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Human Gut-Derived *Prevotella histicola* Suppresses Inflammatory Arthritis in Humanized Mice

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Abstract

Objective—The gut microbiome regulates host immune homeostasis. Rheumatoid arthritis (RA) is associated with intestinal dysbiosis. In this study we used a human gut-derived commensal to modulate immune response and treat arthritis in a humanized mouse model.

Methods—We have isolated a commensal bacterium, *Prevotella histicola*, native to the human gut that 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 the gut epithelial proteins and immune response as well as systemic cellular and humoral immune responses were studied in treated mice.

Results—DQ8 mice when treated with *P. histicola* in prophylactic or therapeutic protocols exhibited significantly decreased incidence and severity of arthritis as compared to controls. The microbial mucosal modulation of arthritis was dependent on the regulation by CD103+ dendritic cells and myeloid suppressors, CD11b+Gr-1, and by generation of T regulatory cells, CD4+CD25+FoxP3+, in the gut, resulting in suppression of antigen-specific Th17 response and increased transcription of IL-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, Zo-1 and Occludin. However, the innate immune response via TLR4 and TLR9 were not affected in treated mice.

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Discussion—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 low/no side effects.

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease that requires 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 suggests that gut microbial composition of naïve *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. MHC 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-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.

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

Our studies suggest that *P. histicola* has immune modulating properties and suppresses inflammatory cytokines. We tested if orally administered *P. histicola* can modulate the immune response in the gut and if that can be translated systemically to control the arthritis in DQ8 mice. Our data suggests that oral feeding of *P. histicola* in a therapeutic protocol (after induction of arthritis) to DQ8 transgenic mice leads to resistance to develop disease 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.

Material and Methods

Isolation and Identification of *Prevotella* Species

Biopsies from individuals were taken from the proximal small bowel and bacterial cultures grown on KV agar plates for isolation of individual colonies. Isolates were cultured on sheep blood agar plates and incubated at 35°C anaerobically for 2 days.

Bacterial genomic DNA was extracted using the QIAamp DNA Mini kit (Qiagen). Real time, rapid cycle Light Cycler PCR with SYBR Green I detection (Roche Applied Science, Indianapolis, IN) was used to amplify 527-bp of the 16S rRNA gene. Universal bacterial 16S rRNA gene primers were used (Microseq® 500 16S rRNA gene PCR kit). PCR cycling was followed by a post-amplification melting curve analysis to verify the amplicon before

sequencing. Sequencing was performed with the BigDye terminator version 1.1 *Taq* kit and an ABI 3730XL DNA sequencer (Applied Biosystems). Bidirectional sequence data were aligned using Sequencher (Gene Codes Corp). The generated consensus sequences were compared to those of the National Center for Biotechnology Information's (NCBI) GenBank database. Identity 99% between the query sequence and the GenBank database with a difference > 0.4% between species was used for identification to 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 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, Rochester, MN in accordance with the Institutional Animal Use and Care Committee. All 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) (100µg of CII emulsified 1:1 with complete Freund's adjuvant) as previously described (10). Mice were monitored for the onset and progression of CIA using a grading system (range 0–3) wherein each paw was scored; 0= no swelling, 1=1 or 2 digit swollen, 2=2 or more digits swollen and 3= swollen paw. The mean arthritic score was determined using arthritic animals only. Mice were divided in 2 groups for reproducibility. Histopathology of representative paws from each group was done to determine arthritis induction. DBA/1 mice were immunized with CII and the paw thickness was measured with calipers before and after induction of arthritis to determine the 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 (KV) (Becton, Dickson) and incubated anaerobically in an anaerobic jar with 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 (TSB) 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 microliters of TSB (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 10 days prior to immunization with CII and continued for 6 weeks post-immunization. For the therapeutic protocol, mice were treated 2 weeks post CIA induction and continued for 6 weeks. Control sham gavage consisted of administering 100ul of bacterial media alone. DBA/1 mice were treated 7 days post immunization. *Prevotella* did not colonize the gut (not shown).

Isolation of Lamina Propria Cells

Intestinal tissue was cut longitudinally using a scalpel blade, and washed six times using CMF solution (88% 1X Hanks balanced salt solution, 10% HEPES-bicarbonate buffer, 2% FBS. A 1 hour collagenase digestion using Complete RPMI-10/collagenase (1.33mg/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 gentamycin.

