intestinal ROR γ t expressors. (Left) Representative flow cytometric dot plot of SI-LP CD4⁺ T cells; summary data for frequencies of (Center) ROR γ t⁺ Foxp3⁻ cells and (Right) ROR γ t⁺ Helios⁻ Treg cells. Numbers in B and E refer to the fractions of cells in the identical gates. (B–E) Mean \pm SEM pooled from two to four independent experiments. BF, *B. fragilis*; Ce, cecum; CH, *C. histolyticum*; Co, colon; IEL, intraepithelial lymphocyte layer; ILN, inguinal lymph node; MLN, mesenteric lymph nodes; PP, Peyer’s patches; SI, small intestine. * P < 0.05 (Mann–Whitney u test); ** P < 0.01 (Mann–Whitney u test); *** P < 0.001 (Mann–Whitney u test).

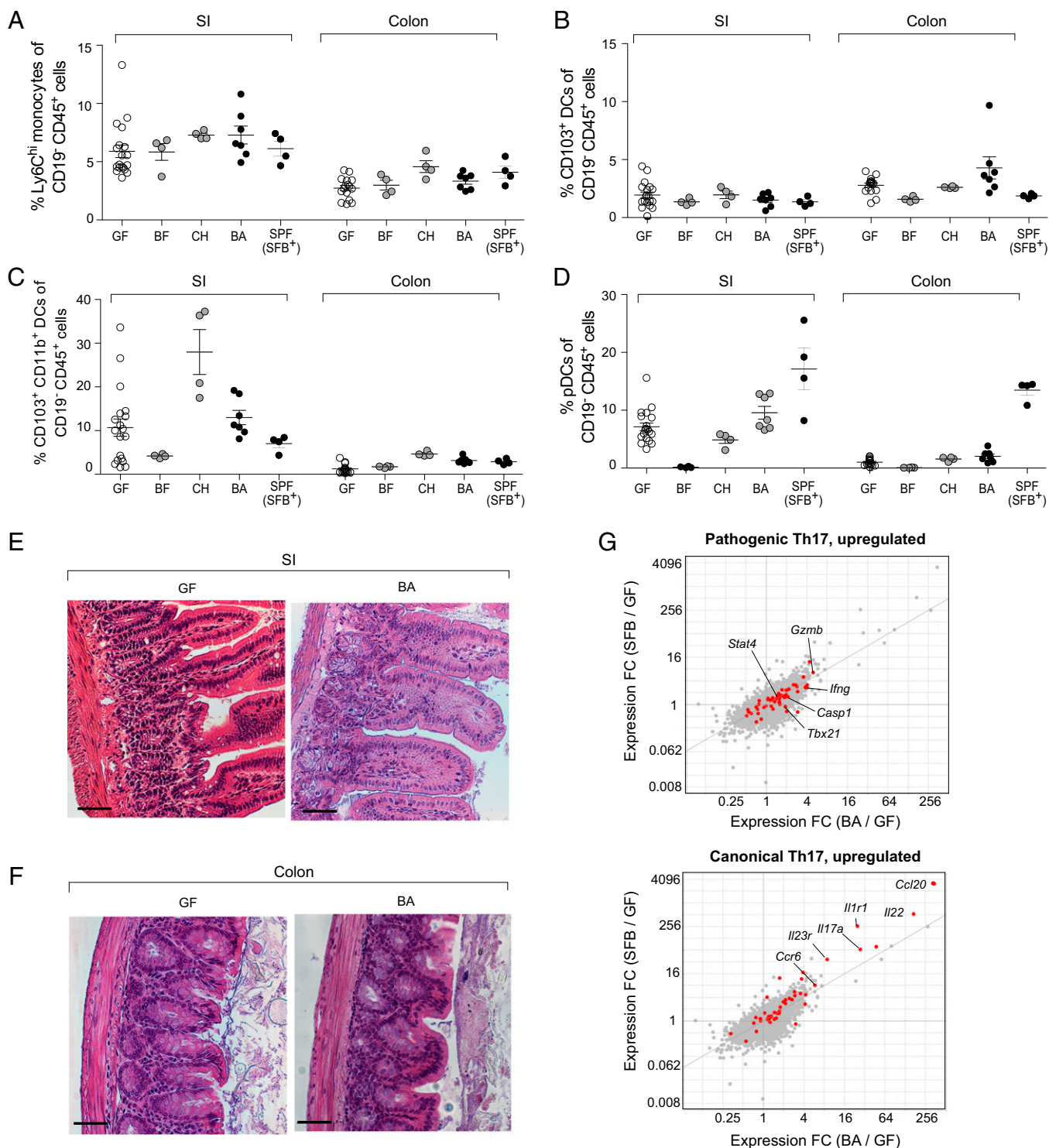


Fig. 2. *B. adolescentis* (BA) does not provoke intestinal inflammation. (A–D) Myeloid cells. Frequencies of the indicated myeloid cell populations in the intestines of mice colonized as indicated. Each symbol represents one mouse. Mean \pm SEM. Data for GF and BA are pooled from at least two independent experiments. *P* value was not significant for all comparisons (Kruskal–Wallis test and Dunn’s multiple comparisons test). (E and F) Histopathology. H&E staining of representative sections of (E) the small intestine (SI) and (F) the colon. (Scale bar: 50 μ m.) (G) Th17 cell phenotype. Fold change (FC)/FC plots comparing transcripts induced by BA vs. SFB in SI-LP CD4⁺ T cells. Red indicates transcripts up-regulated in (Upper) pathogenic Th17 cells or (Lower) canonical Th17 cells. Example genes induced for each Th17 phenotype are indicated (*n* = 3–4 per group). BF, *B. fragilis*; CH, *C. histolyticum*.

species examined, *B. adolescentis* (strain L2-32) promoted the greatest increase in Th17 cell frequencies and numbers in the SI-LP (Fig. 1A and B). In addition to the SI-LP, colonization with *B. adolescentis* significantly increased Th17 cell levels in several

other gut-associated organs, including the cecum, intraepithelial layer, and Peyer’s patches, although these effects were often much milder (Fig. 1C, Upper). In contrast, neither the percentages of Th17 cells in extraintestinal tissues (Fig. 1C, Upper) nor

