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Microbiota-Dependent Involvement of Th17 Cells in Murine Models of Inflammatory Arthritis

Heather Evans-Marin,[1] Rebecca Rogier,[2] Sergei B. Koralov,[1] Julia Manasson,[1] Debbie Roeleveld,[2] Peter M. van der Kraan,[2] Jose U. Scher,[1] Marije I. Koenders,[2] and Shahla Abdollahi-Roodsaz[3]

***Objective*. Intestinal microbiota are associated with the development of inflammatory arthritis. The aim of this study was to dissect intestinal mucosal immune responses in the preclinical phase of arthritis and determine whether the presence of Th17 cells, beyond involvement of the cytokine interleukin-17 (IL-17), is required for arthritis development, and whether the involvement of Th17 cells in arthritis depends on the composition of the host microbiota.**

***Methods*. Mucosal T cell production of IL-17, interferon-γ, tumor necrosis factor α (TNFα), IL-22, and granulocyte–macrophage colony-stimulating factor (GM-CSF) was analyzed by flow cytometry and Luminex assay before arthritis onset in mice immunized to develop collagen-induced arthritis (CIA). Pathogenic features of arthritis in mice with CIA and mice with antigen-induced arthritis were compared between Th17 cell–deficient (*CD4-Cre*$^{+}$ *Rorc*$^{flox/flox}$) and Th17 cell–sufficient (*CD4-Cre*$^{-}$ *Rorc*$^{flox/flox}$) mice. In addition, the impact of intestinal microbiota on the Th17 cell dependence of CIA was assessed.**

***Results*. Lamina propria CD4 T cells were activated before the onset of arthritis in mice with CIA, with marked up-regulation of several cytokines, including IL-17A, TNFα, and GM-CSF. *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice showed a specific reduction in intestinal mucosal levels of Th17 cells and partially reduced levels of IL-17–producing CD8 T cells. However, total levels of IL-17A, mostly produced by γδ T cells and neutrophils, were unaffected. The severity of arthritis was significantly reduced in Th17 cell–deficient mice, suggesting that Th17 cells have additional, IL-17A–independent roles in inflammatory arthritis. Accordingly, antigen-stimulated T cells from Th17 cell–deficient mice produced less IL-17A, IL-17F, and GM-CSF. Importantly, the dependence of CIA on the involvement of Th17 cells was mitigated in the presence of an alternative microbiome.**

***Conclusion*. These data from murine models suggest that activation of mucosal immunity precedes the development of arthritis, and also that Th17 cells have a microbiota-dependent role in arthritis. Therefore, a microbiome-guided stratification of patients might improve the efficacy of Th17-targeted therapies.**

Intestinal microbiota are associated with several autoimmune diseases, including rheumatoid arthritis (RA). Mucosal surfaces such as periodontal, lung, and intestinal tissues are a proposed site of immune activation and breach of tolerance in RA (1). However, the extent and nature of the mucosal immune activation during preclinical arthritis are unclear. Several independent studies have demonstrated perturbed diversity and composition of commensal microbiota in patients with RA compared with healthy individuals (2–5). However, the relationship between dysbiosis and the pathogenesis of RA is not fully understood.

The microbiome profoundly affects the balance between inflammatory CD4+ Th1 cells and Th17 cells and protective Treg cells, both at mucosal surfaces and systemically (6). Specific taxa, such as segmented filamentous bacteria (SFB), enhance differentiation of Th17 cells in the small intestine (SI) lamina propria (7,8). SFB

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[1]Heather Evans-Marin, PhD, Sergei B. Koralov, PhD, Julia Manasson, MD, Jose U. Scher, MD: New York University School of Medicine, New York, New York; [2]Rebecca Rogier, MSc, Debbie Roeleveld, MSc, Peter M. van der Kraan, PhD, Marije I. Koenders, PhD: Radboud University Medical Center, Nijmegen, The Netherlands; [3]Shahla Abdollahi-Roodsaz, PhD: New York University School of Medicine, New York, New York, and Radboud University Medical Center, Nijmegen, The Netherlands (current address: Celgene Corporation, Cambridge, Massachusetts).

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Address correspondence to Shahla Abdollahi-Roodsaz, PhD, Division of Rheumatology, NYU School of Medicine, 301 East 17th Street, Room 1611A, New York, NY 10003. E-mail: sabdollahi@celgene.com.

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colonization exacerbates spontaneous arthritis in interleukin-1 (IL-1) receptor antagonist–deficient and K/BxN mice (9,10). In addition, in SKG mice with increased thymic selection of autoreactive T cells (11), colonization of *Prevotella copri*, a commensal species associated with new-onset RA (2,3), led to increased abundance of SI lamina propria Th17 cells and enhanced arthritis upon concomitant exposure to zymosan (3). Although these studies showed that Th17 cell–inducing intestinal microbiota exacerbate arthritis, it is not known whether involvement of these microbiota-induced Th17 cells is required for the pathogenesis of arthritis.

Inhibition of either IL-17A or its receptor ameliorates arthritis in mice (12–15). Although blockade of IL-17 resulted in clinically relevant improvement based on the American College of Rheumatology 50% improvement response criteria in ~20% of patients with RA, convincing efficacy in the overall population was not observed (16–18). Importantly, Th17 cells produce other proinflammatory mediators, such as IL-17F, IL-22, tumor necrosis factor α (TNFα), and granulocyte–macrophage colony-stimulating factor (GM-CSF), which have IL-17A–independent proinflammatory roles (13,19–21). Therefore, the role of Th17 cells in RA likely extends beyond the production of IL-17A. Previous studies in animal models have assessed the role of Th17 cells in IL-17–deficient mice, and clinical trials have evaluated the effects of IL-17–blocking antibodies. Because several cell types other than Th17 cells produce IL-17 (13,22), the specific contribution of Th17 cells to the pathogenesis of arthritis is unclear.

Enrichment of Th17 cells in vivo strongly depends on the presence and composition of intestinal microbiota (7,8,23). Accordingly, the intestinal mucosa is a major anatomic compartment of Th17 cells (7,8), and the involvement of Th17 cells in arthritis may be highly dependent on the composition of the host microbiota. Our aim was to determine the role of Th17 cells in arthritis pathogenesis and investigate whether the involvement of Th17 cells in arthritis depends on the microbiota present in the gut prior to disease development. In addition, we hypothesized that mucosal immune activation occurs in the preclinical phase and precedes the onset of arthritis.

MATERIALS AND METHODS

Mice. Th17 cell–deficient mice (*CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$) on a C57BL/6 background were generated by crossing B6 (Cg)-Rorc$^{tm3Litt/J}$ with B6.Cg-Tg(Cd4-cre) mice (stock nos. 008771 and 022071; The Jackson Laboratory) (24,25). *CD4-Cre*$^{-}$*Rorc*$^{flox/flox}$ mouse littermates were used as wild-type (WT) control mice. Experimental groups consisted of randomized age- and sex-matched *Cre*$^{+}$ and *Cre*$^{-}$ co-housed littermates. Mice were housed in individually ventilated cages and were provided autoclaved food and water ad libitum. All animal studies were approved by our Institutional Review Board, and were conducted in accordance with institutional guidelines.

Antibiotic treatments and reconstitution with Jackson microbiota. Age- and sex-matched groups of *CD4-Cre*$^{+}$ *Rorc*$^{flox/flox}$ and *CD4-Cre*−*Rorc*$^{flox/flox}$ mice received an antibiotic cocktail of metronidazole, neomycin trisulfate, ampicillin sodium salt, vancomycin, and sucrose that was added to drinking water for 1 week. Microbiota were then reconstituted by oral gavage of a 200-μl aqueous suspension of SFB-free feces from Jackson mice, at 24 hours after cessation of the antibiotics. The SFB-free status of the mice was confirmed by quantitative polymerase chain reaction (qPCR), as reported previously (10,26).

