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Downregulation of Th17 Cells in the Small Intestine by Disruption of Gut Flora in the Absence of Retinoic Acid

Hye-Ran Cha,^{*1} Sun-Young Chang,^{*1} Jae-Hoon Chang,^{*} Jae-Ouk Kim,^{*} Jin-Young Yang,^{*} Chang-Hoon Kim,[†] and Mi-Na Kweon^{*}

Retinoic acid (RA), a well-known vitamin A metabolite, mediates inhibition of the IL-6-driven induction of proinflammatory Th17 cells and promotes anti-inflammatory regulatory T cell generation in the presence of TGF- β , which is mainly regulated by dendritic cells. To directly address the role of RA in Th17/regulatory T cell generation in vivo, we generated vitamin A-deficient (VAD) mice by continuous feeding of a VAD diet beginning in gestation. We found that a VAD diet resulted in significant inhibition of Th17 cell differentiation in the small intestine lamina propria by as early as age 5 wk. Furthermore, this diet resulted in low mRNA expression levels of IL-17, IFN regulatory factor 4, IL-21, IL-22, and IL-23 without alteration of other genes, such as ROR γ t, TGF- β , IL-6, IL-25, and IL-27 in the small intestine ileum. In vitro results of enhanced Th17 induction by VAD dendritic cells did not mirror in vivo results, suggesting the existence of other regulation factors. Interestingly, the VAD diet elicited high levels of mucin MUC2 by goblet cell hyperplasia and subsequently reduced gut microbiome, including segmented filamentous bacteria. Much like wild-type mice, the VAD diet-fed MyD88^{-/-}TRIF^{-/-} mice had significantly fewer IL-17-secreting CD4⁺ T cells than the control diet-fed MyD88^{-/-}TRIF^{-/-} mice. The results strongly suggest that RA deficiency altered gut microbiome, which in turn inhibited Th17 differentiation in the small intestine lamina propria. *The Journal of Immunology*, 2010, 184: 6799–6806.

Intestinal mucosa has a unique and complicated immune system composed of a variety of cell populations (1, 2). Among these, Th17 cells producing IL-17, IL-21, and IL-22 control immunity, inflammation, and infection at mucosal surfaces (3, 4). The lamina propria (LP) of the small and large intestines at steady state contains large numbers of IL-17-secreting Th17 cells and may thus influence intestinal homeostasis (3). Recent studies demonstrated preferential loss of Th17 cells in the gastrointestinal tract of HIV-infected patients (5, 6). In addition, IL-17 is required for host defense against oral *Candida albicans*, *Staphylococcus aureus*, and *Citrobacter rodentium* infection (7, 8). In the T cell-mediated colitis model, the recipient mice provoke disease by inducing IFN- γ and IL-17 secretion dependent on IL-23 production (9, 10). Other studies have shown that the adoptive transfer of IL-17-deficient cells results in a more aggressive colitis in the CD45RB^{high} transfer model, which means that IL-17 plays a protective function in intestinal damages (11).

The development and differentiation of Th17 cells are controlled by local cytokines that include IL-6, TGF- β , IL-21, and IL-23 (4, 12, 13). Several recent studies also proposed that alterations of the composition of commensal bacteria are associated with Th17 cell differentiation in the intestines. In a recent study, Zaph et al. (14) noted that intestinal commensal bacteria are required to limit the frequency of Th17 cells in the large intestine. They proposed that commensal bacteria-dependent IL-25 expression by epithelial cells could suppress Th17 cells by inhibiting expression of macrophage-derived IL-23 (14). In contrast, ATP from commensal bacteria may help induce the differentiation of CD4⁺ T cells into the Th17 cells of the small intestine (SI) (15). Furthermore, differentiation of Th17 cells in the intestines is correlated with the presence of cytophaga-flavobacter-bacteroidetes bacteria and is independent of TLR, IL-21, or IL-23 signaling, but requires appropriated TGF- β activation (16). The composition of intestinal microbiome thus regulates Th17 cell differentiation in the gut.

Retinoic acid (RA), which is specifically secreted by several mucosal compartments, regulates imprinting of mucosal dendritic cells (DCs) for gut T and B cell homing in the Peyer's patches (PP) and mesenteric lymph nodes (MLN) (17, 18). RA is mainly produced by mucosal DCs and induces expression of $\alpha_4\beta_7$ integrin and CCR9 on both CD4⁺ T and B cells and chemotaxis to thymus-expressed chemokine (CCL25) (17, 18). One recent study revealed that RA is also a key regulator of TGF- β -induced T cell differentiation and that RA production by DCs in the LP of the SI (SI-LP) is one requisite for optimal Foxp3⁺ regulatory T (Treg) cell conversion (19, 20). A series of in vitro studies has shown that RA mediates reciprocal Th17 and Treg cell differentiation; RA negatively regulates Th17 cell differentiation, whereas Treg cell generation by mucosal DCs is highly dependent on RA as well as TGF- β (21). Recently, however, Uematsu et al. (22) reported that a low concentration of RA (which is produced by TLR5⁺ DCs in the SI-LP) seems to be necessary for Th17 cell differentiation. Moreover, others have shown that RA depletion in a human primary epithelial cell culture system induces keratinizing squamous differentiation and reduces expression of mucin genes (23).

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The online version of this article contains supplemental material.

Abbreviations used in this paper: AHR, aryl hydrocarbon receptor; Cont, control; DC, dendritic cell; IEC, intestinal epithelial cell; IRF4, IFN regulatory factor 4; LP, lamina propria; MLN, mesenteric lymph node; n.d., not detected; PAS, periodic acid-Schiff; pIgR, polymeric IgR; PP, Peyer's patch; RA, retinoic acid; SFB, segmented filamentous bacteria; SI, small intestine; SIgA, secretory IgA; SI-LP, lamina propria of the small intestine; Treg, regulatory T; VAD, vitamin A deficient.