Intestinal Permeability

All transgenic mice were kept on standard diet. Changes in intestinal permeability were determined using 4-KDa FITC-labeled dextran. Mice were deprived of food for 3 hours, then gavaged with FITC-labeled dextran (0.6 mg/g body weight). Three hours later, mice were bled and serum collected. FITC-dextran was determined at 490 nm. 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 gut, duodenum, jejunum, ileum and colon, were frozen at the termination of the experiments and tested for the expression of tight junction proteins Zonulin-1 (ZO-1) and Occludin by immunofluorescence using purified anti-Zonulin-1 (Life Technologies), Alexa Flour 594 conjugated anti-occludin (Life Technologies), and FITC conjugated anti rabbit IgG (Jackson ImmunoResearch Laboratories). Caco-2 cells (3×10^5) were cultured on 22×22 mm coverslips in 6 well tissue culture plates at 37°C in humidified 5% CO₂ incubator till the cultures were confluent monolayers. They were then incubated with or without *P. histicola* (100 µl of 1×10^8 CFU/ml) for 4 hours, fixed with 10% formaldehyde and evaluated for Zo-1 and Occludin expression.

rtPCR for Cytokine and Chemokine expression

RNA was extracted from cells using RNeasy columns (Qiagen) and cDNA 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 as per manufacturer's instructions. The data was analyzed as per the online resources of the manufacturer.

Collagen Specific ELISA

Mice were bled after CII-immunization before and after treatment with *P. histicola*. Titers of sera IgG antibodies against CII were measured by standard ELISA and are shown as optical density.

T Cell Proliferation Assay

For the T cell proliferation assay, mice were immunized with 200ug of CII emulsified 1:1 in CFA (Difco) intradermally at the base of the tail and proliferation was done as described (10). For some experiments, CD4⁺ cells (5×10^6) sorted from lymph nodes of CII-primed

mice that were treated with *P. histicola* or media only were cultured *in vitro* in the presence or absence of the antigen and CD11c+ dendritic cells (5×10^5) harvested from spleens. Stimulation index of 2 or more was taken as a positive response.

Response to CPG (cytosine connected to guanine through phosphodiester bonds) (1 µg/ml) and LPS (lipopolysaccharide) (5 µg/ml) was tested in mice receiving *P. histicola* treatment for 10 days prior to CII-immunization or 10 days post immunization and followed for 2 weeks of treatment. Also, mice in *in vivo* protocol were tested for response to LPS and CPG.

Flow Cytometry

The expression of DQ in transgenic mice was analyzed by flow cytometry using mAb IVD12 (anti-DQ). Conjugated antibodies for CD3, CD4, CD11c, CD19, CD25, GITR, Gr-1 and B220 (BD Biosciences, CA) were also used. All experiments were done with cells pooled from 2 mice/strain and repeated 2–3 times. Intracellular staining for FoxP3 and IL-10 was performed using specific antibodies obtained (eBioscience, San Diego, CA) as per the manufacturer's instructions. Phycoerythrin-conjugated (PE) rat IgG2a (eBioscience) was used as the isotype control for FoxP3 staining. Analysis was done using the Cell Quest program (Beckton Dickinson).

Cytokines

Cytokines were measured using the Bio-Plex protein array system with the mouse cytokine 23-plex panel as per manufacturer's instructions, and were analyzed with Bio-Plex manager 2.0 software (Bio-Rad laboratories, Hercules, CA). 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 Chi square test. Antibody levels, onset of arthritis, mean scores for arthritic mice and various cells were compared using non-parametric Student's T test.

RESULTS

Gut-Derived *P. histicola* Modulates Immune Response

P. histicola and *P. melanogenica* isolated from the duodenum of an individual were cultured and tested for pathogenic properties. None of the mice developed weight loss greater than 5% of original weight. Also, 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, TNFα, and increased IL-4 and IL-10 compared to sham controls (Figure 1). Also proinflammatory chemokines involved in various autoimmune diseases, GM-CSF and MCP-1, were suppressed demonstrating a pro-biotic effect of *P. histicola* isolate in the DQ8 mice. On the other hand *P. melanogenica* gavaged mice did not show any significant changes in cytokines but did show reduced MIP-1b and MCP-1 levels compared to sham controls.

***P. histicola* Suppresses Arthritis in Susceptible DQ8 Mice by Modulating Cellular and Humoral Response**

P. histicola was tested for treating CIA in DQ8 mice. HLA-DQ8 mice were immunized with CII and some mice were gavaged with *P. histicola* either before immunization (prophylactic) or 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* and CII) as well as therapeutic (CII and *P. histicola*) protocols resulted in statistically significant lower incidences of arthritis ($P<0.05$) as compared to sham controls ($P<0.05$). Mice treated with *P. histicola* developed milder arthritis with delayed onset compared to sham controls. However, *P. melanogenica* did not provide any protection from developing 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 from CIA. Sera from mice 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 antibodies. In addition, the antigen-specific T cell response was lower in treated mice with and without arthritis compared to arthritic controls (Figure 2C, D).