Isolation of lamina propria cells. Mesenteric fat and Peyer's patches were removed from the small intestine and colon. Tissue was incubated with 5 m*M* EDTA to remove epithelial cells, and subsequently was digested with 1 mg/ml collagenase D and 10 μg/ml DNAse I. Lamina propria lymphocytes were harvested at the interphase of a 40%:80% Percoll gradient and utilized in the experiments described below.

Cell cultures and cytokine measurements. SI lamina propria or mesenteric lymph node (LN) cells ($1–2 \times 10^5$ cells/well) were cultured in 96-well round-bottomed plates. Supernatants were collected after 6 hours from cells stimulated with phorbol myristate acetate (PMA) (50 ng/ml) and ionomycin (1 μg/ml), or after 2 days from cells stimulated with collagen (50 μg/ml). Cytokine levels were measured by Luminex assay using Bio-Rad magnetic bead kits specific for mouse cytokine groups 1 and 3, in accordance with the manufacturer's instructions.

Flow cytometry. Prior to flow cytometry staining, cells were restimulated with PMA (50 ng/ml; Sigma), ionomycin, and brefeldin A for 4 hours. Staining protocols and reagents are described in Supplementary Methods and listed in Supplementary Table 1 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract). Cells were fixed in 2% paraformaldehyde and stored at 4°C until acquisition with an LSRII flow cytometer. Analysis of the findings was performed in FlowJo.

Fluorescence-activated cell sorting. Splenocytes were stained with surface markers, and then resuspended in T cell medium and sorted with a FACSAria II using the following parameters: T cell receptor β–positive (TCRβ+), viability dye–negative cells were positively selected, followed by additional positive selection using gating on CD4 and CD8 single-positive cells.

Induction of antigen-induced arthritis (AIA). To induce AIA, mice were treated with 200 μg methylated bovine serum albumin (mBSA) in saline, administered intraarticularly into the footpad, and with 250 ng IL-1β in saline, administered subcutaneously into the footpad, with additional IL-1β treatments at 24 and 48 hours thereafter (27,28). Mice were euthanized on day 7, during the peak of the inflammatory response (27,28).

Induction of collagen-induced arthritis (CIA). CIA was induced via 2 intradermal immunizations with 100 μl of an emulsion consisting of a 1:1 ratio of chicken type II collagen (CII) (4 mg/ml in 10 m*M* acetic acid) and Freund's complete adjuvant, based on previously published protocols optimized for the BL/6 background (29,30). Freund's complete adjuvant was prepared by adding 5 mg desiccated *Mycobacterium tuberculosis* H37RA (Difco) per 1 ml Freund's incomplete adjuvant. Primary immunization was administered in the tail base of mice at ages 10–12 weeks. The mice received a booster in the lower back on day 21, and were

monitored for clinical signs of arthritis. All mice developed mild arthritis.

Assessment of arthritis. The severity of arthritis was scored in a blinded manner, as described previously (31). For histology, ankle joints were isolated and fixed in 4% formaldehyde for 4 days, and thereafter decalcified in 5% formic acid and embedded in paraffin. Tissue sections (7 μm) were stained using hematoxylin and eosin or Safranin O. Synovial inflammation, chondrocyte death, proteoglycan depletion, and erosion of hyaline cartilage were scored in a blinded manner on a scale of 0–3 (31).

DNA qPCR and 16S sequencing. Mouse genomic DNA was extracted from sorted CD4+ and CD8+ splenocytes with a QIAamp DNA Mini kit (Qiagen), in accordance with the manufacturer's instructions. Fecal pellets were collected and stored at −20°C. Extraction of DNA was performed with a PowerLyzer DNA isolation kit (MO BIO Laboratories), in accordance with the manufacturer's instructions. Quantitative PCR was performed on the StepOne System (Applied Biosystems) using a SYBR FAST Master Mix (KAPA Biosystems). The sequencing methods used are extensively described in Supplementary Methods, and a complete list of the primers used is shown in Supplementary Table 2 (http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract).

Statistical analysis. Results are expressed as the mean ± SEM. Statistically significant differences between 2 groups were tested using the Mann-Whitney U test or Student's *t*-test, as appropriate. Statistical comparisons of ≥3 groups were performed using the Kruskal-Wallis test with an uncorrected Dunn's test. Arthritis severity scores were compared using repeated-measures analysis of variance with an uncorrected Fisher's least significant difference test. Statistical analysis was performed using GraphPad Prism version 7.02.

RESULTS

Characterization of mucosal immune activation preceding the onset of arthritis. Modulation of the intestinal immune response by commensal microbiota affects the inflammation patterns and immunopathologic features at distal anatomic sites, including the joints (9,10,32,33). To investigate mucosal immune activation during preclinical arthritis, we assessed intestinal CD4+ T cell cytokine responses before arthritis onset in WT mice 21 days after a single immunization with CII. We found that immunization of the mice with CII induced a robust T cell response in the SI lamina propria, with significantly increased percentages of both Th1 and Th17 cells (Figures 1A and B) (for the gating strategy, see Supplementary Figure 1A [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]).