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Treatment of such cultures with RA leads to restoration of the mucous phenotype and mucin expression (24, 25). Thus, it seems likely that RA orchestrates homeostasis of the intestines by balancing Th17, Treg, and intestinal epithelial cells (IEC).

To examine the homeostatic function of RA *in vivo*, we generated vitamin A-deficient (VAD) mice by continuous feeding of a VAD diet beginning in the gestational period. We found that IL-17-producing CD4⁺ T cells in the SI-LP were significantly decreased in VAD mice. Moreover, there were morphological changes in the IEC of the SI and subsequent reduction of gut microbiome. Our findings strongly suggest that a RA-deficient diet alters gut microbiome by modifying IEC, and that these alterations inhibit Th17 differentiation in the SI-LP.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from Charles River Laboratories (Orient Bio, Sungnam, Korea). Timed pregnant C57BL/6 mice were purchased from the Daehan Biolink (Eumseong, Korea). Polymeric IgR (pIgR) and MyD88^{-/-} and MyD88^{-/-}TRIF^{-/-} mice of B6 background were provided by M. Nanno (Yakult Central Institute for Microbiological Research, Tokyo, Japan) and S. Akira (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan), respectively. All of the knockout mice used in our study were offspring of bleeding heterozygous mice. OVA-specific TCR transgenic OT-II mice of C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). To produce VAD or vitamin A-sufficient mice, pregnant C57BL/6 mice received either a chemically defined diet that lacked vitamin A (AIN-93M; Oriental Yeast, Tokyo, Japan) or a control diet containing retinyl acetate (25,000 IU/kg in the AIN-93M), respectively. These diets started at day 10 of gestation, and pups were maintained on the same diet (17, 18). We used 5–10-wk-old mice that exhibit substantial levels of serum retinol even when fed VAD diets. All mice were maintained under specific pathogen-free conditions in the experimental facility at the International Vaccine Institute (Seoul, Korea).

Flow cytometric analysis

Single-cell suspensions were preincubated with anti-FcR1/III mAb (2.4G2; BD Pharmingen, San Diego, CA). Anti-mouse CD3e PerCP (145-2C11; BioLegend, San Diego, CA), anti-mouse CD4 FITC (RM4-5; BD Pharmingen), anti-mouse IL-17A allophycocyanin (eBio17B7; eBioscience, San Diego, CA), anti-mouse IFN- γ PE (XMG1.2; BD Pharmingen), and anti-mouse Foxp3 allophycocyanin (FJK-16s; eBioscience) Abs were used according to the manufacturers' instructions. Data were obtained using FACSCalibur (BD Immunocytometry Systems, San Jose, CA) with Cell-Quest software (BD Immunocytometry Systems), and the profiles were analyzed using FlowJo flow cytometry software (Tree Star, Ashland, OR).

RT-PCR

Total RNA was extracted from the SI ileum using TRIzol, and cDNA was synthesized by Superscript II reverse transcriptase with oligo(dT) primer (all from Invitrogen, Carlsbad, CA). cDNA from SI ileum of control and VAD mice was diluted semiquantitatively in nuclease-free water at final concentrations of 1 μ g/ μ l, 100 ng/ μ l, and 10 ng/ μ l. The primer sequences for amplification of each transcript are described in Supplemental Table 1. RT-PCR was performed under the following conditions: 95°C (5 min), followed by 35 cycles at 95°C (30 s), annealing (1 min), 72°C (1 min), and final extension at 72°C (10 min). Each relative mRNA expression was determined by the ratio of band intensity to β -actin production. For detection of segmented filamentous bacteria (SFB), we performed real-time PCR using Power SYBR Green PCR master mix (Applied Biosystems, Warrington, U.K.). The real-time PCR program started with an initial step at 95°C for 3 min, followed by 40 cycles at 95°C (10 s) and 40 cycles at 63°C (45 s). PCRs were completed using the genomic DNA from each sample and a SFB-specific primer (Supplemental Table 1).

Bacterial culture

For determination of microbiome, we weighed the SI ileum region and feces, added sterilized PBS (Life Technologies, Carlsbad, CA), and vortexed the samples until homogenous. Each sample was diluted and placed on universal medium for the growth of anaerobic (Luria-Bertani broth [USB, Cleveland, OH]) and aerobic (Bacto agar [BD Pharmingen]) bacteria. The former were grown in an anaerobic chamber, and colonies were counted

after incubation at 37°C for 72 h. Colonies were further classified using a selective medium, such as MacConkey agar (BBL, Baltimore, MD) for *Escherichia coli*, Bacteroides bile esculin agar (BBL) for *Bacteroides fragilis*, enterococcosel agar (BBL) for enterococci and group D streptococci, *Clostridium difficile* selective agar (BBL) for *C. difficile*, and lactobacilli MRS agar (Difco, Franklin Lakes, NJ) for *Lactobacillus* species. All agar plates were made according to the manufacturer's protocol (BBL).

Cell purification

DCs from MLN, PP, or SI-LP were isolated, as described previously (26). For isolation of DCs from SI-LP, tissue pieces were treated with RPMI 1640 containing 2% FBS and 0.5 mM EDTA for 20 min at 37°C to remove epithelial cells. This step was repeated twice. Tissues were then digested with 400 Mandl U/ml collagenase D (Roche Applied Science, Mannheim, Germany) and 10 μ g/ml DNase I (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 containing 10% FBS and digested for 30–90 min at 37°C. CD11c⁺ DCs were enriched by using a Percoll solution (Amersham Biosciences, Uppsala, Sweden), and subsequently by positive selection using anti-CD11c microbeads, according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA).