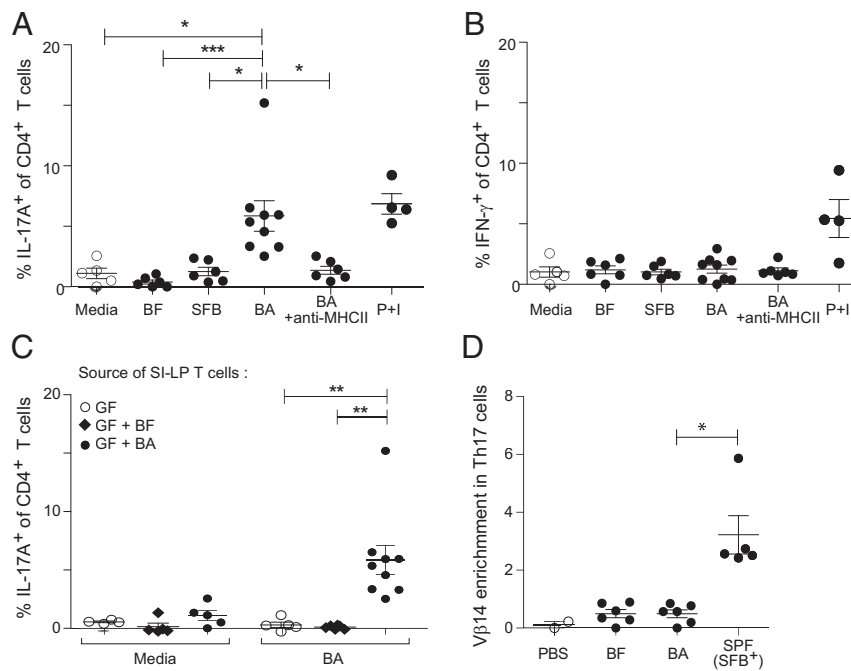


Fig. 3. *B. adolescentis* (BA)-driven intestinal Th17 responses are cognate. (A and B) BA-specific Th17 cells. Frequencies of SI-LP CD4⁺ T cells producing (A) IL-17A or (B) IFN- γ on overnight stimulation by splenic dendritic cells incubated with media alone or lysates prepared from the indicated bacterial species. +Anti-MHC-II (the blocking antibody M5/114.15.2) was added. (C) Frequency of SI-LP CD4⁺ T cells producing IL-17A on stimulation with media alone or BA lysate when T cells were isolated from mice colonized as indicated. (D) The degree of TCRV β 14 enrichment in SI-LP CD4⁺ T cells from SPF mice colonized as indicated, calculated as (percent of V β 14⁺ cells in Th17 fraction)/(percent of TCRV β 14⁺ cells in non-Th17 fraction). Each symbol represents one mouse. Data are pooled from two to four independent experiments. Mean \pm SEM. BF, *B. fragilis*; P + I, PMA and ionomycin. * P < 0.05 (Kruskal-Wallis test and Dunn's multiple comparisons test); ** P < 0.01 (Kruskal-Wallis test and Dunn's multiple comparisons test); *** P < 0.001 (Kruskal-Wallis test and Dunn's multiple comparisons test).

the fractions of Th1 cells in any organ (Fig. 1C, Lower) were substantially altered by *B. adolescentis*. Inoculation of specific-pathogen-free (SPF) mice with *B. adolescentis* recapitulated the immunologic phenotypes observed in monocolonized mice, augmenting Th17 frequencies in the SI-LP while leaving Th1 responses intact, in contrast to the lack of a significant response to a control microbe, *Bacteroides fragilis* (Fig. 1D). [Hereafter, we used either *B. fragilis* or *Clostridium histolyticum* as a control microbe because neither elicited significant SI-LP Th17 cell accumulation relative to GF mice (Fig. 1A and B).]

Because along with Th17 cells some intestinal Tregs express the transcription factor ROR γ t (8, 25), we looked more broadly at ROR γ t-expressing CD4⁺ T-cell populations in the gut. ROR γ t⁺ Foxp3⁻ CD4⁺ T cells and ROR γ t⁺ Foxp3⁺ Tregs were both significantly enriched in the SI-LP and colonic lamina propria (LP) compartments of *B. adolescentis*-monocolonized mice (Fig. 1E). However, ROR γ t⁺ Treg induction was relatively modest, seldom reaching the levels found in SFB⁺ SPF mice or mice monocolonized with human-gut-derived *Clostridium* or *Bacteroides* species (Fig. 1E) (8). In addition, there was no correlation between ROR γ t⁺ Th17 and Treg cell induction across the panel of strains originally screened (Fig. S1A and B). Interestingly, Th17 cell frequencies in the colonic LP were not elevated in *B. adolescentis*-monocolonized mice (Fig. 1C), despite a significant induction of ROR γ t in Foxp3⁻ CD4⁺ T cells (Fig. 1E), implying a disjunction between ROR γ t expression and cytokine production (26). Indeed, the proportion of ROR γ t⁺ T cells producing IL-17A was diminished in the colon relative to the small intestine (Fig. S1C). Unlike the ROR γ t⁺ Treg subset, overall Foxp3⁺ Treg and IL-10⁺ T-cell frequencies were not altered by *B. adolescentis* (Fig. S1D). Hence, *B. adolescentis* preferentially induced Th17 cells in the intestine, with modest concomitant expansion of ROR γ t⁺ Treg cells.

Apart from CD4⁺ T cells, an array of leukocyte subsets also secretes IL-17A and IL-22, often in response to similar cytokine cues, including IL-1 and IL-23 (27). We, thus, investigated the effects of *B. adolescentis* on cytokine production from other immunocyte populations. This microbe mildly increased cytokine production from intestinal $\gamma\delta$ T cells (Fig. S1E) and ROR γ t⁺ type 3 innate lymphoid cells (Fig. S1F and G), although this effect was also detected for other human symbiont bacteria that did not induce Th17 cells in our screen, in line with a previous report (11).

In rodents, SFB colonization leads to robust germinal center (GC) B-cell responses in the Peyer's patches and the subsequent accumulation of T-cell-dependent IgA-producing plasma cells in the SI-LP (28, 29). Additionally, Th17 cells promote IgA class switching in the Peyer's patches (30). We, therefore, assessed the impact of *B. adolescentis* on intestinal B-cell responses but found enhancement of neither the Peyer's patch GC B-cell levels (Fig. S1H) nor SI-LP IgA-producing plasma cell frequencies (Fig. S1I). Taken together, our findings indicate that *B. adolescentis* exerted a potent and specific effect only on CD4⁺ T cells, primarily in the small intestine.