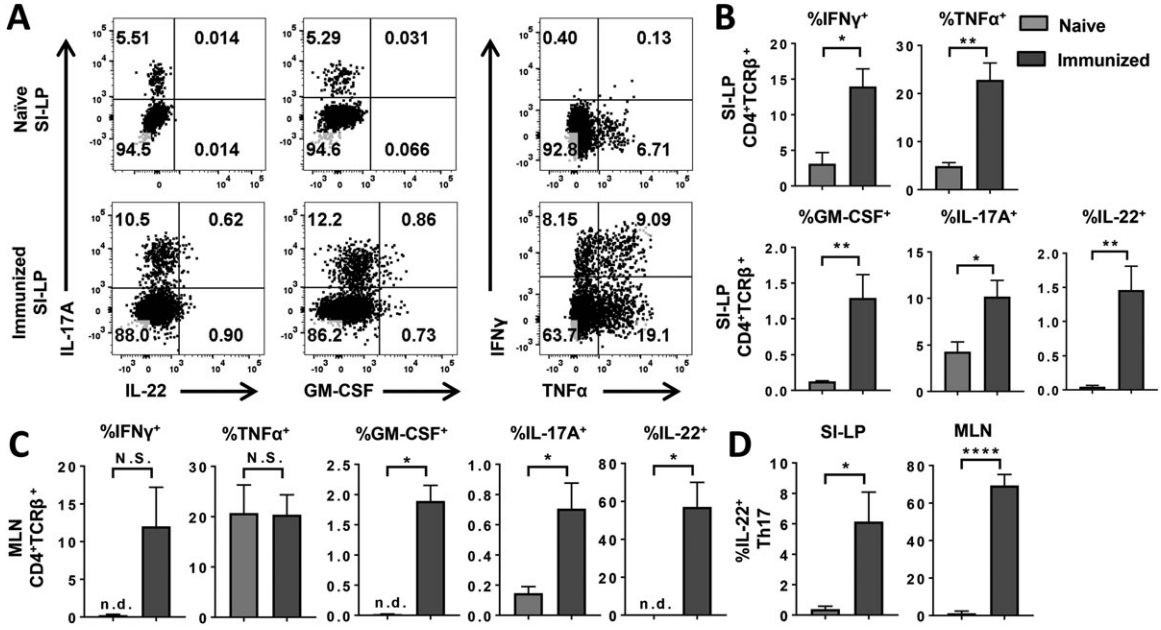

Figure 1. Intestinal mucosal CD4+ T cells are activated during the preclinical phase of arthritis and exhibit Th1 and Th17 cell phenotypes. Small intestine lamina propria (SI-LP) and mesenteric lymph node (MLN) lymphocytes from naive and single-immunized (nonarthritic) wild-type mice were isolated, restimulated with phorbol myristate acetate and ionomycin, and stained for intracellular flow cytometry (see Supplementary Figure 1A [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract] for the gating strategy), and cytokine production by CD4+ T cells was analyzed. **A,** Representative plots from fluorescence-activated cell sorting show CD4+ T cell production of interleukin-17A (IL-17A), IL-22, granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFNγ), and tumor necrosis factor α (TNFα) in the SI-LP of naive and immunized mice. **B** and **C,** Production of IFNγ, TNFα, GM-CSF, IL-17A, and IL-22 by CD4+ T cells was determined in the SI-LP **(B)** and MLNs **(C)** of naive and immunized mice. **D,** Percentages of Th17 cells that coexpressed IL-22 in the SI-LP and MLNs of naive and immunized mice are shown. Results are the mean ± SEM in cells from 4 mice per group, from a representative experiment. * = $P < 0.05$; ** = $P < 0.01$; **** = $P < 0.0001$, by Student's *t*-test. NS = not significant.

In addition, CD4+ T cell expression of TNFα, GM-CSF, and IL-22 was up-regulated in the SI lamina propria after immunization (Figures 1A and B). The numbers of lamina propria CD4+ T cells expressing inflammatory cytokines were increased in mice upon immunization with CII (Supplementary Figure 2A [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]), although not all of the analytes showed a statistically significant change in cytokine levels. The measurement of cytokines in culture supernatants confirmed significantly increased SI lamina propria production of IL-17A and IL-22 (Supplementary Figure 2B [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). Furthermore, we observed higher frequencies of CD4+ T cells that expressed IL-17A, IL-22, and GM-CSF in the mesenteric LNs of immunized mice. T cell interferon-γ (IFNγ) and TNFα production in the mesenteric LNs of immunized mice remained unaffected (Figure 1C and Supplementary Figure 1B [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]).

In the Peyer's patches, percentages of TNFα-, IL-22–, and GM-CSF–producing CD4+ T cells were increased in immunized mice, with no difference in the number of Th1 or Th17 cells (Supplementary Figure 1C [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). We observed no difference in CD4+ T cell cytokine production in the colonic lamina propria of naive mice compared with immunized mice (data not shown), suggesting that mucosal immune activation in response to CII immunization is restricted to the SI lamina propria.

Because Th17 cells produce multiple cytokines (20,22), we examined whether CII immunization specifically altered the production of these cytokines by Th17 cells. SI lamina propria and mesenteric LN Th17 cells from immunized mice exhibited significantly higher coexpression of IL-22 compared with naive mice (Figure 1D). In addition, Th17 cell coexpression of IL-22 and GM-CSF was increased in the Peyer's patches of immunized mice compared with naive mice (Supplementary Figure 1D [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). These data suggest that activation of intestinal CD4+ T cells and increased production of mucosal Th1 and Th17 cells precede the development of arthritis in mice.

Specific reduction of Th17 cells but unaffected IL-17 production by other cellular sources in Th17 cell–deficient mice. Results from previous studies using IL-17 neutralizing antibodies or IL-17–deficient mice suggested that IL-17 has a role in the pathogenesis of arthritis (14,15,34–37). However, because IL-17 is produced by multiple cell types other than Th17 cells, such as

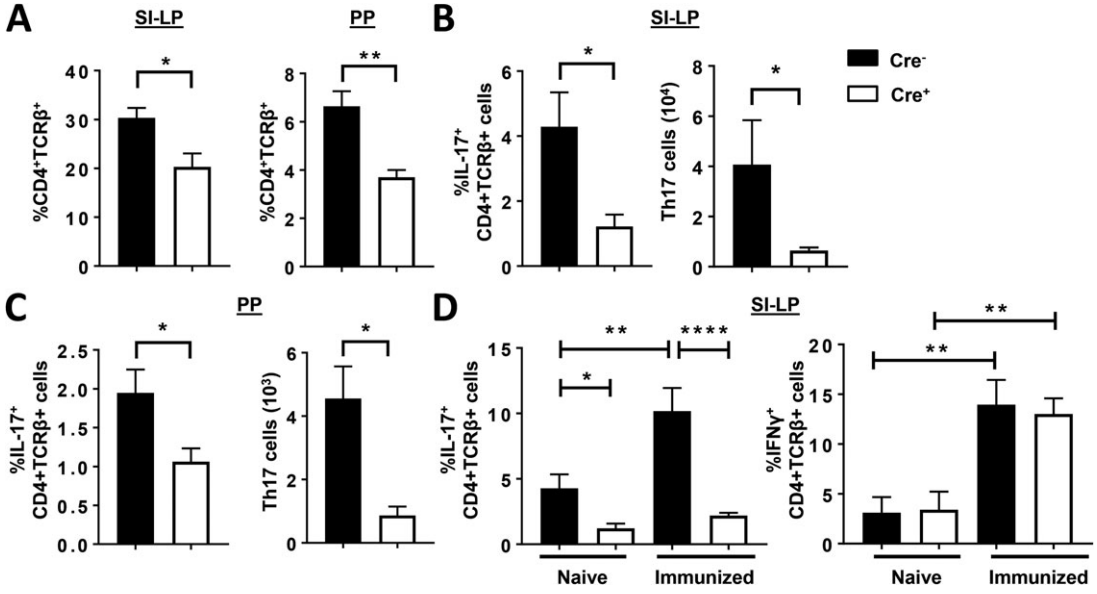

Figure 2. *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice exhibit specific Th17 cell deficiency under naive and immunized conditions. SI-LP and Peyer's patch (PP) lymphocytes from 11–14-week–old male *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ and *CD4-Cre*$^{-}$*Rorc*$^{flox/flox}$ mice were isolated and counted. Cells were then stimulated with phorbol myristate acetate and ionomycin and stained for intracellular flow cytometry. **A–C,** *Cre*$^{+}$ and *Cre*$^{-}$ mice were compared for percentages of CD4+ T cells in the SI-LP and PP **(A)**, and for percentages of IL-17A+ cells within the live CD4+ T cell receptor β–positive (TCRβ+) gate and for absolute numbers of IL-17A+CD4+ T cells in the SI-LP **(B)** and PP **(C)**. **D,** Percentages of IL-17A+ and IFNγ+ cells within the live CD4+TCRβ+ T cell population in the SI-LP were compared between naive and immunized *Cre*$^{+}$ and *Cre*$^{-}$ mice. Results are the mean ± SEM in cells from 4 mice per group, from a representative experiment. * = $P < 0.05$; ** = $P < 0.01$; **** = $P < 0.0001$, by Student's *t*-test or by analysis of variance with Fisher's least significant difference test. See Figure 1 for other definitions.

neutrophils and mast cells (13,38,39), the role of, and requirement for, Th17 cells specifically has not been established. Notably, IL-17 deficiency protects against innate cell–mediated K/BxN serum–transfer arthritis (14,15,40,41). To directly examine the role of Th17 cells in the development of arthritis, we utilized conditional Th17-deficient mice. Th17 ablation in these mice is achieved through a CD4-specific Cre recombinase–induced deletion of a floxed *Rorc* allele, which prevents the expression of retinoic acid receptor–related orphan nuclear receptor γt (RORγt) and thus inhibits differentiation of naive CD4 T cells into Th17 cells (42). *CD4-Cre*$^{+/-}$ *STAT3*$^{flox/flox}$ mice, which is another type of Th17-deficient mouse strain, were not utilized in our studies because production of the Th1 and T follicular helper cell subsets is impaired in these mice (43,44).