In vitro culture conditions

For Ag-specific stimulation, purified CD4⁺CD25⁻ T cells from the spleens of OT-II mice were incubated with 200 nM OVA_{323–339} peptide and DCs of MLN, PP, or SI-LP isolated from control or VAD diet-fed mice for 4 d under Th17-polarizing conditions (TGF- β [1 ng/ml] and IL-6 [20 ng/ml]; R&D Systems, Minneapolis, MN). In some experiments, MLN DCs were cocultured with OT-II CD4⁺ T cells in the presence of RA (1 nM; Sigma-Aldrich) or LE540 (1 μ M; Wako Chemicals, Richmond, VA) and LE135 (1 μ M; Tocris Bioscience, Ellisville, MO). Cells were restimulated with PMA/ionomycin (BD Pharmingen) for 5 h to measure IL-17A intracellularly.

Histology

Randomly selected ileum regions of SI obtained from control and VAD diet-fed mice were washed with PBS and fixed in 4% formaldehyde for 1 h at 4°C. The tissues were dehydrated by gradually soaking in alcohol and xylene and then embedded in paraffin. The paraffin-embedded specimens were cut into 5- μ m sections and stained with H&E or Alcian blue periodic acid-Schiff (PAS; Merck, Nottingham, U.K.). For detection of BrdU incorporation, mice were fed BrdU in drinking water (40 mg/kg; Sigma-Aldrich) for 24 h. The paraffin-embedded SI tissue sections were stained by BrdU staining kit (Calbiochem, San Diego, CA) and viewed with a digital light microscope (Olympus, Tokyo, Japan). To detect IgA Ab-secreting cells in the SI-LP, the frozen sections were stained with anti-IgA FITC Ab (C10-3; BD Pharmingen). To detect MUC2-secreting cells in SI, paraffin-embedded tissue sections were stained with rabbit anti-MUC2 Ab (H-300; Santa Cruz Biotechnology, Santa Cruz, CA), incubated with biotinylated anti-rabbit Ig Ab (Dako, Glostrup, Denmark), and then visualized using Alex 488-conjugated streptavidin (Molecular Probes, Carlsbad, CA). DAPI (Molecular Probes) was used to stain nuclei. The sections were viewed under a confocal scanning laser microscope (Zeiss, Göttingen, Germany).

Antibiotic treatment

Mice were fed 100 μ l of mixed antibiotics orally every day for 5 wk. Mixed antibiotics consisted of vancomycin (10 mg/kg; USB), neomycin (30 mg/kg; Life Technologies, Grand Island, NY), carbenicillin (50 mg/kg; Sigma-Aldrich), and metronidazole (50 mg/kg; Nacalai Tesque, Kyoto, Japan).

Statistics

Data are expressed as the mean \pm SD. Statistical comparisons between experimental groups were performed using Student's *t* test.

Results

VAD diet results in significant reduction of Th17 cell differentiation in SI-LP

To determine the direct role of RA on the homeostasis of intestine *in vivo*, pregnant wild-type mice were fed the VAD diet beginning at day 10 of the gestational period. As reported by others, RA is crucial for gut-homing T and B cells (17, 18); however, we found significantly reduced, but still detectable numbers of CD4⁺ T cells in the SI-LP of VAD mice when compared with control

mice (Fig. 1A, 1B). Mononuclear cells were purified from SI-LP of mice fed either control or VAD diet, and numbers of CD4⁺ T cells secreting IL-17, Foxp3, and IFN- γ with/without stimulation of PMA and ionomycin were determined. We found a significant reduction of Th17 cells in the SI-LP in 5-wk-old VAD mice, but unchanged numbers of Foxp3⁺ and IFN- γ ⁺ CD4⁺ T cells in their SI-LP when compared with age-matched mice fed the control diet (Fig. 1C, 1D). In parallel with intracellular cytokine results, mRNA expression levels of IL-17 were drastically reduced in the SI of VAD diet-fed mice compared with those of control diet-fed mice (Fig. 3). No detectable levels of IL-17-secreting CD4⁺ T cells were seen in other tissues (e.g., spleen, cervical