***B. adolescentis* Does Not Provoke Either Intestinal or Systemic Inflammation.** Because Th17 cells manifest potent proinflammatory effector functions and have been associated with both intestinal and systemic inflammatory diseases, we sought to determine whether expansion of the intestinal Th17 compartment in *B. adolescentis*-monocolonized mice was accompanied by inflammation in the gut or extragut organs. Multiple findings argue against *B. adolescentis* triggering generalized inflammation. First, Th1 cell numbers are often elevated in cases of inflammation and immunopathology, but we saw no increase in gut or systemic Th1 responses (Fig. 1C). Second, the number of CD45⁺ leukocytes and frequencies of various intestinal myeloid subsets that typically expand during colitis or gut infections

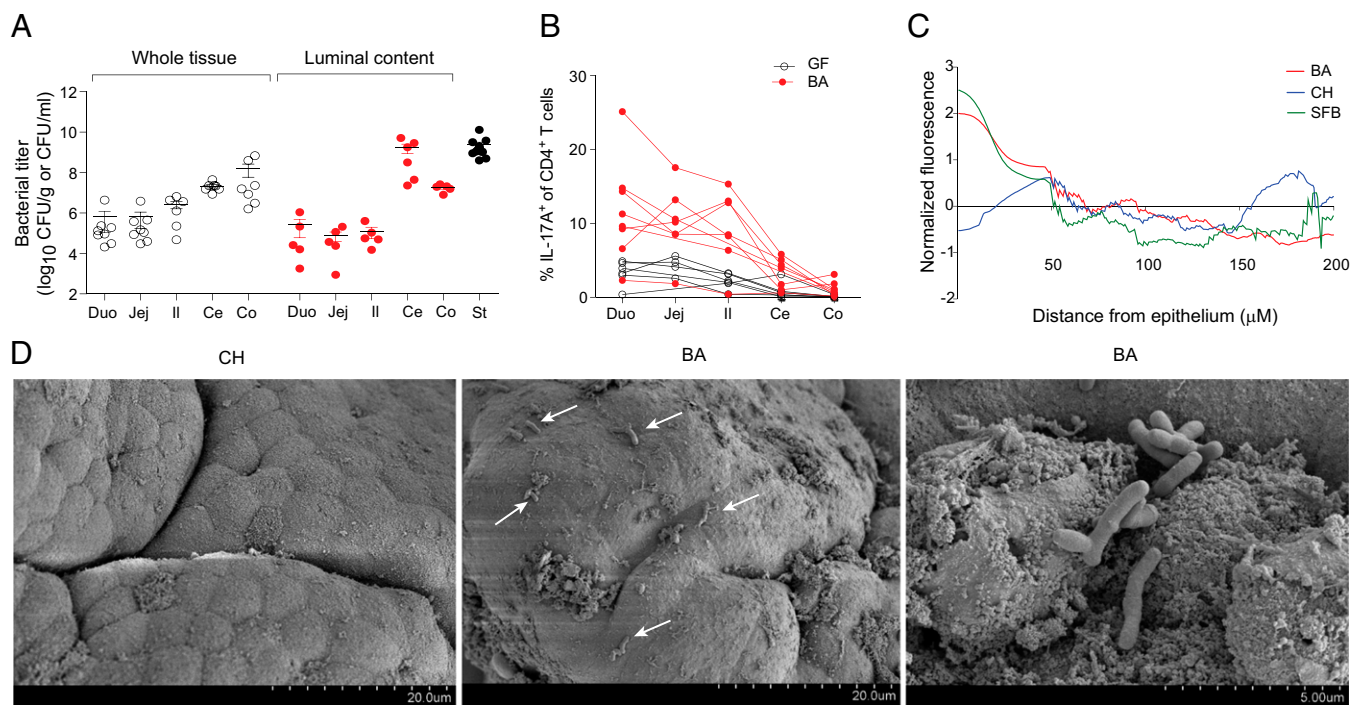


Fig. 4. *B. adolescentis* (BA) colonizes the entire length of the intestines, closely associating with the ileal epithelium. (A) Titers of bacteria associated with various segments of the intestinal mucosa or shed into the corresponding lumen or the stool (St). Normalized to tissue or St weight or the volume of luminal wash. Each symbol represents one mouse. Data are pooled from three to four independent experiments. Mean \pm SEM. (B) Frequencies of Th17 cells along the length of the intestinal LP in mice colonized as indicated. Each line represents frequencies from one mouse. Data are pooled from three independent experiments. (C) FISH quantification. Normalized bacterial fluorescence vs. distance from the epithelial surface of the terminal ileum (Il) from mice mono-colonized as indicated. Data are plotted as the average fluorescence from six to eight total images from two to three fields of view per section from two to four mice per microbe. (D) Representative SEM photograph of the ileal surface in mice colonized as indicated. Arrows indicate sites of bacterial association with the intestinal epithelium. Ce, cecum; CH, *C. histolyticum*; Co, colon; Duo, duodenum; Jej, jejunum.

(31) remained similar in GF and *B. adolescentis*-monocolonized mice (Fig. 2*A–D* and Fig. S2*A* and *B*). Although Ly6C^{hi} monocytes were slightly expanded by *B. adolescentis* in the SI-LP, this increase was not significant, and several other intestinal symbionts produced the same effect without attendant histological signs of inflammation (Fig. 2*A*). Thus, the modest increase in Ly6C^{hi} monocytes driven by *B. adolescentis* likely reflects a physiological response to colonization by broad classes of microbes. Third, histological examination revealed the absence of gross signs of inflammation in the small intestine and colon (Fig. 2*E* and *F* and Fig. S2*C*). Fourth, transcriptional profiling of small-intestinal CD4⁺ T cells showed only a modest up-regulation of genes associated with pathogenic Th17 cells (32) in GF mice on colonization with *B. adolescentis*; this increase was comparable with that elicited by SFB and weaker than the up-regulation of canonical Th17 transcripts observed for both microbes (e.g., *Rorc* and *Ccr6*) (33) (Fig. 2*G*). Therefore, *B. adolescentis* seems to be a bona fide intestinal symbiont akin to SFB in mice, capable of peaceful coexistence in the gut of a healthy host, despite its profound impact on the Th17 compartment.