To confirm that *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice had effective and specific deletion of IL-17 in CD4+ T cells, we compared T helper cell differentiation between *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice and control *CD4-Cre*$^{-}$*Rorc*$^{flox/flox}$ littermates. We observed that the SI lamina propria, Peyer's patches, and mesenteric LNs of *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice had decreased percentages of CD4+ T cells compared with *CD4-Cre*$^{-}$*Rorc*$^{flox/flox}$ control mice (Figure 2A and data not shown). Furthermore, the percentages and absolute numbers of Th17 cells were significantly reduced in the SI lamina propria (Figure 2B) and Peyer's patches (Figure 2C) of *Cre*$^{+}$ mice compared with *Cre*$^{-}$ littermates. Th17 cells were negligible in the mesenteric LNs from both genotypes (data not shown). In contrast to Th17 cells, the percentages and absolute numbers of IFNγ- and TNFα-producing CD4+ T cells were not affected in *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice (data not shown).

To assess the proliferation and apoptosis of Th17 cells, we measured the expression of the proliferation marker Ki-67 on total live CD4− cells, CD4+IL-17− cells, and Th17 cells by flow cytometry. We observed a significant reduction in the levels of Ki-67+ Th17 cells in *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice. There was no difference in Ki-67 expression on CD4− and non-Th17 CD4+ T cells between genotypes (Figure 3A). Therefore, these findings suggest that the *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ genotype leads to reduced proliferation of Th17 cells, but has no effect on other CD4+ T cells or non-CD4 cells. Furthermore, the numbers of annexin V–positive apoptotic cells were increased among CD4+ T cells (both Th17 and non-Th17) in *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice (Figure 3B). These data suggest that loss of Th17 cells in *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice is a reflection of the reduced proliferation and increased apoptosis of Th17 cells.

Because CD4-Cre is expressed at the CD4/CD8 double-positive stage of T cell development, Cre recombinase deletes floxed sequences in mature CD4 and CD8 T cells. To determine the effect of the *CD4-Cre*$^{+}$ *Rorc*$^{flox/flox}$ genotype on CD8 T cells, we assessed deletion of the *Rorc* gene in genomic DNA of sorted TCRβ+CD4+CD8− T cells and TCRβ+CD4−CD8+ T cells (purity of each subset >99%) via real-time PCR. As

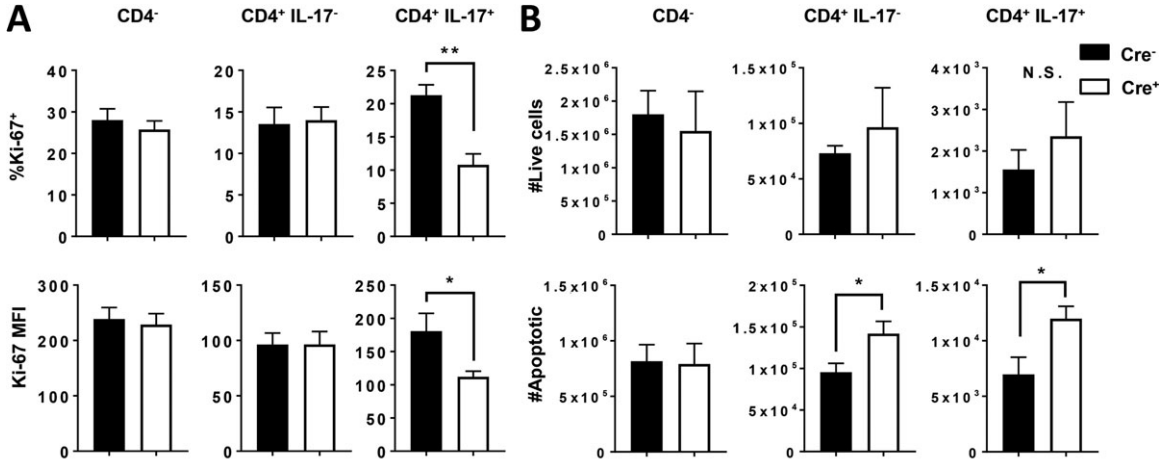

Figure 3. CD4+ T cell apoptosis is increased and Th17 cell proliferation is decreased in *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice compared with *CD4-Cre*$^{-}$*Rorc*$^{flox/flox}$ mice. Flow cytometric analysis was conducted on SI-LP lymphocytes from naive *Cre*$^{+}$ and *Cre*$^{-}$ mice. Cells were first gated into CD4−, CD4+IL-17A−, and CD4+IL-17A+ T cell populations for analysis. **A,** Proliferation of each cell population was determined as the percentage expression and mean fluorescence intensity (MFI) of the proliferation marker Ki-67. **B,** The number of live (annexin V–negative, viability dye–negative) and apoptotic (annexin V–positive) cells within each population was calculated. Results are the mean ± SEM in cells from 4 mice per group. * = $P < 0.05$; ** = $P < 0.01$, by Student's *t*-test. See Figure 1 for definitions.

expected, the relative genomic presence of *Rorc* was significantly deleted in CD4+ T cells from *CD4-Cre*$^{+}$ *Rorc*$^{flox/flox}$ mice ($P = 0.0065$). Expression of *Rorc* was also significantly depleted in CD8+ T cell genomic DNA from *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice, although the difference between genotypes did not reach statistical significance ($P = 0.23$), presumably due to variation (Supplementary Figure 3A [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). On average, TCRβ+CD4+CD8− T cells and TCRβ+CD4−CD8+ T cells sustained expression of 25.8% and 26.7% of their genomic *Rorc* DNA, respectively (Supplementary Figure 3B [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]).

To determine whether production of IL-17 by other cells was altered in *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice, we examined the expression of IL-17 in neutrophils, mast cells, and γδ T cells. Among SI lamina propria TCRβ-negative CD11b+ cells, the proportion of CD117+ mast cells was a mean ± SEM 0.34 ± 0.15%. However, IL-17 expression was not detected in mast cells from naive mice of either genotype. These observations were confirmed in the colonic lamina propria, mesenteric LNs, and Peyer's patches (data not shown). Therefore, it can be concluded that mast cells are not a major cellular source of mucosal IL-17 under naive conditions.

Analysis of CD11b+ cells expressing the surface marker Ly-6G, which is exclusively expressed on neutrophils, showed that a proportion of lamina propria neutrophils coexpressed Rorγt and IL-17. While the percentage of IL-17+Rorγt+ neutrophils was similar between *CD4-Cre*$^{-}$*Rorc*$^{flox/flox}$ and *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice, there was a nonsignificant increase in the percentage of IL-17A+ neutrophils and in the absolute numbers of both total IL-17A+ and IL-17A/Rorγt double-positive neutrophils in *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice (Figure 4A). Furthermore, the numbers of IL-17+TCRγδ+ T cells were nonsignificantly increased in *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice ($P = 0.26$) (Figure 4B). Therefore, these findings suggest that neutrophils and γδ T cells may compensate for the reduced CD4 and CD8 T cell–derived production of IL-17 in the lamina propria of *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice.