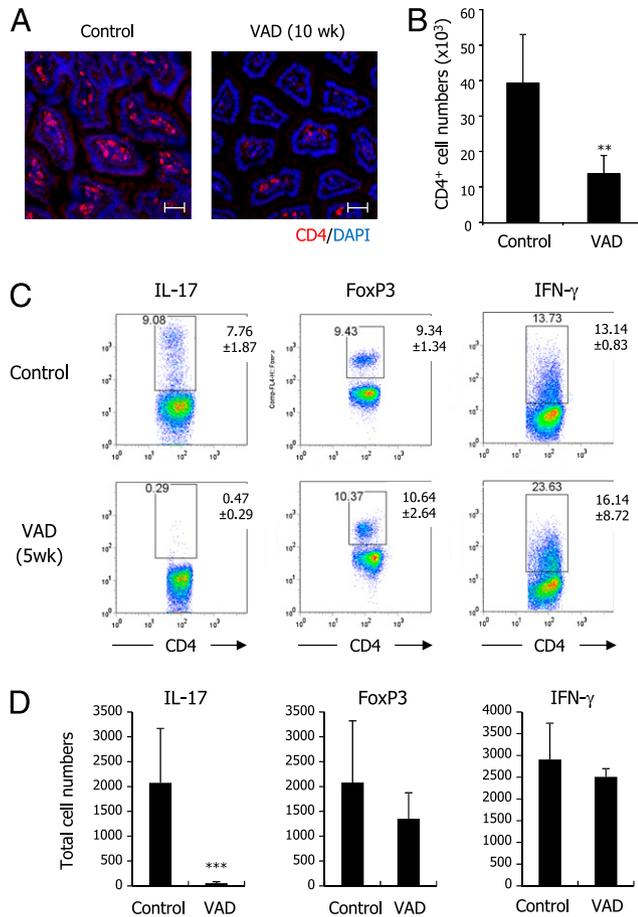


FIGURE 1. Absence of RA results in significantly fewer IL-17-secreting cells in the SI-LP of C57BL/6 mice. *A*, Immunohistochemical staining was used to identify CD4⁺ cells using PE-conjugated anti-mouse CD4 Abs (RM4-5; BD Pharmingen) in the SI-LP of each mouse. Data are representative of two independent experiments. Scale bar, 20 μ m. *B*, Mononuclear cells were isolated from SI-LP of control or VAD mice, and absolute numbers of recovered CD4⁺ cells per mouse were determined by FACS staining. Results are representative of three repetitive experiments. *C*, FACS analysis of IL-17-, Foxp3-, or IFN- γ -producing cells by intracellular staining. Mononuclear cells were isolated from SI-LP of mice fed control or VAD diets. Cells were then stimulated with/without PMA and ionomycin in the presence of Golgi-stop for 4 h and stained for IL-17, Foxp3, or IFN- γ , respectively. Numbers adjacent to boxed areas indicate percentages of IL-17⁺CD4⁺, Foxp3⁺CD4⁺, or IFN- γ ⁺CD4⁺ cells among total CD4⁺ T cells. Mean percentage of IL-17-, Foxp3-, or IFN- γ -positive cells among CD4⁺ T cells is shown in the right corner of each box. *D*, Total recovered IL-17⁺CD4⁺, Foxp3⁺CD4⁺, or IFN- γ ⁺CD4⁺ cells from SI-LP of one control and one VAD mouse (data for one mouse were extrapolated from pooled cells from three mice in each group). Data are representative of five repetitive experiments with at least three mice per group. ***p* < 0.001; ****p* < 0.0001, compared with control diet group.

lymph nodes, MLN, and PP) of VAD diet-fed mice (Fig. 2). These results demonstrate that the VAD diet resulted in inhibition of Th17 cell generation from an early stage without significant alteration of Foxp3⁺ Treg and IFN- γ ⁺ Th1 cell generation in the SI-LP.

VAD diet elicits significantly decreased levels of IFN regulatory factor 4 (IRF4), IL-21, IL-22, and IL-23 mRNA expression in the SI ileum

To address regulatory factors for Th17 cell differentiation in the RA-deficient condition, mRNA levels of potential candidates were determined in the homogenates of the SI ileal region. Together with IRF4, IL-21, IL-22, and IL-23, mRNA levels were significantly reduced in the SI of VAD mice (Fig. 3). However, mRNA levels of the orphan nuclear receptor ROR γ t, TGF- β , IL-6, and aryl hydrocarbon receptor (AHR), which are well known as required factors to induce Th17 differentiation, were identical in control diet- and VAD diet-fed mice (Fig. 3). Furthermore, there were identical levels of IL-25 and IL-27 mRNA in the SI of mice fed control or VAD diets (Fig. 3). Overall, the VAD diet resulted in defects of IL-17-, IRF4-, IL-21-, IL-22-, and IL-23-producing cells without alteration of other positive (i.e., ROR γ t, TGF- β , IL-6, and AHR) and negative (i.e., IL-25 and IL-27) regulatory factors.

Secretory IgA (SIgA) Ab defects of VAD mice are not a direct cause of reduced Th17 differentiation

Before seeking the role of SIgA Ab for Th17 cell differentiation in SI-LP, we first determined the numbers of IgA Ab-producing cells in the VAD mice. Immunohistochemistry (Supplemental Fig. 1a) and ELISPOT (Supplemental Fig. 1b) assay showed significantly reduced numbers of IgA Ab-producing cells. We next fed the VAD diet to pIgR knockout (pIgR^{-/-}) mice and assessed their Th17 cell numbers. As shown in Supplemental Fig. 1c, mice fed the control diet had predominantly Th17 cells in SI-LP; however, like wild-type mice, the VAD diet resulted in significant Th17 cell differentiation defects in the pIgR^{-/-} mice. Overall, these results suggest that defects of Th17 cell differentiation might not be related to lack of SIgA Ab secretions in mice fed a VAD diet.

Inhibition of Th17 differentiation in the SI of VAD mice might be regulated by non-DC factors

To determine the role of DCs for Th17 differentiation in VAD mice, we purified DCs from MLN, PP, and SI-LP of mice fed control or VAD diets and cocultured the DCs with OT-II CD4⁺ T cells in the presence of OVA peptide, TGF- β , and IL-6. All DCs isolated from MLN, PP, and SI-LP of VAD diet-fed mice helped activate CD4⁺ T cells to produce IL-17, unlike those from mice fed the control diet (Fig. 4A). In contrast, Foxp3 expression on CD4⁺ T cells was significantly suppressed by DCs isolated from VAD diet-fed mice, unlike the findings in mice fed the control diet (data not shown). As reported by others (21), addition of RA (1 nM) inhibited IL-17-producing CD4⁺ T cells in the presence of MLN DCs from VAD mice (Fig. 4B). Of interest, IL-17-producing CD4⁺ T cells were significantly induced in the presence of RA antagonists (i.e., LE540 plus LE153) and MLN DCs from VAD mice (Fig. 4B). Thus, it seems likely that DCs might not be directly required for inhibition of Th17 differentiation in the SI of VAD mice.

VAD-deficient diet results in brisk MUC2 expression by increased goblet cells in the SI

Because previous studies demonstrated an indispensable role for vitamin A and its derivatives (i.e., RA) for regulation of growth and differentiation of epithelial cells (23, 24), we analyzed morphological and functional alterations in the IEC of mice fed a VAD diet.

