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Selective Induction of Homeostatic Th17 Cells in the Murine Intestine by Cholera Toxin Interacting with the Microbiota

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Th17 cells play a role as an inflammation mediator in a variety of autoimmune disorders, including inflammatory bowel disease, and thus are widely considered to be pathogenic. However, Th17 cells are present in the normal intestine and show a homeostatic phenotype; that is, they participate in the maintenance of intestinal homeostasis rather than inducing inflammation. We observed an enlarged Th17 population in the small intestine of C57BL/6.IgA−/− mice compared with wild-type mice, which was further amplified with cholera toxin (CT) immunization without causing intestinal inflammation. The increased Th17 induction and the correspondingly 10-fold higher CT B subunit–specific serum IgG response in IgA−/− mice after CT immunization was microbiota dependent and was associated with increased segmented filamentous bacteria in the small intestine of IgA−/− mice.

Oral administration of vancomycin greatly dampened both CT immunogenicity and adjuvanticity, and the differential CT cells. Taken together, we identified a nonpathogenic signature of intestinal homeostatic Th17 cells, which are actively regulated by the expression of a panel of immune-regulatory genes, which was distinctly different from the gene expression profile of pathogenic Th17 cells.

We found that CT induction of homeostatic intestinal Th17 responses was supported not only by segmented filamentous bacteria, but also by other commensal bacteria. Furthermore, transcriptome analysis using IL-17AhCD2 reporter mice revealed a similar gene expression profile in CT-induced intestinal Th17 cells and endogenous intestinal Th17 cells at homeostasis, with upregulated expression of a panel of immune-regulatory genes, which was distinctly different from the gene expression profile of pathogenic Th17 cells. Taken together, we identified a nonpathogenic signature of intestinal homeostatic Th17 cells, which are actively regulated by the commensal microbiota and can be selectively stimulated by CT.

There are trillions of microbes residing in the normal mammalian intestine (1). These microbes include bacteria, archaea, fungi, and viruses, of which bacteria are the most abundantly studied. The role of intestinal microbiota in the induction and modulation of the host immune system has been increasingly recognized in recent years (2, 3). At steady-state, the mammalian intestine is the biggest reservoir of activated effector T cells, which provide protection against potential intestinal pathogens together with other immune cell subsets. Th17 cells are among the most abundant effector CD4+ T cells in the intestinal lamina propria (LP) (4). They are characterized by the expression of master transcription factor Rorγt and the production of cytokines including IL-17A, IL-17F, IL-21, and IL-22 (5). Th17 cells show heterogeneity in their gene expression profile in different research models and conditions. For instance, they are involved in the pathogenesis of an array of autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, experimental autoimmune encephalomyelitis, psoriasis, and inflammatory bowel diseases (IBD). Such pathogenic Th17 cells frequently coexpress IFN-γ and TNF-α (6). In other studies, Th17 cells have been shown to be indispensable in fighting against respiratory fungal infections and intestinal bacterial pathogens via the production of IL-17A and IL-22 (7).

Segmented filamentous bacteria (SFB) are a group of Gram-positive, spore-forming commensal bacteria that form long filaments and preferentially adhere tightly to the epithelium and Peyer’s patches of the terminal ileum. Colonization with SFB strongly induces endogenous Th17 cells, as well as a broad range of other proinflammatory cytokines in the murine small intestine (8, 9). Such induction is considered as a beneficial stimulation of the immune system and is shown to provide mice with better protection against Citrobacter rodentium infection. However, SFB may be only one among a large pool of microbes that are likely to elicit Th17 cells (10), and other bacterial species that could do so still await identification.
Cholera toxin (CT), which is a potent mucosal immunogen and adjuvant, also has the capacity to induce mucosal Th17 cell differentiation after intranasal administration (11). Mucosal delivery of CT induces strong mucosal and systemic humoral immune responses to itself and coadministered protein Ags (12), but does not cause intestinal inflammation. CT does so by activating Gs and the nucleotide-binding oligomerization domain containing 2 expressed in CD11c⁺ cells (13, 14). Th17 cells can convert into T follicular helper cells in the Peyers’ patches, thus enhancing high-affinity intestinal IgA production (15), suggesting an important role of Th17 cells in the induction of Ag-specific IgA in the gut, which in turn is involved in the protection against microbial Ags at the mucosal surface (16).

IgA deficiency is the most commonly seen primary immunodeficiency (17) and has been shown to relate to IBD pathogenesis on genome-wide association studies (18). Interestingly, in mice that are deficient of IgA, we observed increased Th17 cells in their normal small intestine, which can be further augmented with mucosal CT immunization without inducing any inflammation. This increased Th17 and Ab response to CT in IgA⁻/⁻ mice was dependent on intestinal microbiota. Experiments with gnotobiotic mice indicate that not only SFB, but also other commensal bacteria participate in CT induction of the Th17 response. Moreover, we performed mRNA sequencing analysis and found that CT-induced intestinal Th17 cells share a similar expression profile with endogenous intestinal Th17 cells at homeostasis, and both of them are distinctly different from pathogenic Th17 cells.