Treatment with *P. histicola* Changes the Systemic But Not the Innate Immune Response

Treating mice with immunomodulatory agents are known to cause suppression of innate responses leading to infections (11). We assessed whether treatment with *P. histicola* modulates the innate immune responses in *in vivo* model by culturing splenocytes with LPS or CpG. Although immunized and treated mice generated a higher response than naïve 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 regulating cytokines IL-9, IL-13 and IL-12(p40) as compared to sham treated mice (Figure 3C)..

***P. histicola* Treatment Modulates Immune Response in the Gut**

We next evaluated whether *P. histicola* treatment affects the mucosal immune system locally in the gut *in vivo* by determining mRNA transcripts for various cytokines from all parts of the gut (Figure 4). Treated mice as well as sham mice did not show significant changes in cytokine expression in the ileum. The jejunum and colon of treated mice showed most cytokines being suppressed, *P. histicola* treated mice without arthritis showed much higher IL-10 and lower TGF β levels compared to controls. The duodenum had high expression of most cytokines in both groups. Heat maps of intestinally derived mRNA transcripts of Th17 regulatory network revealed that *P. histicola* treatment led to changes in cytokine expression; arthritic control and treated mice showed similarities compared to non-arthritic treated mice (not shown). We further compared the effect of treatment on the duodenum, jejunum, ileum, and colon in the treated, arthritic or non-arthritic, and control groups (Figure 4A–D shows

data with 5 fold or more difference). *P. histicola* treated mice showed suppression of all cytokines in the jejunum (except IL-10), and colon compared to the duodenum (Fig 4 B, D). Cytokines in *P. histicola* treated non-arthritic mice compared to controls showed more than 5-fold reductions in IL-17 and other pro-inflammatory cytokines with an increase in IL-10 (Fig 4 B–D). Interestingly, the major difference between *P. histicola* treated arthritic and non-arthritic 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 (not shown).

Treatment with *P. histicola* Generates Treg Cells via Dendritic Cells and Modulates Antigen Presentation

Next we determined if the *P. histicola* treatment modulates arthritis via IL-10 producing T regulatory cells in the gut and also systemically. Treated mice showed a consistent, although non-significant, increase in the total number of splenic CD4+ Tregs and IL-10 producing Tregs as compared to control mice even though the CD4+ cell numbers were similar (Figure 4E). CD103+ intestinal dendritic cells (DCs) maintain a tolerant state in the intestine by inducing regulatory T 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 CD11c+CD103+ cells were also observed in splenocytes of treated mice, $P<0.05$ (Figure 4G) which might suggest that these DCs could have migrated from the intestine. Splenic DCs from treated mice could modulate the in vitro T cell response by culturing sorted CD4+ cells from the treated and control mice 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, B) suggesting both DCs and T reg cells may be involved in modulation of antigen-specific response. This data corroborates the increased CD103+ DCs and Tregs, 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 as well as 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.

***P. histicola* Treatment Lowers the Gut Permeability**

Arthritis-susceptible humanized mice have enhanced gut permeability (4). Comparison of gut permeability in naïve, treated and control mice showed that. *P. histicola* treated mice had a significantly lower gut permeability compared to controls, $P=0.02$ (Figure 5D). Also, treated mice showed an increase in the expression of tight junction protein Zo-1 in the colon, ileum and jejunum as compared to sham control mice (Figure 5E). *P. histicola* treated arthritic and non-arthritic 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 this 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 post-immunization developed a significantly milder disease

compared to sham controls (Figure 6A), suggesting that amelioration of arthritis by this commensal is not genotype specific.

***P. histicola* Treatment Leads to Increased Expression of Zo-1 by Epithelial Cells**

We next determined the effect of *P. histicola* on human derived epithelial Caco-2 cells by measuring expression of various chemokines and cytokines transcripts (Figure 6). The treatment with *P. histicola* increased Zo-1 and Occludin expression in Caco-2 cells as compared to media control, though it was not significant (Figure 6B). There was an increase in mRNA transcripts in treated epithelial cells for IL17RB, a receptor not associated with production of pro-inflammatory IL-17A, required for production of Th1 cytokine (Figure 6C). However, the most significant increase was observed in the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) while ACTB did not show any difference (not shown).