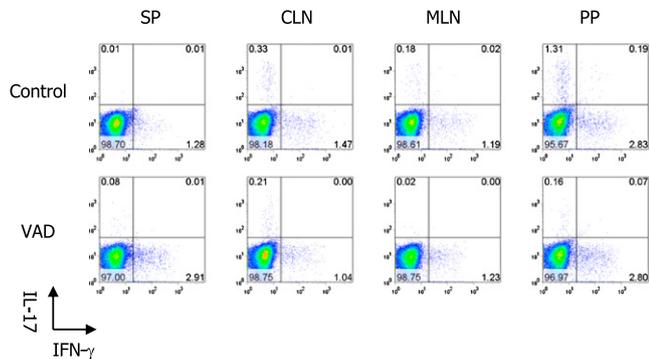
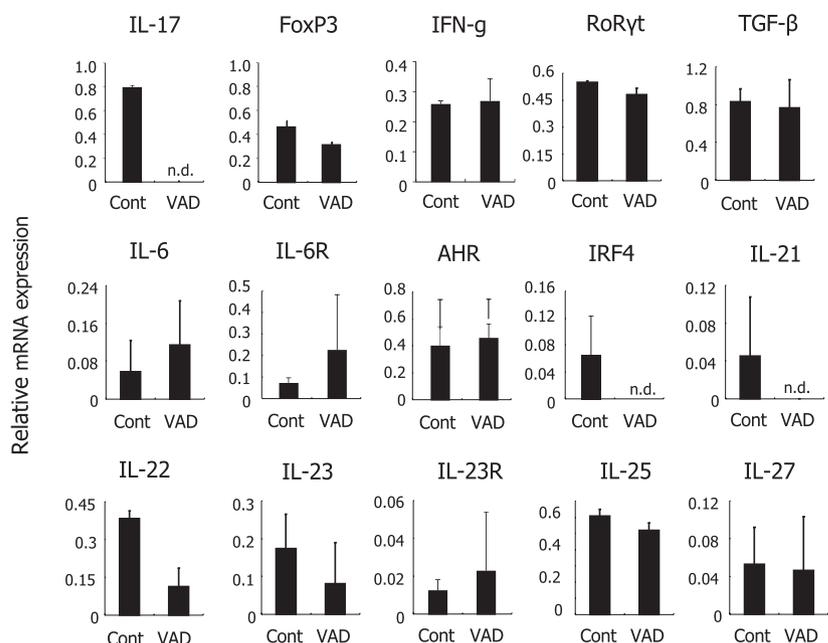


FIGURE 2. Numbers of IL-17-secreting cells in the SP, CLN, MLN, and PP of mice fed control or VAD diets. In contrast to the SI-LP, there were no significant differences in IL-17-secreting cells between control and VAD mice. Cells were stimulated with PMA and ionomycin in the presence of Golgi-stop for 4 h and stained intracellularly with allophycocyanin-conjugated IL-17 and PE-conjugated IFN- γ . Cells were gated CD3⁺CD4⁺ populations. Results are representative of three independent experiments. CLN, cervical lymph node; SP, spleen.

H&E staining revealed significant atrophy in the SI of the VAD mice: shortened villi and increased numbers of goblet cells (Fig. 5A). The ileal regions had more severe changes (i.e., atrophy and increased numbers of goblet cells) (Fig. 5A) than the duodenum and jejunum (data not shown). Alcian blue and PAS staining revealed predominant numbers of PAS⁺ goblet cells in the IEC of SI of the VAD mice (Fig. 5B). Because mucin MUC2 produced by the goblet cells is the major component of the intestinal mucus barrier (27), we further checked expression levels of MUC2 in the VAD mice. In the SI, their mucin MUC-2 expression was dramatically enhanced when compared with control diet-fed mice (Fig. 5C). In addition, S phase-proliferating cells (BrdU⁺ cells) were reduced in the epithelium of the VAD diet-fed mice compared with those of mice fed the control diet (Fig. 5D). Furthermore, there were fewer Paneth cells, which mainly produce defensin, in the SI of VAD mice (Fig. 5E). These pathological changes were seen beginning at age 3–5 wk in VAD mice and slowly progressed with aging. Overall, these results indicate that feeding of a VAD diet provokes severe dysfunction of growth and differentiation of IEC from an early time point, and subsequently enhances mucin production.

FIGURE 3. Absence of RA results in dramatic reduction of mRNA expression of IL-17, IRF4, IL-21, IL-22, and IL-23 in the SI of mice fed VAD diets. For RNA preparation, the SI ileal region of mice fed Cont or VAD diets was isolated, homogenized, and subjected to RT-PCR for cytokine-specific analysis. Each relative mRNA expression was determined by the ratio of band intensity to β -actin production. Results are representative of three repetitive experiments. Cont, control; n.d., not detected.



VAD diet results in disruption of gut microbiome

Previous studies revealed that Th17 cells in the intestine are induced in response to specific components of the commensal microbiome (14–16). Thus, we assessed the microbial ecology of the SI ileum (Fig. 6A) and of feces (Supplemental Fig. 2) of VAD mice. We found that both aerobic and anaerobic bacteria were drastically reduced in both the SI ileum and feces of VAD mice beginning at age 5 wk compared with those from control diet-fed mice. These alterations were maintained until age 15 wk (data not shown). We further analyzed the detailed composition of microbiome by using selective media and found significantly reduced numbers of Firmicutes (i.e., enterococci, *C. difficile*, and lactobacilli) and Proteobacteria (i.e., *Escherichia* species) in the SI ileum of VAD mice compared with those fed the control diet (Fig. 6A). Similar patterns of reduced microbiome were identified in the fecal extract except for *Bacteroides* (i.e., *B. fragilis*) and lactobacilli (Supplemental Fig. 2). In addition, mRNA levels of SFB were drastically reduced in the SI ileum of VAD mice compared with those fed the control diet (Fig. 6B). To further confirm the reduced microbiome levels, we assessed 16S rRNA levels in the SI ileum by use of universal primers that identify all known bacteria (Fig. 6C). Similar to the results we obtained with a selective medium and real-time PCR for SFB mRNA, the levels of 16S rRNA were significantly reduced in the SI ileum of VAD mice when compared with those of control mice (Fig. 6C). To assess a direct role of microbiome on Th17 cell development, after feeding antibiotics to wild-type control mice for 5 wk, we measured Th17 cells in the SI (Fig. 6D). As expected, the antibiotic-treated mice had significantly fewer Th17 cells with levels similar to those of VAD mice (Fig. 6D). Thus, we speculate that significant reduction of both aerobic and anaerobic microbiome caused by a VAD diet might be one crucial factor for eliciting significant reduction of Th17 cell differentiation.

