ARTHRITIS & RHEUMATOLOGY
Vol. 68, No. 12, December 2016, pp 2878–2888
DOI 10.1002/art.39785

Suppression of Inflammatory Arthritis by Human Gut-Derived *Prevotella histicola* in Humanized Mice

Eric V. Marietta, Joseph A. Murray, David H. Luckey, Patricio R. Jeraldo, Abhinav Lamba, Robin Patel, Harvinder S. Luthra, Ashutosh Mangalam, and Veena Taneja

Objective. **The gut microbiome regulates host immune homeostasis. Rheumatoid arthritis (RA) is associated with intestinal dysbiosis. This study was undertaken to test the ability of a human gut-derived commensal to modulate immune response and treat arthritis in a humanized mouse model.**

Methods. **We isolated a commensal bacterium, *Prevotella histicola,* that is native to the human gut and has systemic immune effects when administered enterally. Arthritis-susceptible HLA–DQ8 mice were immunized with type II collagen and treated with *P histicola*. Disease incidence, onset, and severity were monitored. Changes in gut epithelial proteins and immune response as well as systemic cellular and humoral immune responses were studied in treated mice.**

Results. **When treated with *P histicola* in prophylactic or therapeutic protocols, DQ8 mice exhibited significantly decreased incidence and severity of arthritis compared to controls. The microbial mucosal modulation of arthritis was dependent on regulation by CD103+ dendritic cells and myeloid suppressors (CD11b+Gr-1+ cells) and by generation of Treg cells (CD4+CD25+ FoxP3+) in the gut, resulting in suppression of antigen-specific Th17 responses and increased transcription of interleukin-10. Treatment with *P histicola* led to reduced intestinal permeability by increasing expression of enzymes that produce antimicrobial peptides as well as tight junction proteins (zonula occludens 1 and occludin). However, the innate immune response via Toll-like receptor 4 (TLR-4) and TLR-9 was not affected in treated mice.**

Conclusion. **Our results demonstrate that enteral exposure to *P histicola* suppresses arthritis via mucosal regulation. *P histicola* is a unique commensal that can be explored as a novel therapy for RA and may have few or no side effects.**

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease caused by both genetic and environmental factors (1). Among the known genetic factors, the strongest association is with the presence of certain alleles of HLA class II molecules (2). Using transgenic mice expressing RA-associated HLA–DR4/DQ8 genes, we have developed a humanized model of inflammatory arthritis that shares similarities with human disease in sex bias, autoantibody profile, and phenotype (3). Our recent data suggest that the gut microbial composition of naive *0401 and *0402 mice shares similarities with the human mucosal microbiome (4), and the *0401 genotype may be associated with a dysbiosis of the gut microbiome. Major histocompatibility complex polymorphism has been shown to impact gut flora in humans and mice (5–7). Studies in patients with RA have shown dysbiosis, with one study showing decreased *Bacteroides*, *Porphyromonas*, and *Prevotella* species compared to healthy controls (8,9). How certain commensals suppress T cell proliferation is not well understood; further studies are needed to more precisely determine their effects on the immune response in inflammatory diseases.

Supported by the Department of Defense (Congressionally Directed Medical Research Programs grant W81XWH-10-1-0257) and the NIH (National Institute of Arthritis and Musculoskeletal and Skin Diseases grant AR-30752 to Dr. Taneja).

Eric V. Marietta, PhD, Joseph A. Murray, MD, David H. Luckey, BS, Patricio R. Jeraldo, PhD, Abhinav Lamba, Robin Patel, MD, Harvinder S. Luthra, MD, Ashutosh Mangalam, PhD (current address: University of Iowa, Iowa City), Veena Taneja, PhD: Mayo Clinic, Rochester, Minnesota.

Drs. Marietta, Murray, Mangalam, and Taneja are named inventors on a patent for the technology used in this study. Dr. Patel has received grants from nanoMR, BioFire, CheckPoint, Curetis, 3M, Merck, Hutchison Biofilm Medical Solutions, Accelerate Diagnostics, Allergan (Pfizer), and The Medicines Company and has received consulting fees from Curetis, Roche, St. Jude, Thermo Fisher Scientific, and Diaxonhit (less than $10,000 each); she holds a patent on *Bordetella pertussis/parapertussis* polymerase chain reaction with royalties paid by TIB, a patent on a device/method for sonication with royalties paid by Samsung to Mayo Clinic, and a patent on an antibiofilm substance.

Address correspondence to Veena Taneja, PhD, Department of Immunology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. E-mail: taneja.veena@mayo.edu.

Submitted for publication March 14, 2016; accepted in revised form June 7, 2016.

We have isolated a gram-negative anaerobe commensal bacterium, *Prevotella histicola,* native to oral, nasopharyngeal, gastrointestinal, and genitourinary mucosal surfaces. *P histicola* is a recently discovered species with taxonomic similarity to *Prevotella melanogenica* and *Prevotella veroalis*. While commensals like *Bifidobacterium* species and some species of *Prevotella* have been studied for their impact on the immune system, there are no published reports on the biologic effects of *P histicola*.

Our studies suggest that *P histicola* has immunomodulating properties and suppresses inflammatory cytokines. We tested whether orally administered *P histicola* can modulate the immune response in the gut and whether that can be translated systemically to control arthritis in DQ8 mice. Our data suggest that oral feeding of *P histicola* in a therapeutic protocol (after induction of arthritis) to DQ8-transgenic mice leads to resistance to disease development and limits the disease severity. *P histicola* alone did not lead to any enteric or other pathology in transgenic mice. These studies provide experimental support for the exploration of commensals as treatment options for systemic diseases, including RA.

MATERIALS AND METHODS

Isolation and identification of *Prevotella* species. Biopsy specimens of the proximal small bowel were obtained from human subjects, and bacterial cultures were grown on LKV agar plates for isolation of individual colonies. Isolates were cultured on sheep blood agar plates and incubated anaerobically for 2 days at 35°C. Bacterial genomic DNA was extracted using a QIAamp DNA Mini kit (Qiagen). Real-time, rapid-cycle LightCycler polymerase chain reaction (PCR) with SYBR Green I detection (Roche Applied Science) was used to amplify 527 bp of the 16S ribosomal RNA (16S rRNA) gene. Universal bacterial 16S rRNA gene primers were used (Microseq 500 16S rRNA gene PCR kit). PCR cycling was followed by postamplification melting curve analysis to verify the amplicon before sequencing. Sequencing was performed with a BigDye Terminator v1.1 *Taq* kit and an ABI 3730xl DNA Sequencer (Applied Biosystems). Bidirectional sequence data were aligned using Sequencher (Gene Codes). The generated consensus sequences were compared to those of the NCBI GenBank database. Identity ≥99% between the query sequence and the GenBank database with a difference of >0.4% between species was used for identification at the species level.

Transgenic mice. Transgenic mice were generated as described previously (10). All mice used lacked endogenous class II molecules (AEo) and expressed DQB1*0302/DQA1*0301 (DQ8.AEo) on a B6/129 background. Mice of both sexes (8–12 weeks of age) were used in this study and were bred and maintained in the pathogen-free Immunogenetics Mouse Colony at the Mayo Clinic in accordance with the Institutional Animal Use and Care Committee guidelines. All of the experiments included transgene-negative littermates as controls and were carried out with the approval of the Animal Use and Care Committee.

Induction and evaluation of collagen-induced arthritis (CIA). CIA was induced by immunization with type II collagen (CII) (100 μg of CII emulsified 1:1 with Freund's complete adjuvant [CFA]) as previously described (10). Mice were monitored for the onset and progression of CIA, and each paw was scored on a scale of 0–3, where 0 = no swelling, 1 = 1 or 2 swollen digits, 2 = ≥2 swollen digits, and 3 = swollen paw. The mean arthritis score was determined using arthritic animals only. Mice were divided into 2 groups for reproducibility. Histopathologic analysis of representative paws from each group was performed to determine arthritis induction. DBA/1 mice were immunized with CII, and paw thickness was measured with calipers before and after induction of arthritis to determine arthritis severity.