P. histicola* is Novel and Differs from *Prevotella copri

A recent study has shown an expansion of *P. copri* in new onset RA patients (9). We used whole-genome average Nucleotide Identity (ANI) 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 (Figure 6D), the *P. histicola* isolate used in this study is very different from *P. copri* with only 69% ANI. The previously available *P. histicola* strains, JCM_15637_JCVI, JCM_15637_UT and F0411, and the isolate used here have 97% ANI. 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 ORFs between the 2 species showed very low identity in genes when tested by BLASTp.

Discussion

Recent studies have highlighted the impact of dysbiosis in the gut microbiome on systemic inflammatory diseases including RA in patients and arthritis model in transgenic mice (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 various species of *Prevotella* might have different effects on arthritis (8, 9). A comparison with *P. copri*, a disease associated species, suggested that our *P. histicola* isolate as well as other *P. histicola* strains differ from *P. copri*. Moreover, we isolated *P. histicola* from 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 pro-inflammatory 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 with germ-free and specific pathogen-free mice suggesting disruptions to gut microbiota can modulate systemic phenotype further support this contention (17). A role of the gut residing bacteria in causation of arthritis was shown in germ-free mice where a single species could promote expansion of intestinal Th17 cells, resulting in development of arthritis (17, 18).

Further studies showed that inflammatory microbiota driven signals favor maintenance and proliferation of autoimmune CD4⁺ T cells (19). These studies strongly highlight the concept that the gut microbiota plays a role in 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, that is observed with 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 of patients with celiac disease, suggest that not all *Prevotella* species may be suppressive. Arthritis-susceptible HLA-DQ8 mice treated with the two species of *Prevotella* demonstrated that treating mice with *P. histicola* leads to resistance to disease development and limits severity of disease without causing any pathology while *P. melanogenica* did not have this effect. We believe that these studies are relevant to humans as the effect of the treatment can be studied *in vivo* under normal physiological conditions and secondly, the DQ8-restricted effector arm of the immune response has similarities with human disease in 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 of fighting infection is undermined. Our data indicates 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 TLR9, and via LPS, a ligand for TLR4. However, studies to show that mice treated with *P. histicola* are not at risk for developing infectious diseases need to be done.

Commensal bacteria and probiotics have been shown to exert their anti-inflammatory effect through production of IL-10, and the Th2 cytokines IL-25, IL-33 or thymic stromal lymphopoietin (TSLP) as well as via induction of regulatory cells (22–29). There are several putative mechanisms by which luminal applied microbiome therapy could affect inflammation distal from the gut, 1) regulatory cytokines produced by Tregs or suppressive DCs in the gut travel to the target organ, 2) expansion of regulatory cells that traffic to the site of inflammation and 3) change in gut permeability. Our data suggested that all of these 3 mechanisms could be occurring in mice treated with *P. histicola*. A comparison of various parts of the guts demonstrated that treatment with *P. histicola* led to suppression of cytokines in the jejunum, colon and ileum although the duodenum showed an 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 lamina propria (30). Suppressive DCs can stimulate CD4⁺ T cells and reestablish the Th1/Th2 ratio to 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 pathogenesis of RA and CIA in humanized mice (10, 32, 33) it is likely that *P. histicola* suppresses CIA by modulating the immune response to inflammatory cytokines as both these cytokines were produced lower than controls in *in vivo* model. An increase in

IL-10 levels and T regulatory cells in LP and spleen do support this contention and further provide one of the mechanisms by which *P. histicola* modulates arthritis phenotype.

Arthritis as well as the medications used for treating RA, NSAIDS, 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 increase in expression of GAPDH and IL17RB in epithelial cells may be involved in protection from inflammation. Recently, GAPDH-derived anti-microbial peptides (AMPs) 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 rather leads to Th2 response (38, 39). Recent work has suggested that 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 anti-inflammatory response in the context of an inflammatory stimulus.

The hygiene hypothesis suggests 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 GI 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 of autoimmune diseases. Our data suggests 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.

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Figure 1. Oral treatment with *P. histicola* modulates systemic immune response in DQ8.Aβ mice. Serum levels of cytokines and chemokines were tested in naïve DQ8 transgenic mice gavaged with bacterial media alone, Sham (■) *P. histicola* (■) or *P. melanogenica* (▨) every other day for two weeks. *denotes p<0.05 comparison between *P. histicola* treated and sham mice (N=5–7 each experimental group).

Figure 2.