Since cytokine production was relatively low in naive mice, we sought to confirm specific Th17 cell deficiency in antigen-experienced *Cre*$^{+}$ mice. We euthanized naive and CII-immunized *CD4-Cre*$^{-}$*Rorc*$^{flox/flox}$ mice and *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ littermates and measured the proportions of Th1 and Th17 cells by flow cytometry. We found that IL-17A expression was upregulated only in *Cre*$^{-}$ mice upon immunization (Figure 2D). As was observed in naive mice, the abundance of Th17 cells was significantly reduced in immunized *Cre*$^{+}$ mice compared with immunized *Cre*$^{-}$ mice (Figure 2D). In contrast, the numbers of intestinal Th1 cells were similarly increased in both *Cre*$^{+}$ and *Cre*$^{-}$ mice upon immunization (Figure 2D). These data

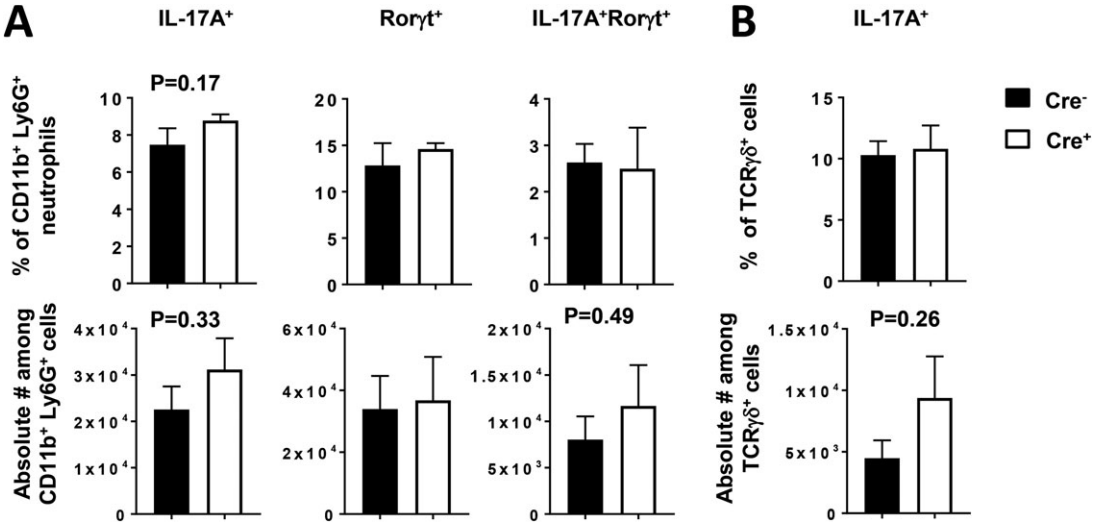

Figure 4. Production of IL-17 by SI-LP neutrophils and γδ T cells is not significantly altered in naive *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ or *CD4-Cre*$^{-}$*Rorc*$^{flox/flox}$ mice. IL-17 production by intestinal neutrophils and γδ T cells from naive *Cre*$^{-}$ and *Cre*$^{+}$ mice was analyzed by flow cytometry. **A,** Acquired cells were gated on neutrophils (CD11b+Ly-6G+), and the percentages and absolute numbers of IL-17A+, retinoic acid receptor–related orphan nuclear receptor γt–positive (Rorγt+), and IL-17A+Rorγt+ neutrophils were determined. **B,** SI-LP mononuclear cells were gated on TCRγδ+ cells, and the percentages and absolute numbers of IL-17+ cells among TCRγδ+ cells were analyzed. Results are the mean ± SEM in cells from 4 mice per group. See Figure 1 for definitions.

confirm that production of Th17 cells, but not Th1 cells, is impaired in *CD4-Cre$^+$Rorc$^{flox/flox}$* mice.

Partial reduction of acute AIA in Th17 cell–deficient mice. To further examine the role of Th17 cells in arthritis, we first used mice with AIA, an acute nonimmunogenic and nonautoimmune model of arthritis (27,28). Development of AIA was previously shown to be dependent on the involvement of IL-17A (28); however, the cellular source of IL-17A and the precise role of Th17 cells have not been elucidated. Comparison of AIA between *CD4-Cre$^+$Rorc$^{flox/flox}$* and *CD4-Cre$^-$Rorc$^{flox/flox}$* mice showed that the severity of arthritis was reduced in *Cre$^+$* mice (Supplementary Figure 4A [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). Control (phosphate buffered saline–injected) contralateral knees displayed minimal arthritis.

Partial protection from arthritis development and progression in *Cre$^+$* mice was confirmed by histology, which showed a 40% reduction in synovial inflammation in *Cre$^+$* mice compared with *Cre$^-$* mice (Supplementary Figure 4B [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). There were no significant differences in cartilage and bone damage between the 2 groups, which is likely attributable to the acute nature of this model.

Analysis of the draining LNs showed a significantly lower frequency of CD4+ T cells in the draining LNs of *Cre$^+$* mice compared with *Cre$^-$* mice (Supplementary Figure 4C [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). The frequency of Th17 cells in the draining LNs was very low and similar in both groups. However, supernatant IL-17A concentrations from draining LN cells stimulated ex vivo with PMA and ionomycin were significantly decreased in *Cre$^+$* mice compared with *Cre$^-$* littermates (Supplementary Figure 4D [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]).

We further investigated the expression of IL-17A by CD4− immune cells during the development of AIA. We found no significant difference in the frequency or mean fluorescence intensity (MFI) of IL-17A within SI lamina propria CD11b+Gr-1+ neutrophils between *Cre$^+$* and *Cre$^-$* mice (Supplementary Figure 5A [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). These findings were confirmed in the mesenteric LNs and draining LNs (data not shown). However, the numbers and frequencies of IL-17+CD8+ T cells were lower, albeit nonsignificantly, in *CD4-Cre$^+$Rorc$^{flox/flox}$* mice with AIA compared with *CD4-Cre$^-$Rorc$^{flox/flox}$* mice with AIA (Supplementary Figures 5B and C [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). Although γδ T cells were not directly examined, we observed that the TCRβ−CD11b−CD11c−Gr-1− cell population (likely representing γδ T cells) from *CD4-Cre$^+$Rorc$^{flox/flox}$* mice with AIA and *CD4-Cre$^-$Rorc$^{flox/flox}$* mice with AIA had similar levels of IL-17A expression (Supplementary Figure 5D [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]).

Our data show that although Th17 cells enhance the development and progression of AIA, other important IL-17–producing cells may contribute to the remaining arthritis observed in *CD4-Cre$^+$Rorc$^{flox/flox}$* mice (Supplementary Figures 4A–D [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). These findings validate our hypothesis that Th17 cells play an important role in the pathogenesis of arthritis and contribute to joint inflammation during acute AIA.

Suppression of CIA development in Th17 cell–deficient mice. To further investigate the role of Th17 cells in chronic autoimmune arthritis, we induced CIA in *CD4-Cre$^-$Rorc$^{flox/flox}$* mice and *CD4-Cre$^+$Rorc$^{flox/flox}$* littermates. All of the mice developed arthritis in at least 1 paw. While the number of affected paws was similar between the groups, the number of affected knee joints was significantly lower in *CD4-Cre$^+$Rorc$^{flox/flox}$* mice (mean ± SEM 1.25 ± 0.16) compared with *CD4-Cre$^-$Rorc$^{flox/flox}$* mice (mean ± SEM 1.78 ± 0.14).

In SFB-specific qPCR analyses, we demonstrated that the mice harbored SFB (data not shown), a taxa known to induce the differentiation of Th17 cells and to exacerbate arthritis in K/BxN mice (9). Nevertheless, studies have yet to demonstrate whether the involvement of these induced Th17 cells is required for arthritis, and the role of IL-17 in SFB-induced exacerbation of K/BxN arthritis has also been a subject of recent debate (45,46). Evaluation of CIA in our SFB-positive mice revealed that conditional Th17 cell–deficient (*Cre$^+$*) mice had significantly lower arthritis severity scores compared with *Cre$^-$* littermate mice beginning 36 days after the primary immunization (Figure 5A). The severity of knee arthritis was also significantly lower in *Cre$^+$* mice compared with *Cre$^-$* mice at the study end point (Figure 5A).