Treatment with commensal bacterium. Organisms (*P histicola and P melanogenica*) were stored at −70°C in skim milk, inoculated onto an Anaerobe Laked Sheep Blood Agar with Kanamycin and Vancomycin (Becton Dickson), incubated anaerobically in an anaerobic jar with an AnaeroPack system (Mitsubishi Gas Chemical America), and incubated at 37°C for 2–3 days. The bacterium was then swabbed into 10 ml of tryptic soy broth and anaerobically incubated for 2 days prior to inoculation. The identity of the organisms was verified by PCR.

Transgenic mice were then orally gavaged on alternate days with 1×10^9 live bacteria suspended in 100 μl of tryptic soy broth (anaerobic) bacterial culture. The dose was chosen based on the fact that a higher dose did not provide any additional benefit. For the preventive protocol, bacteria were administered beginning 10 days prior to immunization with CII and continued for 6 weeks after immunization. For the therapeutic protocol, mice were treated beginning 2 weeks after CIA induction and for 6 weeks thereafter. Control sham gavage consisted of administering 100 μl of bacterial media alone. DBA/1 mice were treated 7 days after immunization. *Prevotella* did not colonize the gut (results not shown).

Isolation of lamina propria cells. Intestinal tissue was cut longitudinally using a scalpel blade, and washed 6 times using CMF solution (88% 1× Hanks' balanced salt solution, 10% HEPES/bicarbonate buffer, and 2% fetal bovine serum). A 1-hour collagenase digestion using complete RPMI-10/collagenase (1.33 mg/ml) solution released lymphocytes from the intestinal tissue. This mixture was passed through a nylon filter, centrifuged, and the pellet containing the lamina propria cells was suspended in complete RPMI-10 with gentamicin.

Intestinal permeability. All transgenic mice were maintained on a standard diet. Changes in intestinal permeability were determined using 4-kd fluorescein isothiocyanate (FITC)–labeled dextran. Mice were deprived of food for 3 hours and then gavaged with FITC-labeled dextran (0.6 mg/gm body weight). Three hours later, mice were bled and serum was collected. Immunofluorescence of FITC was determined at 490 nm excitation and 525 nm emission. Gut permeability was tested in age- and sex-matched treated and control mice 8 weeks after induction of arthritis.

Staining for tight junction proteins. Various parts of the mouse gut, the duodenum, jejunum, ileum, and colon, were frozen at the termination of the experiments and tested for the expression of the tight junction proteins zonula occludens 1 (ZO-1) and occludin by immunofluorescence using purified anti–ZO-1 (Life Technologies), Alexa Fluor 594–conjugated anti-occludin (Life Technologies), and FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch). Caco-2 cells (3×10^5) were cultured on 22 × 22–mm coverslips in 6-well tissue culture plates at 37°C in a humidified 5% CO_2 incubator until the cultures were confluent monolayers. They were then incubated with or without *P histicola* (100 μl of 1×10^8 colony-forming units/ml) for 4 hours, fixed with 10% formaldehyde, and evaluated for ZO-1 and occludin expression.

Reverse transcriptase–PCR for cytokine and chemokine expression. RNA was extracted from cells using RNeasy columns (Qiagen), and complementary DNA (cDNA) was prepared using RNase H reverse transcriptase (Invitrogen) and cDNA generated by standard methods. The expression level of each gene was quantified using the threshold cycle (C_t) method normalized for actin, a housekeeping gene. Affymetrix mouse PAMM073 microarrays were used according to the manufacturer's instructions. Data were analyzed using the online resources of the manufacturer.

Collagen-specific enzyme-linked immunosorbent assay (ELISA). Mice were bled after CII immunization before and after treatment with *P histicola*. Titers of serum IgG antibodies against CII were measured by standard ELISA and are expressed as optical density.

T cell proliferation assay. For the T cell proliferation assay, mice were immunized with 200 μg of CII emulsified 1:1 in CFA (Difco) intradermally at the base of the tail, and proliferation was measured as previously described (10). For some experiments, CD4+ cells (5×10^6) sorted from the lymph nodes of CII-primed mice that were treated with *P histicola* or media alone were cultured in vitro in the presence or absence of the antigen and CD11c+ dendritic cells (DCs) (5×10^5) harvested from mouse spleens. A stimulation index of ≥ 2 was considered to be a positive response. Responses to CpG (1 μg/ml) and lipopolysaccharide (LPS; 5 μg/ml) were tested in mice that received *P histicola* treatment for 10 days prior to CII immunization and in mice that were given *P histicola* 10 days after immunization and treated for 2 weeks every alternate day. Mice in the in vivo protocol (preventive or therapeutic protocol) were also tested for responses to LPS and CpG.

Flow cytometric analysis. The expression of DQ in transgenic mice was analyzed by flow cytometry using IVD12 (anti-DQ) monoclonal antibody. Conjugated antibodies to CD3, CD4, CD11c, CD19, CD25, GITR, Gr-1, and B220 (BD Biosciences) were also used. All experiments were done with cells pooled from 2 mice per strain and repeated 2–3 times. Intracellular staining for FoxP3 and interleukin-10 (IL-10) was performed using specific antibodies according to the recommendations of the manufacturer (eBioscience). Phycoerythrin–conjugated rat IgG2a (eBioscience) was used as the isotype control for FoxP3 staining. The CellQuest program (Becton Dickinson) was used for analysis.

Cytokines. Cytokine levels were measured using a Bio-Plex protein array system with a mouse cytokine 23-plex panel according to the manufacturer's instructions, and were analyzed with Bio-Plex manager 2.0 software (Bio-Rad). Some cytokines were also tested by capture ELISA using commercial kits (BD Biosciences).

Statistical analysis. The difference in the incidence of arthritis between groups was analyzed using a chi-square test. Antibody levels, onset of arthritis, mean scores for arthritic mice, and various cells were compared using Student's nonparametric *t*-test.

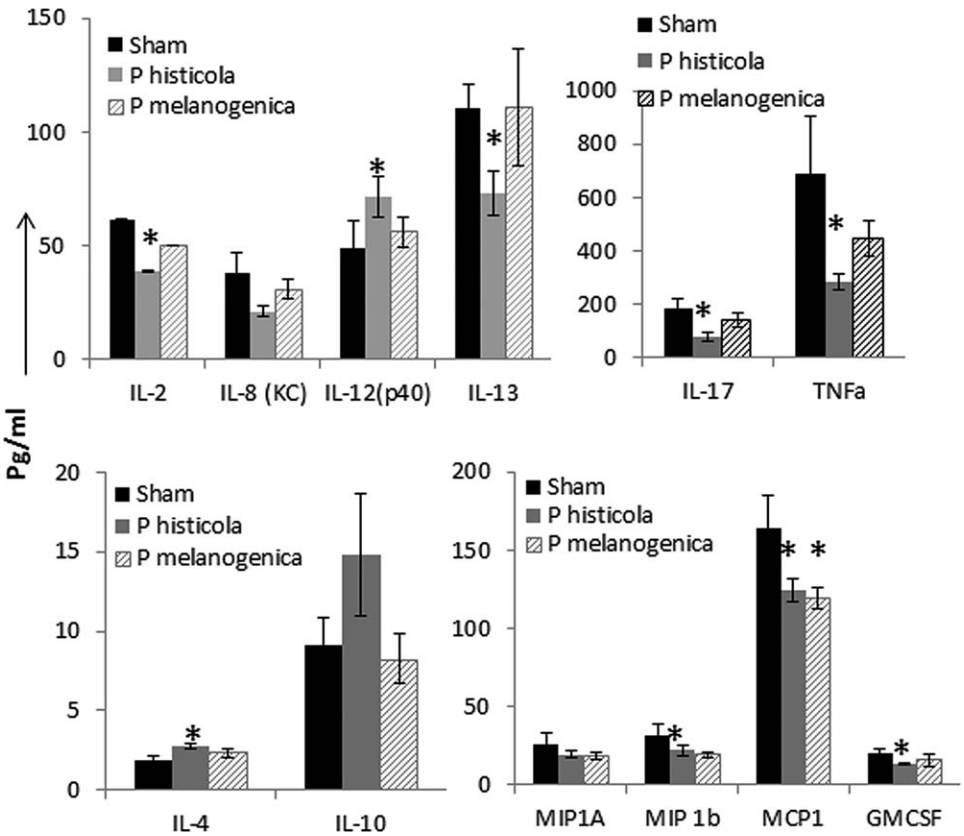

Figure 1. Modulation of systemic immune response in DQ8.AEo mice treated orally with *Prevotella histicola*. Serum levels of cytokines and chemokines were measured in nonarthritic (naive) DQ8-transgenic mice gavaged with bacterial media alone (sham treatment), *P histicola,* or *Prevotella melanogenica* every other day for 2 weeks. Bars show the mean ± SD (n = 5–7 mice per group). * = $P < 0.05$ versus sham-treated mice. IL-12 = interleukin-12; KC = keratinocyte chemoattractant; TNF = tumor necrosis factor; MIP-1α = macrophage inflammatory protein 1α; MCP-1 = monocyte chemotactic protein 1; GM-CSF = granulocyte–macrophage colony-stimulating factor.