MyD88/TRIF-mediated innate immunity is not involved in downregulation of Th17 differentiation in RA-deficient conditions

We further assessed the role of innate immunity for Th17 cell differentiation because innate immunity is essential to maintain homeostasis of the intestine against harsh environments (e.g., commensal and/or pathogenic bacteria) (28). MyD88^{-/-} and MyD88^{-/-}

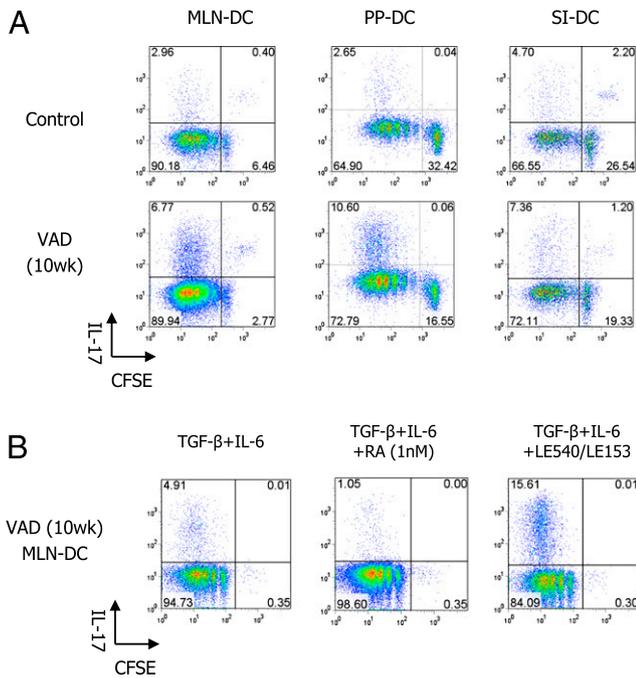


FIGURE 4. RA-deficient DCs activate T cells to secrete IL-17. *A*, DCs were isolated from MLN, PP, or SI of 10-wk-old mice fed control or VAD diets and cocultured for 3 d with CFSE-labeled OT-II CD4⁺ T cells in the presence of OVA peptide. For differentiation into Th17 cells, both IL-6 and TGF-β1 were added. *B*, To measure differentiation into Th17 cells, IL-6, TGF-β1, and RA or RA inhibitors (LE540 + LE153) were added to the cocultured wells with MLN DCs of VAD mice and OT-II CD4⁺ T cells. All results are representative of three repetitive experiments.

TRIF^{-/-} mice were fed control or VAD diets for 10 wk before we isolated mononuclear cells from SI-LP and determined the number of IL-17-secreting CD4⁺ T cells. Much like wild-type mice, the

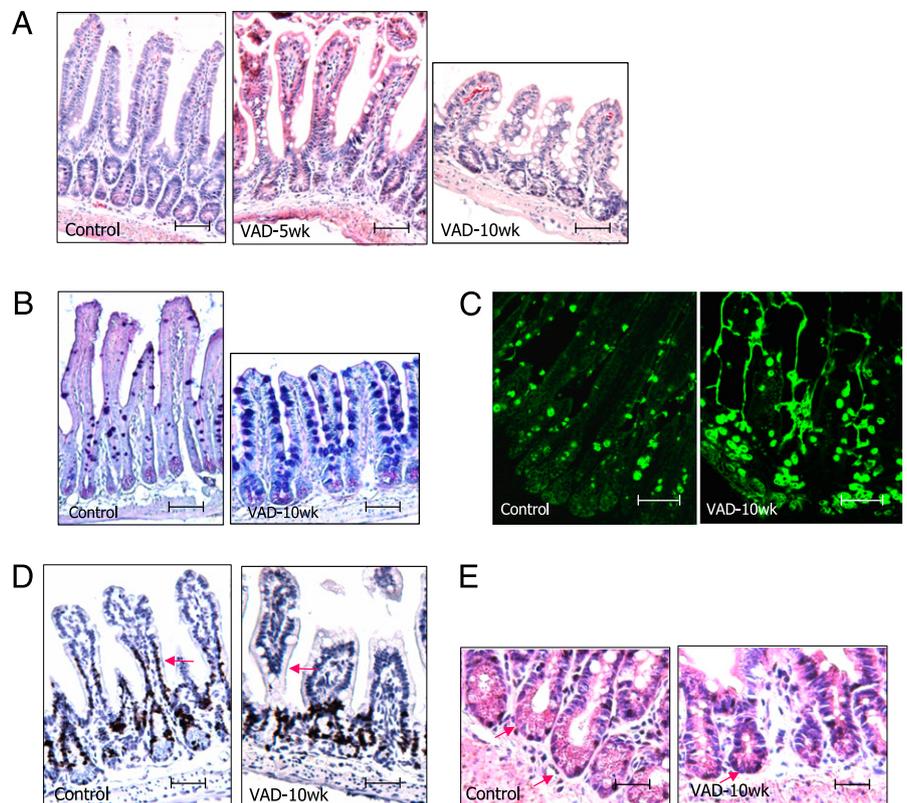
VAD diet-fed MyD88^{-/-} and MyD88^{-/-}TRIF^{-/-} mice had significantly fewer IL-17-secreting CD4⁺ T cells than found in control diet-fed MyD88^{-/-} and MyD88^{-/-}TRIF^{-/-} mice (Fig. 7). Overall, our findings show that a VAD diet leads to a defect of Th17 cell differentiation by MyD88/TRIF in an independent manner.