Gut Th17 Cells Expanded by *B. adolescentis* Are Symbiont-Specific. Recent studies have shown that gut Th17 cells in SFB-bearing hosts are specific for SFB-derived antigens (34, 35). To determine if *B. adolescentis*-induced intestinal Th17 cells are analogously specific for *B. adolescentis*, we isolated CD4⁺ T cells from the small intestine of monocolonized mice and measured their cytokine responses to stimulation by lysates from various bacterial species. IL-17A production was markedly enhanced on restimulation by *B. adolescentis* lysate to levels comparable with those provoked by activation with phorbol 12-myristate 13-acetate (PMA) plus ionomycin but was not augmented by restimulation by *B. fragilis* or SFB lysates (Fig. 3*A*). The increased Th17 response to *B. adolescentis* was also dependent

on MHC class II (MHC-II) molecules (Fig. 3*A*). Consistent with the lack of Th1 cell induction by *B. adolescentis*, IFN- γ production was uniformly low in response to all bacteria tested and remained unaltered by antibody blockade of MHC-II molecules (Fig. 3*B*). *B. adolescentis*-specific Th17 responses were detected only in mice colonized with *B. adolescentis* but not in GF or *B. fragilis*-colonized mice (Fig. 3*C*). Moreover, Th17 cells in SPF mice gavaged with *B. adolescentis* did not display preferential V β 14 T-cell receptor (TCR) chain use as exhibited by SFB-specific Th17 cells (Fig. 3*D*) (35), suggesting that the gut Th17 cells elicited by *B. adolescentis* and SFB were not recognizing a common immunodominant microbial antigen. Collectively, the data indicate that intestinal Th17 cells induced by *B. adolescentis* were symbiont-specific.

***B. adolescentis* Colonizes the Gastrointestinal Tract Widely and Closely Associates with the Epithelium.** The enrichment of Th17 cells in the ileum of SFB-harboring mice corresponds to an overrepresentation of SFB in that intestinal segment (36, 37) and the ability of SFB to form intimate associations with the intestinal epithelium (11). To determine whether *B. adolescentis* occupied an intestinal niche similar to that of SFB, we measured bacterial titers in various intestinal compartments of mice monocolonized with the former. *B. adolescentis* was found in both the gut mucosa and lumen, with the overall bacterial load in the lumen progressively increasing from the duodenum to the colon, reflecting the distribution of overall bacterial burden in SPF mice (38) (Fig. 4*A*). Bacterial loads did not, however, correlate with Th17 levels, because the colon harbored relatively few Th17 cells while hosting very high quantities of *B. adolescentis* (Figs. 1*C* and 4*A* and *B*), although the uniformly high *B. adolescentis* titers throughout the small intestine might explain