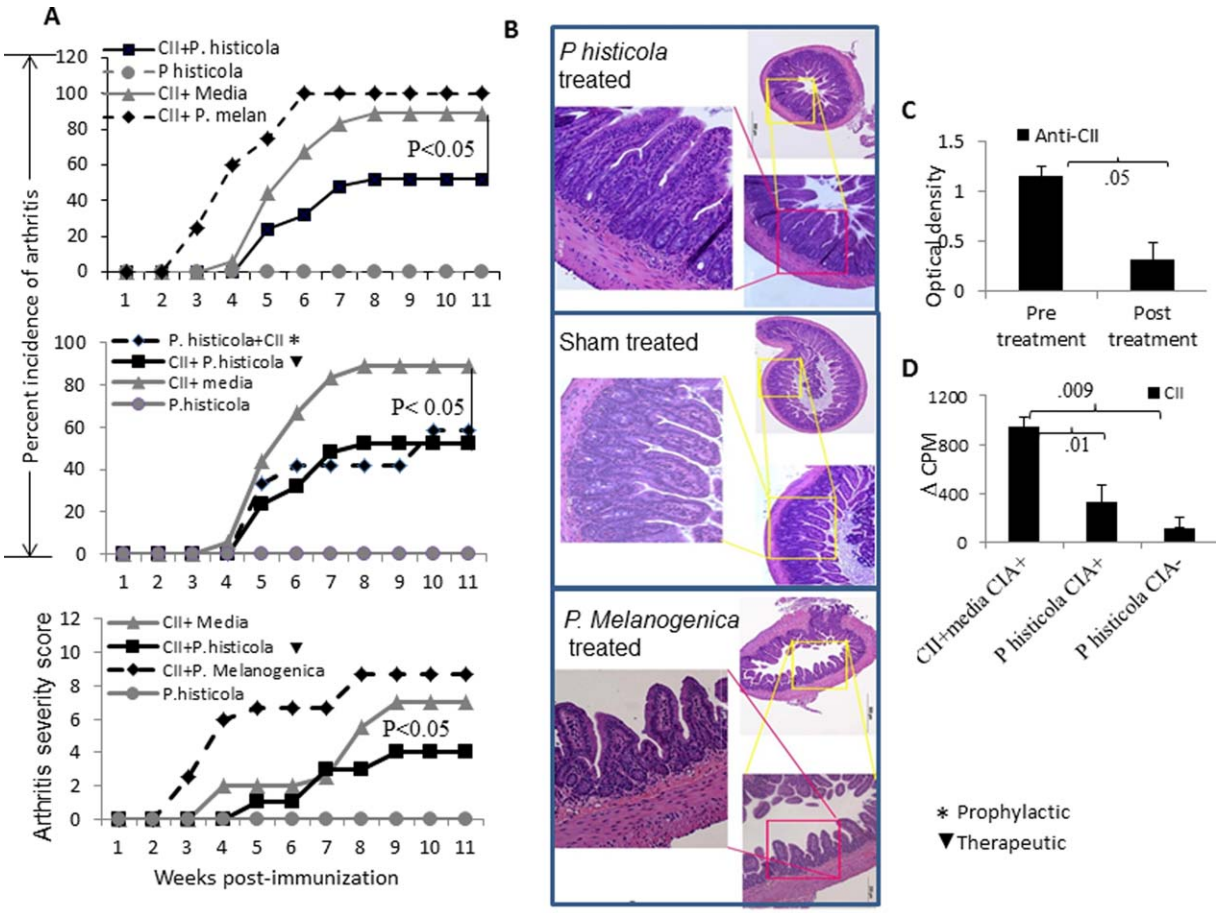

Figure 2. Oral treatment with *Prevotella histicola* protects against collagen-induced arthritis (CIA) in DQ8 mice. *P histicola* was administered to DQ8 mice in a therapeutic protocol (after immunization with type II collagen [CII]) (CII + *P histicola*; n = 21) or as a prophylactic measure (before immunization with CII) (*P histicola* + CII; n = 12). *Prevotella melanogenica* was administered as a species control for the therapeutic protocol (CII + *P melan*; n = 10). Other controls consisted of oral gavage with *P histicola* without CII immunization (*P histicola*; n = 12) and CII immunization with gavage of bacterial culture media alone (CII + media; n = 18). **A,** Incidence of arthritis and arthritis severity score in the experimental groups. $P < 0.05$, CII + media–treated mice versus *P histicola*–treated mice. Values for the arthritis severity score are the mean. **B,** Hematoxylin and eosin staining of the small intestine in a representative mouse from each of the 3 treatment groups (n = 3 mice per group). Images on the left are higher-magnification views (× 60) of the boxed areas in the images on the bottom right (× 10); images on the bottom right are higher-magnification views of the boxed areas in the images on the top right (× 5). **C,** Levels of serum anti-CII (IgG) antibodies before and after administration of *P histicola* to mice in the therapeutic protocol. **D,** T cell proliferative response to CII in vitro at the termination of the experiment at 12 weeks, determined using splenocytes from mice with CIA and those without CIA. In **C** and **D,** bars show the mean ± SD. Numbers above or below horizontal lines are *P* values.

RESULTS

Modulation of immune response by gut-derived *P histicola*. *P histicola* and *P melanogenica* isolated from the duodenum of an individual human subject were cultured and tested for pathogenic properties. Following treatment, none of the mice had weight loss of >5% of original weight. None of the mice developed any gut pathology, villous atrophy, or crypt hyperplasia. Mice gavaged with *P histicola* showed significantly reduced IL-2, IL-17, and tumor necrosis factor levels, and increased IL-4 and IL-10 levels compared to control mice gavaged with bacterial media alone (sham) (Figure 1). Granulocyte–macrophage colony-stimulating factor and monocyte chemotactic protein 1 (MCP-1), proinflammatory chemokines involved in various autoimmune diseases, were also suppressed in *P histicola*–treated mice, demonstrating a probiotic effect of *P histicola* isolate in the DQ8 mice. On the other hand, mice gavaged with *P melanogenica* did not show any significant changes in cytokine levels but did show reduced macrophage inflammatory protein 1β and MCP-1 levels compared to sham-treated controls.

***P histicola* suppresses arthritis in susceptible DQ8 mice by modulating cellular and humoral responses.** *P histicola* was tested for treating CIA in DQ8 mice. HLA–