Histopathologic examination of the ankle joints confirmed significantly less synovial inflammation, proteoglycan depletion, chondrocyte death, and cartilage erosion in *Cre$^+$* mice compared with *Cre$^-$* mice (Figures 5B and C). These data indicate that Th17 cells are required for the development and progression of chronic inflammatory arthritis in the CIA model.

To assess the effects of Th17 cell deficiency on the gut microbiota, we performed high-throughput 16S ribosomal RNA gene sequencing of fecal microbiota from *CD4-Cre$^+$Rorc$^{flox/flox}$* and *CD4-Cre$^-$Rorc$^{flox/flox}$* mice. The results showed that the relative abundance of the genus *Lactobacillus* was increased whereas the genera *Bacteroides*, *Enterococcus*, and *Candidatus arthromitus* (SFB) were reduced in *CD4-Cre$^+$Rorc$^{flox/flox}$* mice compared with

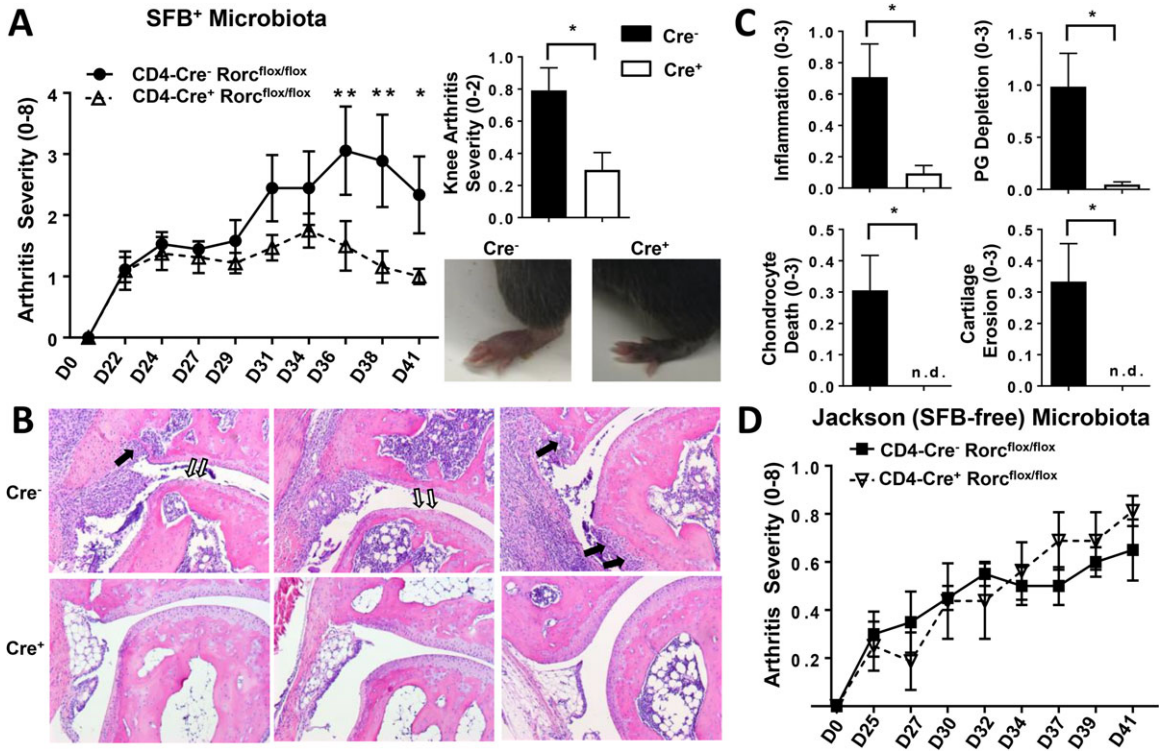

Figure 5. Th17 cells contribute to the progression of collagen-induced arthritis (CIA) in a microbiota-dependent manner. CIA was induced in segmented filamentous bacteria–positive (SFB+) *CD4-Cre$^+$Rorc$^{flox/flox}$* and *CD4-Cre$^-$Rorc$^{flox/flox}$* mice. **A,** Arthritis severity was scored in a blinded manner in *Cre$^+$* and *Cre$^-$* mice 3 times per week, with a maximum score of 2 per limb and 8 per mouse (left panel). During necropsy, mouse knee joints (representative images shown in lower right panels) were scored for arthritis severity on a scale of 0–2 (upper right panel). Results are the mean ± SEM scores of 8–9 mice per group. **B,** Histologic images show features of arthritis in the ankle joints of a representative mouse from each group on day 41 of CIA (same experiment as shown in **A**). Cartilage erosion is indicated by **open white arrows** and bone erosion is indicated by **solid black arrows**. Original magnification × 100. **C,** Histologic sections of the mouse ankle joints were scored on a scale of 0–3 for inflammation, proteoglycan (PG) depletion, chondrocyte death, and cartilage erosion. Results are the mean ± SEM in 16–18 mouse joints per group. **D,** *Cre$^+$* and *Cre$^-$* mice were depleted of native microflora and reconstituted with SFB-free microbiota from Jackson mice before the induction of CIA. Arthritis severity was scored in the same manner as described in **A**. Results are the mean ± SEM from a representative experiment in 4–5 mice per group. * = $P < 0.05$; ** = $P < 0.01$, by Mann-Whitney U test (for 2 groups) or two-way repeated-measures analysis of variance with uncorrected Fisher's least significant difference test (for groups over multiple time points). D = day; ND = not determined.

CD4-Cre$^-$Rorc$^{flox/flox}$ mice (Supplementary Figure 6 and Supplementary Table 3 [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). These differences between the 2 groups were eliminated after correction for multiple testing.

A qPCR analysis of universal bacterial (Eubacteria) and SFB 16S genes showed that, compared with naive mice, mice with CIA had a reduced universal bacterial 16S burden and an increased abundance of SFB (Supplementary Figures 7A and B [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). Comparison of naive *Cre$^+$Rorc$^{flox/flox}$* and *CD4-Cre$^-$Rorc$^{flox/flox}$* mice revealed a nonsignificant trend toward increased abundance of SFB in naive *Cre$^+$* mice, which is consistent with the previously reported findings in *CD4-Cre$^+$STAT3$^{flox/flox}$* mice (47). In contrast, the levels of SFB were significantly decreased in *Cre$^+$* mice compared with *Cre$^-$* mice during the course of CIA (Supplementary Figure 7B [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). Therefore, CD4-Cre–induced deletion of *Rorc* did not lead to a higher SFB burden in *Cre$^+$Rorc$^{flox/flox}$* mice with arthritis.

Evidence that Th17 cell contributions to arthritis are microbiota-dependent. Because the composition of the intestinal microbiota strongly influences the induction of systemic and mucosal Th17 cells, we evaluated whether the requirement of Th17 cells for arthritis development was dependent on the microbiota present in the gut. We depleted the native (SFB+) microbiota of *CD4-Cre$^+$ Rorc$^{flox/flox}$* and *CD4-Cre$^-$Rorc$^{flox/flox}$* mice with broad-spectrum antibiotics, and reconstituted the mice with SFB-free fecal microbiota obtained from Jackson mice (7). After recolonization of the mice with SFB-free

Jackson microbiota, CIA was induced and scores of arthritis severity were assessed. We observed no significant differences in arthritis severity for the duration of the experiment between Cre^+ and Cre^- mice that had been reconstituted with Jackson microbiota (Figure 5D).