Materials and Methods

Mice

C57BL/6 mice and IL-6⁻/⁻ mice were purchased from The Jackson Laboratory. IgA⁻/⁻ mice were kindly provided by Dr. L.N. Mabwuike from Baylor College of Medicine (Houston, TX). 10BiT.Foxp3gfp mice were kindly provided by Dr. C.L. Maynard (University of Alabama at Birmingham [UAB]). IgA⁻/⁻10BiT.Foxp3gfp mice were generated by crossing 10BiT.Foxp3gfp mice with IgA⁻/⁻ mice. IL-17A⁻/⁻ mice were generated and kindly provided by Dr. S.N. Harhour and Dr. C.T. Weaver (UAB). IL-17A⁻/⁻ IL-17C⁻/⁻ mice were generated by crossing IL-17A⁻/⁻ mice with IL-17C⁻/⁻ mice, using the latter as the dam. All mice were on C57BL/6 background and were maintained in the Animal Facility at the UAB under specific pathogen-free (SPF) conditions. Germ-free (GF) mice were purchased from The Jackson Laboratory. IgA⁻/⁻ mice were preserved frozen under dry ice or at −80°C until immediately before oral administration. SFB colonization was performed by oral gavage with 500–400 μl of suspension obtained by homogenizing the fecal pellets from SFB-monocolonized mice in water. Fecal pellets of the recipients were collected 2 wk after gavage, and SFB colonization was confirmed with PCR (primers: forward, 5’-ACG CTA CAT CGT TCT TTC CCG C-3’; reverse, 5’-TCC CCC AAG ACC AAG TTC CCG A-3’). Vancomycin treatment was performed with indicated schedules with 0.5 mg/d in the drinking water. For intragastric immunization, 10 μg of CT and 1 mg of OVA in 500 μl 0.2 mM NaHCO₃ was gavaged on days 0, 7, and 14. Mice were sacrificed on day 28 for examination. Intragastric immunization was performed with 10 μg of CT and 1 mg of OVA in 100 μl of PBS, with the same immunization schedule. Short-chain fatty acid repletion was performed with 100 mM butyrate or 150 mM propionate in the drinking water from day 0 of CT immunization until mice were sacrificed. Eight- to 12-wk-old mice were used in these experiments unless specified otherwise. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee of UAB.

Reagents and materials

Reagents and materials were purchased from the following sources: anti-mouse CD4 (RM4-5), CD25 (PC61) Abs were purchased from BD Biosciences; anti-mouse IL-17A (TC11-18H10.1), IFN-γ (XMG1.2), CD90.1 (OX7) Abs were purchased from BioLegend; anti-mouse Foxp3 (FJK-16s) and IL-22 (1HBPWSR) Abs, as well as Foxp3 staining buffer set, were purchased from eBioscience; anti-mouse CD90.1 (1H7.2) and anti-human CD2 (OKT11) were purchased from the hybridoma laboratory of Epitope Recognition and Immunoreagent Core at UAB; live/dead staining kit was purchased from Invitrogen; mouse LP dissociation kit and regulatory T cell isolation kits were purchased from Miltenyi Biotec; mouse lipocalin-2 (LCN-2) ELISA kit was purchased from R&D Systems; fecal DNA extraction kit was purchased from Zymo Research.

Isolation and in vitro culturing of LP cells

Small and large intestines were removed, sliced, and washed with ice-cold PBS to remove fecal content. After removing epithelium by gentle shaking in Ca²⁺/Mg²⁺-free HBSS supplemented with 5 mM EDTA, 1 mM DTT, and 2% FBS for 40 min at 37°C, the tissue was washed and resuspended in digestion medium following Miltenyi protocol and then incubated for 30 min at 37°C by gentle shaking. Cells were passed through a mesh, centrifuged, and the pellet was resuspended in 40% Percoll and carefully overlaid onto 70% Percoll. The interface containing the LP leukocytes was collected and washed with PBS supplemented with 10% FBS. A total of 10⁶ LP lymphocytes (LPLs) were cocultured with 5 × 10⁷ APCs isolated from naive C57BL/6 mouse spleen (CD4⁻) fraction with the addition of either SFB, CT B subunit (CTB), or OVA CD4 T cell epitope peptide (SFB 568–580, DVQFGSVPKNDT; CTB 89–100, NNKTPHAHAAS; OVA 523–339, ISQAVHAAHAEINAGR) (20–22) for 5 d. Culture supernatant was then collected and measured for cytokine production.

Intracellular staining for cytokine production

As described previously (23), isolated cells were stimulated for 4–5 h with PMA (50 ng/ml; Sigma) and ionomycin (750 ng/ml; Sigma) in the presence of GolgiStop (10 μg/ml; BD Biosciences). After staining of cell surface Ags, cells were fixed and permeabilized using Foxp3 staining buffer set (ebioScience), followed with intracellular cytokine staining. Dead cells were excluded by surface staining with Live/Dead staining kit. Flow cytometry was performed on BD LSR II and analyzed with FlowJo software (V9.3.3, Tree Star).