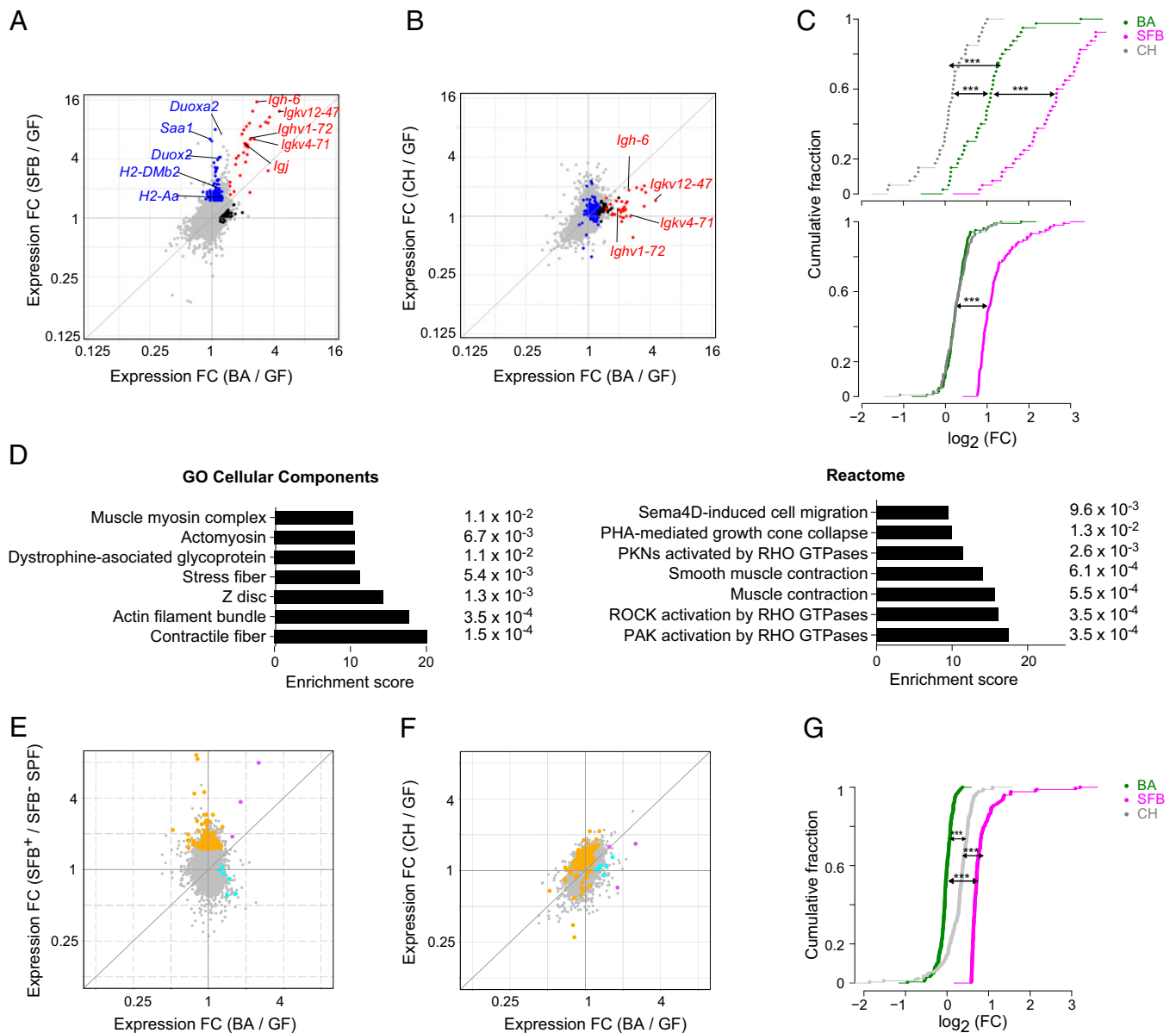


Fig. 5. *B. adolescentis* (BA) and SFB both induce B-cell transcripts, but otherwise, they trigger distinct transcriptional programs. (A and B) Whole-tissue transcriptomes. Fold change (FC)/FC plots comparing ileum tissue transcripts induced by (A) BA vs. SFB (Th17 inducing) or (B) BA vs. *C. histolyticum* (CH; Th17 noninducing). In A, blue indicates genes up-regulated primarily by SFB, black indicates genes up-regulated primarily by BA, and red indicates genes up-regulated by both bacteria. Certain transcripts previously associated with SFB-mediated induction of Th17 cells or encoding Igs are indicated. In B, the genes highlighted in A are again highlighted in the same colors. (C) Statistics for A and B. FC distribution of (Upper) SFB-induced Ig or (Lower) non-Ig RNAs in the ileal transcriptomes of mice colonized as indicated. Only transcripts increased by SFB by at least ≥ 1.6 -fold with a *P* value of < 0.05 were plotted. $***P < 0.001$ (Kolmogorov-Smirnov test). (D) Pathways (from the two databases as indicated) enriched in transcripts specifically up-regulated in the ileum by BA relative to GF mice were determined using Enrichr. *P* values are indicated to the right. (E and F) S-IEC transcriptomes. As per A and B, except that transcripts from isolated S-IECs were examined. In E, orange indicates genes up-regulated primarily by SFB, cyan indicates genes up-regulated primarily by BA, and purple indicates genes up-regulated by both bacteria. In F, the genes highlighted in E are again highlighted in the same colors. (G) Statistics for E and F. Calculated as per C. *n* = 3 (A–D) or 2–4 (E and F) per group. $***P < 0.001$.

the increase in Th17 cells in the duodenum and jejunum as well as the ileum (Fig. 4B).

To visualize the intestinal niche of *B. adolescentis*, we performed FISH on ileum and colon sections from *B. adolescentis*-monocolonized mice and quantified bacterial densities in relation to their distance from the intestinal epithelium (Fig. 4C). Like SFB, *B. adolescentis* associated closely with the ileal but not the colonic epithelium. In contrast, *C. histolyticum*, a human symbiont that did not promote Th17 cell expansion, was found primarily in the ileal lumen. SEM revealed *B. adolescentis* but not *C. histolyticum* to be localized close to the ileal surface in

mice, corroborating the FISH findings (Fig. 4D). The capacity for tight association with the epithelium may thus represent a conserved feature of Th17 cell-inducing microbes.

***B. adolescentis* and SFB Induce Largely Distinct Transcriptional Programs in the Intestine.** Because the effects of SFB and *B. adolescentis* on the host immune system seemed similar, and because both microbes interacted closely with the small-intestinal epithelium, we next profiled gene expression in whole ileal tissue from GF mice and mice monocolonized with *B. adolescentis*, control *C. histolyticum*, or SFB to determine whether *B. adolescentis*

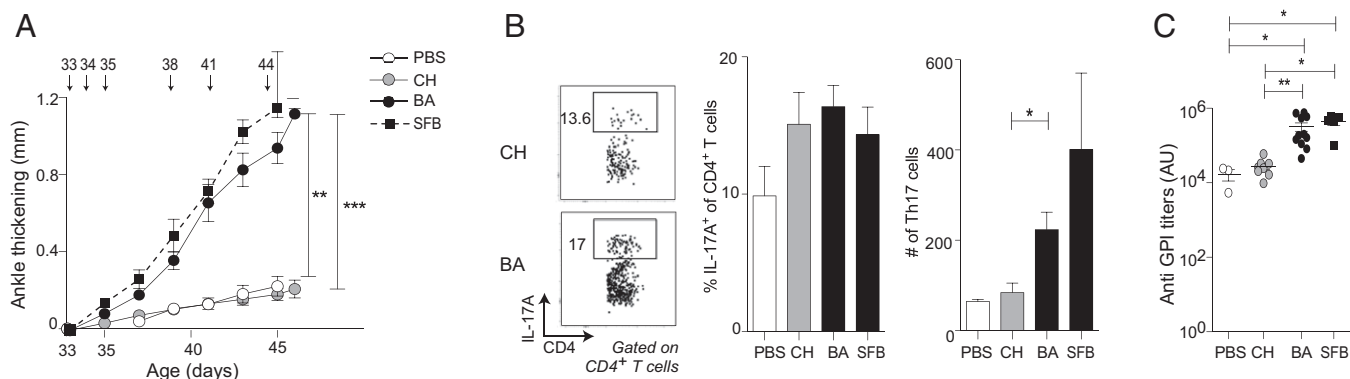


Fig. 6. *B. adolescentis* (BA) exacerbates K/BxN arthritis. K/BxN mice were pretreated with antibiotics from 22 to 32 d of age and subsequently gavaged with PBS, *C. histolyticum* (CH), or BA over 12–13 d. Arrows indicate gavage time points. (A) Ankle-thickening values over the course of bacterial gavage. (B, Left) Representative flow cytometric plot of IL-17A production from SI-LP CD4⁺ T cells 11–13 d after the initial bacterial gavage. Summary data of (B, Center) frequencies and (B, Right) numbers of SI-LP Th17 cells at the same time point. (C) Antigliucose-6-phosphate isomerase titers 11–13 d after initial bacterial gavage. Each symbol represents one mouse [$n = 3$ (PBS), 8 (CH), 11 (BA), and 2–5 (SFB)]. Data for CH and BA are pooled from two to three independent experiments. Mean \pm SEM. GPI, glucose-6-phosphate isomerase. * $P < 0.05$ (Kruskal–Wallis test with Dunn’s multiple comparisons test); ** $P < 0.01$ (Kruskal–Wallis test with Dunn’s multiple comparisons test); *** $P < 0.001$; (Kruskal–Wallis test with Dunn’s multiple comparisons test).

and SFB triggered overlapping intestinal gene programs that might account for their ability to elicit robust Th17 populations (Fig. 5A and B). A substantial number of genes was up- or down-regulated in common by the Th17-cell-inducing bacteria, but we also detected a microbe-specific transcriptional imprint for each of them (Fig. 5A and Tables S1, S2, and S3). RNAs up-regulated by both SFB and *B. adolescentis* were dominated by *Ig* gene transcripts (Fig. 5A, red symbols). This induction likely reflected a robust IgA response in the case of SFB (9, 39), but we did not observe any signs of an enhanced IgA response (Fig. S1G) or a change in B-cell numbers in mice monocolonized with *B. adolescentis*. In this case, the induction of *Ig* transcripts was likely to be a consequence of increased intestinal IgM⁺ plasma cells, because *Igj* and *Igh-6*, encoding the J chain of secretory IgA/IgM and the constant region of the IgM heavy chain, respectively, were among the *Ig* transcripts most strongly induced. Quantitative comparison revealed that the *Ig* transcripts up-regulated by SFB were significantly enriched in *B. adolescentis*- and SFB-colonized mice compared with mice colonized with *C. histolyticum* (Fig. 5C, Upper).