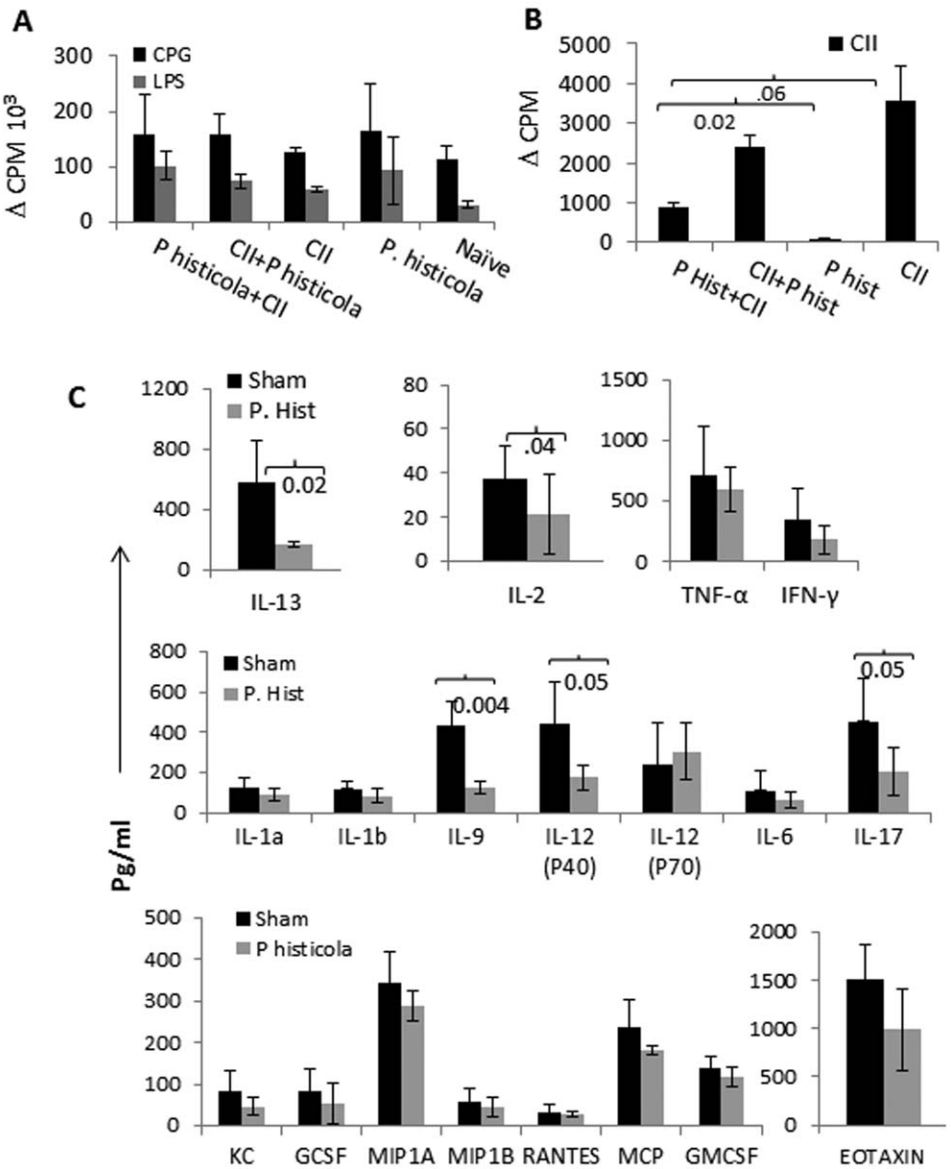

Figure 3. Modulation of immune response in an antigen-specific manner in mice with collagen-induced arthritis (CIA) treated with *Prevotella histicola (P hist)*. **A** and **B,** In vitro proliferative response of splenocytes harvested from DQ8 mice to CpG and lipopolysaccharide (LPS) (**A**) and type II collagen (CII) (**B**). Mice were treated according to a prophylactic protocol (*P hist* + CII), a therapeutic protocol (CII + *P hist*), immunized with CII alone, treated with *P histicola* alone, or left untreated (naive). **C,** Serum cytokine levels in DQ8-transgenic mice with CIA treated with culture media (sham treatment) or with *P histicola* in a therapeutic protocol (*P hist*). Bars show the mean ± SD (n = 4 mice per group). Numbers above or below horizontal lines are *P* values. IFNγ = interferon-γ; G-CSF = granulocyte colony-stimulating factor (see Figure 1 for other definitions).

DQ8 mice were immunized with CII and gavaged with *P histicola* before immunization (prophylactic), *P histicola* after the development of arthritis (therapeutic), or media. Mice gavaged with *P histicola* without CII immunization were used as controls (Figure 2). Both the prophylactic (*P histicola* + CII) and therapeutic (CII + *P histicola*) protocols resulted in significantly lower incidences of arthritis compared to sham control treatment ($P < 0.05$). Mice treated with *P histicola* developed milder arthritis with delayed onset compared to sham-treated controls. However, *P melanogenica* did not provide any protection against CIA. No inflammation in the small intestine or colon of *P histicola*–treated mice was observed, while only mild shortening of villi with mild infiltration occurred with *P melanogenica* treatment (Figure 2B).

We next determined if treatment with *P histicola* modulates the antigen-specific systemic immune response, thereby resulting in protection against CIA. Sera obtained

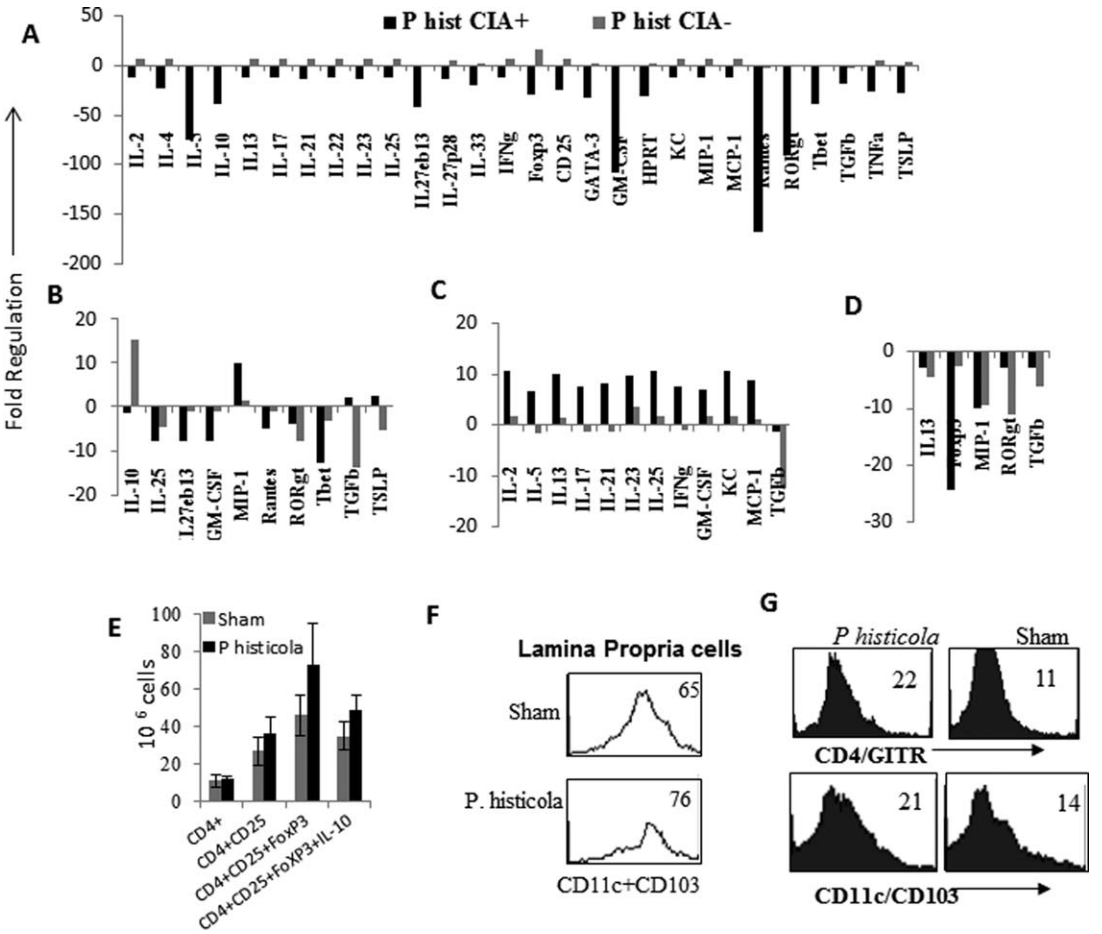

Figure 4. Antiinflammatory effects of *Prevotella histicola* treatment on the mouse intestinal immune system. **A–D,** Fold change in cytokine transcript levels in DQ8 mice with CIA (mice immunized with CII and treated with bacterial media alone [sham treatment] and mice immunized with CII and treated with *P histicola* in a therapeutic protocol) and nonarthritic DQ8 mice treated with *P histicola*, as compared to the control group (untreated mice with CIA), in the duodenum (**A**), jejunum (**B**), ileum (**C**), and colon (**D**) (n = 3 mice per group). **E,** Absolute numbers of Treg cells (CD4+CD25+FoxP3+) producing interleukin-10 (IL-10) in the spleens of mice immunized with CII and treated with *P histicola* or bacterial media alone. Bars show the mean ± SD. **F** and **G,** Fluorescence-activated cell sorting (FACS) histogram of regulatory dendritic cells (CD11c+CD103+) in lamina propria cells (**F**) and splenocytes (**G**). A FACS histogram of CD4+GITR+ Treg cells in the spleens of CII-immunized and treated or sham-treated mice is also shown. Results are representative of 3–4 mice per group. GM-CSF = granulocyte–macrophage colony-stimulating factor; HPRT = hypoxanthine guanine phosphoribosyltransferase; KC = keratinocyte chemoattractant; MIP-1 = macrophage inflammatory protein 1; MCP-1 = monocyte chemotactic protein 1; RORγt = retinoic acid receptor–related orphan nuclear receptor γt; TGFβ = transforming growth factor β; TNFα = tumor necrosis factor α; TSLP = thymic stromal lymphopoietin (see Figure 3 for other definitions).