ELISA and multiplex assay

Ninety-six-well ELISA plates (MaxiSorp; Thermo Fisher Scientific) were coated with capture Ab in PBS or coating buffer at 4°C overnight. Plates were washed and blocked; then samples and standards were added and incubated at room temperature for 2 h. After washing, biotin-labeled detection Abs and streptavidin-HRP were added and incubated for another hour. Followed by final washes, plates were developed with TMB solutions for 15 min and read at 450 nm. Sample concentrations were calculated according to the standard curve. Milliplex mouse Th17 magnetic kit was purchased from Millipore. Multiplex assay was conducted following manufacturer’s instructions. Nine cytokines including IFN-γ, IL-4, IL-5, IL-13, IL-6, IL-17A, IL-21, IL-22, and IL-10 were measured simultaneously.

Histopathologic assessment

Naïve IgA⁻/⁻ mice at 9 mo old and CT-immunized mice at 12 wk old (immunization started at 8 wk old with the schedule described earlier) were sacrificed for histopathologic assessment. At necropsy, the small intestine, cecum, and colon were separated and cut open longitudinally. Tissues were fixed in 10% buffered formalin and paraffin embedded. The sections (5 μm) were stained with H&E. All slides were scored by an experienced veterinary pathologist (T.R. Schoeb, UAB) without knowledge of their origin.

Microbiome sequencing and data analysis

Bacterial genomic DNA from mouse ileal and cecal contents was extracted with ZR Fecal DNA MinPrep kit following manufacturer’s protocol. The V4 region of 16S rRNA gene was targeted for PCR amplification using a modified F525/R806 primer (24). Duplicates were run for all PCR’s. The 150 paired-end sequencing reaction was performed using the MiSeq platform (Illumina) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba (Winnipeg, MB, Canada). The FLASH assembler was used to merge overlapping paired-end Illumina fastq files (25). The output fastq file was then further analyzed by downstream computational pipelines of the open source software package QIIME (26). Taxonomies were assigned to the representative sequence of each OTU using RDP classifier and were aligned with the Greengenes Core reference database using PyNAST algorithms. Within-community diversity (α-diversity) was calculated using

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Sequences were aligned against the Mus musculus reference genome using the Smart-seq2 protocol, modified for 500+ cells of input, processed using the DIRECT purification kit (Life Technologies) following manufacturer’s instructions. mRNA was isolated using Dynabeads mRNA DIRECT purification kit (Life Technologies) following manufacturer’s instructions. Sequences were aligned against the Mus musculus reference genome (mm10) using TopHat + Bowtie (28). HTSeq-count (29) was used to count the transcripts associated with each gene, and a counts matrix containing the number of counts for each gene across different samples and stimulations was obtained. The counts were normalized using the TMM method (30). To analyze differential expression across different stimulations, we analyzed the counts matrix with a generalized linear model, using the edgeR package (31) with a threshold of q ≤ 0.05.

Statistical analysis
Unpaired Student t test was used for the determination of difference between groups unless other methods were stated. Data were presented as mean ± SEM of replicate experiments. The differences were considered statistically significant at p < 0.05 (∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001, ∗∗∗∗p < 0.0001).

Results
IgA+/− mice have increased endogenous intestinal Th17 cells and stronger responses to CT than wild-type mice, but no intestinal inflammation

Intestinal LPLs isolated from 9-mo-old naive C57BL/6.IgA+/− mice were compared with sex- and age-matched naive C57BL/6 wild-type (WT) mice and found to have a significantly enlarged Th17 population in the small intestine compared with WT mice (Fig. 1A–C), a difference not observed in 8-wk-old naive mice (Fig. 1E–G, control group). Correspondingly, there was a significantly higher serum LCN-2 level in elderly IgA+/− mice compared with matched WT mice (Fig. 1D), which is an antimicrobial protein that is downstream of IL-17A stimulation and plays important roles in epithelial homeostasis (32–34).

Next, 8- to 12-wk-old IgA+/− and WT mice were immunized with CT and CIB1 flagellin (a microbiota Ag) intragastrically. Th17 cells in small intestinal LPLs (sILPLs) of both strains increased considerably compared with control mice, and this increase was significantly higher in IgA+/− mice than in WT mice. The induction of Th17 cells in the large intestine was much less profound than that in the small intestine (Fig. 1E–G). Different from a previous report investigating cellular response in the peripheral lymphoid organs after intranasal and i.v. CT immunization (13), we did not observe significant changes of Th1, Th2, or Treg cells either in the mucosal or in the peripheral sites (data not shown). sILPLs isolated from IgA+/− mice secreted almost 10-fold more IL-17A than those from WT mice upon Ag restimulation in vitro (Fig. 1H). Also, IgA+/− mice developed 10-fold higher serum IgG responses against CTB (Fig. 1I). Although Th17 cells have been shown to contribute to pathogenicity in various colitis models, this high percentage of endogenous or CT-induced Th17 cells in the small intestine did not cause spontaneous inflammation in IgA+/− mice in that all sections of intestinal tissue from duodenum to distal colon were normal on histologic evaluation by an experienced pathologist (T.R.S.) (Fig. 1J, data not shown), consistent with a homeostatic role of gut Th17 cells at steady-state.