RNAs specifically enriched in the intestines of SFB⁺ mice included several previously imputed to SFB, such as MHC-II transcripts (40) and mRNAs encoding molecules that augment Th17 responses (e.g., the serum amyloid A family of proteins), *Duox2*, and *Duoxa2* (11, 26) (Fig. 5A, blue symbols). Expression of these transcripts was increased by *B. adolescentis* as well, but the level of induction was much lower than that for SFB and comparable with that provoked by non-Th17-inducing *C. histolyticum* (Fig. 5B and C, Lower). Parsing of the genes specifically induced by *B. adolescentis* (Fig. 5A, black symbols) revealed an enrichment in non-immunologic, particularly muscle-related, pathways that suggested a role for nonhematopoietic intestinal cells in relaying bacterial signals to the host immune system (Fig. 5D).

A clear divergence in gene expression profiles was also observed in small-intestinal epithelial cells (S-IECs) purified from mice colonized with *B. adolescentis* vs. SFB (Fig. 5E and Tables S4–S6). Indeed, the S-IEC transcriptome responses elicited by *B. adolescentis* and *C. histolyticum* were more concordant with each other than with those provoked by SFB (Fig. 5E and F), and RNAs up-regulated by SFB were significantly more enriched in SFB- and *C. histolyticum*-colonized mice compared with *B. adolescentis*-colonized mice (Fig. 5G). Many of the SFB-specific transcripts in S-IECs (e.g., *Duoxa2* and MHC-II transcripts) were unique to SFB in the ileum as well, suggesting a primary role for S-IECs in coordinating the host response to SFB but not to the

other two microbes. Accordingly, the number of transcripts in S-IECs with expression that was differentially regulated by bacterial colonization was far higher for SFB than for *B. adolescentis*, and the identities of *B. adolescentis*-specific RNAs in the ileum and S-IECs were distinct (Tables S1–S6).

***B. adolescentis* Exacerbates Autoimmune Arthritis in a Mouse Model.**

Elevated Th17 cell responses have been associated with autoimmune/inflammatory disease in both mice and humans (19, 20). For example, SFB colonization promotes disease in the K/BxN mouse model of RA, in part by inducing SI-LP Th17 cells that emigrate from the gut to the spleen, where they promote production of autoantibodies against glucose-6-phosphate isomerase (24, 41). Such autoantibodies in and of themselves can induce arthritis and, after they reach high levels, require no further input from Th17 cells. To assess the role of symbiont-induced Th17 populations in autoimmune arthritis, we gavaged SPF K/BxN mice with *B. adolescentis*, *C. histolyticum*, SFB, or PBS. Similar to SFB, *B. adolescentis*, but not PBS or the control microbe *C. histolyticum*, drove more severe arthritis, as evidenced by increased joint thickening (Fig. 6A). Heightened disease in *B. adolescentis*-treated mice was associated with increased numbers (although not frequencies) of SI-LP Th17 cells (Fig. 6B) and elevated titers of antigliucose-6-phosphate isomerase autoantibodies (Fig. 6C). Thus, *B. adolescentis* drives gut-distal Th17-cell-associated disease progression.

Probiotics Containing *Bifidobacterium* Species Can Augment Intestinal Th17 Cell Compartments.

The genus *Bifidobacterium*, of which *B. adolescentis* is a member, is a common component of healthy infant and adult microbiotas and is often included in probiotic formulations because of its purported benefits in promoting gastrointestinal health. The question arose whether such probiotic preparations share *B. adolescentis*’ ability to induce intestinal Th17 cells. We evaluated six probiotic formulations available online, all but two of which (FiveLac and *Bifidobacterium infantis*, which both contain one *Bifidobacterium* strain) contained two or more *Bifidobacterium* species (Table S7). When introduced into GF mice, four of the preparations significantly induced Th17 but not Th1 cell accumulation in the SI-LP (Fig. 7A) comparing values from GF mice. Similar to what was seen with *B. adolescentis*, gavage of Nexabiotic highly induced canonical but not pathogenic signature genes in whole SI-LP tissue. We also tested three of the probiotic preparations in SPF-housed mice, given their complex, more natural microbiotas. One of two Th17-inducing probiotic

mixes reproducibly induced the accumulation of SI-LP Th17 cells in SPF mice without significantly altering Th1 cell frequencies (Fig. 7B). Thus, it seems that Th17 cell induction in the gut may be a feature widely shared by probiotics.

Discussion

We have identified individual symbiont microbes from the human gut that can induce robust Th17 populations in the murine intestine. Interestingly, the mechanisms used by the most potent inducer, *B. adolescentis*, differed from those of the well-known Th17-promoting mouse symbiont SFB. *B. adolescentis* exacerbated autoimmune arthritis, arguing for its pathological relevance. These findings raise several interesting issues meriting additional elaboration.

First, bifidobacteria seem to be common inducers of intestinal Th17 cells. A complex microbial community was insufficient for generating a robust population of Th17 cells in the gut of SPF mice lacking SFB (9, 10) and gnotobiotic mice colonized long term with human fecal contents (42). Although SFB has been detected in multiple vertebrate species (43), there exists only sparse evidence of a related microbe colonizing humans (43–46). A recent study showed that a consortium of 20 symbionts from the feces of an IBD patient could induce Th17 cells in mice but failed to identify the active microbes in healthy people (11).