Oral treatment with *P. histicola* protects DQ8 mice from Collagen Induced Arthritis. *P. histicola* was administered to DQ8 mice in therapeutic protocol (CII+ *P. histicola*) (N=21) or as a prophylactic measure (*P. histicola* + CII), (N=12). *P. melanogenica* was administered as a species control for the therapeutic protocol (CII + *P. melanogenica*) (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) in DQ8 mice. (A) Incidence of arthritis in various experimental groups described above. Difference between CII-immunized and media versus *P. histicola* treated mice, incidence and severity, $P < 0.05$ (B) Hematoxylin and eosin staining of the small intestine is shown from a representative mouse for each of the three treatment groups (*P. histicola* treated, sham (CII+ media) treated, and *P. melanogenica* treated). N=3 each group (C) Levels of serum anti-CII (IgG) antibodies before and after the administration of *P. histicola* using the therapeutic protocol. (D) T cell proliferative response to CII in vitro at the termination of the experiment at 12 weeks using splenocytes in mice with collagen-induced arthritis (CIA+) and without (CIA-).

Figure 3.

Treatment with *P. histicola* modulates immune response in an antigen-specific manner in CIA. Splenocytes harvested from DQ8 mice in different conditions as indicated were tested for in vitro proliferative response to A) CPG and LPS, and B) CII in mice treated with *P. histicola*, prophylactic (*P. histicola*+CII) and therapeutic (CII+*P. histicola*). C) Serum cytokines produced by DQ8 transgenic mice induced for arthritis and treated with culture media (sham) or *P. histicola* in therapeutic protocol (*P. hist.*) (N=4 each group).

Figure 4.

Anti-inflammatory effects of *P. histicola* treatment upon the Intestinal immune system. Fold change in cytokine transcript levels in arthritic (CIA+) and non-arthritic (CIA-) DQ8 mice treated with *P. histicola* treatment (therapeutic) as compared to the control group in the A) duodenum, B) jejunum, C) ileum, and D) colon. Expression levels of cytokine transcripts in duodenum, jejunum, ileum and colon of arthritic mice immunized with CII and gavaged media (Sham), and mice immunized with CII and treated with *P. histicola* in therapeutic protocol, arthritic (group 1) and non-arthritic (group 2) (N=3 each group). Control group was used as the reference. E) the absolute numbers of regulatory T cells (CD4+CD25+FoxP3+) producing IL-10 in the spleens of mice immunized with CII and treated with *P. histicola* or bacterial media alone (sham) Sham. FACS histogram of regulatory DCs, CD11c+CD103, in F) lamina propria cells and G) splenocytes. Also, shown is FACS histogram of CD4+GITR+ regulatory T cells in spleens of CII-immunized and treated or sham mice. (N=3–4 mice per group, experiment representative).

Figure 5.

CD4⁺ cells from *P. histicola* treated mice generate lower antigen-specific T cell response compared to controls. A) In vitro T cell response to CII in splenic CD4⁺ cells of DQ8 mice immunized with CII and gavaged with *P. histicola* (*P. hist.*) with CD11c⁺ cells (DCs) from same mouse or control mouse, CII immunized mice gavaged with bacterial media alone (sham) or vice versa, B) IL-17 production in supernatant from the culture in 5A, and C) regulatory dendritic cells, CD11c⁺CD103⁺ cells and myeloid suppressors, CD11b⁺Gr-1⁺. (N=4 each group) were enumerated from splenocytes from sham and *P. histicola* treated mice. D) Gut permeability, done by FITC-Dextran, in naïve (N=12) and CII-immunized mice treated with *P. histicola* (N=12) or not (Sham) (N=8). E) Expression of ZO-1 in intestinal sections of control and *P. histicola* treated DQ8 mice induced for arthritis. F) Expression of Occludin in arthritic DQ8 mice (a), arthritic mice administered *P. histicola* (b) and naïve mice treated with *P. histicola* (c). Sections are shown at 60× magnification. Mean florescence intensity (MFI) of expression is depicted below.

Figure 6.

P. histicola treatment suppresses arthritis by increasing expression of tight junction protein.

A) DBA/1 mice treated with *P. histicola* develop milder arthritis compared to controls.

DBA/1 mice were induced for arthritis and treated with *P. histicola* or media 7 days post immunization (N=10) and monitored for arthritis. From day 30 onwards, a significant difference in paw severity was observed between treated and control mice.

P. histicola treated Caco-2 cells show increase expression of B) Zo-1 (left panel) and Occludin (right panel) and merged (middle panel) and C) fold expression of mRNA transcripts of certain cytokines in treated as compared to Caco-2 cells in media only.

D) The ANI plot comparing whole-genome average nucleotide identity as calculated using the windowed blast method and maximum.

E) Likelihood phylogenetic tree of the 16S rRNA gene of all the available different genomes and the *P. histicola* isolate from the present study.