Human Gut-derived Commensal Bacteria Suppress Central Nervous System Inflammatory and Demyelinating Disease

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SUMMARY

The human gut is colonized by a large number of microorganisms (~10^{13} bacteria) that support various physiologic functions. A perturbation in healthy gut microbiome might leads to the development of inflammatory diseases including multiple sclerosis (MS). Therefore, gut commensals can provide promising therapeutic options for treating autoimmune diseases such as MS. We report identification of human gut–derived commensal bacteria, *Prevotella histicola*, which can suppress an autoimmune disease in HLA class-II transgenic model of experimental autoimmune encephalomyelitis (EAE); an animal model of MS. *P. histicola* suppresses disease through modulation of systemic immune responses. *P. histicola* challenge led to a decrease in pro-inflammatory Th1 and Th17 cells, and increase in the frequencies of CD4+FoxP3+ regulatory T cells, tolerogenic dendritic cells, and suppressive macrophage. Our study provides evidence that administration of gut commensals may regulate a systemic immune response and may, therefore, have a possible role in the treatment strategies for MS.

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INTRODUCTION

The human gut is colonized by a large number of microorganisms ($\sim 10^{13}$ bacteria) that support various physiologic functions (Sender et al., 2016). Recent research envisages humans as holobionts having evolved with our microbiome with our gut microbiome being vital to human health (Charbonneau et al., 2016; Honda and Littman, 2016). The increased prevalence of inflammatory diseases in developed countries has been attributed to an altered gut microbiome that is characteristically linked with the disease state. Therefore, gut commensals capable of restoring the healthy microbiome could be a promising therapy for treating inflammatory diseases such as multiple sclerosis (MS). Although several therapies are available, none cure the disease and many are not well tolerated.

The hypothesis that MS is an autoimmune disease caused by myelin-specific CD4 T-cells comes from experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Gold et al., 2000). CD4 T cell repertoire is selected in humans by human leukocyte antigen (HLA)-class II molecules, and MS patients show increased frequency of particular HLA class II haplotypes such as HLA-DR2/DQ6, DR3/DQ2, and DR4/DQ8 (Dyment et al., 2005; Zivadinov et al., 2007). We have used transgenic mice expressing human class II genes and lacking endogenous class II genes to identify disease-susceptible and resistant class II alleles (Luckey et al.; Mangalam et al., 2008). Transgenic mice expressing HLA-DR3 and DQ8 genes (HLA-DR3.DQ8) develop EAE and have severe brain and spinal cord pathology (Mangalam et al., 2009a; Mangalam et al., 2004).

During recent decades, the incidence of autoimmune diseases in developed countries has increased steadily (Bach, 2002; Okada et al., 2010). Numerous hypotheses have been suggested to explain this phenomenon, including alterations of the gut microbiome due to decreased exposure to parasites, antibiotics, western diet, and other environmental factors (Rook, 2012). Additionally, western diets are associated with an abundance of the *Bacteroides* enterotype, whereas the *Prevotella* enterotype is more prevalent in persons with a carbohydrate-rich, high-fiber, agrarian diet (Wu et al., 2011).

As certain commensal bacteria residing in the intestine might have immunomodulatory properties, we cultured proximal small bowel biopsies from Coeliac disease patients to isolate and characterize gut commensal with the ability to modulate immune response (Marietta et al., 2016). We isolated *Prevotella histicola (P. histicola)*, *Prevotella melaninogenica (P. melaninogenica)*, and *Capnocytophaga sputigena (C. sputigena)* and investigated the ability of these anaerobic, gram-negative *Bacteroidetes* commensals to modulate PLP$_{91-110}$-induced EAE in HLA-DR3.DQ8 transgenic mice. Here, we report that a strain of *P. histicola* can suppress or prevent disease in EAE. We further show that *P. histicola* suppresses disease through downregulation of pro-inflammatory Th1/Th17 response and induction of regulatory CD4$^+$FoxP3$^+$ regulatory T cells (Tregs).

RESULTS

Treatment with *P. histicola* suppressed PLP$_{91-110}$-induced EAE in HLA-DR3.DQ8 transgenic mice

Previously, we showed that double-transgenic mice expressing human HLA-DR3 and DQ8 (HLA-DR3.DQ8) develop EAE with CNS pathology (Mangalam et al., 2009a). Therefore, we tested the immunomodulatory capabilities of *P. histicola*, *P. melaninogenica*, and *C. sputigena* in HLA-DR3.DQ8 transgenic mice (See Figure S1A). Among three bacteria tested, *P. histicola* treated group had higher levels of IL-10 and TGF-β compared to medium or *C. sputigena*. Mice treated with *P. melaninogenica* had higher levels of IL-10 but no change in levels of TGF-β or TNF-α. Next, we tested if these commensals modulated EAE in HLA-DR3.DQ8 transgenic mice.

EAE was induced in HLA-DR3.DQ8 transgenic mice (Mangalam et al., 2009a) and 7 days post-immunization, animals were challenged with 1×10^9 CFU/ml bacteria or medium (Lavasani et al., 2010). Mice were gavaged every other day with *P. histicola, P. melaninogenica, C. sputigena*, mouse *E. coli* (control), or medium alone and monitored for disease incidence and severity. *P. histicola* treated mice had reduced disease incidence, with only 5 of 20 mice (25%) developing EAE compared with 100% EAE incidence (20/20) in medium fed mice (*P*<0.005) (Figure 1A, 1B, and Table 1). Challenge with *P. melaninogenica* had a mild suppressive effect but the cumulative disease score was not different from control groups. Whereas all mice in the *C. sputigena* or *E. coli* treated group developed disease. Because only *P. histicola* challenged (13.6±18.9 vs 76.75±7.7; *P*<0.001) (Figure 1B) but not *P. melaninogenica* challenged mice had less cumulative disease, we focused on *P. histicola* for later experiments. Further, disease onset was delayed in *P. histicola* challenged mice compared with medium challenged mice (17.5±0.3 vs 10.6±0.2 days; *P*<0. 005) (Table 1). To address whether colonization with *P. histicola* alone can modulate the disease, we depleted microbial flora of mice using broad acting antibiotics for three weeks (Rakoff-Nahoum et al., 2004) and gavaged with medium or *P. histicola*. *P. histicola* challenged group developed milder EAE compared to medium-treated group (See Figure S1B). These data suggests that *P. histicola* alone can modulate EAE in HLA-DR3.DQ8 mice.