Although *B. adolescentis* was 1 of 3 microbes (of a total of 39) in our screen that was able to robustly induce intestinal Th17 cells, we think it highly plausible that many other symbiont species, including other bifidobacteria, can act singly or in concert with other microbes to promote Th17 cell accumulation in the human gut. Given the tremendous diversity of the human microbiota (47), our screen was performed limited to testing a fraction of it. Even with this restricted scope, we succeeded in identifying three bacterial species spanning distinct phyla that could induce intestinal Th17 cells to a degree comparable with that of SFB. Wider sampling would almost certainly unveil more microbes with this property. Indeed, most of the bifidobacteria-containing probiotics that we tested potently expanded Th17 cells, arguing that this property might be a relatively common bifidobacterial trait. Relatedly, as exemplified by SFB in rodents, some intestinal symbionts show host specificity, consequent to millennia of coevolution (11, 42). By design, our screen elides symbiont strains capable of inducing Th17 cells in humans but unable to do so in mice. Moreover, some symbionts might exert their effects on the host immune system only in the presence of other microbes, a restriction that might apply to certain bifidobacterial species, which could account for the prevalence of Th17 induction by probiotic mixes. Hence the results from our screen likely underestimate the true number and diversity of Th17-promoting human symbionts.

Bifidobacteria are ubiquitous symbionts, well-represented in the gut microbiota of healthy humans across age and geography (47). In infants, they are among the first colonizers of the intestine, and their abundance serves as a biomarker of a healthy microbiota (47, 48). With age, the frequency of bifidobacteria in the gut wanes, and the dominant species change, although members of the genus remain a substantial component in the adult (48, 49). A metagenomic sequencing study of gut microbes from 124 adults identified several *Bifidobacterium* strains as dominant symbionts, with *B. adolescentis* exceeding 10% in relative abundance in two-thirds of the individuals (50). Thus, *B. adolescentis*, along with other bifidobacteria, is well-poised to be a universal Th17-inducing symbiont in humans throughout ontogeny into adulthood.

Second, *B. adolescentis* induced Th17 cells by a mechanism that clearly diverged from that of SFB. Overall, SFB triggered more pronounced transcriptional changes than those elicited by *B. adolescentis*, *C. histolyticum*, or the vast majority of human symbionts that we have tested. A prosaic explanation for this

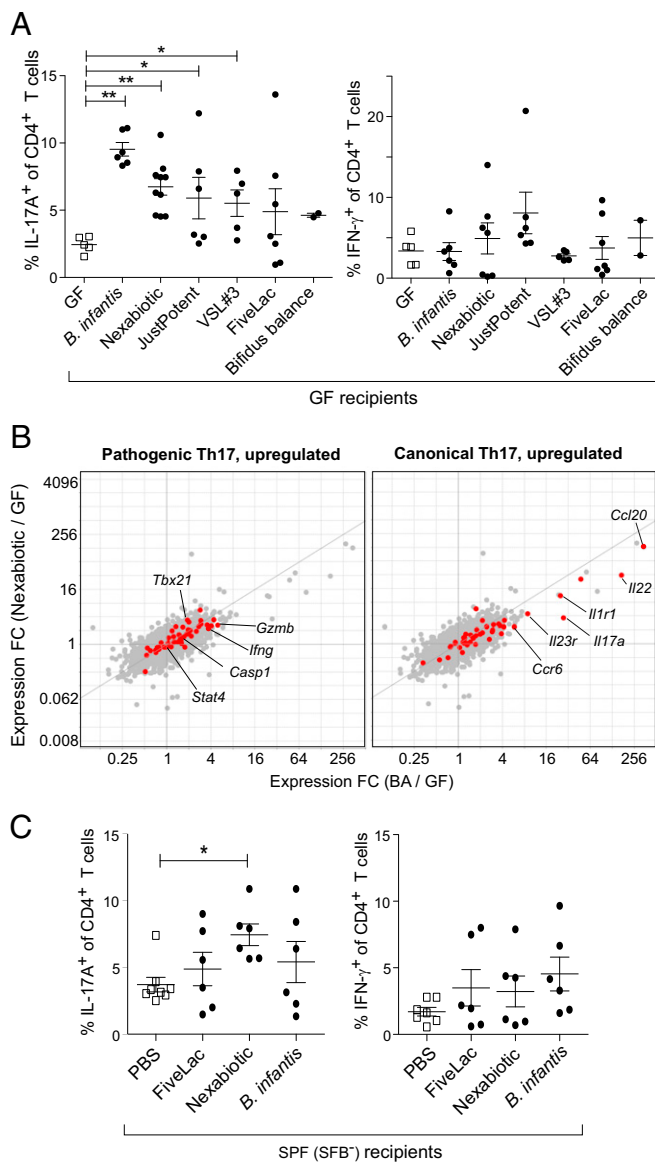


Fig. 7. Some probiotic formulations containing bifidobacterial species also elicit Th17 populations in the SI-LP. (A) Frequencies of SI-LP (Left) Th17 or (Right) Th1 cells in GF mice colonized with the indicated probiotic mixes. (B) Th17 cell phenotype. Fold change (FC)/FC plots comparing transcripts induced by *B. adolescentis* (BA) vs. the probiotic mix, Nexabiotic, in SI-LP CD4⁺ T cells. Symbols in red and labels are as per Fig. 2G ($n = 2\text{--}3$ per group). (C) Frequencies of SI-LP (Left) Th17 and (Right) Th1 cells in SPF (SFB⁻) mice gavaged with PBS or one of three other probiotic preparations in A. Full names and corresponding abbreviations of probiotics can be found in *SI Materials and Methods*. Each symbol represents one mouse. Data are pooled from two to three independent experiments. Mean \pm SEM. * $P < 0.05$ (Mann-Whitney u test); ** $P < 0.01$ (Mann-Whitney u test).

divergence is that SFB is a murine symbiont and thus better adapted to interact with the mouse host, thereby inducing more profound gene expression changes. Related or not to the host species, *B. adolescentis* was located in considerable quantities in the intestinal lumen in close association with the epithelium, whereas SFB was found almost exclusively attached to the ileal surface. In addition, the association of SFB seemed tighter, actually penetrating the epithelium in places. SFB and *B. adolescentis* seemed to mobilize distinct cell types and transcriptional programs to induce Th17 responses. The transcriptional changes effected by *B. adolescentis* on S-IECs were relatively subtle and

distinct from those on whole ileal tissue, where the up-regulated RNAs were enriched in non-S-IEC-related pathways, such as muscle contraction and interactions with the ECM. These pathways could potentially regulate the activity of mechanosensitive integrins and cytokines (e.g., TGF- β) relevant to Th17 cell differentiation and trafficking. Interestingly, DNA from SFB-like microbes was recently enriched in the gut of human IBD patients, associated with cavernous fistulous tracts running between muscle bundles (46). The enrichment for muscle-related pathways in whole-tissue SI-LP preparations from mice colonized with *B. adolescentis* hints at a more general relationship between Th17-inducing microbes and intestinal muscle tissue. In contrast, S-IECs seemed to be critical drivers of the ileal transcriptional response to SFB, in line with previous studies (10, 11). Gut microbes also produce metabolites that can access the stroma and immunocytes in the LP without directly interacting with the gut epithelium (51), and intestinal antigen presenting cells can extend their dendrites into the lumen to sample bacteria directly (52). These mechanisms, in particular those accomplished by intestinal immunocytes, might explain the relatively modest impact of *B. adolescentis* on the transcriptomes of S-IECs and the ileum (where leukocytes are vastly outnumbered by nonhematopoietic cells).