Intestinal microbiota contribute to the immune differences between IgA+/− and WT mice

It has been shown that long-term isolated breeding of mutant and WT mice can cause marked compositional differences in their gut microbiota (35), which plays a critical role in immune system development. Thus, we asked whether the gut microbiota or the lack of IgA accounted for the enlarged endogenous and CT-induced Th17 responses in IgA+/− mice. First, we tested whether the gut microbiota in IgA+/− mice differed from WT mice by comparing the bacterial composition in the ileum and cecum of age- and sex-matched adult mice. Pie charts of the phyla show that IgA+/− mice had higher proportions of Firmicutes than WT mice in both ileum and cecum (Fig. 2A). In contrast, WT mice had more Bacteroidetes, Tenericutes, and Actinobacteria. Principal coordinate analysis shows that bacteria in IgA+/− mice cluster separately from WT mice in both ileal and cecal samples (Fig. 2B), indicating that the composition of the intestinal microbiota of IgA+/− mice differs from that of WT mice. Because Firmicutes and Bacteroidetes are the two major phyla of intestinal commensals, and are closely related to intestinal immune homeostasis, we compared the ratio of Firmicutes over Bacteroidetes in IgA+/− and WT mice. In both ileum and cecum, this ratio was significantly higher in IgA+/− mice (Fig. 2C). Further analysis showed that a large part of Firmicutes in the ileum of IgA+/− mice was SFB, comprising ∼40% of the total detected bacteria, much higher than that in WT mice (Fig. 2D). Because SFB is a potent inducer of endogenous Th17 cells in the small intestine, we asked whether differences in microbiota composition, in particular elevated SFB in IgA+/− mice, could promote stronger CT-induced Th17 and Ab responses. To test this hypothesis, we equalized the intestinal microbiota of these two strains by crossing maternal IgA+/− mice with male WT mice and then interbreeding of WT littermates to WT mice and then interbreeding of WT mice with female IgA+/− mice to male WT mice and then interbreeding of the heterozygous first generation (F1) mice to obtain littermate IgA+/−/IgA+/− mice and WT mice in the second generation (Fig. 2E). The intestinal microbiota of all offspring were transmitted from maternal IgA+/− mice and remained equivalent in adulthood (Supplemental Fig. 1). Homozygous IgA+/− and WT littermates in the F2 generation were immunized with CT and OVA intragastrically. Surprisingly, the previously seen differences in intestinal Th17 induction and Ag-specific serum IgG responses did not occur (Fig. 2F, 2G), indicating that it was the microbiota composition in IgA+/− mice that led to the elevated host Th17 responses. Furthermore, naive littermate IgA+/− and WT mice had comparable levels of serum LCN-2 as well (Fig. 2H). Interestingly, because offspring WT mice were colonized with intestinal microbiota from the original IgA+/− mothers, their immune responses upon CT immunization resembled that of IgA+/− mice, but not the parent WT strain, providing strong evidence that IgA-deficient mice are not intrinsically colonized by higher levels of Firmicutes, including SFB, which is different from a previous report (36).

Selective ablation of Gram-positive bacteria impairs the Th17 response to CT immunization

To investigate how intestinal microbiota modulates immune responses to CT immunization, we treated IgA+/−.10BiT.Foxp3gfp

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FIGURE 1. IgA−/− mice have increased endogenous intestinal Th17 cells and stronger responses to CT than WT mice, but no intestinal inflammation. (A–C) siLPLs and large intestinal LPLs (liLPLs) were isolated from naive 9-mo-old IgA−/− and WT mice. Representative plots from repeated experiments in (A) show IL-17A and IFN-γ expression in CD4+ LPLs, and combined data (n = 5 per group) of the percent of IL-17A expression in CD4+ LPLs (B) and absolute numbers are shown in (C). (D) Serum LCN-2 level was measured with ELISA (n = 19 for IgA−/−; n = 16 for WT C57BL/6). (E–G) Eight- to 12-wk-old mice were immunized intragastrically with 10 mg of CT and 100 μg of CBir1 flagellin in 500 μl 0.2 M sodium bicarbonate on days 0, 7, and 14; LPLs were isolated when mice were sacrificed on day 28 and from control naive mice. Representative plots in (E) show IL-17A and IFN-γ expression in CD4+ LPLs, and combined data (n = 4 per group; two-way ANOVA) of the percent of IL-17A expression in CD4+ LPLs (F) and absolute numbers are shown in (G). (H) APCs were isolated from naive C57BL/6 mouse spleen and pulsed with 50 μg of CTB or CBir1 flagellin, respectively, for 4 h. Then pulsed APCs were cultured with siLPLs isolated from immunized mice for 5 d. IL-17A level in the culture supernatants was measured with ELISA (n = 6 per group). (I) CTB- and CBir1-specific IgG responses (Figure legend continues)
mice, which were derived from IgA−/− mice and express a Thy1.1 (CD90.1) reporter for IL-10 expression and a GFP reporter for Foxp3 expression (37), with antibiotics in their drinking water for 2 wk to ablate Gram-positive, spore-forming bacteria, including SFB. Ablation of SFB was confirmed with PCR using DNA extracted from their fecal pellets (data not shown). IgA−/−.10BiT.Foxp3gfp mice receiving vancomycin or regular water were immunized intragastrically with CT and OVA as described earlier. On day 28 of immunization, mice receiving vancomycin developed 1 log less anti-CTB and anti-OVA IgG responses in their sera compared with mice kept on regular water (Fig. 3A), indicating that Gram-positive intestinal bacteria are actively regulating systemic immune responses to CT/OVA immunization.