P. histicola challenge did not cause any pathology in the upper gut (See Figure S1C). To investigate which part of the gut *P. histicola* colonize naïve mice (8–12 weeks old) were treated with either medium or *P. histicola*. Utilizing qPCR and *Prevotella* specific primers, we observed higher colonization in the stomach and jejunum/ileum (See Figure S1D). Thus our data suggest that *P. histicola* might colonize the upper gut of HLA-DR3.DQ8 transgenic mice.

Disease-suppressive effects of *P. histicola* required viable bacteria

To determine whether *P. histicola* suppression of disease required whole bacteria or bacteria-derived soluble factors, we investigated the ability of cell-free *P. histicola* culture supernatant (PH-CS) to suppress EAE. The PH-CS–treated group had a disease incidence of 67% (10/15) compared with a 27% incidence rate in the *P. histicola* – challenged group (See

Table S1). Mice challenged with PH-CS had a greater risk of developing EAE than mice given live *P. histicola* (hazard ratio, 3.3 [95% CI, 1.03–10.5]; *P*=0.04), indicating that PH-CS had a modest effect on disease incidence (Supplemental Figure 2). The risk of EAE development was significantly lower in the live-bacterium group (hazard ratio, 7.7 [95% CI, 2.5–24]; *P*=0.005). We tested live *P. histicola* in doses that ranged from 1×10^7 to 1×10^9 CFU/ml and observed a dose-dependent effect with optimal suppression at 1×10^8 CFU/ml (See Table S2).

Treatment with *P. histicola* reduced inflammation and demyelination in the CNS

Analysis of CNS tissues from medium challenged HLA-DR3.DQ8 transgenic mice showed severe inflammation and demyelination in the brain and spinal cord compared to the *P. histicola* challenged groups (Figure 1C). Quantitative analysis of spinal cord inflammation and demyelination showed that *P. histicola* challenged animals had fewer regions with inflammation and demyelination (Figure 1D). Groups receiving other bacteria (*E. coli* and *C. sputigena*) had severe CNS inflammation and demyelination similar to the medium challenged group. Thus, treatment with *P. histicola* reduced CNS inflammation and demyelination compared to the medium only group.

P. histicola administration downregulated PLP$_{91-110}$–specific T-cell and cytokine response

To determine the effect of *P. histicola* on antigen-specific T cell responses, we isolated splenocytes from mice given bacteria or medium and stimulated with the PLP$_{91-110}$ peptide. *P. histicola* challenged mice had less antigen-specific T cell response compared with medium or *E. coli* challenged group (Figure 2A). Splenocytes from *P. histicola* challenged mice produced less proinflammatory cytokines IL-17 and IFN-γ after stimulation with PLP$_{91-110}$, whereas anti-inflammatory cytokine IL-10 was increased (Figure 2B). Levels of tumor necrosis factor (TNF)-α was similar among different groups (Figure 2B). Animals given control bacteria (*E. coli)* showed cytokine levels similar to the medium only group.

P. histicola treatment reduced the blood brain barrier (BBB) and gut permeability and downregulated CNS inflammation

The *P. histicola* challenged group had reduced BBB permeability as measured by FITC-albumin compared with the medium only group (Figure 2C). Mice with EAE also had increased gut permeability, however, challenge with *P. histicola* restored (Figure 2D). Groups receiving *E. coli* had both increased BBB and gut permeability similar to the medium treated group (2C and D). CNS tissue from the *P. histicola* treated mice had reduced cellular infiltration compared with the control groups (Figure 2E) with lower levels of total and CD4 T cell specific IFN-γ and IL-17 expressing cells compared with the medium or *E. coli* groups (Figure 2F, G, and H). Thus, *P. histicola* decreased BBB permeability and reduced frequency of pro-inflammatory Th1 and Th17 cells in CNS.

P. histicola suppresses EAE through an increase in CD4+FoxP3+Tregs

To identify the cell population(s) responsible for the disease-protective effect, we characterized the cellular profile of bacterially challenged and untreated animals. *Prevotella* challenged mice had an increased frequency of CD4+FoxP3+ Tregs in the spleen and

mesenteric lymph nodes (MLNs) (Figure 3 A–B). To test whether *P. histicola* could directly induce a Treg population, naïve HLA-DR3.DQ8 transgenic mice were challenged with 1×10^8 CFU/ml *P. histicola* or medium on alternate days for 7 doses. *P. histicola* treated animals had higher levels of $CD4^+CD25^+FoxP3^+$ Tregs in splenocytes, MLNs, and cervical lymph nodes (CLNs) compared with the medium treated group (See Figure S3). $CD4^+CD25^+$ Treg cells isolated from *P. histicola* challenged animals also showed higher suppression of PLP_{91-110}-specific $CD4^+$ T effector cells compared with the Tregs isolated from naïve, medium, or *E. coli* treated mice (Figure 3C). Therefore, *P. histicola* increased regulatory $CD4^+$ T cell numbers and also enhanced their suppressive function.