The composition of the fecal microbiota observed in these mice was determined by 16S sequencing (Supplementary Figure 8 [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). At the end point of the experiment, only 2 taxa in the microbiota differed significantly ($P < 0.05$) between *CD4-Cre*$^+$*Rorc*$^{flox/flox}$ mice and *CD4-Cre*$^-$*Rorc*$^{flox/flox}$ mice reconstituted with Jackson microbiota (Supplementary Table 4 [http://onlinelibrary.wiley.com/doi/10.1002/art.40657/abstract]). The significance of the difference was eliminated after correction for multiple testing. SFB were not detected by qPCR in the feces from Jackson microbiota–reconstituted mice at the end point of the experiment. These data suggest that the Th17 cell dependency of inflammatory arthritis relies on the microbiota-specific activation of mucosal Th17 cell immunity.

Disparate roles of Th17 cells and IL-17A in arthritis pathogenesis. Having established a contextual, microbiota-dependent role of Th17 cells during arthritis pathogenesis, we examined cytokine expression in the SI lamina propria and draining LNs of *CD4-Cre*$^+$*Rorc*$^{flox/flox}$ and *CD4-Cre*$^-$*Rorc*$^{flox/flox}$ mice with CIA. Consistent with our previous observations (see Figure 2), the frequency and MFI of IL-17A expression were decreased in SI lamina propria CD4+ T cells from Cre^+ mice compared with Cre^- mice during CIA (Figure 6A). However, quantification of IL-17A from the supernatants of SI lamina propria cells stimulated with PMA and ionomycin revealed that total SI lamina propria IL-17A production was not significantly affected in Th17 cell–deficient mice, and tended to be higher than that in Th17 cell–sufficient mice ($P = 0.63$) (Figure 6B). These findings suggest that non-Th17 cells, such as neutrophils and γδ T cells (see Figure 4), are the likely sources of IL-17 in *CD4-Cre*$^+$*Rorc*$^{flox/flox}$ mice.

Similarly, the production of GM-CSF and IFNγ from SI lamina propria cells stimulated with PMA and ionomycin was not significantly different between the 2 genotypes (for GM-CSF, mean ± SEM 4.36 ± 1.50 pg/ml in Cre^- mice versus 3.92 ± 1.26 pg/ml in Cre^+ mice; for IFNγ, 5.56 ± 0.88 pg/ml in Cre^- mice versus 5.31 ± 1.37 pg/ml in Cre^+ mice). IL-17F and IL-22 were undetectable in culture supernatants of SI lamina propria cells (data not shown). Moreover, draining LN cells from *CD4-Cre*$^-$*Rorc*$^{flox/flox}$ and *CD4-Cre*$^+$*Rorc*$^{flox/flox}$ mice with CIA

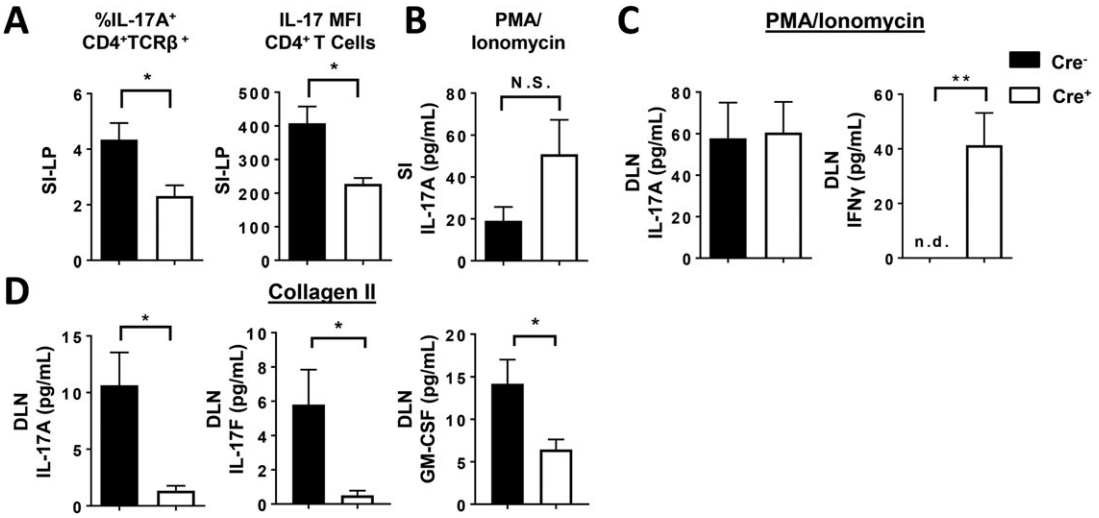

Figure 6. Protection from collagen-induced arthritis (CIA) in mice is dependent on the involvement of Th17 cells, but not the activity of interleukin-17A (IL-17A). CIA was induced in *CD4-Cre*$^+$*Rorc*$^{flox/flox}$ and *CD4-Cre*$^-$*Rorc*$^{flox/flox}$ mice, and cells from the small intestine lamina propria (SI-LP) and draining lymph nodes (DLNs) of these mice were analyzed for cytokine production. **A,** Percentages of Th17 cells and the mean fluorescence intensity (MFI) of IL-17A expression were determined by flow cytometry in SI-LP CD4+ T cells from Cre^+ and Cre^- mice. Results are the mean ± SEM in cells from 4 mice per group. **B,** Production of IL-17A was determined in SI-LP cells cultured with phorbol myristate acetate (PMA) and ionomycin. Results are the mean ± SEM of 2–3 replicate experiments in cells from 4–6 mice per group. **C,** Production of IL-17A and interferon-γ (IFNγ) was determined in DLN lymphocytes (2×10^5 cells/well) that were stimulated with PMA and ionomycin for 6 hours. Results are the mean ± SEM of 2–3 replicate experiments in cells from 4–8 mice per group. **D,** Production of IL-17A, IL-17F, and granulocyte–macrophage colony-stimulating factor (GM-CSF) was determined in supernatants of DLN lymphocytes (2×10^5 cells/well) that were stimulated with type II collagen for 2 days. Results are the mean ± SEM of 1–2 replicate experiments in cells from 5–8 mice per group. All cytokines were measured by Luminex cytokine array. * = $P < 0.05$; ** = $P < 0.01$, by Mann-Whitney U test. NS = not significant.

showed no significant difference in IL-17A production after stimulation with PMA and ionomycin (Figure 6C). Interestingly, the PMA and ionomycin–stimulated draining LNs of Cre^+ mice had significantly higher concentrations of IFNγ compared with draining LNs from Cre^- mice (Figure 6C). We observed no difference between the 2 genotypes in draining LN cell production of GM-CSF (mean ± SEM 10.56 ± 1.68 pg/ml in Cre^- mice versus 12.59 ± 2.72 in Cre^+ mice) or IL-17F (15.68 ± 3.76 pg/ml in Cre^- mice versus 13.14 ± 3.96 pg/ml in Cre^+ mice). IL-22 was not detectable in the draining LNs. However, upon stimulation with CII to determine antigen-specific cytokine responses, the levels of IL-17A, IL-17F, and GM-CSF were all significantly lower in the draining LNs of Cre^+ mice compared with Cre^- mice (Figure 6D). Production of TNFα by CII-stimulated draining LNs was similar between the 2 groups, and IFNγ and IL-22 were not detectable following stimulation with CII (data not shown).

These data indicate that during CIA, there is no difference in the total production of IL-17A, IL-17F, and GM-CSF by PMA and ionomycin–stimulated draining LN cells between Cre^- mice and Cre^+ mice. However, antigen-specific cytokine production is significantly reduced in Cre^+ mice when compared with Cre^- mice, a finding that suggests the potential involvement of Th17 cells. Thus, we may infer that the production of multiple cytokines, including IL-17A, IL-17F, and GM-CSF, by Th17 cells drives arthritic joint inflammation in this murine model.