Discussion

RA maintains intestinal immune homeostasis by inducing Foxp3⁺ Treg cells and inhibiting Th17 cell generation in the gut through DCs (mainly in in vitro culture systems) (17–21). Surprisingly, our present study showed that a VAD diet completely depleted Th17 cell differentiation and that Foxp3⁺ Treg cells and IFN-γ⁺ Th1 cells were unchanged in the SI-LP. In an in vitro culture system, however, RA-deficient DCs obtained from MLN, PP, or SI-LP of VAD mice could induce more Th17 cells than those from mice fed a control diet. Conversely, RA inhibited the generation of Th17 cells induced by DCs together with TGF-β and IL-6. These contradictory results between in vitro and in vivo systems imply that non-DC factors could be involved in regulation of Th17 differentiation by RA in vivo.

Intestinal epithelium is in continuous contact with potential pathogens and beneficial commensal bacteria. Epithelial cell recognition of luminal microorganisms is crucial for maintaining immune homeostasis in the gut (29). In one study, translocation and colonization of *E. coli* were significantly increased in VAD-fed rats (30). Others reported that rotavirus infection caused almost complete destruction of the tips of villi in the SI of VAD-fed rats (31) and that the commensal bacterial burden in the rat gut was increased by decreased mucin (i.e., MUC2) expression in the absence of vitamin A (32). In other studies, RA directly affected the differentiation of epithelial cells with mucosal phenotypes and enhanced mucin expression by epithelial cells (25, 31, 33). In our present study, however, VAD mice had significantly fewer commensal bacteria in both the SI ileum and fecal extracts (Fig. 6A

FIGURE 5. Absence of RA results in altered SI pathophysiology. *A*, H&E staining demonstrates shortened villi and increased numbers of goblet cells in the SI ileum of mice fed VAD diets. *B*, Numbers of goblet cells (blue) were confirmed by Alcian blue-PAS staining. *C*, Expression levels of MUC2 were determined in the SI ileum by staining with biotinylated MUC-2 mAb, followed by FITC-conjugated streptavidin. *D*, Patterns of BrdU-incorporating cells in the SI of VAD and control mice. *E*, H&E staining shows fewer Paneth cells in the SI of mice fed VAD diets. Data are representative of two independent experiments. Scale bar, 20 μm, except for *C* (50 μm).



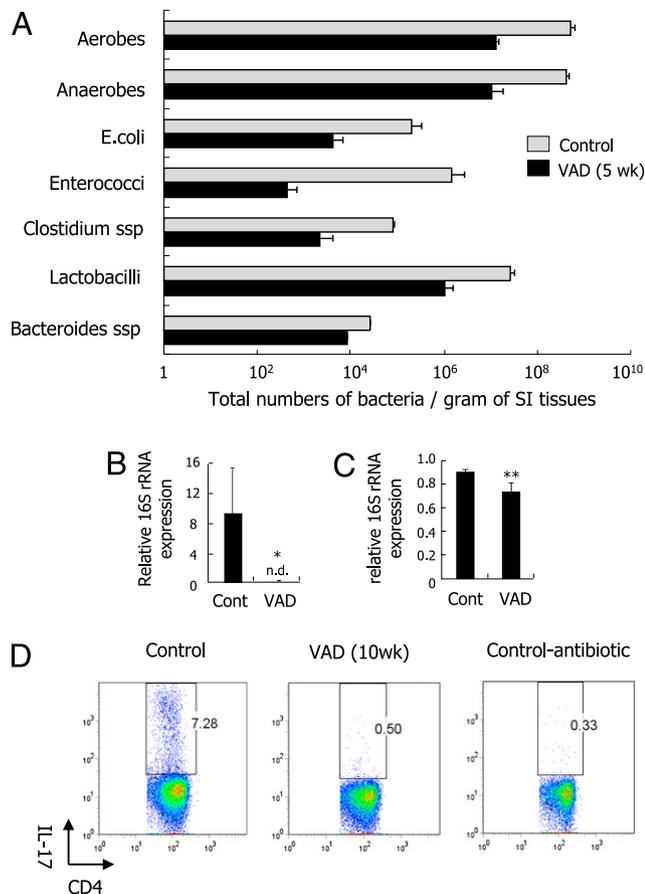


FIGURE 6. Absence of RA results in disruption of numbers and composition of gut microbiome. Each microorganism was determined in the SI ileum of 5-wk-old mice fed Cont or VAD diets by using selective medium (A). For detection of SFB (B) and bacteria 16S rRNA (C), real-time PCR and PCR were performed using SFB-specific and universal primers (Supplemental Table 1), respectively. D, After control mice were fed drinking water containing antibiotics for 5 wk, numbers of IL-17⁺CD4⁺ T cells were assessed, as described in the legend of Fig. 1. Results are representative of three repetitive experiments. **p* < 0.05; ***p* < 0.001. Cont, control; n.d., not detected.

and Supplemental Fig. 2). These mice were characterized by increased numbers of goblet cells with high levels of MUC2 protein expression in the SI-LP (Fig. 5C). Consistent with these alterations, another group showed that lack of vitamin A significantly changed mucin dynamics in rats as reflected by the enlarged goblet cell cup area relative to controls. VAD rats had decreased

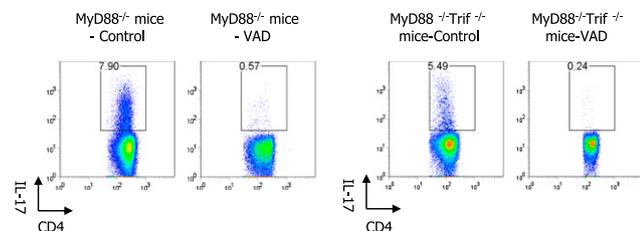


FIGURE 7. MyD88/TRIF signaling is not involved in reduction of IL-17 differentiation in the absence of RA. Both MyD88^{-/-} and MyD88^{-/-}TRIF^{-/-} mice were fed control or VAD diets from the gestational period, and mononuclear cells were isolated from the small intestine LP. IL-17- and Foxp3-positive cells were determined after coculture with and without PMA and ionomycin, respectively. Percentages of IL-17⁺ cells among CD4⁺ T cells are indicated. Results are representative of three repetitive experiments.