P. histicola challenge increased the frequency and activity of tolerogenic DCs

Next, we analyzed levels of $CD103^+CD11c^+$ tolerogenic DCs in MLNs, and splenocytes among different treatment groups. *P. histicola* treated animals had increased levels of $CD103^+CD11c^+$ DCs in the spleen and MLNs compared with medium treated group (Figure 3D). $CD11c^+$ cells from *P. histicola* treated animals had lower antigen presentation capacity compared with $CD11c^+$ DCs isolated from naïve, medium or *E. coli* treated mice (Figure 3E). Lipopolysaccharide-stimulated $CD11c^+$ DCs from medium treated mice produced high levels of IL-23 and low levels of IL-10, whereas *P. histicola* treated animals produced low levels of IL-23 and high levels of IL-10 (Figure 3F). $CD11c^+$ DCs isolated from *E. coli* treated group behaved similarly to medium treated group (Figure 3F). We also observed that *P. histicola* challenge led to induction of $CD11b^+Gr-1^{med}$ macrophages (See Figure S4A) and CD11b+ cells from *P. histicola* challenge group had reduced antigen presentation capacity compared to medium group (See Figure S4B). Macrophages from *P. histicola* treated animals produced higher IL-10 and lower IL-12 (See Figure S4C) compared with medium or *E.coli* treated groups. Thus, *P. histicola* treated animals had an increased frequency of tolerogenic antigen presenting cells and decreased antigen presentation capacity compared with medium treated animals.

Splenocytes from *P. histicola* treated animals suppressed PLP_{91-110}–induced EAE

Next, we tested whether adoptive transfer of immune cells from *P. histicola* treated mice would ameliorate EAE. We transferred splenocytes (1×10^7) from EAE-immunized animals treated with either *P. histicola* or medium into immunized mice 5 days after immunization (Mangalam et al., 2012). The group receiving splenocytes from *P. histicola* treated animals had lower disease incidence and severity compared with the untreated mice or those receiving splenocytes from medium treated animals (See Figure S5). Thus, our data indicated that the disease-suppressive effect of *P. histicola* was transferable.

P. histicola challenge caused shift in gut microbiota

Finally, to investigate whether treatment with *P. histicola* can alter gut microbiota composition, fecal samples were collected from pre (naïve) and post-immunized (EAE) mice receiving either medium or *P. histicola*. Mice with EAE had a distinct fecal microbiota compared to naïve mice (Figure 4A); however, challenge with *P. histicola* shifted gut microbiota composition closer to pre-immunized (naïve) mice. Naïve mice had a higher relative abundance of certain genera such as *Prevotella, Lactobacillus*, and *Sutterella* which were reduced in the EAE group receiving medium. However, mice induced for EAE and

treated with *P. histicola* had increased abundance of *Prevotella, Lactobacillus*, and *Sutterella* (Figure 4B and C). Thus *P. histicola* challenge restored gut microbiota to pre-immunized state.

DISCUSSION

We have identified a commensal bacterium *Prevotella histicola (P. histicola)* from the human upper gastrointestinal tract that had a systemic suppressive effect distant from the small intestine. *P. histicola* inhibited the development of EAE in HLA-DR3.DQ8 transgenic mice, a preclinical model of MS. We observed that *Prevotella histicola* treatment markedly attenuated inflammation, demyelination, and reduced BBB permeability compared to medium or control bacteria. We demonstrated that *Prevotella histicola* suppressed disease by downregulating pro-inflammatory cytokines IFNγ and IL-17, inducing CD4$^+$CD25$^+$FoxP3$^+$ Tregs, tolerogenic DCs, and suppressive macrophages. These observations suggest that *Prevotella histicola* monotherapy is effective when administered to HLA transgenic mice, a model of the chronic disease. Finally, that disease protection induced by *Prevotella histicola* was transferable into other animals; strengthening our hypothesis that *Prevotella histicola* protects the mice from EAE by modulating the systemic immune response. Our study shows that a human commensal from the upper gastrointestinal tract possesses potent disease-protective characteristics and provide proof of principle that human commensal gut bacteria protect against neuroinflammation.

Recent studies have indicated that human commensal bacteria (i.e., the microbiome) are a new frontier of biologic discovery with great therapeutic potential. Examples of bacterial therapeutics for ameliorating EAE include *B. fragilis* (Round and Mazmanian, 2010; Surana and Kasper, 2012) and mixtures of *Lactobacillus* strains (Lavasani et al., 2010). The disease-protective effects of *B. fragilis* have been attributed to its production of polysaccharide A (PSA) (Ochoa-Reparaz et al., 2010). However, live *P. histicola* was needed for maximal disease protection in our study, which suggests that the interaction of the living bacteria with the gut mucosal immune systems may be required. The ability of *P. histicola* to suppress EAE in mice with depleted gut flora suggests a direct role for *P. histicola* in disease suppression. However we cannot rule out potential additional indirect effects through modulation of other microbes because *P. histicola* treatment was also associated with a shift in gut microbiota to more closely resemble a healthy mouse with an increase in the relative abundance of *Prevotella*, *Sutterella* and *Lactobacillus* at the genus level. Although both *Prevotella* and *Sutterella* genera had been shown to be increased in RRMS patients on disease modifying treatments (Jangi et al., 2016), it is currently unclear whether the species of *Prevotella* (i.e. *P. histicola)* used in our study is part of the observed alteration of *Prevotella* at the genus levels in patients with MS. Previous study has shown that *B. fragilis*, a gut commensal can influence host behavior by modulation of gut microbes (Hsiao et al., 2013). Thus it is possible that *P. histicola* induced changes in gut microbiome might also contribute to its disease protective effect either directly, indirectly or both.