before and after treatment were tested for the presence of anti-CII antibodies in mice used in the therapeutic protocol. Mice treated with *P histicola* showed a significant reduction in anti-CII antibody levels. In addition, the antigen-specific T cell response was lower in treated mice with arthritis and treated mice without arthritis compared to arthritic controls (Figures 2C and D).

Changes in the systemic, but not the innate, immune response in mice treated with *P histicola*. Treating mice with immunomodulatory agents is known to cause suppression of innate responses, leading to infections (11). We assessed whether treatment with *P histicola* modulates the innate immune responses in an in vivo model by culturing splenocytes with LPS or CpG. Although immunized and treated mice generated a higher response than non-arthritic mice, administration of *P histicola* did not significantly change the response to CpG or LPS (Figure 3A). A near-significant decrease in the antigen-specific T cell response to CII was observed in treated mice, suggesting that *P histicola* suppresses CIA by changing the systemic immune response rather than causing immune suppression (Figure 3B). Treated mice showed significantly lower levels of serum IL-17 as well as the regulatory cytokines IL-9, IL-13, and IL-12(p40) compared to sham-treated mice (Figure 3C).

Modulation of immune response in the gut in mice treated with *P histicola*. We next evaluated whether *P histicola* treatment affects the mucosal immune system

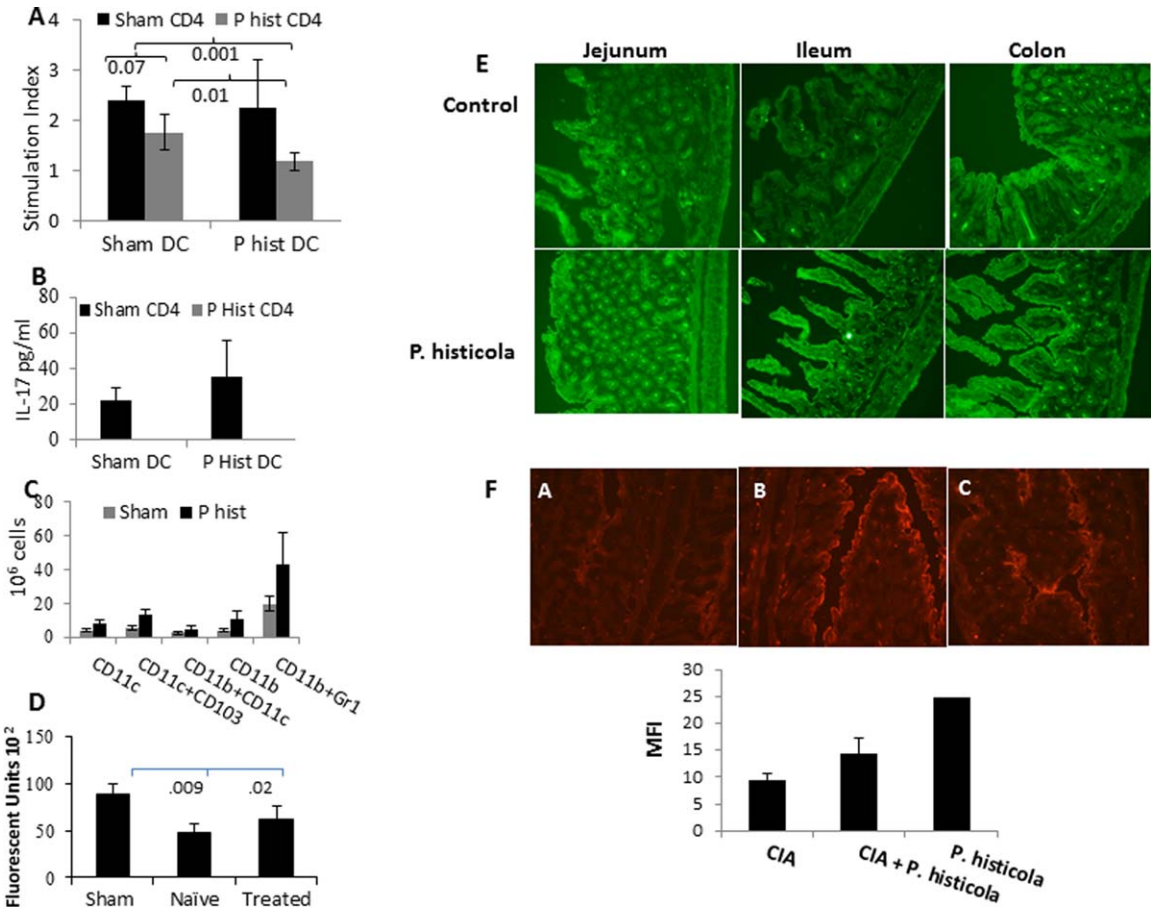

Figure 5. Lower antigen-specific T cell responses in CD4+ cells from *Prevotella histicola (P hist)*–treated mice compared to controls. **A,** In vitro T cell response to type II collagen (CII) in splenic CD4+ cells from DQ8 mice immunized with CII and gavaged with bacterial media alone (sham treatment) and DQ8 mice immunized with CII and gavaged with *P histicola*, cultured with CD11c+ cells (dendritic cells [DCs]) from the same mouse or a control mouse in a crisscross manner (DCs from *P histicola*–treated mice were cultured with CD4+ cells from sham-treated and *P histicola*–treated mice. Similarly, DCs from sham-treated mice were cultured with CD4+ cells from *P histicola*–treated and sham-treated mice). **B,** Interleukin-17 (IL-17) production in supernatants from the culture described in **A**. **C,** Numbers of regulatory DCs (CD11c+CD103+) and myeloid suppressor cells (CD11b+Gr-1+) in splenocytes from sham-treated and *P histicola*–treated mice (n = 4 mice per group). **D,** Gut permeability, determined using fluorescein isothiocyanate–labeled dextran, in nonarthritic (naive) mice (n = 12), CII-immunized mice treated with *P histicola* (n = 12), and CII-immunized mice treated with bacterial media alone (sham; n = 8). **E,** Expression of zonula occludens 1 in intestinal sections from DQ8 mice with CIA treated with bacterial media alone (control) and DQ8 mice with CIA treated with *P histicola*. **F,** Expression of occludin in DQ8 mice with CIA (**A**), DQ8 mice with CIA treated with *P histicola* (**B**), and naive mice treated with *P histicola* (**C**). The mean florescence intensity (MFI) of expression is shown. Bars in **A, B, C, D,** and **F** show the mean ± SD. Numbers below horizontal lines are *P* values. Original magnification × 10 in **E** and × 60 in **F**.

locally in the gut in vivo by determining mRNA transcripts for various cytokines from all parts of the gut (Figure 4). *P histicola*–treated mice as well as sham-treated mice did not show significant changes in cytokine expression in the ileum. In the jejunum and colon of *P histicola*–treated mice, most cytokines were suppressed. *P histicola*–treated mice without arthritis showed much higher IL-10 and lower transforming growth factor β levels compared to controls. The duodenum had high expression of most cytokines in both groups. Heatmaps of intestinally derived mRNA transcripts of the Th17 regulatory network revealed that *P histicola* treatment led to changes in cytokine expression; arthritic control and treated mice showed similarities compared to nonarthritic treated mice (data not shown).