Two-week treatment with vancomycin did not reduce intestinal Th17 on its own (data not shown); however, when we treated parent IgA−/−.10BiT.Foxp3gfp mice with vancomycin for 1 mo before breeding and during gestation, offspring had greatly decreased intestinal Th17 cells, as well as IL-17A and IL-10 double-expressing cells in all parts of their small intestine compared with sex- and age-matched SPF IgA−/−.10BiT.Foxp3gfp mice in the sera were measured with ELISA (n = 4 per group). (G) CTB- and OVA-specific IgG responses (n = 4 per group) and (H) LCN-2 level in the sera of littermate IgA−/− and WT mice (n = 6 per group) were measured with ELISA. All of the results are representative of at least two independent experiments. *p < 0.05, **p < 0.01.
FIGURE 3. Selective ablation of Gram-positive bacteria impairs the Th17 response to CT immunization. (A) IgA<sup>−/−</sup>.10BiT.Foxp3<sup>Cre</sup> mice were treated with vancomycin (Vanc) in the drinking water for 2 wk before intragastric CT/OVA immunization; Ag-specific IgG responses in the sera on day 28 of immunization were measured with ELISA (<i>n</i> = 6 per group). (B) IgA<sup>−/−</sup>.10BiT.Foxp3<sup>Cre</sup> parents were treated with vancomycin in the drinking water for 1 mo before breeding and continuously during gestation, IL-17A and IL-10 expression in CD4<sup>+</sup>LPLs in 6-wk-old offspring and sex- and age-matched SPF IgA<sup>−/−</sup>.10BiT.Foxp3<sup>Cre</sup> mice are shown in representative flow cytometric plots, whereas combined data (<i>n</i> = 3 per group, each sample was a pool of two to three mice) of the percent of IL-17A<sup>+</sup> and IL-17A<sup>+</sup>Thy1.1<sup>+</sup> in CD4<sup>+</sup>LPLs and absolute numbers are shown in (C). (D) Same as described in (B), IgA<sup>−/−</sup>.10BiT.Foxp3<sup>Cre</sup> mice were born from vancomycin-treated parents and maintained separately from other mice in the same facility. SFB presence in the offspring was measured twice, 4 wk apart, using PCR. Eight-week-old SFB-absent IgA<sup>−/−</sup>.10BiT.Foxp3<sup>Cre</sup> mice were immunized with 10 μg of CT in 50 μl 0.2 M sodium bicarbonate intragastrically on days 0, 7, and 14; naive IgA<sup>−/−</sup>.10BiT.Foxp3<sup>Cre</sup> mice from the same litter with and without the presence of SFB were used as controls. IL-17A and IL-17A<sup>+</sup>IL-10<sup>+</sup> expressions in CD4<sup>+</sup>LPLs are shown (<i>n</i> = 6 per group). (E) C57BL/6 mice were pretreated with vancomycin in the drinking water for 10 d and then received intracolonic CT immunization on days 0, 7, and 14. Indicated short-chain fatty acids (butyrate [B] and propionate [P]) were added to the drinking water together with vancomycin from the beginning of immunization until day 28. Fecal anti-CTB IgA level on day 28 was measured with ELISA (<i>n</i> = 4 per group; one-way ANOVA). All of the results are representative of at least two independent experiments. *<i>p</i> < 0.05, **<i>p</i> < 0.01, ***<i>p</i> < 0.001, ****<i>p</i> < 0.0001.
(Fig. 3B, 3C). Th17 cells in the colonic LP or other T cell subsets in the intestine were not significantly affected, indicating that Gram-positive bacteria ablated by vancomycin are mainly present in the small intestine and locally modulate siLPLs, consistent with our microbiome sequencing data showing SFB outgrowth in the terminal ileum of parent IgA−/− mice (Fig. 2D).

To test whether SFB is playing a role in modulating siLPLs in response to intragastric CT immunization, we immunized 8-wk-old IgA−/−.10B1.Foxp3gfp mice born from vancomycin-treated parents lacking SFB and divided the small intestine equally into three parts when examined. Using naive littermate controls that either had SFB or not, we found that SFB alone was able to induce endogenous Th17 cells as well as IL-17A and IL-10 double-producing cells in the small intestine, but this induction was the most prominent in the middle and lower thirds. In contrast, CT immunization increased Th17 cells in all parts of the small intestine, but did not induce IL-17A and IL-10 double-producing cells (Fig. 3D).