P. histicola challenge was associated with elevated levels of IL-10, CD4$^+$FoxP3$^+$ Tregs, tolerogenic DCs and suppressive macrophages. The disease-protective effect of other human commensals such as *B. fragilis* and a mixture of *Lactobacillus* species in EAE have been

attributed to their ability to induce CD4$^+$FoxP3$^+$ Tregs and IL-10 (Lavasani et al., 2010; Ochoa-Reparaz et al., 2010). A role of tolerogenic DCs observed in our study is consistent with earlier studies (Ochoa-Reparaz et al., 2010) showing a role for CD11c$^+$ DCs in suppressing disease in a PSA-treated EAE model. *P. histicola* increased levels of tolerogenic CD103$^+$ DCs producing high levels of IL-10 and low levels of IL-23.

Both IL-17 and IFN-y are the major pro-inflammatory cytokines associated with the pathology of MS. *P. histicola* treatment suppressed myelin antigen–specific T cell recall response and reduced IL-17 and IFN-γ in the periphery as well as in CNS. Thus, *P. histicola* might mediate its effect through downregulation of pro-inflammatory cells of both Th1 and Th17 phenotypes. Once inflammatory cells are activated in the periphery, their movement across the BBB and into the CNS is an essential step for initiating inflammation and demyelination in the brain and spinal cord. Reduced BBB permeability and milder pathology in the brain and spinal cord in *P. histicola* treated mice suggests that *P. histicola* mediates its suppressive effect through modulation of inflammatory cells trafficking to CNS.

Our study demonstrates that *P. histicola* can suppress EAE in mice, however it is currently unclear whether *P. histicola* supplementation will be effective as a treatment for MS. Interestingly, genus *Prevotella* are reduced in relapsing remitting MS (RRMS) patients compared with healthy controls (Chen et al., 2016; Miyake et al., 2015) and increased in RRMS patients on disease modifying treatments (Jangi et al., 2016). Thus human MS studies suggest that individual or a combination of *Prevotella* strains might have immunomodulatory properties; however, it is unknown whether these changes at genus level correlate with changes in *P. histicola* or some other species of *Prevotella*.

In summary, we report the association of a human commensal bacterium with disease-protective abilities in an animal model of MS. Our data indicate that *P. histicola*, a human commensal, has immunomodulatory and anti-inflammatory capabilities that suppress PLP$_{91-110}$-induced EAE in HLA-DR3.DQ8 transgenic mice. The disease protection was due to the ability of *P. histicola* to induce regulatory and suppressive immune subsets. Our future studies will study whether *P. histicola* modulates human immune cells directly or via its interaction with intestinal epithelial cells. This work demonstrates that *P. histicola* can manipulate the systemic immunity and organ-specific disease far away from its localization in the gut.

EXPERIMENTAL PROCEDURE

Mice

HLA-DR3.DQ8 double-transgenic [DQ8 (DQA1*0103, DQB1*0302)-DR3 (DRB1*0301] mice lacking MHC class II genes (AE$^{-/-}$), were generated as previously described (Das et al., 2000; Mangalam et al., 2009b) and are referred as HLA-DR3.DQ8 transgenic mice in the text. Mice of both sexes were studied. All mice were bred and maintained in the pathogen-free Immunogenetics Mouse Colony of Mayo Clinic (Rochester, Minnesota) and University of Iowa in the accordance with NIH and institutional guidelines. All experiments were approved by the Institutional Animal Care and Use Committee at Mayo Clinic, Rochester, and University of Iowa, Iowa City.

Isolation and culture of bacteria

Prevotella and similar anaerobic gram-negative Bacteroidetes bacteria were isolated from the duodenum of treated coeliac disease patients, and their identity was confirmed using 16S rRNA specific PCR followed by sequencing as well as whole genome sequencing as described previously (Marietta et al., 2016).

Flow Cytometry

Expression of HLA-DR and HLA-DQ molecules on peripheral blood leukocytes, lymph node cells, and splenocytes were analyzed by flow cytometry. Additional details are provided in the supplemental methods.

Disease induction and scoring

For disease induction, 8–12 week-old transgenic mice were immunized subcutaneously in both flanks with 100 μg of PLP_{91-110}. Disease severity was scored as described previously (Mangalam et al., 2009a) with additional details provided in the supplemental methods.

Treatment of animals with commensal bacteria

HLA-DR3.DQ8 transgenic mice were treated with *P. histicola, P. melaninogenica, C. sputigena*, and mouse-specific *E. coli.* or media by oral gavage, starting 7 days after immunization. Animals were gavage-fed on alternate days with 1×10^8 colony-forming units (CFU) in 100 μL of culture medium or medium alone for a total of 7 doses. Mice were evaluated for disease incidence, duration, and severity for 4 weeks after immunization.

T cell proliferation and cytokine assay

T-cell recall response was measured in splenocytes and lymph nodes from immunized mice using standard thymidine incorporation methods (Das et al., 2000).

Gut flora depletion and colonization with *Prevotella*

Gut flora was depleted by giving broad spectrum antibiotic cocktail (0.5 g/L vancomycin, 1g/L neomycin sulfate, 1g/L metronidazole, 1g/L ampicillin) in drinking water as described previously (Rakoff-Nahoum et al., 2004). After three weeks of antibiotic treatment, animals were placed on sterile water for 3 days before challenged with *P. histicola* (10^8 CFU) or medium on alternate days for the total of seven doses. One week after the last dose, EAE was induced and disease was monitored (Mangalam et al., 2009a).