We further compared the effect of treatment on the duodenum, jejunum, ileum, and colon in the treated (arthritic or nonarthritic) and control groups. Figures 4A–D show data with 5-fold or more difference in cytokine transcript expression between the groups. *P histicola*–treated mice showed suppression of all cytokines in the jejunum (except IL-10) and colon compared to the duodenum (Figures 4B and D). Cytokines in *P histicola*–treated nonarthritic mice showed >5-fold reductions compared to controls in IL-17 and other proinflammatory cytokines, with

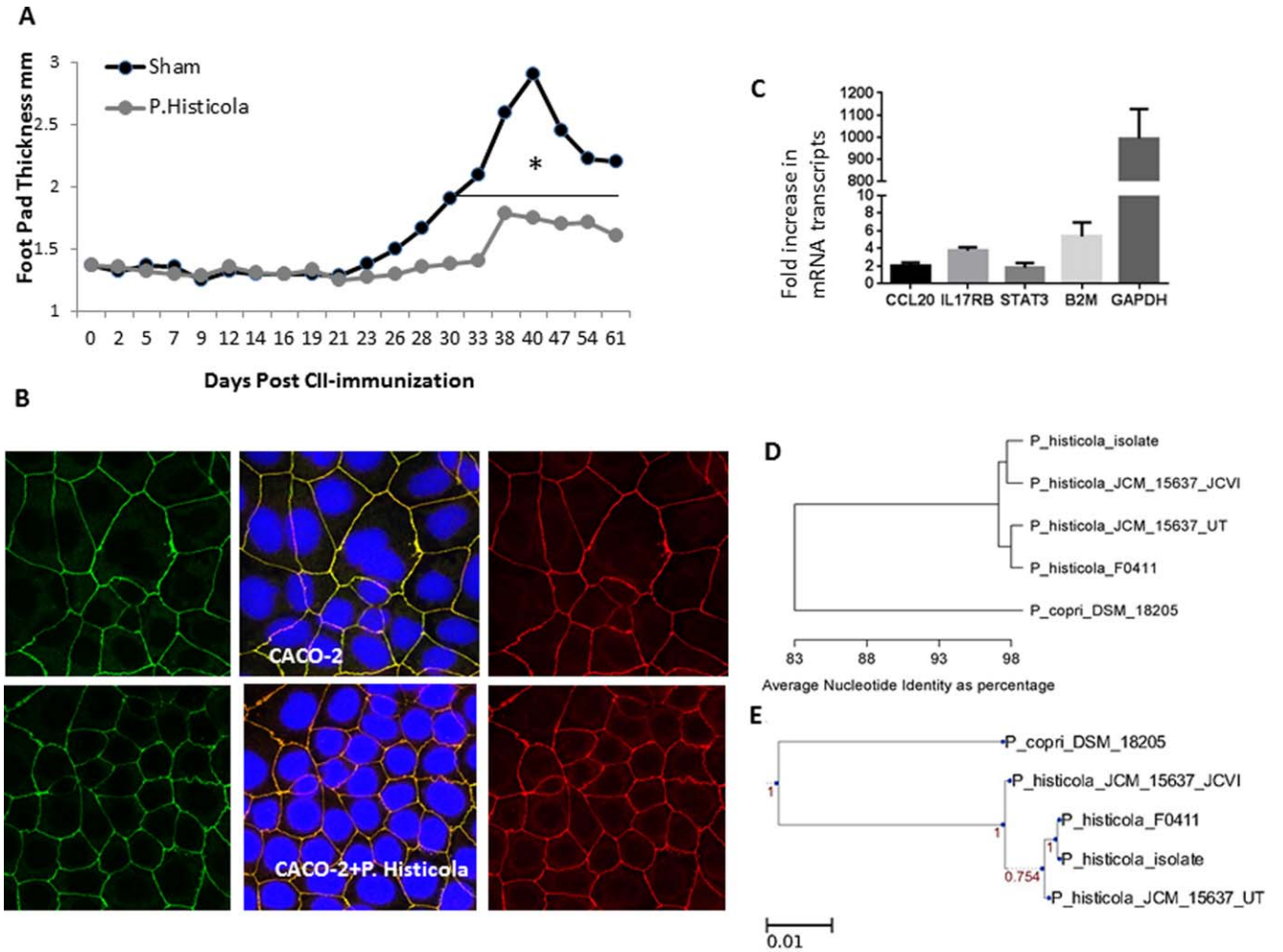

Figure 6. *Prevotella histicola* treatment suppresses arthritis by increasing expression of tight junction protein. **A,** Arthritis severity, determined by paw thickness, in sham-treated and *P histicola*–treated DBA/1 mice. Arthritis was induced in DBA/1 mice by immunization with type II collagen (CII), and mice were treated with bacterial media alone (sham) or *P histicola* 7 days after immunization and monitored for arthritis. From day 30 on, a significant difference in arthritis severity (indicated by the asterisk) was observed between treated and control mice. Values are the mean (n = 10 mice per group). **B,** Increased expression of zonula occludens 1 (left) and occludin (right) in Caco-2 cells treated with *P histicola* compared to control cells in media alone. The middle panels show merged images. Original magnification × 63. **C,** Fold change in expression of mRNA transcripts for cytokines in *P histicola*–treated Caco-2 cells compared to Caco-2 cells in media alone. Bars show the mean ± SD. IL-17RB = interleukin-17 receptor B; B2M = β_2-microglobulin. **D,** Average nucleotide identity plot comparing whole-genome average nucleotide identity as calculated using the windowed blast method and maximum. **E,** Likelihood phylogenetic tree of the 16S ribosomal RNA gene of all of the available different genomes and the *P histicola* isolate from the present study. Bar indicates expected substitutions per site; values are bootstrap confidence values.

an increase in IL-10 levels (Figures 4B–D). Interestingly, the major difference between *P histicola*–treated arthritic and nonarthritic mice was the increase in the levels of cytokines in the duodenum and decrease in the ileum along with an increase in the regulatory cytokines, IL-10 and FoxP3, in the jejunum and colon of the latter (data not shown).

Treatment with *P histicola* generates Treg cells via DCs and modulates antigen presentation. Next we determined if *P histicola* treatment modulates arthritis via IL-10–producing Treg cells in the gut and systemically. Treated mice showed a consistent, although nonsignificant, increase in the total number of splenic CD4+ Treg cells and IL-10–producing Treg cells compared to control mice even though the CD4+ cell numbers were similar (Figure 4E). CD103+ intestinal DCs maintain a tolerant state in the intestine by inducing Treg cell differentiation (12). Mice treated with *P histicola* had increased numbers of CD103-expressing DCs in the lamina propria ($P < 0.05$) (Figure 4F). Increased numbers of CD11c+ CD103+ cells were also observed in the splenocytes of

treated mice ($P < 0.05$) (Figure 4G), which suggests that these DCs could have migrated from the intestine.

Splenic DCs from treated mice modulated the in vitro T cell response when sorted CD4+ cells from the treated and control mice were cultured in a crisscross manner in the presence or absence of CII (Figure 5A). Antigen presentation by DCs from *P histicola*–treated mice showed a significantly lower response, and CD4+ cells produced undetectable levels of IL-17 (Figures 5A and B), suggesting that both DCs and Treg cells may be involved in modulation of the antigen-specific response. These data are consistent with the increased numbers of CD103+ DCs and Treg cells, and altered cytokine profile in the gut and periphery (Figure 4). *P histicola*–treated mice showed an increase in absolute numbers of both CD103+ DCs and myeloid CD11b+Gr-1+ cells, suggested to be suppressors (13,14) (Figure 5C), although the differences were significant only in percentages and not absolute numbers.

Decreased gut permeability in mice treated with *P histicola*. Arthritis-susceptible humanized mice have enhanced gut permeability (4). Comparison of gut permeability in naive, *P histicola*–treated, and sham-treated control mice showed that *P histicola*–treated mice had a significantly lower gut permeability compared to controls ($P = 0.02$) (Figure 5D). Also, *P histicola*–treated mice showed an increase in the expression of tight junction protein ZO-1 in the colon, ileum, and jejunum as compared to sham-treated control mice (Figure 5E). *P histicola*–treated arthritic and nonarthritic mice had much higher expression of the tight junction protein occludin compared to sham-treated mice (Figure 5F), suggesting that *P histicola* regulates tight junction proteins.