Consistent with previous reports (38, 39) that intracolonic CT immunization induces systemic humoral immune responses, our data show that intracolonic CT immunization induced intestinal Th17 cells as well. The Ab response to intracolonic CT immunization was also microbiota dependent, because vancomycin-treated C57BL/6 mice had impaired anti-CTB fecal IgA as well as total fecal IgA after immunization (Fig. 3E, data not shown). Addition of the bacterial fermentation products butyrate or propionate to the drinking water (40–42) partially rescued the production of anti-CTB fecal IgA, as well as total fecal IgA (Fig. 3E), indicating that decreased immune responses to CT after ablation of Gram-positive bacteria was partially due to deficiency of bacterial metabolic products in the intestine. Collectively, these findings indicate that Gram-positive bacterial ablation with vancomycin impairs the host intestinal Th17 response and its related Ab responses to CT immunization. SFB specifically induces an IL-17A+IL-10+ population in the distal small intestine, indicating that the intestinal microbiota can modulate the host Th17 response in some ways that are different from CT.

**Bacterial colonization enhances host Th17 and Ab responses to CT**

To better define the effect of SFB versus other microbes on the ability of CT to induce Th17 cells, we used gnotobiotic mice. GF and ASF colonized C57BL/6 mice (43) were selectively colonized with SFB to further test whether SFB alone is sufficient to modulate immune responses against CT or whether other commensal bacteria also contribute to this regulation. In both GF and ASF mice, SFB colonization alone induced Th17 cell differentiation in the small intestine and cecum (Fig. 4A). However, the increase of Th17 cells in the small intestine in SFB monocolonized mice versus GF mice was not statistically significant (Fig. 4A, 4B). ASF colonization did not induce Th17 cells by itself and largely suppressed Th17 cell differentiation in both small intestine and cecum (data not shown). In the absence of SFB, CT/OVA immunization significantly induced Th17 cells in the small intestine and cecum of ASF mice, but only slightly in GF mice, indicating that single or multiple bacteria belonging to ASF also have the potential to regulate Th17 response upon CT immunization (Fig. 4A, 4B). SFB predominantly enhanced Th17 cell induction in response to CT immunization in monocolonized mice, although not significantly in ASF mice, which already had a higher level (Fig. 4A). Cytokine production (including Th1-, Th2-, and Th17-related cytokines) was measured by coculturing of isolated siLPLs with naive APCs and either SFB, CTB, or OVA CD4 T cell epitope peptide (see Materials and Methods). Intragastric CT immunization specifically induced LP IL-17A response (Fig. 4C) and slightly suppressed IFN-γ response, without a significant effect on IL-4, IL-5, IL-13 response (data not shown). Although peptide restimulation resulted in increased IL-6 production in CT-immunized groups, IL-6 appeared not to be the sole pathway that contributed to Th17 differentiation in vivo, because there was no difference in Th17 cell induction or Ab responses with CT immunization in IL-6−/− mice (Supplemental Fig. 2). Upon SFB colonization, both GF and ASF mice receiving CT/OVA immunization significantly increased IL-17A production specific to SFB peptide stimulation, to a much greater extent than either CTB or OVA peptide, indicating that CT serves as an adjuvant for the proliferation of Th17 cells that are predominantly reactive to SFB. Collectively, these data provided clear evidence that CT induction of intestinal Th17 response is dependent on the gut microbiota. Indeed, siLPLs isolated from CT-immunized SFB WT mice responded significantly more strongly than those of any group of gnotobiotic mice (Supplemental Fig. 3A, 3B), indicating that a more diverse intestinal microbiota provides optimal support for the development of host Th17 response to CT immunization. Although CT elicits strong B cell and T cell responses to itself, tetramer staining showed that most of the induced Th17 cells are not CTB-specific, indicating that CT induces Th17 response in a polyclonal fashion (Supplemental Fig. 3C, 3D).

SFB colonization is a strong inducer of both fecal and serum IgA in gnotobiotic mice, whether naïve or immunized (Fig. 4D). In contrast, ASF colonization enhanced fecal secretory IgA levels, but not serum IgA, indicating that different commensals have distinct mechanisms to regulate host IgA production. Surprisingly, in contrast with our vancomycin experiment data, SFB colonization did not increase host Ab responses to CT or OVA in either GF mice or ASF mice; that is, SFB did not act as an adjuvant for the response to these Ags. However, regardless of the presence of SFB, ASF-colonized mice developed greater CTB- and OVA-specific Ab responses upon immunization than GF mice both mucosally and systemically (Fig. 4E), indicating that the decreased Ab responses upon CT immunization in vancomycin-treated mice were due to vancomycin-sensitive commensal bacteria other than SFB. These experiments suggested that, in addition to SFB, which is a known potent inducer for endogenous Th17 cells in the intestine, other commensal bacteria are also involved in the regulation of host Th17 and Ab responses upon stimulation. **CT-induced intestinal Th17 cells share the same transcriptome profile with endogenous homeostatic Th17 cells, and both differ from pathogenic Th17 cells**