Isolation of Tregs, dendritic cells, and macrophages

Tregs, dendritic cells (DCs), and macrophages were isolated from splenocytes using commercial cell isolation kits (Miltenyi Biotec Inc; San Diego, CA). Additional details are provided in *SI methods*.

In vitro functional analysis of Tregs, DCs, and macrophages

Suppressive abilities of $CD4^+CD25^+$ Tregs isolated from *P. histicola* or medium treated HLA-DR3.DQ8 transgenic mice were analyzed by coculturing Tregs (5×10^4 cells/well) with PLP_{91-110}-specific $CD4^+$ T cells (5×10^4 cells/well) in the presence of irradiated antigen-

presenting cells (APCs) loaded with antigen. Additional details are provided in *SI methods*. Lipopolysaccharides from *E. Coli 026:B6* (Sigma-Aldrich, St. Louis, MO, USA) was used to stimulate macrophages and dendritic cells.

Adoptive transfer of splenocytes

The splenocytes were collected from EAE-immunized animals treated with either *P. histicola* or medium and transferred (1×10^7 cells/mouse) intravenously into PLP$_{91-110}$-immunized HLA-DR3.DQ8 transgenic mice 5 days after immunization. The timing for adoptive transfer studies was based on our previous study (Mangalam et al., 2012).

Microbiome Analysis

Fecal pellets were collected from pre and post-immunized mice. Microbial DNA extraction, 16S amplicon preparation (V3-V5 region), and sequencing were done as described previously (Chen et al., 2016). R1 and R2 fastq reads were merged using Paired-End read merger (PEAR) (Zhang et al., 2014), merged reads were converted to fasta and merged fasta sequences were processed by Cloud Virtual Resource (CloVR) (Angiuoli et al., 2011) to form operational taxonomic units (OTUs) at 97% similarity and histograms were generated using METAGENassist (Arndt et al., 2012).

Pathology

Brain and spinal cord sections were analyzed for inflammation and demyelination after staining with modified hematoxylin and eosin as described previously (Mangalam et al., 2009a). Additional details are provided in *SI methods*.

Statistical analysis

Differences in proliferation or cytokine levels between groups was assessed by a one-way analysis of variance with multiple comparisons of the means when more than two groups were analyzed, or by Student's t-test when only two groups were analyzed if their data were normally distributed. The Kaplan-Meier method was used to estimate the probability of survival. The log-rank test was used to compare outcomes of different treatment groups. Hazard ratios (HR) for developing disease (and 95% confidence intervals [CI]) calculated from the estimated coefficients in Cox proportional hazards regression models. All statistical analyses were done with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA) or SAS version 9.3 (SAS Institute, Cary, NC). A significance level of 0.05 was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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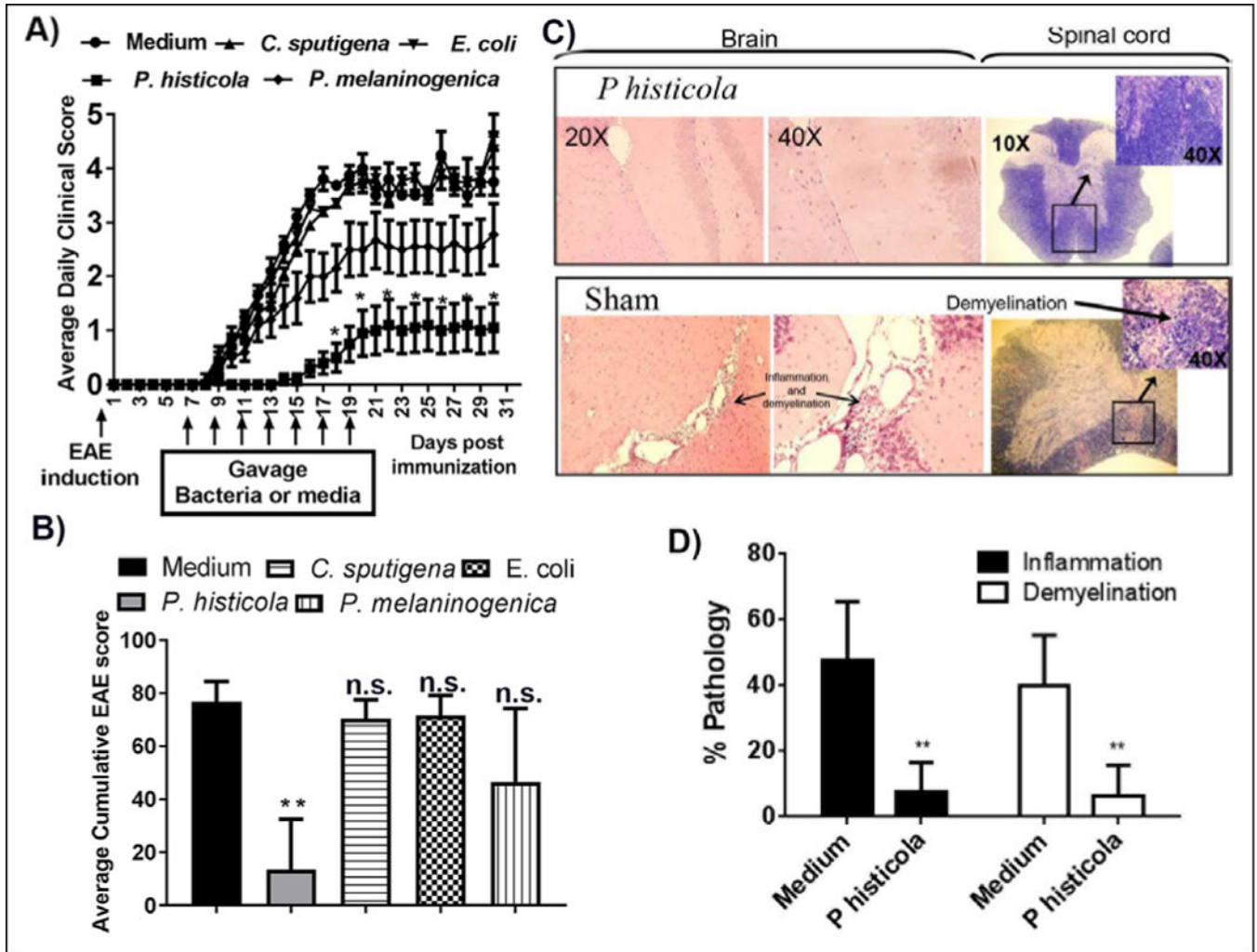
Figure 1. Effect of various human commensals on PLP$_{91-110}$-induced EAE in HLA-DR3.DQ8 transgenic mice