Suppression of arthritis in *P histicola*–treated DBA/1 mice. To confirm our findings, we also tested *P histicola* therapy in a commonly used mouse model of arthritis, DBA/1 mice. All CII-immunized mice developed arthritis. However, mice treated with *P histicola* 7 days after immunization developed significantly milder disease compared to control mice that received sham treatment (Figure 6A), suggesting that amelioration of arthritis by this commensal is not genotype specific.

Increased expression of ZO-1 by epithelial cells treated with *P histicola*. We next determined the effect of *P histicola* on human-derived epithelial Caco-2 cells by measuring the expression of various chemokine and cytokine transcripts (Figure 6). Treatment with *P histicola* increased ZO-1 and occludin expression in Caco-2 cells as compared to media control, though the difference was not significant (Figure 6B). *P histicola*–treated epithelial cells showed an increase in mRNA transcripts for IL-17 receptor B (IL-17RB), a receptor not associated with production of proinflammatory IL-17A, which is required for production of Th1 cytokine (Figure 6C). However, the most significant increase was observed for the expression of GAPDH, while no difference was observed for ACTB (data not shown).

***P histicola* is novel and differs from *P copri*.** A recent study showed an expansion of *P copri* in patients with new-onset RA (9). We used whole-genome average nucleotide identity to compare differences between the sequences from various known *P histicola* strains and *P copri* with the *P histicola* isolate used in this study. As shown in Figure 6D, the *P histicola* isolate used in this study is very different from *P copri*, with only 69% average nucleotide identity. The previously available *P histicola* strains, JCM_15637_JCVI, JCM_15637_UT, and F0411, and the isolate used here have 97% average nucleotide identity. We assembled and generated a Maximum Likelihood phylogenetic tree of the 16S ribosomal RNA gene of all the different genomes tested, which suggested that the *P histicola* isolate used in this study is novel. Comparison of the functional open-reading frames between the 2 species showed very low identity in genes when tested by BLASTp (Figure 6E).

DISCUSSION

Recent studies have highlighted the impact of dysbiosis in the gut microbiome on systemic inflammatory diseases, including RA in humans and a transgenic mouse model of arthritis (4,9,15,16). Interestingly, while one study showed an association with *Prevotella* species, another showed a lack of *Prevotella* (*Bacteroidetes* phylum) in fecal samples, suggesting that various species of *Prevotella* might have different effects on arthritis (8,9). A comparison with *P copri*, a disease-associated species, suggested that the *P histicola* isolate used in this study and other *P histicola* strains differ from *P copri*. Moreover, we isolated *P histicola* from the duodenum while other studies used stool samples; commensals isolated from different sites may have different functions.

In transgenic mice, dysbiosis of the gut microbiome is associated with proinflammatory conditions locally, implicating a bottom-up approach (driven by the gut microbiome), such that the adaptive immune system may be modulated by the gut immune system (4). Studies in germ-free and specific pathogen–free mice suggesting that disruptions to gut microbiota can modulate the systemic phenotype further support this contention (17). A role of gut-residing bacteria in the causation of arthritis was shown in germ-free mice, where a single species could promote expansion of intestinal Th17 cells, resulting in the development of arthritis (17,18). Further studies showed that inflammatory microbiota-driven signals favor maintenance and proliferation of autoimmune CD4+ T cells (19). Those studies strongly highlight the concept that the gut microbiota play a role in the causation of arthritis, thereby suggesting that commensals can be used for modulating immune responses locally and systemically.

We took advantage of the HLA-transgenic mouse model to test if a gut-derived commensal, which is observed in lower numbers in arthritis-susceptible transgenic mice and human RA (4,8), can be used for treating CIA as a preclinical model for RA. Our hypothesis was that it would modulate dysbiosis and result in immune homeostasis in the gut that can be translated systemically for modulating disease outcome. Our additional data with *P melanogenica,* which belongs to the *Bacteroidetes* phyla and was also isolated from duodenal biopsy samples from patients with celiac disease, suggest that not all *Prevotella* species may be suppressive. In arthritis-susceptible HLA–DQ8 mice, *P histicola* treatment led to resistance to disease development and limited the severity of the disease without causing any pathology, while *P melanogenica* did not have this effect. We believe that these studies are relevant to humans, since the effect of the treatment can be studied in vivo under normal physiologic conditions, and since the DQ8-restricted effector arm of the immune response is similar to human disease with regard to phenotype and autoantibody profile (3,10,20,21).

The major drawback of the available biologic drugs used for treating RA is that they suppress the immune response such that an individual's capability to fight infection is undermined. Our data indicate that treatment with *P histicola* does not lead to the suppression of innate responses via CpG, sequences found in bacterial and viral genomes that bind to Toll-like receptor 9 (TLR-9), and via LPS, a ligand for TLR-4. However, studies evaluating whether mice treated with *P histicola* are at risk of developing infectious diseases are needed.

Commensal bacteria and probiotics have been shown to exert their antiinflammatory effect through production of IL-10, and the Th2 cytokines IL-25, IL-33, or thymic stromal lymphopoietin, as well as via induction of regulatory cells (22–29). There are several putative mechanisms by which luminally applied microbiome therapy could affect inflammation distal from the gut: regulatory cytokines produced by Treg cells or suppressive DCs in the gut may travel to the target organ, there may be an expansion of regulatory cells that traffic to the site of inflammation, and gut permeability may change. Our data suggested that all 3 of these mechanisms could be occurring in mice treated with *P histicola*. A comparison of various parts of the gut demonstrated that treatment with *P histicola* led to suppression of cytokines in the jejunum, colon, and ileum, although the duodenum showed increased expression in comparison to controls, suggesting that *P histicola* may control immune response differently in various parts of the gut.

This change in expression of cytokines was associated with an increase in lamina propria Treg cells, CD103+ DCs, and CD11c+F4/80+ cells, the latter having been shown to produce IL-10 and induce differentiation of T cells into Treg cells in the lamina propria (30). Suppressive DCs can stimulate CD4+ T cells and reestablish the Th1:Th2 ratio at a "normal" level (31). Our data demonstrated that *P histicola* has a potent modulatory effect upon the systemic production of inflammatory cytokines. IL-13 and IL-17 are involved in the pathogenesis of RA and CIA in humanized mice (10,32,33), and it is likely that *P histicola* suppresses CIA by modulating the immune response to inflammatory cytokines, since both of these cytokines were produced at levels lower than in controls in an in vivo model. An increase in IL-10 levels and Treg cells in lamina propria and spleen do support this notion and further provide one of the mechanisms by which *P histicola* modulates the arthritis phenotype.

Arthritis and the nonsteroidal antiinflammatory drugs used to treat RA are associated with increased gut permeability (34,35). The observations of lowered gut permeability and increased expression of ZO-1 in treated compared to control mice as well as in epithelial cells suggest that *P histicola* protects by preserving gut epithelium integrity in the context of inflammation. Further, *P histicola*–mediated increases in the expression of GAPDH and IL-17RB in epithelial cells may be involved in protection against inflammation. Recently, GAPDH-derived antimicrobial peptides have been identified (36,37). IL-17RB is a cytokine receptor that does not bind IL-17A, known to be involved in RA, and is not associated with RA pathogenesis but rather leads to a Th2 response (38,39). Recent work has suggested that a transient increase in colonic permeability in the presence of normal commensal organisms may provide protection against subsequent colitis, again suggesting the importance of commensal organisms in immune homeostasis and a beneficial or antiinflammatory response in the context of an inflammatory stimulus.

The hygiene hypothesis suggests that a reduced bacterial burden has led to an increase in autoimmunity. However, the colon, which is replete with large quantities of bacteria, is less likely to be affected by this hygiene than the upper gastrointestinal tract, which is the first portal of entry of foreign bacteria and bacterial products. Administering commensals to exact an effect on systemic immune responses through their interaction with the small intestine may be more germane for modifying systemic autoimmune responses and could provide an experimental framework to explain how the increase in environmental hygiene could result in an increase in autoimmune diseases. Our data suggest that *P histicola* has potent probiotic properties, at least in this mouse model, and should be explored further for its beneficial effects for treating inflammation.