MUC2, but increased MUC3 mRNA expression in the jejunum, ileum, and colon (30). These contradictory results of the role of RA on mucin expression might be due to differences between in vitro and in vivo systems, in detection of mRNA or protein level, or to different composition of commensal bacteria, different vitamin A concentrations in the diet, and to variances between mice and rats. Further studies will be required to elucidate the differences. Overall, our data clearly show that a VAD diet can change the phenotype and functionality of IEC and increase mucin expression and subsequently alter the number and composition of commensal microbiome.

Our results suggest that low populations of Th17 in VAD mice might be ascribable to reduced microbes following abnormal epithelial changes, especially of goblet cells. ATP derived from commensal microbiome induces Th17 generation via a unique LP CD70^{high}CD11c^{low} cell that expresses cell surface ionotropic (P2X) and metabotropic (P2Y) receptors (15). In that study, germ-free mice possessed fewer Th17 cells in the colonic LP; this was accompanied by less ATP secretion (15). However, ATP levels were not detectable in the fecal extracts of mice fed control or VAD diets (Supplemental Fig. 3). Differentiation of Th17 cells also correlated with the presence of cytophaga-flavobacter-bacteroidetes bacteria in the intestine, indicating that the quality, but not quantity of commensal bacteria is important for Th17 generation (16). Recently, two unique studies demonstrated an important role of SFB on the induction of intestinal Th17 cells (34, 35). Of note, 16S rRNA levels of SFB were significantly reduced in the SI ileum of VAD mice compared with control mice (Fig. 6B). Thus, Th17 generation is closely related to commensal microbiome, including SFB, although its precise role in Th17 differentiation, even in relation to ATP involvement, remains controversial.

Th17 lineage cells produce IL-17A, IL-17F, IL-21, IL-22, and IL-23 to communicate with immune cells. TGF-β, IL-6, and IL-21 are differentiation factors, and IL-23 is the growth and stabilization factor of naive T cells that helps differentiate Th17 cells (4, 33). In the current study, we found significantly lower levels of IL-21 and IL-23 in the presence of identical levels of TGF-β and IL-6. IL-21 has been reported to initiate an alternative pathway to induce proinflammatory Th17 cells (36). More recently, IL-23 was found not to be involved in the initial differentiation of Th17 cells, although it appears to be essential for the sustained differentiation of Th17 cells (4). Of note, IL-23p19-deficient mice have limited numbers of Th17 cells. Moreover, prolonged culture of Th17 cells in vitro requires the addition of IL-23 (37). In contrast, recent studies show that IRF4 is essential for IL-21-mediated induction, differentiation, and stabilization of Th17 cells (38). It seems plausible that a VAD diet keeps Th17 cells from differentiation to growth and stabilization by eliminating IL-21, IL-22, IL-23, and IRF4 activation.

In contrast, both Th1 and Th2 lineage-specific cytokines, such as IFN-γ, IL-4, and IL-12 antagonize Th17 cell differentiation (39, 40). Th17 cell development is also inhibited by IL-25 (41), IL-27 (42), IFN-αβ (43), as well as by IL-2 (44). In addition to cytokines, RA (21) and ligands of the AHR (45) negatively regulate Th17 generation reciprocally with enhanced Foxp3⁺ Treg cells. In inflammatory conditions, flagellin activates TLR5⁺ LP DCs to induce Th17 cell differentiation dependent on RA (22). These cytokines and environmental factors affect directly or indirectly Th17 cell differentiation via APC. In the current study, Foxp3⁺ Treg cells in the SI-LP were not reciprocally increased in 5-wk-old VAD mice (Fig. 1C, 1D). In addition, non-DC factors might play a crucial role in the regulation of Th17 versus Treg generation in the VAD mice (Fig. 4). Of note, we found no significant difference in mRNA expression of both IL-25 and IL-27 in the SI of VAD and control

diet-fed mice. Although GATA3 mRNA expression was slightly decreased in the SI-LP of VAD mice compared with control diet mice, there was no strong evidence of Th1- or Th2-dominant responses in the VAD mice (Supplemental Fig. 4). When all of our findings are considered together, the mechanism of Th1, Th2, and Treg activation is not expected to be affected in depiction of Th17 generation in VAD mice.

Th17 cells are pivotal in autoimmune diseases, inflammatory bowel diseases, and infections (4, 33, 46). Moreover, malnutrition, from which many children in developing countries suffer, may lead to impairment of many aspects of host defense (47). Our results in this study clearly demonstrate that nutrients, such as vitamin A, regulate development and maintenance of Th17 cells in the intestines. Thus, future studies that seek cures for inflammatory and infectious diseases should consider the roles of nutrients and disease.

We found that lack of vitamin A in the mouse diet suppresses Th17 cell generation in the gut via reduced commensal microbes, including SFB following altered IEC phenotypes. These results provide new insight into the relationship between RA and Th17 cell populations *in vivo*. Although found throughout the body, Th17 cells are most abundant in the gut at steady state, where the immune response is tightly regulated. Th17 cells play a double role: They are both pathogenic and protective to intestinal inflammation dependent on the situation. RA shapes immune homeostasis of the gut by orchestrating the regulatory factors for Th17 cells. Further definition of the molecular mechanisms that regulate Th17 cells in the absence of RA is essential to determine the regulatory network of Th17 cell development in the gut *in vivo*.

Disclosures

The authors have no financial conflicts of interest.

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