Animals were immunized with PLP$_{91-110}$ peptide and treated with either bacteria or medium starting at day 7 post-immunization and every other day for a total of 7 doses. **A)** Only *P. histicola* treated mice had late disease onset and lower disease incidence. No significant effect was observed in the groups receiving *P. melaninogenica*, *C. sputigena*, or *E. coli*. Animals were scored daily for disease, and the daily mean disease score for each group is plotted. Error bars represent the standard error of mean. **B**) *P. histicola* treated animals had a lower average cumulative disease score compared with medium treated animals. The data in figures A and B represent average cumulative scores of 3 experiments performed at different times with n=10 mice per group. **C)** Mice were sacrificed on day 30 post-immunization and the brain and spinal cord were examined by histopathology. Representative photomicrographs show mild to severe inflammation and demyelination in medium treated mice compared with *P. histicola* treated mice. **D)** Quantitative analysis of spinal cord pathology similarly showed increased inflammation and demyelination in medium treated groups compared with the *P. histicola* treated group. The data in figures C and D represent 1 of 3 experiments performed at different time points (n=5 mice per group). A single asterisk

indicates p 0.05, double asterisks indicate p 0.005, and 'n.s.' indicates not significant, when compared to medium treated group.
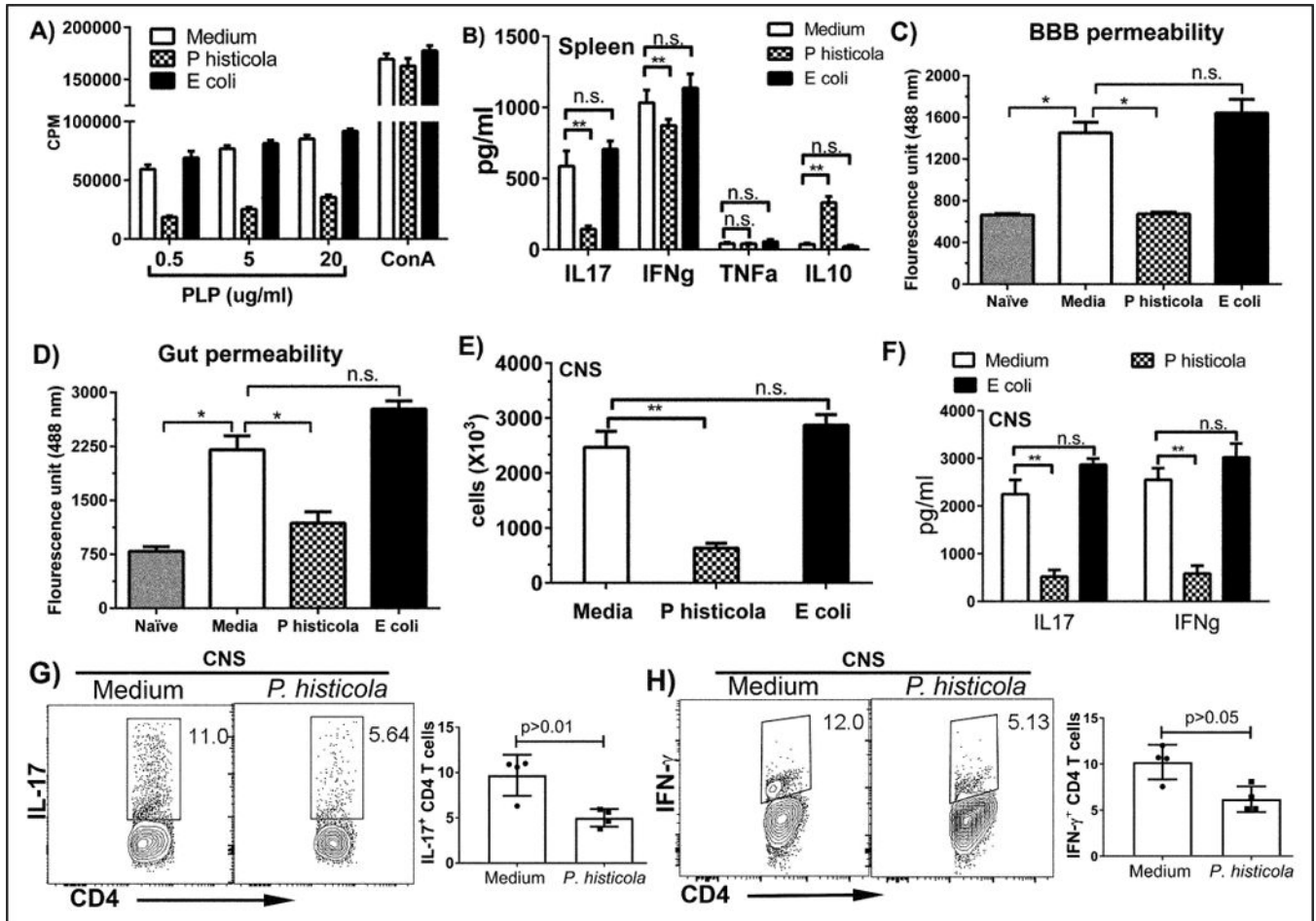
Figure 2. *P. histicola* treated mice show decreased T cell recall response, CNS inflammation and cytokine levels