ACKNOWLEDGMENTS

We thank Dr. Chella David for providing transgenic mice and for helpful suggestions in this study and Dr. Susan Barton for help in isolating, and Melissa J. Karau for help in culturing, *Prevotella histicola*.

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. Taneja 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. Murray, Patel, Luthra, Mangalam, Taneja.
Acquisition of data. Marietta, Luckey, Lamba.
Analysis and interpretation of data. Marietta, Murray, Jeraldo, Taneja.

REFERENCES

1. Klareskog L, Padyukov L, Lorentzen J, Alfredsson L. Mechanisms of disease: genetic susceptibility and environmental triggers in the development of rheumatoid arthritis. Nat Clin Pract Rheumatol 2006;2:425–33.
2. Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis: an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 1987;30:1205–13.
3. Behrens M, Trejo T, Luthra H, Griffiths M, David CS, Taneja V. Mechanism by which HLA-DR4 regulates sex-bias of arthritis in humanized mice. J Autoimmun 2010;35:1–9.
4. Gomez A, Luckey D, Yeoman CJ, Marietta EV, Berg Miller ME, Murray JA, et al. Loss of sex and age driven differences in the gut microbiome characterize arthritis-susceptible 0401 mice but not arthritis-resistant 0402 mice. PLoS One 2012;7:e36095.
5. Toivanen P, Vaahtovuo J, Eerola E. Influence of major histocompatibility complex on bacterial composition of fecal flora. Infect Immun 2001;69:2372–7.
6. Vaahtovuo J, Toivanen P, Eerola E. Bacterial composition of murine fecal microflora is indigenous and genetically guided. FEMS Microbiol Ecol 2003;44:131–6.
7. De Palma G, Capilla A, Nadal I, Nova E, Pozo T, Varea V, et al. Interplay between human leukocyte antigen genes and the microbial colonization process of the newborn intestine. Curr Issues Mol Biol 2010;12:1–10.
8. Vaahtovuo J, Munukka E, Korkeamaki M, Luukkainen R, Toivanen P. Fecal microbiota in early rheumatoid arthritis. J Rheumatol 2008; 35:1500–5.
9. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. Elife 2013;2:e01202.
10. Taneja V, Behrens M, Mangalam A, Griffiths MM, Luthra HS, David CS. New humanized HLA–DR4–transgenic mice that mimic the sex bias of rheumatoid arthritis. Arthritis Rheum 2007;56:69–78.
11. Winthrop KL. Risk and prevention of tuberculosis and other serious opportunistic infections associated with the inhibition of tumor necrosis factor. Nat Clin Pract Rheumatol 2006;2:602–10.
12. Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, et al. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. J Exp Med 2008;205:2139–49.
13. Marigo I, Dolcetti L, Serafini P, Zanovello P, Bronte V. Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells. Immunol Rev 2008;222:162–79.
14. Del Rio ML, Bernhardt G, Rodriguez-Barbosa JI, Forster R. Development and functional specialization of CD103+ dendritic cells. Immunol Rev 2010;234:268–81.
15. Hakansson A, Molin G. Gut microbiota and inflammation. Nutrients 2011;3:637–82.
16. Vaahtovuo J, Munukka E, Korkeamaki M, Luukkainen R, Toivanen P. Fecal microbiota in early rheumatoid arthritis. J Rheumatol 2008; 35:1500–5.
17. Wu HJ, Ivanov, II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity 2010;32:815–27.
18. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell 2009;139:485–98.
19. Chappert P, Bouladoux N, Naik S, Schwartz RH. Specific gut commensal flora locally alters T cell tuning to endogenous ligands. Immunity 2013;38:1198–210.
20. Taneja V, Taneja N, Behrens M, Griffiths MM, Luthra HS, David CS. Requirement for CD28 may not be absolute for collagen-induced arthritis: study with HLA-DQ8 transgenic mice. J Immunol 2005;174: 1118–25.
21. Taneja V, Taneja N, Paisansinsup T, Behrens M, Griffiths M, Luthra H, et al. CD4 and CD8 T cells in susceptibility/protection to collagen-induced arthritis in HLA-DQ8-transgenic mice: implications for rheumatoid arthritis. J Immunol 2002;168:5867–75.
22. Liu YJ, Soumelis V, Watanabe N, Ito T, Wang YH, Malefyt Rde W, et al. TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. Annu Rev Immunol 2007;25:193–219.
23. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001;19:683–765.
24. Saenz SA, Taylor BC, Artis D. Welcome to the neighborhood: epithelial cell-derived cytokines license innate and adaptive immune responses at mucosal sites. Immunol Rev 2008;226:172–90.
25. Braat H, van den Brande J, van Tol E, Hommes D, Peppelenbosch M, van Deventer S. Lactobacillus rhamnosus induces peripheral hyporesponsiveness in stimulated CD4+ T cells via modulation of dendritic cell function. Am J Clin Nutr 2004;80:1618–25.
26. Pessi T, Isolauri E, Sutas Y, Kankaanranta H, Moilanen E, Hurme M. Suppression of T-cell activation by Lactobacillus rhamnosus GG-degraded bovine casein. Int Immunopharmacol 2001;1:211–8.
27. Pessi T, Sutas Y, Saxelin M, Kallioinen H, Isolauri E. Antiproliferative effects of homogenates derived from five strains of candidate probiotic bacteria. Appl Environ Microbiol 1999;65:4725–8.
28. Schultz M, Linde HJ, Lehn N, Zimmermann K, Grossmann J, Falk W, et al. Immunomodulatory consequences of oral administration of Lactobacillus rhamnosus strain GG in healthy volunteers. J Dairy Res 2003;70:165–73.
29. Konieczna P, Groeger D, Ziegler M, Frei R, Ferstl R, Shanahan F, et al. Bifidobacterium infantis 35624 administration induces Foxp3 T regulatory cells in human peripheral blood: potential role for myeloid and plasmacytoid dendritic cells. Gut 2012;61:354–66.
30. Denning TL, Wang YC, Patel SR, Williams IR, Pulendran B. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. Nat Immunol 2007;8:1086–94.
31. Surana NK, Kasper DL. The yin yang of bacterial polysaccharides: lessons learned from B. fragilis PSA. Immunol Rev 2012;245:13–26.
32. Hitchon CA, Alex P, Erdile LB, Frank MB, Dozmorov I, Tang Y, et al. A distinct multicytokine profile is associated with anti-cyclical citrullinated peptide antibodies in patients with early untreated inflammatory arthritis. J Rheumatol 2004;31:2336–46.
33. Chabaud M, Lubberts E, Joosten L, van Den Berg W, Miossec P. IL-17 derived from juxta-articular bone and synovium contributes to joint degradation in rheumatoid arthritis. Arthritis Res 2001;3:168–77.
34. Bjarnason I, Takeuchi K. Intestinal permeability in the pathogenesis of NSAID-induced enteropathy. J Gastroenterol 2009;44 Suppl 19:23–9.
35. Edwards CJ. Commensal gut bacteria and the etiopathogenesis of rheumatoid arthritis. J Rheumatol 2008;35:1477–14797.
36. Branco P, Francisco D, Chambon C, Hebraud M, Arneborg N, Almeida MG, et al. Identification of novel GAPDH-derived antimicrobial peptides secreted by Saccharomyces cerevisiae and involved in wine microbial interactions. Appl Microbiol Biotechnol 2014;98:843–53.
37. Seo JK, Lee MJ, Go HJ, Kim YJ, Park NG. Antimicrobial function of the GAPDH-related antimicrobial peptide in the skin of skipjack tuna, Katsuwonus pelamis. Fish Shellfish Immunol 2014;36:571–81.
38. Hwang SY, Kim HY. Expression of IL-17 homologs and their receptors in the synovial cells of rheumatoid arthritis patients. Mol Cells 2005;19: 180–4.
39. Petersen BC, Budelsky AL, Baptist AP, Schaller MA, Lukacs NW. Interleukin-25 induces type 2 cytokine production in a steroid-resistant interleukin-17RB+ myeloid population that exacerbates asthmatic pathology. Nat Med 2012;18:751–8.