To test the hypothesis that intestinal Th17 cells under steady-state conditions are different from pathogenic Th17 cells, we used IgA−/−.IL-17A+CD2 reporter mice, in which the IL-17A promoter directs the transcription of a bicistronic mRNA such that IL-17A-producing cells are marked by surface membrane expression of the human CD2 molecule (Supplemental Fig. 4A; S.N. Harbour, H. Turner, C.L. Maynard, C.L. Zindl, R.D. Hatton, and C.T. Weaver, manuscript in preparation), allowing us to recover IL-17A-producing cells for RNA-Seq. Of note, almost none of the IL-17A–producing cells coexpress Foxp3 (Supplemental Fig. 4B). We isolated CD4+CD2+ (Th17) and CD4+CD2− (non-Th17) cells from the small intestine of either naïve or CT-immunized IgA−/−.IL-17A+CD2 mice and used splenic CD4+CD25+ cells from naïve mice as negative controls for gene expression profiling. Comparing CD4+ cells isolated from naïve and CT-immunized IgA−/−.IL-17A+CD2 intestines, we found that Th17 cells induced by CT only have one gene differently expressed compared with
endogenous LP Th17 cells; similarly, non-Th17 cells isolated from CT-immunized mice only had two genes differently expressed compared with non-Th17 cells isolated from naive mice. Thus, although mucosal CT immunization enlarges the Th17 population in the intestinal LP, CT-induced Th17 cells share the same expression profile as endogenous intestinal Th17 cells.

**FIGURE 4.** Bacterial colonization enhances host Th17 and Ab responses to CT. (A–E) GF and ASF mice were colonized with SFB by oral gavage of fecal pellets collected from SFB monoassociated mice. Colonization was confirmed by PCR with recipients’ fecal pellets. Intragastric CT immunization was given 2 wk after colonization on days 0, 7, and 14. Mice were sacrificed on day 28, when intestinal LPLs were isolated and sera as well as fecal samples were collected. (A) Combined data of IL-17A expression in CD4+ LPLs in GF and ASF mice (n = 4 per group; two-way ANOVA), respectively, whereas absolute numbers of CD4+IL-17A+ cells in the LPLs are shown in (B). (C) Isolated LPLs were cultured with indicated peptides and naive APCs for 5 d, and IL-17A production in the culture supernatants was measured with multiplex assay (n = 4 per group; two-way ANOVA). (D) Total IgA level in the feces and sera and (E) CTB- and OVA-specific fecal IgA and serum IgG levels were measured with ELISA (n = 5 per group; Student t test and one-way ANOVA). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
The gene expression of Th17 cells isolated from the healthy intestine or CT-immunized intestine is very different from pathogenic Th17 cells induced with IL-6+IL-23+IL-1β, as well as Th17 cells isolated from the LP of colitic tissues (Fig. 5, data not shown). In particular, a series of genes related to Th17 pathogenicity, including Tnf, Ifng, Il23a, Il24, Il12rb1, and Gzmb, were significantly less expressed in endogenous and CT-induced intestinal Th17 cells compared with pathogenic Th17 cells, whereas genes involved in anti-inflammatory activities, such as Clda4, Icos, Il22, Ahr, Maf, and Ikrf3, showed at least 4-fold higher expression in homeostatic intestinal Th17 cells, consistent with previously reported Th17 signatures (44–47). Additionally, functional analysis reveals a stronger immune-regulatory profile of homeostatic intestinal Th17 cells, whereas pathogenic Th17 cells highlight pathways that are involved in various inflammatory diseases. In summary, comparison of gene expression levels shows great similarity between CT-induced Th17 cells and endogenous Th17 cells isolated from the healthy small intestine, and both of them are distinct from the signature of pathogenic Th17 cells.

Discussion
In this study, we showed that endogenous Th17 cells present in the normal intestine have a homeostatic phenotype. The increase of this homeostatic population and the elevated Th17 response upon CT immunization in IgA
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2 mice are dependent on intestinal microbiota. Using gnotobiotic mouse models, we found that SFB, ASF, and other commensal bacteria are actively involved in the regulation of CT immunogenicity and adjuvanticity. Furthermore, our RNA-Seq data indicate that CT-induced intestinal Th17 cells share a similar gene expression profile with endogenous Th17 cells in the intestine, both of which are quite different from the signature of pathogenic Th17 cells.

The age-related increase of intestinal Th17 cells in IgA
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2 mice suggested an environmental effect of Th17 cell development, in this case most likely the intestinal microbiota. Indeed, with activation-induced cytidine deaminase knockout mice, Suzuki et al. (36) concluded that the lack of hypermutated IgA resulted in the excessive overgrowth of anaerobic bacteria in the intestine, the most predominant being SFB. Although conflicting data have been reported with Igh-J
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2 mice (48), neither of them were perfect models for the study of IgA deficiency. Targeted deletion of the IgA C region in our IgA
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2 mice leads to specific deficiency of IgA, thus providing a more appropriate model of the effects of IgA deficiency on microbiota (49). Our study shows that increased Firmicutes, including SFB, led to the exaggerated intestinal Th17 responses and humoral Ab induction upon CT immunization in IgA
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2 mice. However, this microbiota difference is not intrinsic to IgA deficiency, in that crossing the WT with IgA
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2 mice equalized the intestinal microbiota in the offspring, indicating a familial transmission, rather than IgA deficiency, caused microbiota shift (35, 36, 50).