A) *P. histicola* treated animals had reduced T cell proliferative response to PLP$_{91-110}$ peptide in vitro compared with the medium or *E. coli* treated group. Response to Concanavalin A was similar among three groups. The data presented are the mean±SD CPM. **B) Splenocytes stimulated with antigen from** *P. histicola* treated mice had reduced levels of inflammatory cytokines IL17 and IFN-γ, and increased levels of anti-inflammatory cytokine IL10 compared with medium treated mice. The data presented are the average of 2 independent experiments (n=4 mice per group). **C)** Medium treated HLA-DR3.DQ8 transgenic mice had compromised BBB permeability (increased FITC uptake in the brain). Challenge with *P. histicola* reduced BBB permeability and reduced gut permeability. **E)** Cellular infiltration into the CNS was reduced in *P. histicola* treated mice compared with medium or *E. coli* treated mice. **F)** CNS inflammatory cells from *P. histicola* treated group had reduced levels of inflammatory cytokines IFN-γ and IL-17 as measured by ELISA (F) and flow cytometry (G and H) compared with the medium treated group. The data represent 2 separate experiments performed in triplicate (n=5 mice per group). A single asterisk indicates p 0.05; double asterisks indicate p 0.005 and 'n.s.' indicates not significant, when compared to medium treated group.
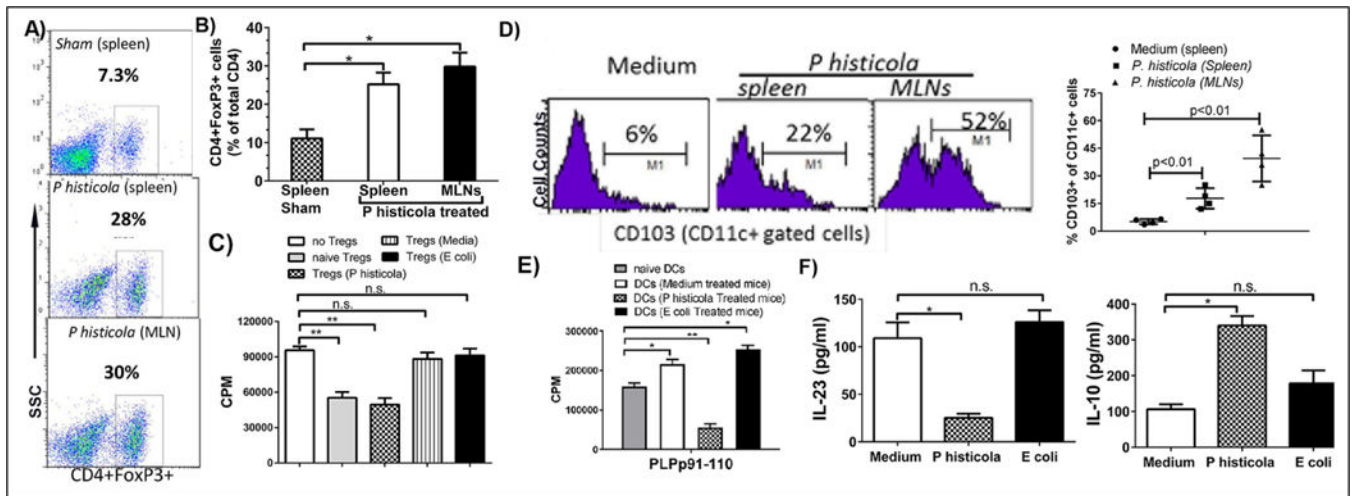
Figure 3. *P. histicola* challenge increased the frequency and activity of CD4+FoxP3+ regulatory T cells and tolerogenic DCs