DISCUSSION

The intestinal microbiome has emerged as a key determinant of health and disease. Culture-independent high-throughput sequencing techniques have revealed that the diversity and significant overrepresentation of *Prevotella copri* are reduced in patients with new-onset, untreated RA (2,3). Another study found an association of treatment-naive RA with a cluster of metagenomic linkage groups related to *Clostridium asparagiforme*, *Gordonibacter pamelaeae*, *Eggerthella lenta*, and *Lachnospiraceae* (4). Furthermore, an increased abundance of *Collinsella*, *Eggerthella*, and *Faecalibacterium* was identified in patients with longstanding, treated RA (mean disease duration 81.6 months) (5). However, the pathophysiologic relevance of these alterations in the microbiota in new-onset or established RA is not fully understood.

A recent study demonstrated increased lamina propria Th17 cell differentiation in SKG mice humanized with fecal microbiota from patients with new-onset RA as compared with mice harboring fecal microbiota from healthy controls (3). This coincided with more severe arthritis in the SKG mice when the mice were co-exposed to the fungal component zymosan (3). However, a requirement for the involvement of microbiota-induced Th17 cells was not demonstrated. While several studies have demonstrated that experimental arthritis can be exacerbated by SFB (9,32,45,46), it is unclear whether these arthritogenic effects are due to Th17 cell induction or due to T follicular helper cell induction and autoantibody production (45). Microbiome perturbations occur during CIA, and the composition of the microbiota differs between both naive and arthritic CIA-susceptible and -resistant mouse strains (48–50). Tuftsin phosphorylcholine, a natural glycoprotein with tolerogenic potential, attenuates the development of CIA and prevents dysbiosis in mice (48,51). Studies on microbiota-dependent differentiation of Th17 cells in the K/BxN mouse model of arthritis have demonstrated that Th17 cell induction in SFB-colonized mice occurs around weaning and precedes the onset of arthritis (9,46). Because age-dependent increases in SI lamina propria Th17 cells are also observed in naive mice, a side-by-side comparison between age-matched prearthritic mice and naive control mice, as was performed in the present study, is crucial to demonstrate that the increase in Th17 cells is characteristic of preclinical arthritis in mice.

Our findings demonstrate initiation of mucosal Th1 and Th17 cell responses and marked production of TNFα, GM-CSF, and IL-22 by mucosal CD4 T cells just before the clinical onset of arthritis. Furthermore, increased production of GM-CSF and IL-22 by SI lamina propria Th17 cells during preclinical arthritis is an additional novel finding. These cytokines are implicated in the pathogenesis of RA and may have IL-17–independent proinflammatory and osteoclastogenic functions (13,20–22). Therefore, our observations suggest that activation of intestinal mucosal T cells during the immune-priming phase of RA may contribute to early, preclinical processes.

Further studies using Th17 cell–deficient mice showed that Th17 cells are required for the progression of arthritis and affect several features of structural joint pathology in 2 murine arthritis models, mBSA-induced arthritis (AIA) and CIA. AIA is an acute nonimmunogenic and nonautoimmune model of arthritis, whereas CIA is chronic, immunogenic, and autoimmune. Chronic acute autoimmunity models frequently demonstrate differential immunogenicity, cellular involvement, and mechanisms of pathogenesis. Therefore, these 2 models provide complementary insights into the role of Th17 cells in arthritis. The known role of other cellular sources of IL-17 in arthritis (52) emphasizes the value of investigating the specific, differential contribution of Th17 cells as a main source of IL-17 and other proinflammatory mediators.

In our comparison of the effects of CD4-induced *Rorc* deletion between the acute AIA and CIA models, we found that the AIA model is less dependent on Th17 cells. This aligns with previous observations of the role of the cytokine IL-17A in these 2 models (28,35,53,54). This may be attributable to the B cell and antibody dependence of CIA as compared to acute AIA, because Th17 cells also assist B cells and promote antibody production. Conversely, AIA could reflect a predominant role of other IL-17–producing cells, such as γδ T cells. Examining γδ T cell–specific *Rorc* deletion during CIA and AIA is an avenue for future study.

Since total IL-17 production upon stimulation of T cells with PMA and ionomycin was not affected in arthritis-resistant Th17-deficient mice, it is likely that the role of Th17 cells in arthritis expands beyond that of the cytokine IL-17. Moreover, the cytokine profile of collagen-stimulated cells from the joint-draining LNs indicated that antigen-experienced T cells produced IL-17A, IL-17F, and GM-CSF, whereas the levels of these were significantly reduced in Th17 cell–deficient mice. GM-CSF activates the monocyte/macrophage system as well as neutrophils, and promotes inflammation (20). Inhibition of GM-CSF or its receptor led to a reduction in the RA disease activity score and in the C-reactive protein levels in arthritis patients in clinical trials (20,55,56). GM-CSF and IL-17A act synergistically to induce matrix metalloproteinases, RANKL, and IL-23 in the synovium, and simultaneous expression of both IL-17A and GM-CSF leads to complete destruction of the joint structure in mice (57). Accordingly, simultaneous blockade of IL-17A and GM-CSF is more effective than blocking either cytokine alone (57). Therefore, we attribute the reduced arthritis severity in Cre^+ mice to the simultaneous reduction of IL-17A, IL-17F, and GM-CSF production by antigen-specific Th17 cells.

Th17 cell production of multiple proinflammatory cytokines with synergistic effects during arthritis suggests that modulation of Th17 cell development may be more effective than IL-17 blockade. This may extend to other rheumatic inflammatory diseases, such as psoriatic arthritis, in which Th17 cells play a pathogenic role (58). In addition, direct effects of Th17 cells on B cell activation and isotype class switching as well as facilitation of antibody production have been reported, and may represent other important IL-17–independent roles for Th17 cells (59,60).

While T cell IL-22 expression was increased in immunized mice during preclinical arthritis (Figures 1B–D), IL-22 was not detectable in culture supernatants derived from the SI lamina propria or draining LNs of mice with fulminant CIA. Therefore, the role of IL-22–producing mucosal T cells in arthritis remains unclear, and may be restricted to the preclinical phase.

Differentiation of the Th17 cell lineage is mediated by both RORα and RORγt transcription factors (61). Intact RORα expression in CD4-expressing cells may explain why the Th17 cell deficiency in *CD4-Cre*$^+$ *Rorc*$^{flox/flox}$ mice is incomplete. Studies of Rorα/Rorγt double-deficient mice with fully impaired Th17 cell development (61) would help clarify whether arthritis is fully inhibited in the complete absence of Th17 cells.

Th17 cells and IL-17 production are strongly induced by intestinal microbiota (8,9,23). Therefore, the microbiota composition strongly influences the dominant immune processes underlying disease in certain patients. Th17 cell frequencies are elevated in RA, particularly in patients with poor responses to TNFα inhibitors (13,62). Our data show that in the absence of specific murine Th17 cell–inducing microbiota, namely SFB, the Th17 cell dependence of arthritis is mitigated. Therefore, Th17 cells contribute to the pathogenesis of arthritis only in the context of specific microbiota. This suggests that the composition of the microbiota in each patient may be a major factor affecting the involvement of the Th17/IL-17 pathway in mucosal immunity and RA. Thus, the microbiome profile of a patient may be a valuable biomarker for predicting the efficacy of Th17 cell– or IL-17–targeted therapies. We therefore speculate that microbiome-guided stratification of patients with inflammatory arthritis may improve therapeutic efficacy.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Abdollahi-Roodsaz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Evans-Marin, Rogier, Manasson, Roeleveld, van der Kraan, Koenders, Abdollahi-Roodsaz.

Acquisition of data. Evans-Marin, Abdollahi-Roodsaz.

Analysis and interpretation of data. Evans-Marin, Rogier, Koralov, Scher, Koenders, Abdollahi-Roodsaz.

ADDITIONAL DISCLOSURES

Author Abdollahi-Roodsaz is currently an employee of Celgene Corporation.

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