CT has been known as a potent mucosal immunogen and adjuvant for several decades; we confirm and extend a recent report linking these CT properties with the intestinal microbiota (14). With SFB as a known stimulator of endogenous Th17 cells in the intestine, we found that in addition, other commensal bacteria such as those belonging to the ASF can also participate in the regulation of intestinal Th17 and Ab responses to CT mucosal

FIGURE 5. Gene expression profile of endogenous, CT-induced, and pathogenic Th17 cells. RNA-Seq was performed with CD4+CD2+ cells isolated from the small intestine of naive and CT-immunized IgA
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2 mice (a pool of at least six mice per sample), and these were compared with the transcriptome of pathogenic Th17 cells induced with IL-23 and IL-1β. (A) Heatmap shows transcripts differentially expressed in Th17 cells from different origins and (B) shows the principal component analysis on their gene expression profiles, where each point represents an individual sample.
immunization. When the CTB subunit binds to the GM1 ganglioside present on cell membranes, CT induces strong activation of STAT3 signaling and massive production of IL-6 (23, 55–57). However, IgA/IgG molecules do not notice any differential expression of Cd5l, Cd177, Icos, or Cd15s compared with endogenous and CT-induced Th17 cells, consistent with what has been shown by Harbour et al. (67) that Th17 convert into Th1 cells and contribute to the pathogenesis of colitis. Most recently, Gauzden et al. (47) and Wang et al. (68) published their studies of Th17 cell pathogenicity using the single-cell RNA-Seq technique, in which they found an intestinal Th17 cell signature similar to ours, although we did not notice any differential expression of Cd5l in our RNA-Seq data. The ratio of homeostatic Th17 cells to pathogenic Th17 cells might be critical in overt inflammation; it is an important topic of future research to understand whether these are separate lineages, and if not, what triggers the conversion from homeostatic Th17 cells to pathogenic Th17 cells and/or Th1 cells, and what determines the conversion from Th17 to Treg cells. This will provide us with new insights into the pathogenesis, prevention, and treatment of diseases of dysregulated immunity and inflammation.

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Disclosures

The authors have no financial conflicts of interest.

References

HOMEOSTATIC TH17 CELLS IN THE INTESTINE


SUPPLEMENTARY DATA AND LEGENDS
Figure S1. IgA−/− and WT littermates share similar intestinal microbiota. (A) Ileal and cecal contents were collected from adult IgA−/− and WT littermates (including 5 male and 4 female mice each group), as well as from 2 IgA−/− mothers of the parent strain. Bacterial genomic DNA was extracted for 16s rDNA microbiome sequencing. Pie chart shows percent of total bacteria at the phylum level. (B) Principal coordinate analysis shows ileal and cecal bacterial composition of IgA−/− and WT littermates and their IgA−/− mothers. Each dot represents an individual mouse. (C) Combined diversity of ileal and cecal contents of IgA−/− and WT littermates.
Figure S2. IL-6 does not play a significant role in CT-induced immune responses. 8-12-week-old IL-6−/− and WT littermates were immunized intragastrically with 10µg CT and 1mg OVA in 500µl 0.2M sodium bicarbonate on days 0, 7, and 14. LPLs were isolated when mice were sacrificed on day 28. Representative plots in (A and B) show the percentage of different cell subsets in the siLPLs. (C-F) Total and antigen-specific IgA and IgG responses in stool pellets and sera were measured with ELISA.
Figure S3. Host intestinal Th17 response to CT immunization is dependent on microbiota diversity and the majority of CT-induced Th17 cells are not CTB-specific. SPF, GF, ASF, and SFB colonized GF and ASF WT mice were immunized intragastrically with 10µg CT in 500µl 0.2M sodium bicarbonate on days 0, 7, and 14, siLPLs were isolated when mice were sacrificed on day 28. Combined data of IL-17A expression in CD4+ LPLs in the small intestine is shown in (A). (B) APCs were isolated from naïve C57BL/6 mouse spleen and either pulsed with 50µg CTB for 4 hours or directly co-cultured in the presence of 1µg CTB peptide with siLPLs isolated from immunized mice for 5 days. IL-17A level in the culture supernatants was measured with ELISA. (C) IgA+ mice were immunized intragastrically with CT and OVA. Upon sacrifice, siLPLs were isolated and fluorescently stained with a MHC class II tetramer. FACS plots show CTB tetramer positive populations among activated and/or IL-17A producing cells in CD4+ and CD8+ T cells, respectively. Compared to naïve IgA+ mice (data not shown), the absolute number of CTB-tetramer positive cells in the small intestinal lamina prorla alone was increased by 40-fold from ~100 to ~4000 per mouse. Aggregated percentages of different populations are shown in (D).
**Figure S4. IL-17A producing cells co-express hCD2 but not Foxp3.** (A) C57BL/6.IL-17AhCD2 mice were immunized intragastrically with CT. Upon sacrifice, siLPLs were isolated and restimulated with PMA and ionomycin in the absence of Golgi stop. FACS plot shows co-expression of cell surface hCD2 on IL-17A producing cells. (B) siLPLs isolated from 9-month-old IgA−/−.10BiT.Foxp3fp mice were restimulated with PMA and ionomycin with Golgi stop for 3.5 hours. FACS plot shows co-expression of GFP (Foxp3) on IL-17A producing cells.