Third, Th17 cells seem to have a yin-yang role in human health. Mice devoid of IL-17 signaling manifest alterations in their microbiotas and suffer from increased intestinal permeability and bacterial translocation to systemic sites after infectious insults of the gut (15, 16, 53). Additionally, loss of Th17 populations during infections by either simian virus or HIV has been associated with intestinal dysbiosis, systemic microbial translocation, and disease progression (53–55). Moreover, SFB confers heterologous protection from the murine enteropathogen *C. rodentium* (10). Hence, symbiont-driven intestinal Th17 cells seem to bolster host mucosal defenses via various mechanisms, including the augmentation of barrier integrity, the provision of cross-protective defenses against pathogens during early stages of infection, and sculpting of the gut microbiota.

However, microbiota-dependent Th17 responses have been implicated in IBD and other extraintestinal autoimmune disorders, including psoriasis, multiple sclerosis, and RA. Elevated Th17 frequencies have been observed in the intestinal mucosa of IBD patients (20), and increased IL-17A titers can be detected in the synovial fluid of people afflicted with RA (56, 57). Variants in genes important for Th17 cell differentiation and function (e.g., *IL23R* and *CCR6*) have also been associated with the severity of these diseases (58–60). Furthermore, dysbiosis is concomitant with new-onset, treatment-naïve IBD and RA, implying a potential etiological role for the intestinal microbiota (21, 22). Of note, the relative abundances of *B. adolescentis* and several *Bifidobacterium* species were profoundly altered in the microbiotas of pediatric (22) and adult IBD subjects (Fig. S3) (50), albeit in opposite directions, with an enrichment of bifidobacteria in the microbiotas of the latter cohort of patients. In support of a pathogenic role for symbiont-driven Th17 cells in inflammatory diseases, we observed an exacerbation of spontaneous autoimmune arthritis in mice gavaged with *B. adolescentis* but not with the non-Th17-inducing microbe, *C. histolyticum*.

In this context, how should one interpret the induction of Th17 cells by several probiotic formulations in widespread use as ad hoc nutritional supplements? Our in vivo data are in concert with findings on cultured human immunocytes (61). One interpretation is that this induction is part of their favorable action in the setting of gastrointestinal infection and dysbiosis (62), where Th17 cells elicited by probiotics might evince anti-infectious benefits. However, one might also consider that these Th17 cells contribute in an unrecognized manner to the frequency or exacerbation of chronic inflammatory diseases linked to Th17 responses, such as RA or multiple sclerosis.

Materials and Methods

Mice. Unless otherwise stated, SPF C57BL/6J (B6) mice were obtained from the Jackson Laboratory and housed under SPF conditions at Harvard Medical School. GF mice were bred and maintained in sterile isolators at Harvard Medical School. Manipulations of mice are detailed in *SI Materials and Methods*. Experiments were conducted according to the guidelines of the Harvard Medical School Institutional Animal Care and Use Committee.

Bacteria and Probiotics. Bacteria were cultured as previously described (8). Bacteria and probiotics used are detailed in *SI Materials and Methods*.

Isolation of S-IECs, Intraepithelial Lymphocytes, and Intestinal LP Leukocytes. S-IECs, intraepithelial lymphocytes, and leukocytes were processed as previously described (8) and are further detailed in *SI Materials and Methods*.

Antibodies and Flow Cytometry. Single-cell suspensions from intestinal tissues and lymphoid organs were stained with antibodies for flow cytometry and analyzed as detailed in *SI Materials and Methods*.

Antigen Presentation Assays. Antigen presentation assays are detailed in *SI Materials and Methods*.

Measurement of Bacterial Titers. Bacterial titers from monoclonized mice were measured as detailed in *SI Materials and Methods*.

Histopathology. Histopathology of intestinal sections was scored as detailed in *SI Materials and Methods*.

FISH and SEM. FISH and SEM were performed on intestinal sections as previously described (63, 64) and are detailed in *SI Materials and Methods*.

K/BxN Murine Arthritis Model and ELISA. Three-week-old K/BxN mice of both sexes were pretreated with antibiotics [1 g/L ampicillin (Sigma), 1 g/L neomycin (Fisher Scientific), 1 g/L metronidazole (Sigma), 0.5 g/L vancomycin (Amresco)] for 10 d, rested for 1 d, and subsequently gavaged with PBS or bacteria (10^8 cfu of *C. histolyticum* or *B. adolescentis*) for 3 consecutive days and every 3 d thereafter until the time of euthanasia. Ankle thickness was measured with a caliper (J15 Blet Micrometer) as previously described (24). All mice were housed at the SPF animal facility at the University of Arizona. Antiglucose-6-phosphate isomerase antibody titers were measured as previously described and further detailed in *SI Materials and Methods*. (41).

Gene Expression Profiling and Analysis. Microarray or RNA sequencing analysis was performed on whole ileal tissue, S-IECs, or SI-LP CD4⁺ T cells of monoclonized mice as detailed in *SI Materials and Methods*.

Comparison of the Microbiotas of Healthy Vs. IBD Subjects. Publicly available metagenomic profiling reads from 124 adults from the MetaHIT database (50) were analyzed as detailed in *SI Materials and Methods*.

Statistics. Unless otherwise stated, significance was assessed using the Mann-Whitney *u* test or the Kruskal-Wallis test with Dunn's multiple comparisons test (Prism 6; Graph-Pad). *P* values were deemed significant if less than 0.05. To compare ankle thickening, the area under the curve was calculated for each mouse followed by the Mann-Whitney *u* test between groups. Mean \pm SEM was routinely used.

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