A) *P. histicola* treated mice had an increased frequency of CD4+FoxP3+ Tregs compared with the medium or *E. coli* treated group. Numbers in scatter plots indicate the percentage of positive cells. **B)** Histogram plot of Treg frequency in the *P. histicola* treated and untreated groups. The data presented are the average of 2 independent experiments (n=4 mice per group). **C)** CD4+CD25+ Tregs from *P. histicola* treated mice had increased suppressive activity compared with the medium or *E. coli* treated group. **D)** The *P. histicola* treated group had increased levels of CD103+CD11c+ DCs in the spleen and MLNs compared with the medium treated group. Numbers in the histogram indicate the percentage of CD103+ cells gated from the CD11c+ population. Data are from 1 of 3 experiments performed at different times. **E)** Co-culture of CD11c+ cells from the *P. histicola* treated group with PLP specific CD4 T cells resulted in reduced proliferation compared to medium treated or naïve group. The data are presented as the mean±SD CPM and are the average of 2 independent experiments with n=3 mice per group. **F)** CD11c+ DCs from the *P. histicola* treated group produced low IL-23 and high IL-10 compared with the medium treated group. The data are presented as the mean±SD CPM and are the average of 2 independent experiments (n=3 mice per group). A single asterisk indicates p 0.05, double asterisks indicate p 0.005 and 'n.s.' indicates not significant.
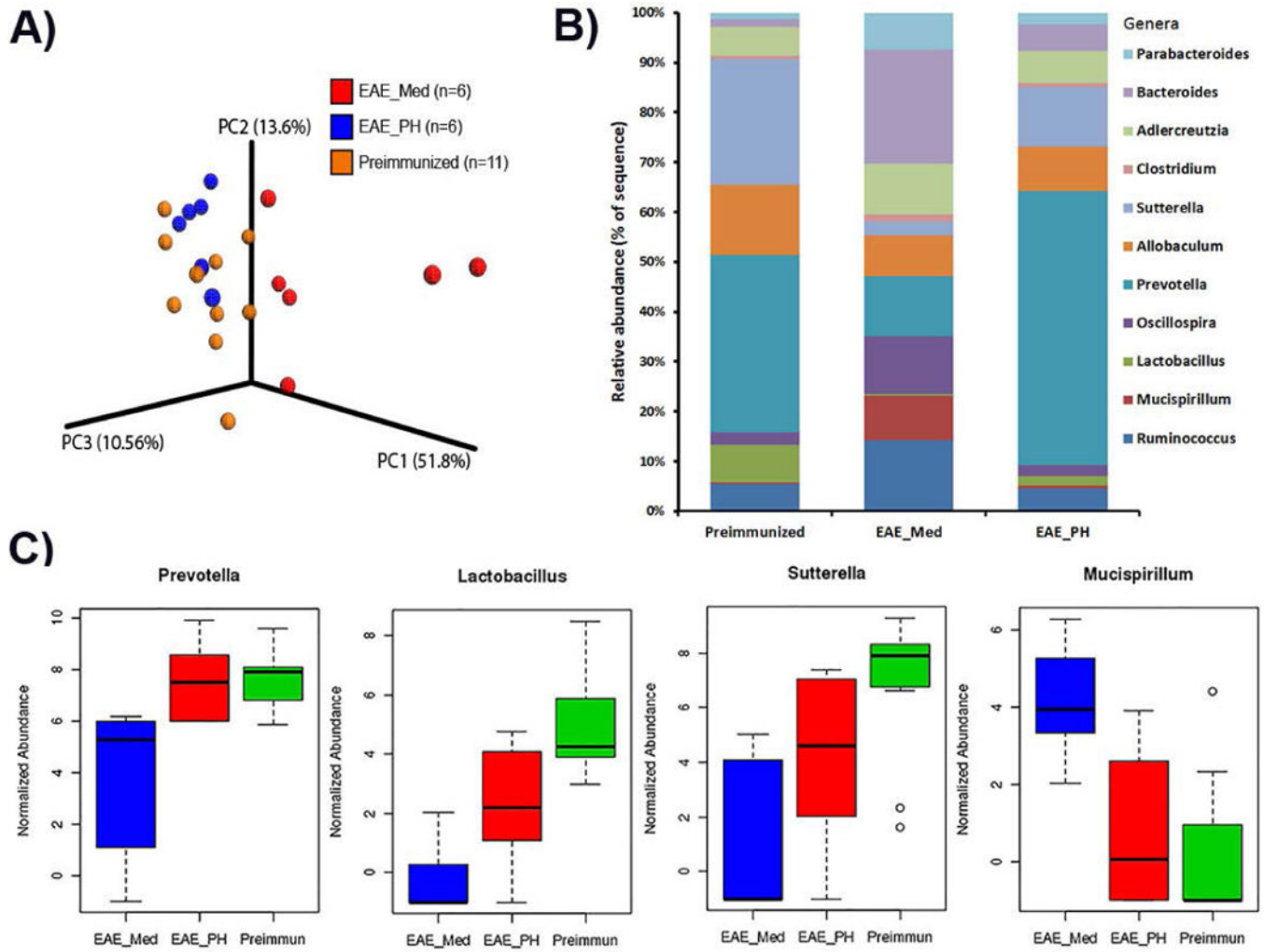
Figure 4. *P. histicola* challenge caused shift in gut microbiota

A) HLA-DR3.DQ8 transgenic mice with EAE had a distinct gut microbiome profile compared to those from pre-immunized (naïve mice). The *P. histicola* treated group had gut microbiota profile similar to pre-immunized (naïve mice) group. Weighted UniFrac-based 3D PCoA plot based on all OTUs from fecal samples of mice from, pre-immunized (n=6), medium treated EAE group (n=11), and *P. histicola* treated group (n=6). **B)** Relative abundance of gut microbiota at the genus levels in pre-immunized (naïve mice), EAE mice treated with medium, and EAE mice treated with *P. histicola*. C) Box plot showing normalized relative abundance of bacteria (genus-level profile) showing difference between groups. Mice with EAE had loss of certain genera especially *Prevotella, Sutterella*, and *Lactobacillus* compared to pre-immunized (naïve) mice. *P. histicola* challenge restored abundance of *Prevotella, Sutterella*, and *Lactobacillus*. The difference between groups were analyzed using one-way ANOVA (Kruskal-Wallis rank sum test) and FDR-adjusted p<0.05.

Table 1

Effect of commensal bacteria on PLP$_{91-110}$ induced EAE in HLA-DR3.DQ8 transgenic mice

Treatment	Disease incidence (%)	Disease free, Mean ± SE, days[A]	Hazard ratio (95% CI)	p-value
Medium fed	20/20 (100%)	10.6 ± 0.2	1 (ref)	
P. histicola	5/20 (25%)	17.5 ± 0.3	0.03 (0.01–0.09)	<0.0001
C. sputigena	20/20 (100%)	11.4 ± 0.4	0.6 (0.3–1.2)	0.15
P. melaninogenica	16/20 (80%)	12.5 ± 0.4	0.2 (0.1 –0.5)	<0.0009
E. coli	20/20 (100%)	11 ± 0.3	0.8 (0.4 – 0.6)	0.47

[A]Estimated by the log-rank test